How severe RNA virus infections such as SARS-CoV-2 disrupt tissue and organ barriers—Reconstitution by mesenchymal stem cell-derived exosomes

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1. COVID-19 and the contribution by immune effector cells

1.1 SARS-COV-2

SARS-Cov-2 is a coronavirus with a single-strand, positive-sense RNA genome with multiple open reading frames that codes for 10 proteins including the nucleocapsid and the Spike protein, which is important for infection [1]. SARS-Cov-2 primarily infects type II alveolar epithelial cells (AECs) because they have a high concentration of ACE2 receptors, which have a strong interaction with the viral Spike protein, facilitating internalization of the virus [2,3]. COVID-19, the viral respiratory illness that results from SARS-Cov-2 infection, initially presents with mild symptoms for several days concurrent with the highest levels of viral shedding [4]. The inflammatory damage of COVID-19 follows as the natural immune response to the virus causes the release of high levels of inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), in a sustained pattern distinct from bacterial sepsis or influenza [5,6]. The rapid clinical deterioration about 7 days after initial onset of symptoms suggests that the respiratory failure in COVID-19 results from a unique pattern of immune dysregulation characterized by macrophage activation syndrome (MAS) or profound depletion of CD4 lymphocytes, CD19 lymphocytes, and natural killer (NK) cells. The persistent immune response, despite falling

viral titers in this inflammatory phase, leads to progressive organ and tissue injury, suggesting that the immune-mediated damage is more significant than the viral cytopathic damage [7,8].

2. Immune-mediated pathogenesis

SARS-Cov-2 induces immune dysregulation in a different pattern than influenza or SARS-CoV. Chemokines (CCL2 [MCP 1], CCL3 [MIP1α], CXCL1, CXCL5, and CXCL10) are significantly upregulated in COVID-19 and act to recruit macrophages, neutrophils, and effector T cells [9]. These inflammatory conditions are linked to defective antigen presentation. Individual viral antigens may be responsible for lymphocyte apoptosis which further drives monocytes to produce high levels of TNF-α and IL-6. TNF-α activates endothelial cells to recruit leukocytes, stimulates neutrophils, and increases the levels NF-κB, AP-1, IL-8, and caspases, which induce apoptosis of target tissues [10]. The ratio of lymphocytes to neutrophils appears to be an important predictor for outcome of COVID-19. Finally, advanced cases of COVID-19 respiratory failure are characterized by features of immune dysregulation or MAS.

Over-production of IL-6 promotes immune dysregulation with inhibition of HLA-DR expression on CD14 monocytes contributing to the impaired T cell response. IFN-γ produced by CD4+ TH1, NK, and CD8+ T cells normally increases MHC class I and II to promote killing of infected cells by T cells, increased macrophage activity and production of IgG antibodies, but there are very low levels of IFN-γ detected in COVID-19. Immune dysregulation caused by COVID-19 features lower counts of CD4+ T cells, CD8+ T cells, and NK cells than at the intermediate immune state. Recently, SARS-CoV2 specific genes have been identified which counter-regulate appropriate interferon responses in COVID-19. Infection by SARS-CoV-2 is characterized by fewer CD4+ T cells but more NK cells and B cells than H1N1 influenza. IL-17 production, indicating Th17 function, is downregulated in COVID-19 patients with immune dysregulation. There is also some evidence to suggest that SARS-Cov-2 infects and induces apoptosis of T lymphocytes, such as CD4+ TH1 cells that activate macrophages, CD4+ TH17 cells that activate neutrophils, and CD8+ cytotoxic T cells that kill infected cells. NK cells that kill infected or damaged cells are also reduced in SARS-CoV and SARS-CoV2 infection possibly resulting from the rapidly propagating virus [11]. In some cases of COVID-19, elevated levels of IL-1β promote a MAS with a pattern that may be similar to secondary hemophagocytic lymphohistiocytosis [12,13].

3. Acute lung injury, increased endothelial permeability, and loss of organ barrier function

The massive release of inflammatory mediators termed cytokine storm can cause an acute lung injury (ALI) characterized by disruption of junctions between cells, damage to AECs, damage to pulmonary capillary endothelial cells, and loss of alveolar fluid clearance mechanism [14,15]. Inflammatory mediators (cytokines and chemokines) released by type II AECs increase vasodilation, leukocyte adhesion, and capillary permeability [16,17]. Proteolytic enzymes and reactive oxygen species released by neutrophils and macrophages within the alveoli damage alveolar cells and the extracellular matrix [18,19]. Disruption of the junctions between the cells in the alveolar–capillary barrier promotes recruitment of nonspecific immune cells (e.g., neutrophils, macrophages) and allows exudative leak from the pulmonary capillaries [20]. In this context, the detrimental role of the kallikrein-bradykinin system is likely responsible for extensive fluid loss and inflammation for endothelial barriers. Neutrophils, macrophages, and other immune cells thus evade from the circulation, accumulate in the lungs, and increase their release of inflammatory mediators creating a positive feedback loop.

Viral inhibition of ACE2-mediated inactivation of des-Arg bradykinin and proinflammatory cytokine-mediated upregulation of bradykinin receptor type 1 (B1) on endothelial cells may cause pulmonary angioedema consistent with the radiographic findings of COVID-19 [21], which is in part because of increased expression of bradykinin receptor 2 (B2). The diffuse alveolar damage and angioedema could progress to acute respiratory distress syndrome with accumulation of proteinaceous fluid in alveoli that interferes with arterial oxygenation. This proteinous fluid consists of gelatinous hyaluronic acid and explains the opaque signs of COVID-19 lung images (Fig. 3.1). Diffuse thrombosis in the pulmonary vasculature and ventilator pressure support may also increase pulmonary artery pressure causing pulmonary hypertension, which could have secondary cardiovascular effects.

4. Endogenous repair systems

Endogenous repair systems that regulate the immune response and stimulate tissue regeneration exist to promote recovery from ALI but may
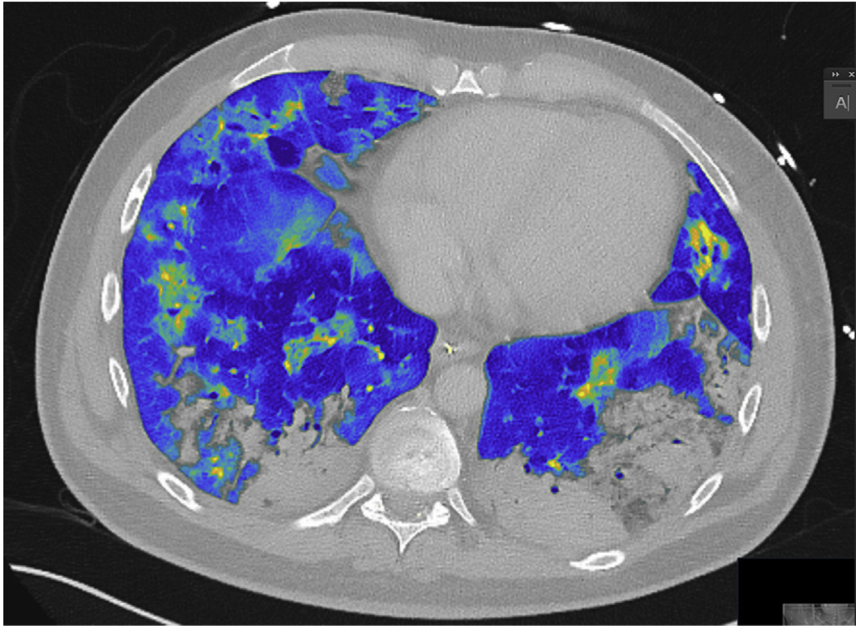
Figure 3.1 Mechanisms of action of mesenchymal stem cell-derived exosomes in acute respiratory distress syndrome [70].

be overwhelmed by the cytokine storm of COVID-19. The natural regulatory mechanisms of the immune system include production of antiinflammatory cytokines such as IL-10, TGF-β3, and IL-1ra, as well as immune cells with more regulatory phenotypes such as M2 macrophages and regulatory T cells [22]. Antiinflammatory cytokines (IL-10) promote phenotypes by polarizing M1 macrophages, which release high levels of inflammatory mediators, into tissue regenerative M2 macrophages, which release much lower levels of inflammatory mediators and also improve phagocytosis of cell debris, and danger ligands [23–25]. Antiinflammatory cytokines (TGF-β3) promote polarization of T lymphocytes from TH1, which produce high levels of inflammatory mediators to Treg cells, which regulate the cytotoxic activity of cytotoxic T lymphocytes (CTLs) and NK cells [26]. Regenerative cytokines including Ang-1, KGF, HGF, VEGF, EGF, Tsp1, S1P promote repair of the junctions between cells in the alveolar–capillary barrier, repair of the damaged alveoli and bronchioles by type II AECs and bronchoalveolar stem cells, and repair of pulmonary capillaries by epithelial cells [27–30].

5. The role of mesenchymal stem cells

In newborns and young children, mesenchymal stem cells (MSCs) are found in almost every organ and tissue. In adults, these large amounts of MSCs have been shown to be restricted to fat tissues including the bone marrow (BM). In addition to their potential to function as progenitors, MSCs have demonstrated the capacity to modulate the immune response and promote tissue regenerat ion. MSCs exert their paracrine effects through the release of soluble mediators including anti-inflammatory and regenerative cytokines and extracellular vesicles containing transcriptionally active RNA species, such as miRNA, lncRNA, and effector species. These soluble mediators and RNA species could potentially arrest the inflammatory response, repair the damage to the alveoli and pulmonary capillaries, and allow the immune system to clear the virus [20,30–36]. A Chinese study at Shanghai University on COVID-19 treatment using a master cell bank created from stromal progenitor cells harvested from lipoaspirate successfully treated seven patients who recovered, while three patients treated with placebo progressed to severe viral pneumonia or death [37]. An Israeli study of COVID-19 treatment using a clonal cell line of expanded, placental, MSCs treated six critically ill COVID-19 patients under a compassionate use program and demonstrated improvement in respiratory parameters and a 100% survival rate [38]. In the Israel study, four patients had other organ failures (cardiovascular, renal failure) and the placental MSCs therapy promoted recovery of the other organ failures as well as improvement in unrelated pre-existing conditions. Nearly 1000 clinical trials investigating administration of MSCs derived from sources such as BM, adipose tissue, cord blood, and others have also demonstrated a well-established safety profile of MSCs [39].

6. Extracellular vesicles: Exosomes and small microvesicles

MSCs exert the vast majority of their paracrine effects through the release of EVs, vesicles of roughly 50–1000 nm in diameter that are secreted by all cell types [40]. Small EVs (sEVs, 30–200 nm diameter, (Fig. 3.2), harvested using different protocols from cell culture supernatants of MSCs grown under diverse culture conditions, have been reported to be therapeutically active in various preclinical models [41]. EVs further may be responsible for the tissue regenerative effects observed with atopically transplanted
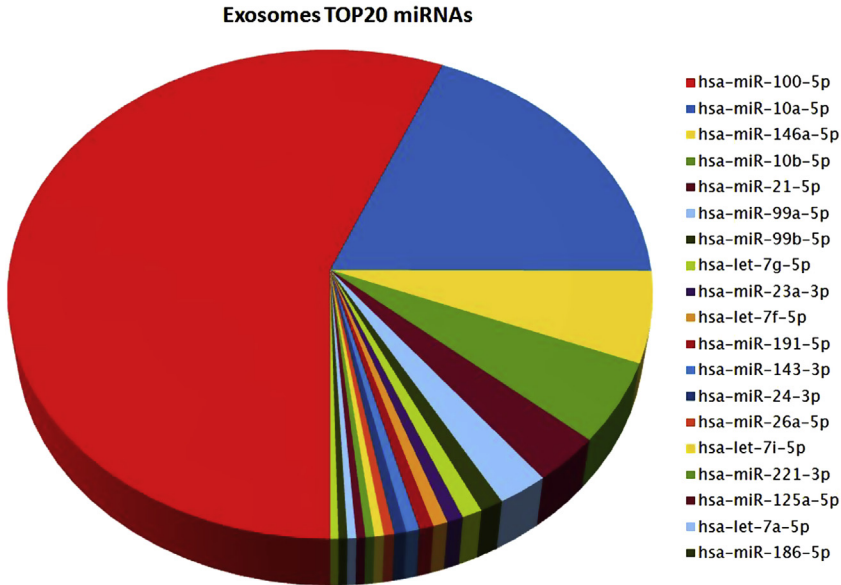
Exosomes TOP20 miRNAs

- hsa-miR-100-5p
- hsa-miR-10a-5p
- hsa-miR-146a-5p
- hsa-miR-10b-5p
- hsa-miR-21-5p
- hsa-miR-99a-5p
- hsa-miR-99b-5p
- hsa-let-7g-5p
- hsa-miR-23a-3p
- hsa-let-7f-5p
- hsa-miR-191-5p
- hsa-miR-143-3p
- hsa-miR-24-3p
- hsa-miR-26a-5p
- hsa-let-7i-5p
- hsa-miR-221-3p
- hsa-miR-125a-5p
- hsa-let-7a-5p
- hsa-miR-186-5p

Figure 3.2 Expression of miRNA in purified mesenchymal stem cell-derived exosomes (small extracellular vesicles).

MSCs in various animal models. The origin of MSC-sEVs, a population of exosomes and small microvesicles produced by MSCs, suggests a similar safety profile to their parent cells that could expedite the development of clinical applications.

MSCs-EVs are internalized by target cells through endocytosis, and delivery of RNA species influences the behavior of these cells. Noncoding RNA such as miRNA, abundant in MSC-sEVs, target specific processes and pathways within target cells. The RNA content of MSC-sEVs confers their biological properties, which include being anti–inflammatory (e.g., miR1, miR100, miR181c), immunomodulatory (e.g., miR146a), proangiogenic (e.g., let-7, miR29), prosynthetic (e.g., miR92-3p, miR140-5p), antiapoptotic (e.g., miR21, miR199a), antifibrotic (e.g., miR21, miR23a, miR125b), and tumor suppressive (e.g., miR15a, miR145) (Fig. 3.2). It is important to note that the biological effects of MSC-sEVs are not the result of any single factor, but rather the combined activity of the abundant RNA moleculesaffecting multiple cellular pathways in the context of target cell behavior prior to internalization.

7. Tissue reconstitutive mechanisms by mesenchymal stem cell-small extracellular vesicles in COVID-19

Internalization of MSC-sEVs by cells exhibiting pathologic behavior may influence cellular pathways to regulate the hyperinflammatory response

to SARS-CoV-2 and promote regeneration of damaged pulmonary and other tissues. MSC-sEVs may positively regulate expression of proteins such as TGF-β3, IL-10, IL-4, TNFR, IL-1ra, and HGF that could modulate the excessive immune response that contributes to tissue damage in COVID-19 [42—53]. MSC-sEVs could also promote alveolar tissue regeneration through Enhanced expression of angiopoietin-1 after endocytosis of MSC-sEVs could also promote alveolar tissue regeneration and decrease alveolar-capillary barrier permeability in ALI [28,29]. MSC-sEV induced expression of KGF (FGF-7) may also contribute to repair of AECs and increased alveolar fluid clearance [27]. EGF and HGF expression induced by MSC-sEVs has been demonstrated to be mitogenic for type II AECs and promoted alveolar regeneration [54]. One small cohort study in humans demonstrated direct evidence of a therapeutic benefit of MSC-sEVs in the treatment of COVID-19 with significant improvements in absolute neutrophil count, lymphopenia and acute phase reactants including C-reactive protein, ferritin, and D-dimer. Another phase I study of exosomes bearing CD24 also demonstrated complete recovery in all 30 patients with moderate to severe COVID-19, and recovery within three to five days in 29 of these patients.()

Transfer of MSC-sEV miRNA has also demonstrated beneficial effects on pathologic cellular processes that are believed to contribute to the pathogenesis of COVID-19. miR-455-3p inhibited the activation and cytokine production of macrophages challenged with lipopolysaccharide (LPS) both in vivo and in vitro and reduced levels of IL-6, G-CSF, IL-17, IL-10, IP-10 (CXCL10), MCP-1 (CCL2) [55]. miR-146a-5p targeted the expression of IRAK-1 and TRAF-6, significantly suppressed LPS-mediated TNF-α, IL-6, and IL-1β induction in alveolar macrophages, and increased IL-10, M2 macrophage polarization, and phagocytosis [56—60]. miR-223 targeted the transcription factor Pknox1 decreasing IL-1β, TNF-α, IL-6, and NF-κB, suppressed the proinflammatory activation of macrophages and promoted the alternative antiinflammatory M2 phenotype [61,62]. miR-511-3p targeted Rho-associated coiled-coil containing protein kinase 2 (Rock2), which is a serine threonine kinase that phosphorylates IRF4, and thus promoted the expression of M2-related genes [63]Fig. 3.2, MiR-100, an mTOR inhibitor, positively regulated autophagy, attenuated bleomycin-induced cellular apoptosis in type II AECs and reduces the levels of proinflammatory cytokines IL-6, IL-8, and TNF-α [64]. miR-30b-3p and miRNA-21-5p reduced apoptosis of AECs ALI induced by LPS and ischemia-reperfusion injury, respectively [65,66]. miR-615-5p is an
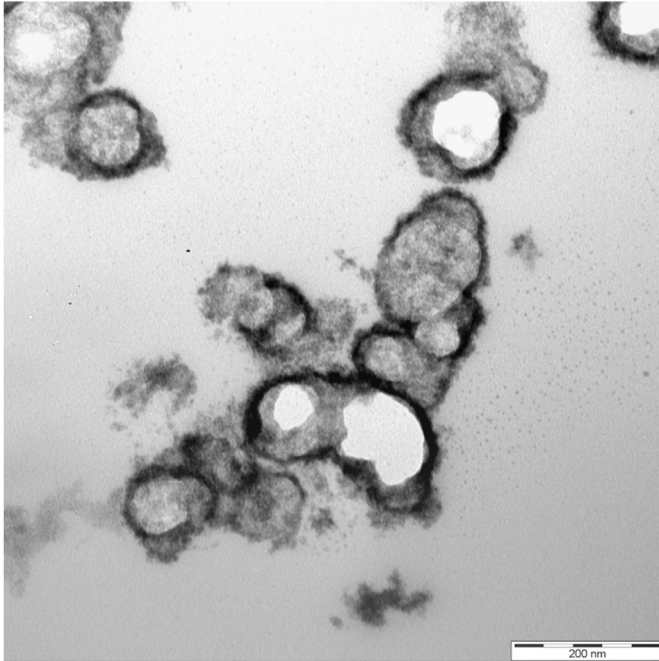
Figure 3.3 Electron microscopic image of cryo-fixed, highly purified exosome preparation from mesenchymal stem cells.

antiangiogenic microRNA targeting IGF2 (insulin–like growth factor 2) and RASSF2 (Ras–associating domain family member 2) that interfered with eNOS (endothelial nitric oxide synthase) signaling which contributes to endothelial leakage [67]. Furthermore, nine miRNA molecules identified by bioinformatic analysis to be complementary to the SARS-Cov-2 viral genome could be candidates to interfere with viral RNA transcription or protein translation essential for viral replication [68,69]. An example of miRNAs identified from EVs of placental derived mesenchymal stem cells is shown Fig. 3.3.

Modulation of the hyperinflammatory immune response through reduction of inflammatory mediators, increased expression ofanti-inflammatory mediators, decreased influx of inflammatory cells (neutrophils, M1 macrophages), increased polarization into M2 macrophages providing high capacity to eliminate tissue debris (Fig. 3.3), and differentiation of a regulatory T cell phenotype could arrest the progression of the lung damage in COVID-19. Consequently, intercellular junctions between alveolar cells

and pulmonary capillary endothelial cells could be regenerated along with AEC repair. Degradation of hyaluronic acid accumulating in ARDS lungs and improved mucociliary activity are likely to improve clearance of alveolar fluid and debris. Also, if the nine human miRNA molecules found to be complementary to the SARS-Cov-2 RNA genome can demonstrate any direct or indirect activity to inhibit viral replication, as in the cases of other RNA viruses such as influenza and hepatitis C, MSC-sEVs could interfere with viral replication [71–78].

Investigation of MSC-sEVs in preclinical models of ALI and ARDS have demonstrated significant reduction in inflammatory mediators, inflammatory cell influx, AEC apoptosis, bacterial load, and viral replication, as well as significant improvement in anti–inflammatory mediators, alveolar–capillary barrier permeability, monocyte phagocytosis and alveolar-arterial oxygen gradient [25,28,29,79–81]. Preclinical studies of MSC exosomes as therapy for influenza virus-induced ALI (similar to COVID-19 pulmonary disease) in a clinically relevant swine model demonstrated inhibition of virus-induced apoptosis in AECs, inhibition of influenza virus replication through miRNA transfer, decreased virus shedding, decreased virus replication in the lungs, and decreased production of proinflammatory cytokines [82].

Early clinical studies demonstrating 100% clinical recovery rate from COVID-19 with systemic administration of adipose-derived stromal progenitor cells and placental MSCs, whose mechanism of action is transfer of EVs, also suggest potential efficacy of MSC-sEVs produced by these types of cells. One small cohort study in humans demonstrated direct evidence of a therapeutic benefit of MSC-sEVs in the treatment of COVID-19 with significant improvements in absolute neutrophil count, lymphopenia and acute phase reactants including C-reactive protein, ferritin, and D-dimer. Another phase I study of exosomes bearing CD24 also demonstrated complete recovery in all 30 patients with moderate to severe COVID-19, and recovery within three to five days in 29 of these patients. (https://www.jpost.com/health-science/tel-aviv-hospital-cures-29-of-30-covid-19-patients-in-days-it-says-658024)

8. Source of exosomes

Numerous peer-reviewed preclinical and clinical studies have demonstrated promising therapeutic bioactivity of MSC exosomes for more

hundreds of different clinical indications including COVID-19 and acute lung injury [83–86]. The vast majority of these studies have focused on bone marrow-derived MSC (BM-MSC) exosomes, perhaps because they were the first type to be isolated. The remainder of these studies have evaluated adipose tissue- (AT-MSC), umbilical cord- (UC-MSC) and placenta-derived (P-MSC) exosomes to a lesser extent [90]. Comparative studies of these types of MSC exosomes have demonstrated that their cargo and bioactivity differ significantly [91]. It is important to note that the type and state of the producer cells significantly influences the bioactivity of the exosomes they release. Placental MSCs have demonstrated greater immunomodulatory effects and regenerative capacity than bone marrow, adipose and umbilical cord derived MSCs [92,93]. Also, whereas placental MSCs are isolated from perinatal donor tissue usually discarded as medical waste, bone marrow- and adipose-derived MSCs are most commonly isolated from adult donor tissue. Perhaps as a result of changes associated with aging or environmental exposure, BM-MSC and AT-MSC exosomes have demonstrated some tumorigenic and pro-metastatic effects in preclinical studies [94, 95, 96, 97]. As suggested by preclinical studies in which RNAse treatment of MSC exosomes abrogates their bioactivity, this malignant behavior may correlate with the miRNA cargo of these types of MSC exosomes. The miRNA content of BM-MSC exosomes differs significantly from that of P-MSC exosomes [98]. In the case of BM-MSC exosomes, the most abundant microRNA species is miR-1246, which has demonstrated significant oncogenic and metastatic effects. This microRNA species is not present in biologically significant quantities in P-MSC exosomes. Instead, the abundant miRNAs identified in PMSC exosomes participate in important tissue reconstitution, anti-inflammatory pathways and tumor suppression [98]. MSC exosomes hold tremendous potential for the reconstitution of natural tissue barriers, such as the alveolar-capillary barrier that is disrupted in COVID-19, as well as those barriers disrupted in many other clinical conditions. Knowing that the producer cell type and environment strongly influence the character and bioactivity of the exosomes secreted, the development of exosome-based therapeutics for conditions such as COVID-19 and acute lung injury will require optimal producer cell selection, standardized biomanufacturing protocols and rigorous quality management standards. Orthogonal exosome characterization methods including mass spectrometry proteomics and lipidomics, RNA

sequencing, nano-flow cytometry and electron microscopy will help to confirm optimal purity and consistency of these products and enable the development and eventual clinical use upon regulatory approval of this next generation of biopharmaceuticals.

The miRNAs of certain mesenchymal stem cell–derived exosomes participate in important tissue reconstitution and anti–inflammatory pathways (Fig. 3.3).

9. Mesenchymal stem cell-small extracellular vesicles as investigational new drug

Isolated MSC–sEVs derived from cultures of P-MSCs of fetal origin can be primed with an optimal combination of IL-6, IFN-γ, IL-1β, and Poly (I:C) or overexpressing specific miRNA could be an excellent drug candidate for clinical trials such as for COVID-19. The investigational study participant population could be inclusive of for patients suffering from post COVID-19 disease, because MSC exosomes may be able to arrest the progression of fibrosis in pulmonary disease, and may influence the detrimental progression of autoimmunity [99].

10. Exosome enrichment

The process of exosome enrichment for in–vitro, ex–vivo (whole blood assays), and the application in-vivo requires highly purified and well-defined material. Among different biochemical and molecular methods, electron microscopy performed under most stringent conditions of biological structure preservation is highly valid (Fig. 3.4).

In vivo, exosomes may attenuate inflammation and eventually reconstitute endothelial barrier function in acute respiratory distress syndromes, primarily by reconstituting the balance of pro- (M1) and antiinflammatory macrophages (M2) as schematically demonstrated (Fig. 3.3), In this illustration the preferential release of exosomes targeted by MSC-derived exosomes may constitute a highly relevant amplification mechanism occuring at the site of damage and inflammation. This local amplification mechanism would be followed by resolution of thrombotic elements in small vessels of other organs as well.
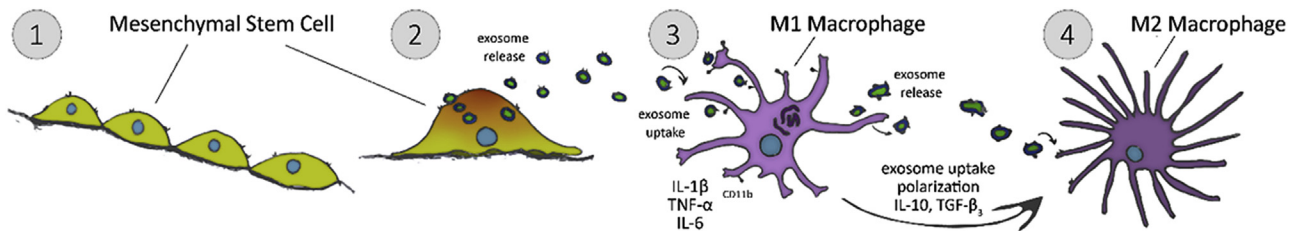
Figure 3.4 COVID-19 lung damage and acute respiratory distress syndromes.

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