Extracellular Vesicle Biology in the Pathogenesis of Lung Disease

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The human body is comprised of trillions of cells that are well organized into different functional compartments, tissues, and organs. To maintain homeostasis, coordinated intercellular communication is required. Some well established systems for communication between cells and their environment include the endocrine system and the complex circulation and neurological systems. Direct exchange of information through gap junctions and membrane nanotubes are also used by adjacent neighboring cells. In the last several years, investigators have identified cell-free nucleic acids in circulation within extracellular lipid vesicles, termed extracellular vesicles (EVs) (1). EVs represent small membrane phospholipid-based particles that are released by every cell in the human body. Harboring a variety of materials, including RNA, DNA, lipids, and proteins, EVs often reflect the cell of origin, and have emerged as important vehicles for the intercellular communication in human disease and maintenance of homeostasis (2). These initial observations have led to a large series of studies aimed at further defining the biogenesis, selective packaging, characterization of contents, and potential clinical applications of EVs in human health and disease. This is clearly evidenced by the exponential growth of the number of articles in the literature over the past few years. EVs are released by different cell types, and are largely defined by their physical characteristics instead of molecular properties. Therefore, it can be challenging to determine the precise function of EVs. For example, the initial observation of the release of transferrin receptor–containing lipid vesicles during the maturation of reticulocytes suggested that EVs may be essential for cells to remove unwanted or excessive molecules (3). We have now realized that EVs may have biological functions other than waste management. The mechanisms by which EVs can mediate intercellular signaling and changes in the micro- and macroenvironment are complex. When recipient cells absorb EVs from the extracellular environment, the contents can affect their biological activities. EVs may alter many physiopathological processes, including antigen presentation, cell proliferation, cell–cell communication, and others. For example, activated T cells within the lung could have their activities dampened through major histocompatibility complex (MHC) class II molecule–containing exosomes from dendritic cells (DCs) (4). Cancer cells use exosomes to promote angiogenesis and maintain or modify the microenvironment. CD147-positive EVs from epithelial ovarian cancer cells promoted angiogenesis *in vitro* (5). In addition, tumor-derived EVs converted fibroblasts or mesenchymal stem cells (MSCs) into myofibroblasts, a key player in the tumor microenvironment that facilitates tumor cell growth and invasion (6). The initiation and progression of lung diseases are often dependent on a well orchestrated interaction between environmental stressors, the immune system, genetic drivers, and communication between lung cells. EVs have emerged as potential vehicles for the transfer of molecular contents in the lung, thus providing improved understanding for the molecular underpinnings of lung disease and an opportunity for novel biomarkers and therapeutics.

EV Nomenclature and Biogenesis

Although the nomenclature for EVs continues to evolve, they may be fundamentally divided into subgroups based on size and biogenesis (1). Thus, it is essential to be able to purify EVs so that their molecular content can be analyzed and biological functions assessed. Given the overlap that exists between subgroups, the term EVs is often used (Figure 1). EVs can be grouped into three main types: microvesicles (MVs), apoptotic bodies, and exosomes (Table 1). MVs, also known as ectosomes or shedding vesicles, are about 100–1,000 nm in diameter and are produced by shedding or outward budding of the plasma membrane from cells. Once MVs are generated, they are encapsulated as part of the cytosol. MVs, therefore, have a membrane structure similar to that of the cell of origin. Exosomes were first described more than 30 years ago by Pan and Johnstone (3) when they detected transferrin receptor–containing vesicles released by reticulocytes during the maturation of red blood cells. Exosomes are about 30–150 nm in diameter and produced within cells through inward budding of the endosomal membrane (Figure 1). The endosome system is an intracellular molecular transport system that drives both sorting and transportation of molecules between the plasma membrane, lysosomes, and trans-golgi

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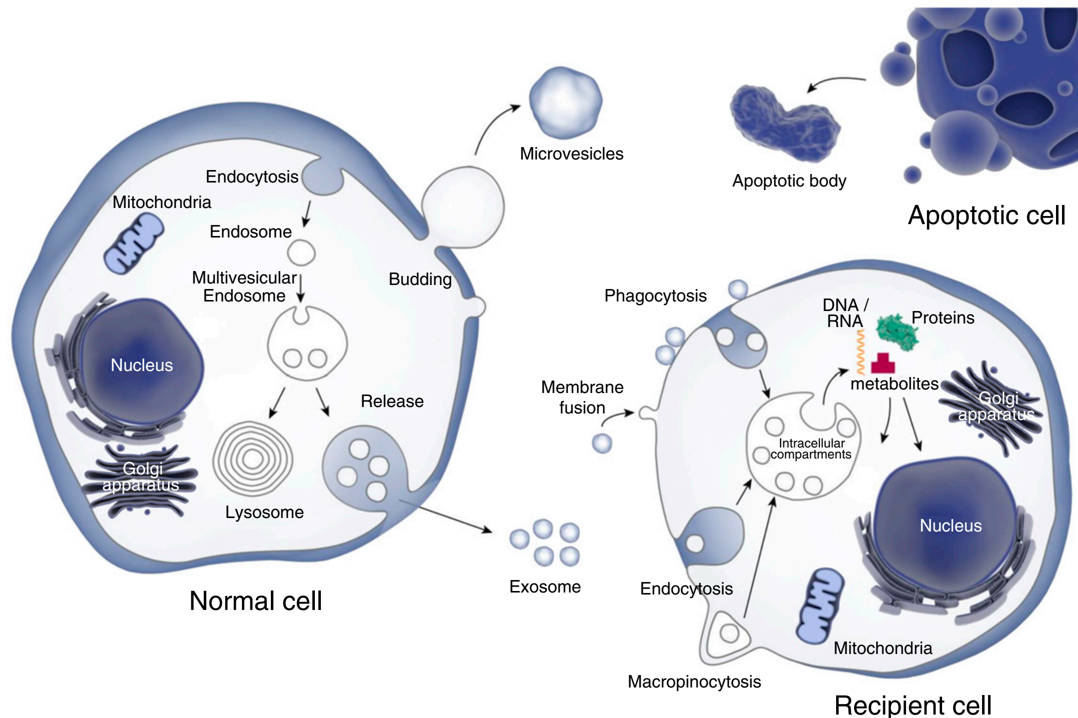

Figure 1. Extracellular vesicle biogenesis. Extracellular vesicles are divided into three main types: exosomes, microvesicles, and apoptotic bodies. Exosomes are formed in multivesicular endosomes and contain molecules including nucleic acids, proteins, lipids, and metabolites. The exosomal content can be transferred to other cells through different processes, including endocytosis, phagocytosis, macropinocytosis, or direct membrane fusion.

network. Exosomes and their contents can be either processed through the lysosome or accumulate within multivesicular bodies and then be released to the extracellular environment once the multivesicular bodies fuse with cell membrane. The molecular contents of exosomes also often reflect those of its cell of origin. For example, exosomes released from DCs are enriched with CD1, CD80, CD86, and MHC class I and II molecules that are involved in the immune response (7). Exosome populations are enriched for tetraspanin membrane proteins, such as CD9, CD63, CD81, and vesicle-trafficking proteins, including tumor susceptibility (TSG) 101 and programmed cell death 6–interacting protein, and heat shock proteins (Hsps), like Hsp70 family A member 4 (HSPA4) (8). The CD63, programmed cell death 6 interacting protein and TSG101 are also enriched in the endosomal membrane. Apoptotic bodies (also called apoptotic blebs or apoptotic vesicles) are 50–5,000 nm in diameter and released from apoptotic cells (9). The density of apoptotic bodies ranges from 1.16 to 1.28 g/ml, overlapping with that of exosomes. In addition, the size of an apoptotic body is similar to that of a platelet. Apoptotic bodies contain DNA, and have been implicated as a vehicle for horizontal oncogene transfer. A number of proteins, including histones, thrombospondins, complement component C3, and annexin (ANXA) V, have been identified in apoptotic bodies (10).

Researchers continue to unravel the complexities of EV biogenesis. Changes in environmental stressors (e.g., hypoxia,

Table 1. Extracellular Vesicle Classification

Particle	Microvesicle	Exosome	Apoptotic body
Diameter, nm	100–1,000	30–150	50–5,000
Density, g/ml	Undetermined	1.13–1.19	1.16–1.28
Biogenesis	Shed from cell membrane	Inward budding of endosomal membrane	Released from blebs of apoptotic cells
Protein	VCAM1, ARF6, FLOT2, integrin, CD40, MMPs	CD9, CD63, CD81, TSG101, PDCD6IP, HSPA4	Thrombospondins, C3, ANXA5, histones

Definition of abbreviations: ARF6 = adenosine diphosphate–ribosylation factor 6; ANXA = annexin; C3 = complement component C3; FLOT = flotillin; HSPA4 = shock protein family A member 4; MMP = matrix metalloproteinase; PDCD6IP = programmed cell death 6–interacting protein; TSG101 = tumor susceptibility 101; VCAM = vascular cell adhesion protein.

pH, temperature) have all been identified as regulators of EV release (11–13). Proper EV release and function are dependent upon sorting, packaging, directed release, and uptake. The mechanisms that drive such processes are also slowly coming to light. For example, EV microRNA (miRNA) content does not always match that of the parent cell. Researchers have determined that an Exo motif of miRNA sequences can dictate miRNA sorting into EVs (14). This concept was further validated with the identification of a protein called synaptotagmin-binding, cytoplasmic RNA-interacting protein, the binding of which to select sequences within exosomal enriched miRNAs can guide packaging and release (15).

EV Isolation Characterization

EV Isolation

There are several different methods used for exosome purification, with most based on the physical characteristics of EVs (Table 2). One of the issues in studying EVs is overcoming the hurdle of obtaining pure populations of subgroups, as there is substantial overlap of characteristics between different lipid vesicles. For example, the density of exosomes overlaps with that of apoptotic bodies, and the range of particle size for exosomes overlaps with that of MVs.

Ultracentrifugation. Ultracentrifugation, combined with a density gradient method, results in a fairly pure exosome population. This method requires multiple rounds of sequential centrifugation to separate vesicles based on size (16). The drawbacks of this method include the long purification process, larger sample volume requirement, and low throughput. There is also a suggestion that the centrifugation forces are insufficient to pull down a significant fraction of exosomes in some samples, and may disrupt exosome structure and fuse lipid vesicles together.

Exosome precipitation kits. There are several precipitation-based commercial kits for exosome purification. These are most often based on water-clouding agents, such as polyethylene glycol to precipitate certain-sized particles. This method is quick, does not require ultracentrifugation, and results in very high throughput. It can also handle smaller sample volumes.

Immunoaffinity. Exosomes may be isolated by targeting common cell surface proteins, such as CD63. However, not all exosomes contain CD63, yielding only a fraction of the exosome population.

Size-exclusion column. Based on exosome particle size, size-exclusion columns yield relatively pure vesicles, with minimal protein contamination, within about 20 minutes. In addition, physical force is reduced, thus preserving shape. This method may still result in coelution with other subtypes, including MVs and apoptotic bodies (17).

EV Characterization

Through various high-throughput profiling platforms, we have gained insight into the spectrum of molecular contents of EVs. As most of these technologies require significant amount of materials, most profiling studies are based on *in vitro* cell line–secreted EVs. Based on ExoCarta (www.exocarta.org)—a database dedicated to cataloging exosome content—more than 5,000 different proteins, 2,000 RNAs, 1,300 miRNAs, and 800 lipid molecules have been identified (18).

Proteins. Protein content can be effectively analyzed to elucidate EV function. Mass spectrometry (MS)–based methods, such as one- or two-dimensional liquid chromatography–tandem MS and matrix-assisted laser desorption ionization–time of flight/time of flight MS are commonly used platforms to analyze the spectrum of proteins in EVs (19). Recently, using one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis coupled with liquid chromatography–tandem MS, researchers studied the proteome of different types of vesicles and identified exosome-enriched proteins, including TSG101, syntenin-1, eps 15 homology domain–containing 4, ANXA11, and a disintegrin and metalloproteinase metallopeptidase domain 10 (20). To compare molecular contents from different samples, technologies, such as stable isotope labeling with amino acids in cell culture combined with MS can be used (21). Stable isotope labeling with amino acids is based on the metabolic incorporation of an isotopically "light" or "heavy" form of amino acids into proteins, the MS analysis of which results in not just identifying proteins in the EV, but also providing quantitative information on relative protein abundance. In addition to global profiling approaches, a targeted proteomics approach, called multiple (or selected) reaction monitoring, is often used to quantify specific proteins in EVs. The protein abundance determined by selected reaction monitoring is based on the spike-in of a known concentration of heavy labeled peptide standard (22). It is sensitive, specific, and does not require high-affinity antibodies to assess protein concentration. Other technologies,

Table 2. Isolation Methods for Extracellular Vesicles

Purification Method	Ultracentrifugation	Precipitation	Immunoaffinity	Size Exclusion Column
Time required	About 24–48 h	About 50 min	About 1 h	About 20 min
Purity	High	Low	High	Medium
Volume needed	About 1 ml	100–200 μl	Variable	100–200 μl
Other issues	• Centrifugation force may change the nature of lipid vesicle • Inconsistent results	• High protein contamination • Polyethylene glycol may affect downstream analysis	• Obtain just a fraction of the exosome population	• Exosomes obtained, most maintain original structure • Suitable for functional studies • May be contaminated with different types of vesicles

including antibody array, have also been used to profile protein content in EVs.

RNA. EVs contain a wide variety of RNA, including full-length protein coding mRNA, noncoding RNAs, such as miRNAs, long intergenic noncoding RNA, transfer RNAs, P-element induced Wimpy testis RNAs, ribosomal RNAs, vault RNAs, and cytoplasmic Y RNAs (23). Most of the RNAs in EVs are less than 700 nt, which suggests that some of the protein coding mRNAs and long intergenic noncoding RNAs are probably degraded RNA. Unlike cellular RNAs, RNAs from EVs lack significant amounts of ribosomal RNA, obviating the need for a ribosomal RNA depletion step (24). Commonly used technologies to assess the RNA, for both small and large RNA molecules in EVs, include complementary DNA microarrays, quantitative reverse transcription–polymerase chain reaction (RT-PCR), and next-generation sequencing (NGS). Quantitative RT-PCR is sensitive, and requires a small amount of RNA, but can only assess the concentration of specific RNA transcripts. Some of the RNAs in EVs are degraded transcripts, which may limit the assay. Microarray requires a higher concentration of RNA and, therefore, is not commonly used in EV RNA profiling. Recently, the NGS approach has gained significant interest, given low template requirements, ability to assess degraded RNA fragments, and lack of a requirement of prior knowledge on the identity of RNA in the sample. NGS requires specialized informatics tools to process the sequence reads, determine the sequence read identity, and quantify the level of specific transcripts. In addition, the library construction process, especially for small RNA, is long and may introduce significant variability.

Lipids. Due to its sensitivity and selectivity, MS is commonly used for lipid profiling (25). Unlike other biomolecules, lipids do not have a general guideline on ionization efficiency and rules of fragmentation in MS; therefore, it is difficult to develop standard methods for global lipid analysis. Electrospray ionization and matrix-assisted laser desorption ionization are the commonly used ionization technologies, because they do not induce significant fragmentation.

One limitation to current technologies for EV characterization of content lies in the heterogeneity of EV populations that exist in body fluids. Thus, detection of "disease- or cell-specific" EVs and signal may be masked by larger numbers of EVs originating from a multitude of cell types. To overcome these challenges, novel platforms for targeted EV capture and characterization, including lipid biospheres and lipoplex-based nanochips, are under development (26, 27)

EVs and Intercellular Communication in the Lung

The exchange of genetic information mediated through EV transfer between cells of the lung has become an increasingly recognized mechanism for initiating the immune response, development of disease, and maintenance of homeostasis. The environmental cues that drive the release of EVs from the lung and shuttling of contents include hypoxia, LPS, pollutants, cigarette smoke, and microbial pathogens. However, although our knowledge of the underlying mechanisms for cell-specific interactions and downstream signaling is minimal, it appears that EV release is cell, stimulus, and disease specific. Multiple cell types, including epithelial cells, macrophages, mast cells, and endothelial cells, have been identified as mediators of EV intercellular communication.

Immune Response

Early studies revealed that exosomes derived from human tracheobronchial epithelium harbor typical EV characteristics and epithelial-specific markers, including mucins (28). The release of epithelial-derived EVs in the setting of environmental exposures may be a critical step in the triggering the immune response, and serve as a link to the development of diseases of the airway, including chronic obstructive pulmonary disease (COPD) and asthma. For example, epithelial cell–derived EVs promote leukotriene conversion of leukotriene C4 (LTC4) to LTD4 in monocytic cells, potentially contributing to tissue remodeling and bronchoconstriction (29). Stimulated monocytes induce apoptosis through EV transfer of caspase-1 (30). In the case of environmental allergens, dust-derived EVs drive a neutrophilic response in the lung, characterized by T-helper cell type 1 (Th1) and Th17 accumulation in the lung (31). In addition, preclinical models of inhalation of LPS led to an increase in EVs within bronchoalveolar lavage fluid (BALF) that, in turn, drives a Th1 and Th17 response (32). The molecular contents that facilitate an inflammatory response are varied. For example, select miRNAs, including miR-146 and miR-155 within immune cells, can regulate the inflammatory response (33). Reciprocal inflammatory signaling may occur between cell types of the lung, with epithelial and alveolar macrophages (AMs) representing one of the best studied examples (34) (Figure 2). AMs release EV-based suppressor of cytokine signaling (SOCS) proteins (SOCS1 and SOCS2) to abrogate activation of cytokine-induced signal transducers and activators of transcription in alveolar epithelial cells (35). The mechanisms underlying AM EV-selective packaging of SOCS proteins remain largely unknown. There is a suggestion that exposure to infectious agents may induce SOCS3 release from AM-derived EVs (36). *In vivo* hyperoxia treatment resulted in a release of primarily epithelial-derived EVs, both in circulation and in BALF. Exposure of AMs to these same epithelial cell–derived EVs led to a potent induction in proinflammatory cytokines *in vitro* and neutrophil and macrophage accumulation *in vivo* (37). These observed effects were partially mediated through a caspase-3–Rho-associated protein kinase pathway (37).

The relative contribution of subfamily members of EVs to intercellular transfer of contents and downstream signaling remains largely unknown, but there are likely to be both overlapping and divergent functions. In the setting of oxidative stress after hypoxia exposure in mice, the majority of EVs in both BALF and *in vitro* were MVs and epithelial in origin (38). Furthermore, these miRNA-enriched MVs induced a potent inflammatory (tumor necrosis factor α) and migratory response in recipient macrophages.

Lung Cancer

EVs have perhaps been best studied in the hallmarks of cancer biology and cancer initiation and progression. In lung cancer, EVs and their contents can drive angiogenesis, proliferation, epithelial–mesenchymal transition, and chemosensitivity (39–43) (Figure 2). Novel studies demonstrate that tumor-derived exosomes carry cell surface integrins that direct organ-specific metastatic deposition (44). Exosomes expressing integrin (ITG) α6β and ITGα6αβ1 were more likely to target lung cells, whereas ITGαvβ5 targets cells in the liver (44). One particularly intriguing role for EVs has been as a biological link between benign and malignant disease. For example, exosomes derived from plasma of mice exposed to intermittent hypoxia and plasma from patients with obstructive sleep

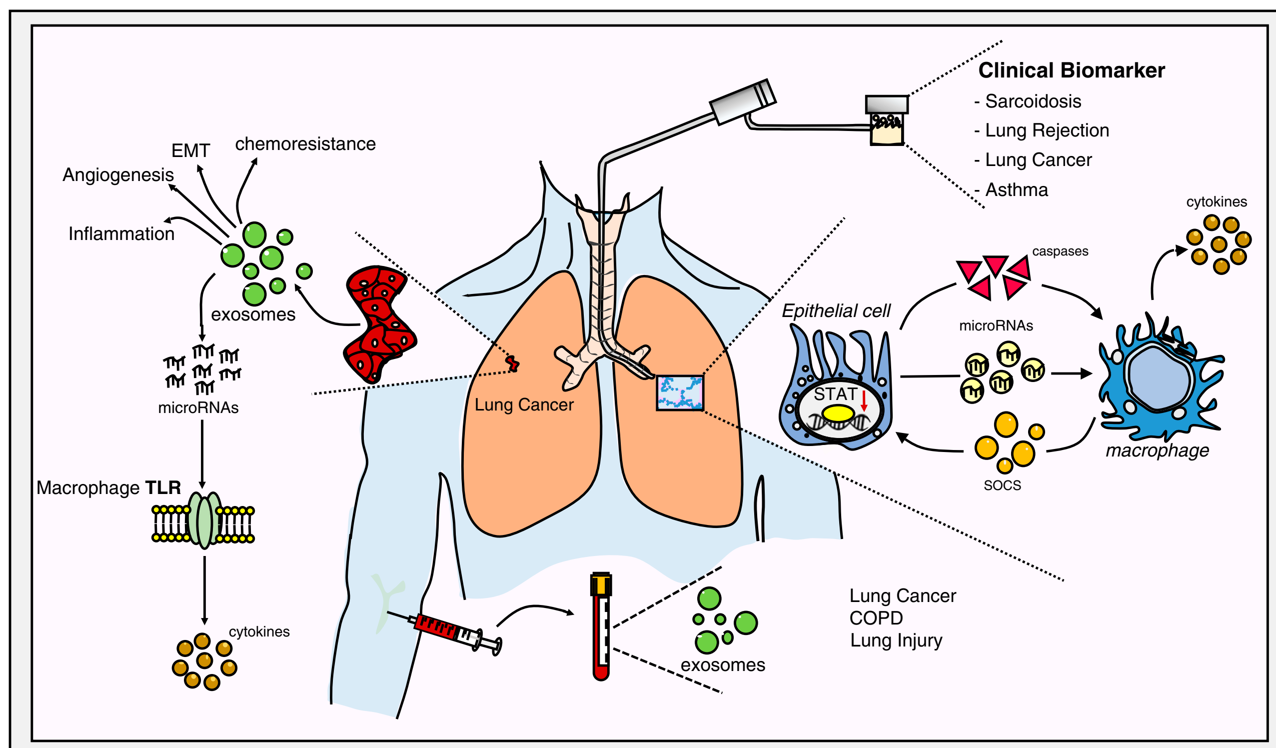

Figure 2. Applications for extracellular vesicles in lung disease. COPD = chronic obstructive pulmonary disease; EMT = epithelial–mesenchymal transition; SOCS = suppressor of cytokine protein signaling; STAT = signal transducers and activators of transcription; TLR = Toll-like receptor.

apnea enhanced malignant properties of recipient cancer cells (45).

Toll-Like Receptor Signaling

The mechanisms by which EV contents facilitate downstream signaling are not fully understood. However, a recent and exciting development in the field of EV biology involves the recognition that EV content may function as ligands for Toll-like receptors (TLRs). Investigators demonstrated that miRNAs released by cancer cell–derived EVs can induce macrophage-mediated production of proinflammatory cytokines through binding of TLR8 (46) (Figure 2). This novel link may also serve as a mechanism for the development of chronic lung disease. LPS-mediated TLR4 activation was shown to increase the release of exosome-packaged proteases, including prolyl endopeptidase from airway epithelial cells (47).

Noninvasive Biomarkers of Lung Disease

A number of clinical diagnostic applications have been developed based on the concentration changes of specific molecules in circulation. The observed release of EVs in blood and other body fluids after environmental stressors, or in the setting of disease states, suggests that they provide a less-invasive means for disease monitoring, making them particularly compelling candidates for clinical biomarkers. The presence of these cell-free molecules in body fluids are likely the result of normal or pathological conditions associated with cell death; therefore, disease states may affect this spectrum of circulating molecules. The reported number of exosomes ranges from 0.84 to 4.32×10^8 particles/ml of plasma and contains about 40–60 ng of RNA (48). The exosome has a density that ranges between 1.10 and 1.14 g/ml, and a molecular weight of approximately 3.3×10^7 g/mole. Furthermore, an independent study by Chevillet and colleagues (49) suggested that the copies of miRNAs in body fluid exosomes, when isolated by differential ultracentrifugation, are minimal, raising questions regarding their biological significance.

Lung Cancer

Several studies have suggested the utility of blood-based EV signatures as potential biomarkers in lung cancer. A high-throughput analysis of circulating exosomes resulted in the identification of a panel of miRNAs that could distinguish between malignant and benign nodules of the lung (96.0% sensitivity; 60.0% specificity; area under the curve, 76.0%) (50). An independent study determined that a six-miRNA panel in plasma could distinguish between patients with adenocarcinoma of the lung and healthy control subjects (51). Finally, patterns of exosome-bound proteins in circulation have been correlated with lung cancer survival (52). Although encouraging, such studies are limited by small cohort sizes, and thus require larger independent validation.

Acute Lung Injury

Few studies to date have investigated the impact of lung injury on circulating EV populations. *In vivo* murine exposure to 4 hours of high–tidal volume mechanical ventilation resulted in physiological characteristics of acute lung injury and a marked increase in the release of endothelial (platelet endothelial cell adhesion molecule/ANXA5$^+$)–derived microparticles (300–1,000 nm) in circulation (53).

COPD

Cigarette smoke exposure leads to a potent induction of endothelial-derived microparticles, which may signify endothelial injury. In COPD, elevated subpopulations of microparticles in circulation correlated with presence of COPD and exacerbations (54). Environmentally derived EVs may also elicit a response in the periphery linked to disease activity or risk. In one small study, investigators demonstrated that elevated serum IgG against dust-derived EVs was a risk factor for the development of lung cancer, COPD, and asthma (55).

EVs are detectable in BALF; however, the cell origin of BALF EVs remains unclear. One of the first reports of EVs in BALF by Admyre and colleagues (56) described BALF EVs that express MHC class I and II, CD53, and CD63, suggesting possible antigen-presenting cell origin. The molecular contents of BALF EVs may be applied as diagnostic biomarkers of clinical disease.

Sarcoidosis

Using mass spectroscopy, Martinez-Bravo and colleagues (57) conducted an analysis of protein expression in BALF-derived EVs from patients with sarcoidosis versus control subjects. They identified 690 proteins, including several up-regulated inflammatory proteins. An independent study demonstrated elevated numbers of BALF-derived EVs from patients with sarcoidosis inducing a proinflammatory response in recipient cells (58).

Lung Transplant

A recent study among patients undergoing lung transplantation demonstrated that BALF EVs in those with acute lung rejection carried distinct RNA transcripts linked to both the innate and adaptive immune responses (59). Comparing patients with and without chronic rejection, investigators identified higher levels of red blood cell– and epithelial cell–derived EVs among those with chronic rejection and an association with poor survival (60).

Asthma

Several studies have demonstrated that BALF-derived EVs in asthma harbor distinct properties that drive inflammation. Epithelial-derived exosomes were detectable in BALF of subjects with asthma and stimulated both CXCL-8 and LTC4 release in recipient epithelial cells (61). In addition, BALF EVs derived from subjects with asthma induced cytokine and leukotriene production in recipient epithelial cells (62). Preclinical murine models for asthma also exhibited a marked increase in epithelial-derived EVs that can induce IL-13–mediated monocyte proliferation (63).

EVs as Targeted Therapeutics

The clinical use of EVs for therapy is in the near future. One such example involves the use of EVs as modifiers of response to immunotherapy for lung cancer. Investigators have explored the use of DC-derived exosomes (Dex) as a means for augmenting natural killer and T cell function in the setting of immunotherapy (64). Treatment of patients with advanced-stage non–small cell lung cancer with Dex resulted in an increased natural killer cell function in a subgroup of patients, suggesting potential for Dex as an adjuvant therapeutic strategy for immunotherapy (64). Aptamer-based targeting represents another potential avenue for augmenting the immune response. Targeting cancer-derived Hsp70-enriched EVs attenuated myeloid-derived suppressor cell activation (65)

EVs have also been applied as therapies in pulmonary hypertension (PH). Aliotta and colleagues (66) demonstrated that the exosome fraction of circulating EVs from mice treated with monocrotaline, when delivered by tail vein injection, induced PH-caused anatomical changes consistent with PH in healthy mice. Conversely, intravenous exosomes derived from MSCs both prevented and reversed monocrotaline-induced PH.

The use of MSCs represents an exciting and novel direction for therapeutics in lung disease. For example, MSCs have been shown to reduce inflammation, permeability, and bacterial growth in *in vivo* models of pneumonia, and reduce the Th2/Th17–mediated airway hyperresponsiveness in models of allergic airway inflammation. *In vivo* administration of MSC-conditioned media and EVs reduced Th2/Th17 inflammation and hyperresponsiveness of the airway after *Aspergillus* hyphal extract exposure (67). Immunization with *Staphylococcus aureus*–exposed EVs conferred significant protective effects on the immune system in the setting of both lethal and sublethal exposure to *S. aureus* infection (68). Translation of MSCs as therapies in human disease is most evident in acute lung injury (69, 70). However, the challenges inherent to isolation and purification of MSCs and potential toxicity may limit the potential for large-scale human studies. The effects of MSC-derived conditioned media on lung cellular repair and inflammation mirrors that of primary MSCs, suggesting the presence of therapeutic components within the media (71, 72). MSC-derived EVs are protective in abrogating inflammation and improving alveolar fluid clearance in preclinical models of lung injury (73). Although the underlying mechanisms have yet to be full elucidated, an independent study showed that MSC-derived EV miRNAs could mediate both autophagy and mitophagy in macrophages (74).

Conclusions: The Future of EV Biology in Lung Disease

Since their initial discovery over 30 years ago, EVs have assumed an increasingly prominent role as mediators of intercellular communication in human disease. To date, investigators have demonstrated that EV-mediated transfer of RNAs and proteins contribute to the lung immune response to environmental insults and biology fundamental to cancer initiation and progression. We have observed early studies targeting EVs in augmenting the immune response to cancers. Such exciting potential should be tempered by the fact that our current understanding of their role in the lung is minimal, and there remains a great deal to be learned. Standardized protocols for the isolation, quantification, and characterization of EV family members will be essential in parsing out the relative contributions of each subtype to disease. The majority of studies to date have focused on smaller exosomes, with minimal attention to the potential contribution from larger MVs and apoptotic bodies. Second, the observed stimulus, cell- and disease-specific release of EVs, highlights the complex "networks" underlying EV-mediated biology. Third, we are just beginning to understand that the mechanisms by which select EV subpopulations and their contents are packaged and released. Finally, the identification and relative stability of EVs in body fluids provides us with an opportunity to develop novel, clinically informative biomarkers, but will also depend

on improved understanding of EV member contributions to disease pathogenesis and characterization of EV contents. However, further stoichiometric studies are required to better quantify body fluid–based EV contents and their potential contribution to downstream pathophysiology. Although it is still early, one should be optimistic about the future for EV biology in lung disease and the potential for additional intense investigation to eventually impact lung health. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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