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Title: **Extracellular Vesicles in Acute Respiratory Distress Syndrome: Understanding Protective and Harmful Signaling for the Development of New Therapeutics**

Running Title: ***Extracellular vesicles in ARDS***

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1. Abstract

Acute respiratory distress syndrome (ARDS) is a severe respiratory condition characterized by increased lung permeability, hyper-inflammatory state, and fluid leak into the alveolar spaces. ARDS is a heterogeneous disease, with multiple direct and indirect causes that result in a mortality of up to 40%. Due to the ongoing Covid-19 pandemic, its incidence has increased up to ten-fold. Extracellular vesicles (EVs) are small liposome-like particles that mediate intercellular communication and play a major role in ARDS pathophysiology. Indeed, they participate in endothelial barrier dysfunction and permeability, neutrophil, and macrophage activation, and also in the development of a hypercoagulable state. A more thorough understanding of the variegated and cell-specific functions of EVs may lead to the development of safe and effective therapeutics. In this review, we have collected evidence of EVs role in ARDS, revise the main mechanisms of production and internalization and summarize the current therapeutical approaches that have shown the ability to modulate EV signaling.

Keywords: acute respiratory distress syndrome (ARDS), Acute Lung Injury (ALI); extracellular vesicles (EVs), alveolo-capillary permeability, therapeutics, EVs biogenesis, EVs internalization.

2. Introduction

Acute respiratory distress syndrome (ARDS) is a severe respiratory condition characterized by cough, difficulty breathing and shortness of breath (Diamond and Sanghavi, 2023). ARDS was first described in 1967. Formal criteria were established in 1994 and included a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen ($PaO_2/FiO_{2)}$ less than 200 mmHg. In 2011 the definition was revised into what is now referred to as the "Berlin definition." (Ashbaugh *et al.*, 1967; Walsh and Van Patten, 1994; ARDS Definition Task Force et al., 2012). Criteria include: 1) acute onset; 2) presence of bilateral opacities; 3) respiratory failure not of cardiac origin; and 4) a notable impairment of oxygenation reflected by the PaO_2/FiO_2 ratio <300 mmHg. The current therapeutic approaches include assisted ventilation, corticosteroid therapy, proper fluid management and veno-venous extracorporeal life-support (Menk *et al.*, 2020). However, the mortality of ARDS is still remarkably high and can reach 45% in severe cases (Bellani *et al.*, 2016). ARDS incidence ranges from 64.2 to 78.9 cases/100,000 person-years in the US, but due to the ongoing SARS-CoV-2 pandemic, it has increased up to ten-fold, highlighting the need for new therapeutic interventions (Rawal *et al.*, 2018; Diamond Sanghavi, 2023). Unfortunately, hundreds of drug candidates that have been tested clinically in the past decade have produced minimal or even negative results. This limited therapeutic success may be explained by the fact that the pathophysiology of ARDS is complex, heterogeneous, and incompletely understood. It is normally triggered by a pulmonary insult or systemic injury, which elicits a strong inflammatory response that impacts the alveolo-capillary structures. This results in increased lung vascular permeability, fluid leak into the alveolar spaces, and consequent inefficient exchange of oxygen and CO2, resulting in acute respiratory failure (Rawal *et al.*, 2018). Animal models of ARDS have demonstrated that multiple insults, such as lipopolysaccharides, bacteria, or microemboli, play a role in increasing pulmonary endothelial permeability and fluid extravasation in the lungs (Matthay *et al.*, 2019). Injury of the alveolo-capillary barrier is indeed a crucial step in the pathophysiology of ARDS and has been attributed to increased levels of cytokines, chemokines, adhesion molecules, damage-associated molecular patterns (DAMPs), and thrombin (Meyer *et al.*, 2021). These mediators cause the transition of the endothelium to a leaky state, that allows migration of inflammatory cells and fluid into the alveolar structures (Sun *et al.*, 2013; Millar *et al.*, 2016). This process involves cytoskeletal rearrangements in endothelial cells and disruption of the tight junctions between neighboring endothelial cells, which ultimately causes the breakdown of monolayer integrity (Dudek and Garcia, 2001; London *et al.*, 2010). The endothelial dysfunction is further exacerbated by the apoptotic mechanisms induced by the immune cells recruited to the areas of pulmonary inflammation (Fujita *et al.*, 1998; Abadie *et al.*, 2005; Gill *et al.*, 2015).

An additional mechanistic framework that can contribute to disruption of the endothelial and epithelial barriers is the one provided by the new paradigm of cell-cell communication via extracellular vesicles (EVs). It is broadly recognized that EVs are a key component of the intracellular signaling network that takes place in multicellular organisms (Sanwlani & Gangoda, 2021).

EVs are small liposome-like particles that contain proteins, nucleic acids, lipids, and various metabolites that exert their signaling properties through the release of their cargo into recipient cells. As of 2023, the Vesiclepedia repository includes nearly 350,000 proteins, 27,500 mRNAs,

10,500 microRNAs (miRNAs), and 639 lipids found in EVs, based on over 1,250 studies in more than 40 species (Kalra *et al.*, 2012). miRNA, which are a type of non-coding RNA that aid in the regulation of gene expression and thus determine EVs' capability to modulate cellular immune responses (Sanwlani and Gangoda, 2021), increase or decrease inflammation, and impact on tissue repair and proliferation (Buzas *et al.*, 2014; Oggero *et al.*, 2019; Li *et al.*, 2021; Takeuchi, 2021; Spiers *et al.*, 2022). The lung, is one of the organs that benefit from the immunoregulatory activity of EVs under homeostatic conditions (Haggadone and Peters-Golden, 2018; Letsiou and Bauer, 2018; Su *et al.*, 2020). Also, EVs may take on a preventive anti-pathogenic role or modulate differentiation in lung epithelial cells (Ismail *et al.*, 2013, Fujita *et al.*, 2018). However, though normally protective and homeostatic in nature, the activity of these EVs can change drastically during inflammation, shifting the content of their cargo and promoting inflammatory cascades, endothelial hyperpermeability and hypoxia (**Fig. 1**).

Indeed, circulating EVs in patients with ARDS express on their surface Sphingosine 1-phosphate receptor (S1PR3) which has been suggested as a possible new clinically-relevant biomarker (Sun *et al.*, 2012). These patients display an increased number of circulating EVs, of mainly neutrophil and endothelial origin, compared to healthy controls (Li *et al.*, 2015a), and have been shown to play a critical role in inducing cellular permeability of the lung endothelium (Densmore *et al.*, 2006), as well as propagation of the inflammatory cascade by eliciting production of IL-1β and TNF-α (Buesing *et al.*, 2011). Thus, by modulating cellular homeostasis during health and promoting inflammatory cascades in ARDS, EVs may act as a "double-edged sword"; the beneficial or harmful signals propagated through EVs may depend on the body's systemic responses. A thorough understanding of their effects in health and disease, as well as tracking their cellular origin and uptake by cells, is necessary for the development of a new class of therapeutics that target EV signaling. In this review, we have provided an overview of the profound shift in EV population numbers and cargo observed in leukocytes, platelets, epithelial and endothelial cells during ARDS. In addition, we described the current drug candidates that target biogenesis and uptake of EVs that could represent novel targeted approaches for mitigating lung pathology.

3. Extracellular vesicles; a short history

Some of the first reported observations referencing EVs can be traced back to 1945 and the work of Dr. Erwin Chargaff, when he described encountering a particulate fraction that partitioned at 31,000 x g and which expressed elevated clotting potential (Chargaff and West, 1946). In 1967, Dr. Peter Wolf described and published electron microscopy images of his experience interacting with minute particulates separable via high-speed centrifugation derived from, but distinct from, platelets. He referred to these particles as "platelet dust" (Wolf, 1967). The work of Dr. Neville Crawford would follow suit, publishing additional vesicular images and demonstrating the capability to carry lipids, contractile proteins, and adenosine triphosphate (Crawford, 1971). Nunez and Gershon next contributed in 1974 by becoming some of the first to describe multivesicular bodies in proximity to an apical membrane, and to propose a mechanism of exosome formation and release via fusion with the plasma membrane (Nunez *et al.*, 1974). Finally in 1983, two papers, published the same week, reported that the transferrin

receptors of reticulocytes are associated with small ~50 nM vesicles before being released into the extracellular space (Pan and Johnstone, 1983). Notwithstanding these important historic hallmarks, the field has witnessed tremendous growth for the past 2 decades, when the pathways of EV generation, function and potential therapeutic applications started to emerge. Currently, the field continues to rapidly expand and, as such, has encountered numerous successes, but is also hindered by challenges and areas of disagreement. One of the main challenges remains understanding the mechanism by which EV circulate between donor and recipient cells and how the release of their cargo impacts cellular signaling in recipient cells.

4. Extracellular vesicles classification

EVs can circulate through blood and lymphatic vessels or via the extracellular medium of tissues, and upon reaching the intended destination they are internalized and deliver their cargo to the recipient cells (Gurung *et al.*, 2021; Dilsiz, 2022). There are several cellular mechanisms for uptake, including membrane fusion, phagocytosis, receptor-mediated endocytosis, and micropinocytosis (McKelvey *et al.*, 2015). EVs are subdivided into exosomes and microvesicles (Akers *et al.*, 2013).

Most exosomes are formed when endocytosis or the trans-Golgi network generates a region of intracellular space encapsulated by a lipid-bilayer called an endosome (Teng & Fussenegger, 2020). Enzyme-mediated invagination of this space in proximity to genetic material or proteins leads to additional, smaller encapsulated regions called intraluminal vesicles, each containing various blends of the aforementioned materials. The endosome is then ferried to the cell membrane for release by a series of complexed proteins, known as endosomal sorting complexes required for transport machinery (ESCRT), where the intraluminal vesicles are secreted to the extracellular space (van Niel *et al.*, 2022).

Microvesicles may form from plasma membrane lipid rafts or by enzyme mediated evagination of the cell's plasma membrane. This outward budding and fission of the plasma membrane occurs as a result of regulated interactions between the process of phospholipid redistribution and cytoskeletal protein contraction (D'Souza-Schorey and Clancy, 2012). Initiation of the process is generally regulated by the activity of flippases and floppases, whereby they mediate remodeling of the inner or outer leaflets of the plasma membrane via mobility of phospholipids (Zwaal and Schroit, 1997; Bevers *et al.*, 1999; Leventis and Grinstein, 2010). After phosphatidylserine is translocated to the outer leaflet of the membrane, the membrane will begin to bud outward and form vesicles that are pinched off and released via cytoskeletal actin-myosin contraction (Hugel *et al.*, 2005; McConnell *et al.*, 2009; Muralidharan-Chari *et al.*, 2009).

Some authors also consider apoptotic bodies as part of the EV classification. They form during apoptosis (Elmore, 2007), enclosing the remains of nuclear chromatin into membrane vesicles (Kerr *et al.*, 1972), and have larger sizes ranging from 500 to 4000 nm (Ihara *et al.*, 1998; Hristov *et al.*, 2004; Taylor *et al.*, 2008).

More recently, other EV subpopulations have been characterized, including secretory amphisomes, exophers, and autophagosomes. While exophers are big (~4um) vesicles released by cells and able to contain organelles such as mitochondria and lysosomes,

amphisomes and autophagosomes are both involved in cellular autophagy. Indeed, amphisomes are intermediate organelles produced through the fusion of endosomes with autophagosomes. They belong to the retrograde cellular signaling and further fuse with lysosomes for cargo degradation (Ganesan and Cai, 2021). Finally, the newly discovered migrasomes are EV that are formed and released from retracting fibers of cells and play a role in either disposing of damaged organelles or delivering molecules that promote cellular proliferation and tumor growth (Yu and Yu, 2022). As the number of EV biogenesis pathways is growing, the diversity of EV populations follows and offers opportunities to link them with specific mechanisms that will ultimately improve the nomenclature and the reporting related to EV biology. In the following sections, we will focus on homeostatic and pathologic roles of EVs from different cellular sources on lung function and in acute lung injury.

5. Role of EVs in lung physiology and acute lung injury

5.1. Endothelial cell-derived EVs

Endothelial cells produce EVs that have been associated both with protective and harmful effects. We have previously shown that endothelial-derived EVs isolated in physiological and inflammatory conditions carry different cargos and exert opposing effects on the coronary vascular endothelium. In some cases, endothelial cell derived EVs promote healing after injury, while in others, they elicit increased permeability and loss of monolayer integrity (Carter *et al.*, 2022). Some of the beneficial, homeostatic effects of exosomes released from endothelial progenitor cells (EPC) include reduction of inflammatory cell recruitment, cytokine/chemokine expression, reactive oxygen species (ROS) production, and protein concentrations in bronchoalveolar lavage (BAL) fluid in animal models of lung injury (Wu *et al.*, 2018; Zhou *et al.*, 2019). Additionally, EPC-derived exosomes downregulate expression of multiple regulatory genes involved in apoptosis, DNA repair, and senescence, facilitating proliferation and migration of endothelial cells (Liu *et al.*, 2022). This mechanism occurs by transferring EVs rich in miR-126, which modulates Sprouty-related, EVH1 domain-containing protein 1 (SPRED1), with consequent activation of Raf/ERK signaling and antiapoptotic effects (Kerr *et al.*, 1972). However, endothelial-derived EVs can have detrimental effects in acute lung injury (ALI) and ARDS. Intravenous injection of endothelial EVs isolated from animals exposed to dust particulates was found to elevate BAL fluid and plasma TNFα and IL-1β levels (Li *et al.*, 2015a). EVs of endothelial origin released during inflammation contain miRNA that can promote the expression of VEGFRβ in the recipient pericyte cells, suggesting they play a role in inflammation-induced endothelial permeability. Therefore, limiting production or internalization of endothelial exosomes may constitute an attractive new therapeutic target (Yamamoto *et al.*, 2015). In most cases of sepsis and ARDS, injury to the endothelium is the earliest pathological incident (Matthay *et al.*, 2019). The endothelium can sustain injury via stimuli such as plasminogen activated inhibitor-1 (PAI-1) and mechanical stress, resulting in a release of EVs (Densmore *et al.*, 2006; Letsiou *et al.*, 2015). These EVs can inhibit the release of nitric oxide and arteriole vasodilation [64]. They have also been demonstrated to cause inflammatory lung injury in vivo by promoting alveolar neutrophilic infiltration, pulmonary edema, increased production of TNF-α, and increasing lung endothelial permeability (Buesing *et al.*, 2011; Li *et al.*,

2015b). Endothelial microparticles are released as a result of ALI and have been demonstrated to carry enriched levels of moesin, a protein similarly involved with increased endothelial permeability (Letsiou *et al.*, 2015). Thus, during inflammation EVs display profound shifts in their cargo content; instead of maintaining cellular and tissue homeostasis they promote the inflammatory cascade, increased permeability, and cytokine release (**Fig. 2**).

5.2 Epithelial cell-derived EVs

EVs produced by epithelial, bronchial, and alveolar cells have mostly demonstrated protective effects on the epithelium and immune cell infiltration and activation. During hyperoxia, lung epithelium-derived EVs are released and can be found in BAL fluid, a mechanism mediated by endoplasmic reticulum stress (Moon *et al.*, 2015). Macrophages exposed to these EVs have displayed an increased expression of macrophage inducible protein-2 (MIP-2). When mice were treated intranasally with these same EVs, an augmented migration of macrophages and neutrophils was observed. The alveolar macrophage activation is largely induced by EVs containing caspase-3, which stimulates macrophages through the Rho associated coiled-coil protein kinase 1 (ROCK1) pathway (Moon *et al.*, 2015).
Wnts are a family of hydrophobic, Cys-rich, secreted glyco-lipoproteins that control developmental processes, stem cell proliferation, and pulmonary repair (MacDonald *et al.*, 2009; Kikuchi *et al.*, 2011; Patel *et al.*, 2019). Wnt-5A and its target genes in ARDS were found to be increased in marked collagen deposition, supporting the theory that Wnt-5A and the β-catenin pathway aid in lung repair (Königshoff *et al.*, 2008; Crosby and Waters, 2010). Modulation of Wnt signaling has been related to the production of EVs by human bronchial epithelial cells. These EVs contain a particular cargo of miRNAs (O'Brien *et al.*, 2018). One example of miRNA target gene is TGF-β which effects on myofibroblast differentiation and epithelium senescence (Kadota *et al.*, 2021). Exposure to acid causes the lung epithelium to release microvesicles that contain significantly elevated amounts of miRNA encapsulated as their cargo, including miR-17 and miR-221 (Lee *et al.*, 2017). Streptococcus pneumoniae is a common cause of ALI and ARDS. Pneumolysin, released from epithelial cells, represents an innate mechanism of protection against this pathogen. One of the mechanisms of pneumolysin is related to its effects on inducing alveolar epithelial cells to release microvesicles containing mitochondria, that when absorbed by neutrophils, play a role in modulating ROS production, cellular activation, and the consequent injury to cells and tissues (Letsiou *et al.*, 2021).

5.3 White Blood Cell-derived EVs

Leukocytes or White Blood Cells (WBCs) are the main effectors of the immune response and participate in the defense against bacteria, viruses, parasites, and toxins. Besides their protective role, during ARDS, WBCs can exert a series of deleterious effects by uncontrollably producing inflammatory cytokines such as TNF-α, IL-6 and IL-1β that damage alveoli or endothelial cells, and participate in the accumulation of BAL fluid (Huppert *et al.*, 2019). WBCs have been shown to produce EVs in both resting conditions and during immune responses to inflammatory stimuli. Resting granulocytes release EVs with an anti-inflammatory profile, able to

decrease IL-1β, TNFα, IL-6, IL-8, IL-10, IL-12, but increasing the levels of TGF-β and resolving mediators (Kolonics *et al.*, 2021). EVs derived from granulocytes have also been shown to modulate the production of ROS in a stimulus-dependent manner. Conversely, when WBCs encounter foreign pathogens marked with opsins such as antibodies, they ramp up EV production, and the makeup of the EV cargo changes. In this situation, the EVs cause augmentation of ROS production and increasing expression of E-selectin and Vascular Cell Adhesion protein1 (VCAM-1) (Kolonics *et al.*, 2021). Other para-physiological and pathological insults can also stimulate granulocyte-related production of EVs. Indeed, during exercise, WBCs, platelets, endothelial cells, and antigen presenting cells release significantly more EVs than in resting conditions (Brahmer *et al.*, 2019). Immortalized cells, isolated from patients with acute monocytic leukemia, generate a larger number of EVs during cell death compared to normal cells, a mechanism that could be responsible for the strong inflammation observed in these patients and the spread of malignant cells through the body (Baxter *et al.*, 2019).

Leukocytes represent a diverse population of cells, which in turn have varied EV cargo. Alveolar macrophage-derived EVs, for example, carry various miRNAs, Long non-coding RNAs (LncRNA), and effector molecules such as CCL3, IFN-γ, TNF-α, and ERAP1 (Soni *et al.*, 2016; Danesh *et al.*, 2018). Granulocyte-derived EVs carry factors that have been shown to activate monocytes and also to upregulate TNF-α (Jong *et al.*, 2017). EVs produced by natural killer cells contain cytotoxic proteins that act directly on pathogens and targeted cells (Tucher *et al.*, 2018). Finally, EVs produced by T-lymphocytes carry a cargo composed of multiple cytoskeletal remodeling proteins such as gamma- and beta- actins, 14-3-3 protein theta, myosin heavy chain 9, protein phosphatase 1 regulatory subunit 7, and major vault protein (Tucher *et al.*, 2018). Taken together, this data suggests that different cells of the immune system produce EVs with specific cargos and function, and while macrophage-, granulocyte- and NK cell-derived EVs target pathogens and promote the immune response, EVs produced by regulatory T-lymphocytes, after resolving inflammation, may deliver cytoskeletal components in an attempt to restore endothelial or tissue homeostasis (Bayless and Johnson, 2011; Kása *et al.*, 2015; Yadunandanan Nair *et al.*, 2022). It is clear then, that EVs represent a crucial and cell-specific communication mechanism responsible for the maintenance of homeostatic balance during resting conditions, but that could be easily activated when necessary and participate in the inflammatory and immune responses.

5.4. **Platelet-derived EVs**

Platelets may also contribute to the pathogenesis of ARDS. While primarily known for their involvement in coagulation, platelet signaling is involved in the modulation of different immune response mechanisms, some of which are mediated by the release of EVs. Platelet-derived EVs represent the majority of EVs found in the blood under normal physiological conditions (Kerris *et al.*, 2020). Their EV release is mediated by thrombin, or other physiologic agonists, able to activate platelets (Heijnen *et al.*, 1999; French *et al.*, 2020). Some of these vesicles will have procoagulant effects, carrying prothrombin, annexin-V, and factor X (Kerris *et al.*, 2020; Puhm *et al.*, 2021). Interestingly, patients with ARDS display lung EVs containing Tissue Factor (TF), the

main initiator of the pro-coagulant cascade. This may be responsible for the hypercoagulable state observed in patients with ARDS (Bastarache *et al.*, 2009).
Other platelet-derived EVs will carry different cargo and take on more immunomodulatory roles, beginning with the promotion of vasodilation to increasing the population of inflammatory cells able to reach the site of infection by upregulating cyclooxygenase-2 expression (Barry *et al.*, 1999). Platelet EVs have been shown to activate leukocytes or to communicate with mast cells by carrying mitochondria that are converted to inflammatory mediators, transporting cytokines, or upregulating molecules that stimulate lymphocyte function like CD40 (endothelial ligand) (Sprague *et al.*, 2008; Boudreau *et al.*, 2014; Yadav and Kor, 2015; Puhm *et al.*, 2021). Platelet EVs will further assist the arriving leukocytes by releasing EVs carrying P-selectin, a molecule that mediates cell adhesion (Kuravi *et al.*, 2019). As inflammation progresses, activated platelets will activate other platelets, in turn creating a positive feedback loop (Yadav and Kor, 2015). As the alveoli are infiltrated and the endothelium becomes more permeable, fluid leaks in, preventing gas exchange. The platelets continue to release EVs with miRNA-223 which may moderate the severity of the ongoing immune response and endothelial permeability (Laffont *et al.*, 2013; Miyazawa *et al.*, 2019; Roffel *et al.*, 2020), miRNA-24 to possibly induce apoptosis in damaged endothelium (Fiedler *et al.*, 2011; Michael *et al.*, 2017), and miRNA-126 to promote Hypoxia inducible factor 1α (HIF-1α) (Alique *et al.*, 2019; Gasperi *et al.*, 2019).

6. **Therapeutic approaches for EVs**

Our assertion thus far has been that EVs contribute to the pathology of ARDS. In the next section we will discuss several drug candidates or approved therapies that are reported to inhibit EV biogenesis, release, or uptake (**Fig. 3**).

6.1. **Drugs that affect biogenesis**

As previously discussed, EV biogenesis can occur independently of, or through, the ESCRT pathway. Thus, modulation of the ESCRT pathway has been considered as a therapeutic approach. Manumycin A inhibits Ras activation, whose effectors include ESCRT complex components (Zheng *et al.*, 2012; Datta *et al.*, 2017). Imatinib also has an inhibitory effect on Ras activation, but more indirectly by targeting receptor kinases that mightinitiate the signaling pathway (Margolis and Skolnik, 1994; Neshat *et al.*, 2000; Lee and Wang, 2009, Mineo *et al.*, 2012; Steegmann *et al.*, 2012; Iqbal and Iqbal, 2014; Abbaspour Babaei *et al.*, 2016). Clopidogrel inhibits p38 MAP kinase, which would otherwise activate EEAP1, a protein that assists in the recruitment of ESCRT complexes to the endosome surface (Ryu and Kim, 2011). The role of sulfisoxazole is somewhat controversial. Some reports indicate a significant decrease in EVs released by breast cancer cells due to decreased expression of ESCRT genes, while other studies have demonstrated the opposite effect (Im *et al.*, 2019; Fonseka *et al.*, 2021).
Other non-ESCRT-targeted drugs include Imaprine and GW4869, which inhibit enzymes that participate in invagination of the endosome to form the smaller bodies known as intraluminal

vesicles, and also facilitate budding when translocated to the cell membrane (Bianco *et al.*, 2009; Essandoh *et al.*, 2015; Vilette *et al.*, 2015; Deng *et al.*, 2017; Kosgodage *et al.*, 2017; Menck *et al.*, 2017).

Drugs focusing more heavily on EV export include Calpeptin, which targets calpains, a group of proteins whose many roles also include cytoskeletal remodeling, one of the processes involved in budding, and EV release (Yano *et al.*, 1993). Calpains are activated by ERK, so preventing activation of ERK should lead to an indirect prevention of calpain activity, which is accomplished by U0126 inhibition of MEK1/2, the signaling molecules upstream of ERK (Li *et al.*, 2010; Jin *et al.*, 2022). Calpains require a certain concentration threshold of calcium in order to function, thus dimethyl amiloride, an inhibitor of sodium/calcium channel proteins, can also prevent their activation and participation in EV release (Savina *et al.*, 2003; Chalmin *et al.*, 2010). Regardless of the production pathway, biogenesis requires accessible lipids to modulate the EV structure (Skotland et al., 2020). Indomethacin capitalizes on this, by inhibiting lipid transporter proteins, removing access to lipids, and subsequently decreasing EV biogenesis (Aung *et al.*, 2011; Koch *et al.*, 2016).

A different approach has been that of targeting proteins involved in cytoskeletal reorganization, thus impeding the actin re-organization required for EV release. Among these, ROCK proteins are inhibited by Y27632 and Bisindolylmaleimide I (Tramontano *et al.*, 2004; Sapet *et al.*, 2006; Abid Hussein *et al.*, 2007; Latham *et al.*, 2013; Kim *et al.*, 2014; Stratton *et al.*, 2015). NSC23766 also targets cytoskeletal remodeling, but by inhibiting the activity of Rho small GTPase family protein Rac1 (Wang *et al.*, 2017). Cytochalasin D targets the cytoskeleton more directly, binding to the edges of actin filaments, blocking polymerization and any subsequent participation in budding of the plasma membrane (May *et al.*, 1998; Khan *et al.*, 2011). Pantetheine blocks a separate process involved in budding, specifically the translocation of phosphatidylserine from the intracellular facing portion of the phospholipid membrane to the outer membrane leaflet (Kavian *et al.*, 2015).

6.2. **Drugs that interfere with EV uptake**

Several of the uptake pathways utilize interactions with EV surface characteristics such as surface charge (Fröhlich, 2012; Mulcahy *et al.*, 2014), thus any environmental stimulus that disrupts these interactions may influence the specific method of uptake. With the understanding that zeta potential represents a measurement of EV surface charge (Yu *et al.*, 2014), compounds such as timolol maleate and brinzolamide have demonstrated the ability to decrease EV uptake, supposedly by decreasing the negativity of the EV surface charge (Tabak *et al.*, 2021).

Being one of the major uptake pathways, endocytosis presents a viable target for inhibiting the uptake of extracellular vesicles. There are several mechanisms by which endocytosis occurs. One such mechanism is facilitated by heparan sulfate proteoglycans, whereby the molecule acts as a plasma membrane signaling receptor for initiating caveolin-dependent endocytosis (Christianson *et al.*, 2013; Christianson and Belting, 2014). Heparin is a mimetic of heparan sulfate, which prevents endocytosis and subsequently inhibits EV uptake by competitively binding to the ligands on the EV surface (Sarrazin *et al.*, 2011; Meneghetti *et al.*, 2015; Huang *et al.*, 2020; Tu *et al.*, 2021). Another necessary component of caveolin- and clathrin-dependent

endocytosis is dynamin2, a protein that assists the invaginated region with scission from the cell membrane (Ehrlich *et al.*, 2004; Ferguson and De Camilli, 2012; González-Jamett *et al.*, 2013). Dynasore exploits this interaction to prevent endocytosis by non-competitively inhibiting the enzymatic activity of dynamin 2 and blocking up to 70% of EV uptake (Newton *et al.*, 2006; Kirchhausen *et al.*, 2008). Genistein also targets this interaction, as it can not only facilitate actin network disruption, but also inhibit membrane recruitment of dynamin (Pelkmans *et al.*, 2002; Costa Verdera *et al.*, 2017). Another archetype of endocytosis is mediated by clathrin proteins (Kirchhausen, 2000). Recent reports indicate that protein tyrosine phosphatases (PTPs), specifically protein of regenerative liver-1, -2, and -3 (PRL-1, -2, -3) participate in the early endosome formation, via interaction of their prenylated moiety with the plasma membrane (Zeng *et al.*, 2000). It was further demonstrated that a loss of function in endoplasmic reticulum-localized non-receptor protein-tyrosine phosphatase 1B (PTP1B) led to hyperphosphorylation of *N*-ethylmaleimide-sensitive factor (NSF), which attenuates further vesicle fusion and EV uptake via disassembly of the SNARE complex during initial vesicle fusion (Sangwan *et al.*, 2011). Novel PTP inhibitors, with potent and effective profile of activity have been proposed as novel drug candidates for the treatment of inflammation and lung injury (Lazo *et al.*, 2023). The clathrin-mediated endocytosis is further targeted by chlorpromazine, which blocks the generation of clathrin-coated pits at the plasma membrane, thus decreasing EV uptake (Wang *et al.*, 1993; Escrevente *et al.*, 2011). Another mechanism of EV internalization is through phagocytosis (Feng *et al.*, 2010). Wortmannin has proved successful in targeting phosphoinositide 3-kinases (PI3Ks), a critical mediator of the phagocytic processes, whose inhibition blocks phagosome formation and consequent EV uptake (Stephens *et al.*, 2002; Liu *et al.*, 2005; Bastos-Amador *et al.*, 2012; Abliz *et al.*, 2015). Drugs that modulate EV biogenesis and uptake are summarized in **Table 1**.

7. **Conclusion**

Extracellular vesicles are critical and novel mediators of intercellular communication that contribute to the maintenance of tissue homeostasis and, at the same time, participate in multiple disease pathophysiologies. In ARDS, circulating EVs can evoke endothelial hyperpermeability, activation of the inflammatory cascade, and priming of the immune system, thus playing a major role in the signaling responsible for disease development and progression. In contrast, EVs of alveolar-epithelial origin seem to exert a protective effect on lung structures during disease. Multiple analeptic approaches have been proposed to combat EV pathologic signaling by targeting their production or their absorbance by recipient cells. Inhibition of EV uptake and/or release may represent an innovative strategy to target ARDS. Further investigation of EV production may also lead to the development of detailed interventions able to modulate the EV cargo, and thus modulating their dangerous effects without modifying their concentration and release. Ancillary research is necessary to produce cell-specific, selective, efficacious and safe therapeutic drug candidates.

List of abbreviations:
ARDS: acute respiratory distress syndrome;

EVs: Extracellular Vesicles;
PaO_2: partial pressure of oxygen;
FiO_2: fraction of inspired oxygen;
SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus-2:
COVID-19: SARS-CoV-2 related disease;
CO2: carbon dioxide;
DAMPS: damage-associated molecular patterns;
miRNA: microRNA;
S1PR3: Sphingosine 1-phosphate receptor;
IL-1β: interleukin 1 beta;
TNF-α: tumor necrosis factor Alpha;
ESCRT: endosomal sorting complexes required for transport machinery;
ROS: reactive oxygen species;
BAL: Bronchoalveolar lavage;
EPC: endothelial progenitor cells;
DNA: Deoxyribonucleic acid;
SPRED1: Sprouty-related, EVH1 domain-containing protein 1;
Raf: Rapidly Accelerated Fibrosarcoma;
ERK: Extracellular signal-regulated kinases;
ALI: Acute Lung Injury;
VEGFRβ: Vascular endothelial growth factor receptor beta;
PAI-1: plasminogen activated inhibitor-1;
MIP-2: macrophage inducible protein-2;
ROCK1: Rho associated coiled-coil protein kinase 1;
Cys: cysteine;
Wnts: Wingless and Int-1;
WBC: White blood cells;
IL-6: interleukin 6;
TGF-β: Transforming growth factor β
VCAM-1: Vascular Cell Adhesion protein1;
LncRNAs: Long non coding RNAs;
CCL3: chemokine ligand 3;
IFNγ: Interferon gamma;
ERAP1: endoplasmic reticulum aminopeptidase 1;
NK: natural killer cells;
TF: Tissue factor;
CD40: Cluster of Differentiation 40;
HIF-1α: Hypoxia inducible factor 1α;
MEK: mitogen-activated protein kinase kinase;
PTPs: protein tyrosine phosphatase;
PRL-1,2,3: protein of regenerative liver 1,2,3, also known as PTP type IVA;
PTP1B: protein-tyrosine phosphatase 1B;
NSF: *N*-ethylmaleimide-sensitive factor;
SNARE: SNAp Receptor;

PI3Ks: phosphoinositide 3-kinases;
RAS: rat Sarcoma virus;
aSMase: Acid Sphingomyelinases;
nSMase: Neutral sphingomyelinase;
NHE: Plasma membrane Na+/H+ exchanger isoforms;
ABCA3: ATP-binding cassette sub-family A member 3;
PKC: Protein Kinase C;
HMGCR: HMG-CoA reductase;
HSPG: Heparan sulfate proteoglycans;
AP2: Adaptor Protein 2;

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Figure legends:

FIGURE 1

Figure 1. Extracellular vesicle (EV) mediates endothelial permeability in ARDS. (**A**) Schematic representation of the alveolo-capillary structures in health and disease. During ARDS, the endothelial monolayer is inflamed, allowing the migration of neutrophils, monocyte-macrophages, and fluid into the alveolar spaces, resulting in inflammation, release of cytokines and respiratory dysfunction. (**B**) Focus on the endothelial monolayer at the blood-gas interface. Circulating inflammatory EVs are absorbed by endothelial cells and mediate endothelial barrier dysfunction in ARDS.

FIGURE 2

Figure 2. In ARDS, each cell type produces specific extracellular vesicles (EV). Endothelial cell-derived EVs contain inflammatory cytokines, vascular growth factor β, protease activator factor 1 and moesin that promote endothelial barrier function and apoptosis; Alveolar cell-derived EVs mediate macrophage recruitment and defense against pathogens; Activated neutrophil-derived EVs mediate cell adhesion, inflammatory reaction, and immune system activation; Platelet-derived EVs mediate coagulation and neutrophil recruitment.

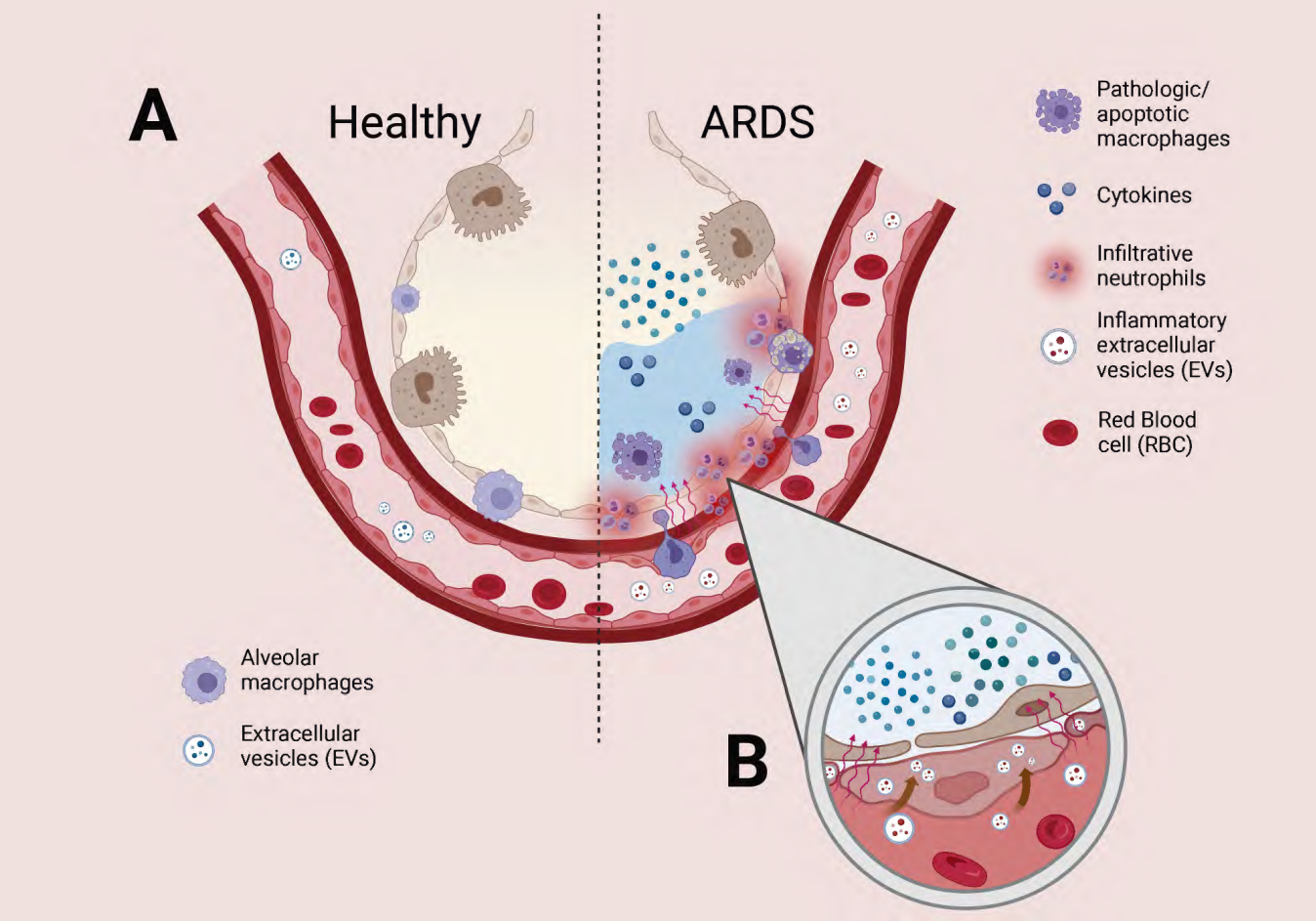
FIGURE 3

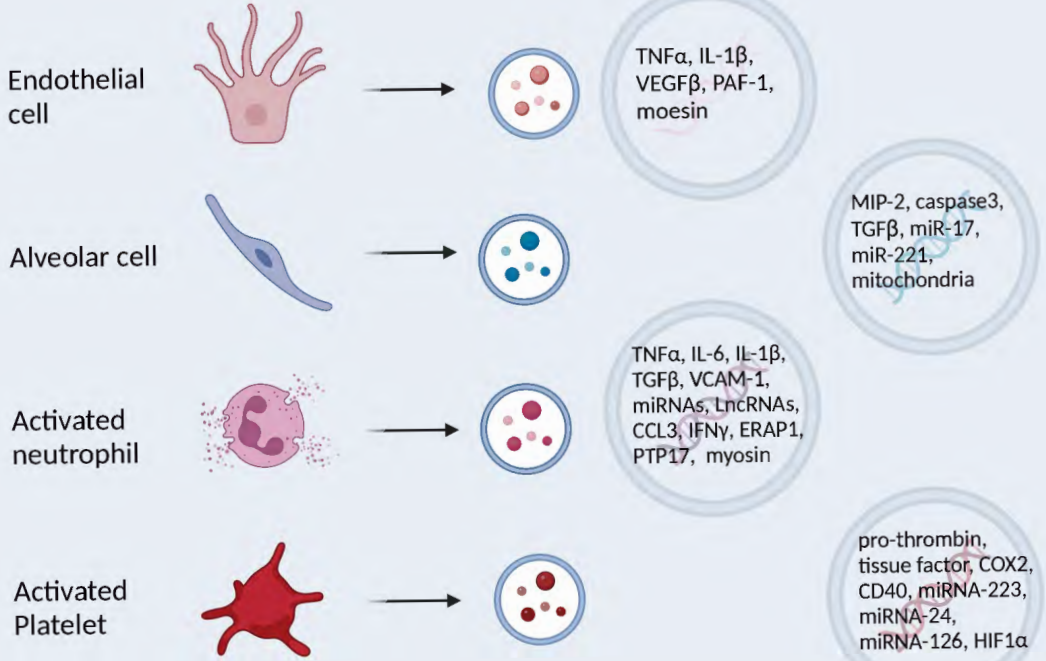
Figure 3. Schematic representation of the main drug-targeted approach to modulate extracellular vesicle (EV) signaling. (*left*) Drugs that modulate EV biogenesis and release; (*right*) therapeutic interventions that modulate EV uptake and absorption (right).

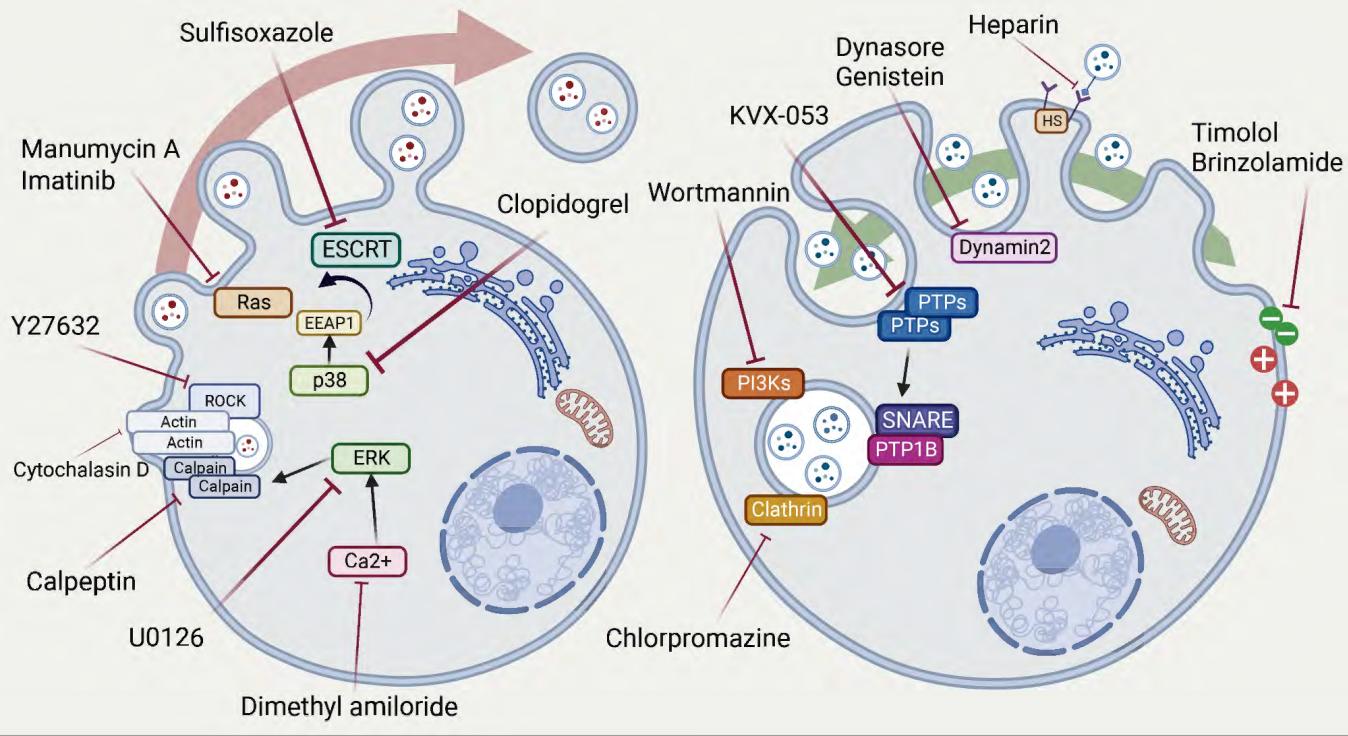
TABLE 1

Drugs modulating EVs biogenesis	Targeted Pathway	Target	Studies
Manumycin A	ESCRT pathway	RAS	(Zheng *et al.*, 2012, Datta *et al.*, 2017)
Imatinib	ESCRT pathway	BCR-ABL1	(Margolis & Skolnik, 1994, Neshat *et al.*, 2000, Lee & Wang, 2009, Mineo *et al.*, 2012, Steegmann *et al.*, 2012, Iqbal & Iqbal, 2014, Abbaspour Babaei *et al.*, 2016)
Clopidogrel	ESCRT pathway	p38 MAPK	(Ryu & Kim, 2011)
Sulfisoxazole*	ESCRT pathway	ETA	(Im *et al.*, 2019, Fonseka *et al.*, 2021)
Imipramine	Non-ESCRT pathway	aSMase	(Bianco *et al.*, 2009, Deng *et al.*, 2017, Kosgodage *et al.*, 2017)
GW4869	Non-ESCRT pathway	nSMase	(Essandoh *et al.*, 2015, Vilette *et al.*, 2015, Menck *et al.*, 2017)
Calpeptin	Non-ESCRT pathway	Calpain	(Yano *et al.*, 1993)
U0126	Non-ESCRT pathway	MEK 1/2	(Li *et al.*, 2010, Jin *et al.*, 2022)
Dimethyl Amiloride	Non-ESCRT pathway	NHE 1/2/3, NCX	(Savina *et al.*, 2003, Chalmin *et al.*, 2010)
Indomethacin	Non-ESCRT pathway	ABCA3	(Aung *et al.*, 2011, Koch *et al.*, 2016)
Y27632	Non-ESCRT pathway	ROCK 1/2	(Tramontano *et al.*, 2004, Sapet *et al.*, 2006, Abid Hussein *et al.*, 2007, Latham *et al.*, 2013, Kim *et al.*, 2014)
Bisindolylmaleimide I	Non-ESCRT pathway	PKC	(Stratton *et al.*, 2015)
NSC23766	Non-ESCRT pathway	Rac1	(Wang *et al.*, 2017)
Cytochalasin D	Non-ESCRT pathway	Actin	(May *et al.*, 1998, Khan *et al.*, 2011)
Pantetheine	Non-ESCRT pathway	ACC, HMGCR	(Kavian *et al.*, 2015)
Drugs modulating EVs uptake			
Timolol Maleate	General Uptake	βR 1/2	(Fröhlich, 2012, Mulcahy *et al.*, 2014, Yu *et al.*, 2014, Tabak *et al.*, 2021)
Brinzolamide	General Uptake	CA-II	(Fröhlich, 2012, Mulcahy *et al.*, 2014, Yu *et al.*, 2014, Tabak *et al.*, 2021)
Heparin	Caveolin-dependent Endocytosis	HSPG	(Sarrazin *et al.*, 2011, Meneghetti *et al.*, 2015, Huang *et al.*, 2020, Tu *et al.*, 2021)
Dynasore	Caveolin-dependent Endocytosis	Dynamin-2	(Newton *et al.*, 2006, Kirchhausen *et al.*, 2008)
Genistein	Caveolin-dependent Endocytosis	Tyrosine Kinase	(Pelkmans *et al.*, 2002, Costa Verdera *et al.*, 2017)
Chlorpromazine	Clathrin-mediated Endocytosis	AP2	(Wang *et al.*, 1993, Escrevente *et al.*, 2011)
Wortmannin	Phagocytosis	PI3K	(Stephens *et al.*, 2002, Liu *et al.*, 2005, Bastos-Amador *et al.*, 2012, Abliz *et al.*, 2015)

Table1. Drugs that modulate EV signaling.


A
Healthy
ARDS
Pathologic/ apoptotic macrophages
Cytokines
Infiltrative neutrophils
Inflammatory extracellular vesicles (EVs)
Red Blood cell (RBC)
Alveolar macrophages
Extracellular vesicles (EVs)
B


Endothelial cell
TNFα, IL-1β, VEGFβ, PAF-1, moesin
Alveolar cell
MIP-2, caspase3, TGFβ, miR-17, miR-221, mitochondria
Activated neutrophil
TNFα, IL-6, IL-1β, TGFβ, VCAM-1, miRNAs, LncRNAs, CCL3, IFNγ, ERAP1, PTP17, myosin
Activated Platelet
pro-thrombin, tissue factor, COX2, CD40, miRNA-223, miRNA-24, miRNA-126, HIF1α


Sulfisoxazole
Manumycin A
Imatinib
Clopidogrel
ESCRT
Ras
EEAP1
Y27632
p38
ROCK
Actin
Actin
Calpain
Calpain
Cytochalasin D
ERK
Calpeptin
Ca2+
U0126
Dimethyl amiloride
Heparin
Dynasore
Genistein
KVX-053
HS
Timolol
Brinzolamide
Wortmannin
Dynamin2
PTPs
PTPs
PI3Ks
SNARE
PTP1B
Clathrin
Chlorpromazine