EXTRACELLULAR VESICLES AS IMMUNOMODULATORY MEDIATORS IN INFLAMMATORY PROCESSES

EDITED BY: Danièle Noël, Paula Barbim Donate, Fausto Almeida, Yann Lamarre and Karina Pino-Lagos

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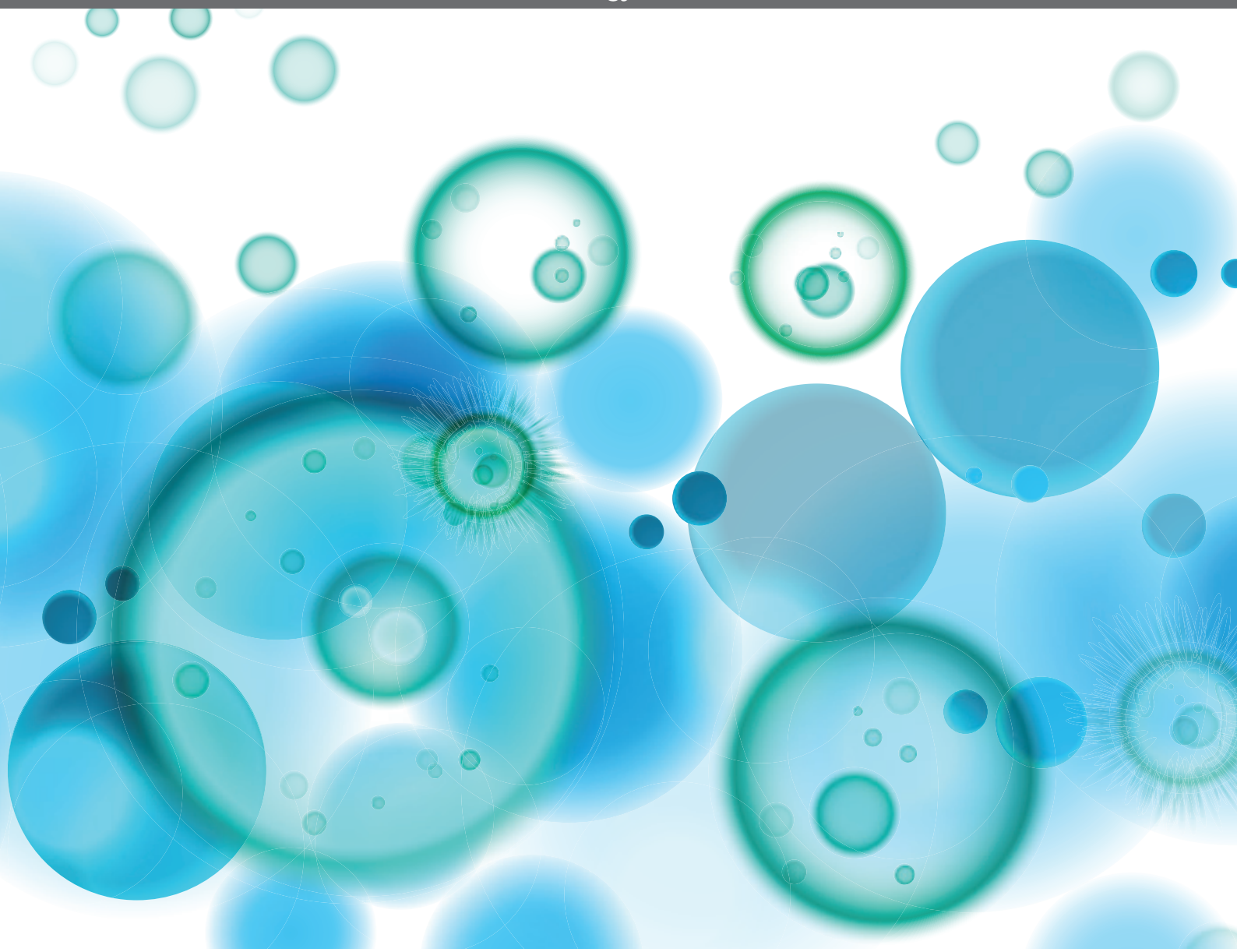

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EXTRACELLULAR VESICLES AS IMMUNOMODULATORY MEDIATORS IN INFLAMMATORY PROCESSES

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Mesenchymal Stromal Cell Derived Membrane Particles Are Internalized by Macrophages and Endothelial Cells Through Receptor-Mediated Endocytosis and Phagocytosis

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Mesenchymal stromal cells (MSC) are a promising therapy for inflammatory diseases. However, MSC are large and become trapped in the lungs after intravenous infusion, where they have a short survival time. To steer MSC immunoregulatory therapy beyond the lungs, we generated nm-sized particles from MSC membranes (membrane particles, MP), which have immunomodulatory properties, and investigated their internalization and mode of interaction in macrophages subtypes and human umbilical vein endothelial cells (HUVEC) under control and inflammatory conditions. We found that macrophages and HUVEC take up MP in a dose, time, and temperature-dependent manner. Specific inhibitors for endocytotic pathways revealed that MP internalization depends on heparan sulfate proteoglycan-, dynamin-, and clathrin-mediated endocytosis but does not involve caveolin-mediated endocytosis. MP uptake also involved the actin cytoskeleton and phosphoinositide 3-kinase, which are implicated in macropinocytosis and phagocytosis. Anti-inflammatory M2 macrophages take up more MP than pro-inflammatory M1 macrophages. In contrast, inflammatory conditions did not affect the MP uptake by HUVEC. Moreover, MP induced both anti- and pro-inflammatory responses in macrophages and HUVEC by affecting gene expression and cell surface proteins. Our findings on the mechanisms of uptake of MP under different conditions help the development of target-cell specific MP therapy to modulate immune responses.

Keywords: mesenchymal stromal cells, membrane particles, extracellular vesicles, endocytosis, phagocytosis, macrophages, endothelial cells, immunomodulation

INTRODUCTION

Mesenchymal stromal cells (MSC) are self-renewing cells found in several postnatal organs and tissues from which they can be easily isolated and expanded in *in vitro* conditions (1, 2). Their immunomodulatory and regenerative properties enable MSC to be used as an potential therapy for several diseases, including inflammatory bowel diseases (3, 4), rheumatoid arthritis (5),

atherosclerosis (6), and kidney injury (7). However, culture-expanded MSC are large and become trapped in the pulmonary vascular network after intravenous administration (8–11). MSC are no longer detected in the lungs after 24 h and their cellular debris is phagocytized and distributed to other sites of the body (12). Moreover, MSC transplantation can lead to practical complications resulting from the use of living cells, including immune responses, thrombosis, tumor formation, and transmission of infections (2, 13–15).

To steer MSC therapy beyond the lungs, we generated nm-sized vesicles from MSC membranes (membrane particles, MP). MP have a spherical shape and are composed of MSC outer cell membranes and organelles (unpublished data). Because of their small size and vesicle shape, MP are potentially capable of overcoming the pulmonary barrier. These particles contain the membrane-bound proteins of MSC, several of which have immunomodulatory, metabolic, and adhesion functions. We previously reported that MP possess similar immune regulatory properties as MSC with respect to the modulation of monocyte function after being taken up by these cells (16). We also found that MP, like naturally occurring extracellular vesicles, are efficiently taken up by endothelial cells and modulate their function (unpublished data), and (17, 18). However, the specificity and mechanisms of MP uptake by target cells remain unclear. Thorough understanding of the mechanisms of MP uptake by different cell types is of great importance for the use of MP for immune and regenerative therapy.

The mechanisms of particle uptake involve protein interactions that facilitate subsequent endocytosis. The internalization process can be divided into receptor-mediated endocytosis, phagocytosis/macropinocytosis, and passive penetration (19, 20). Endocytosis is mediated by specific cell surface receptors. These are transmembrane proteins that interact with specific extracellular molecules on vesicles and subsequently initiate endocytosis, resulting in heparan sulfate proteoglycans (HSPG)-, dynamin-, clathrin-, and caveolin-mediated endocytosis (21, 22). Phagocytosis and macropinocytosis are mediated by the polymerization of actin and phosphoinositide 3-kinases (PI3K), which allow the insertion of the cell membrane in the formation of phagosomes (23–25). Moreover, the properties of particles in combination with characteristics of the cellular and extracellular environments, such as temperature, exposure time, inflammatory environment, and type of receptor cells, can govern the localization of particles in the target cells (19, 24).

The ability of MP to interact with host cells, deliver their biological effect, and provoke an immunological and regenerative response is dependent on their uptake. Understanding the mechanisms of uptake allows steering and conditioning of their uptake and thereby control of their potential therapeutic effects. Here, we characterized human MP uptake and internalization by macrophages subtypes and endothelial cells, which are among the first cell types to be exposed to infused MP and play a crucial role in immune responses, and examined their function under quiescent and inflammatory conditions.

MATERIALS AND METHODS

Isolation and Culture of MSC

MSC were obtained from subcutaneous adipose tissue from 13 healthy human donors that became available during the living kidney donation procedure. All donors provided written informed consent as approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam (protocol no. MEC-2006-190). MSC were isolated and phenotypically characterized by the expression of CD13, CD73, CD90, and CD105 and the absence of CD31 and CD45 as described previously (16). MSC were cultured in minimum essential medium-α (MEM-α) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (P/S), 2 mM L-glutamine, and 15% fetal bovine serum (FBS) (all Lonza, Verviers, Belgium). Cultures were kept at 37°C, 5% CO_2, and 95% humidity. At 90% confluence, adherent cells were collected from culture flasks by incubation in 0.05% trypsin-EDTA (Life Technologies, Bleiswijk, The Netherlands) at 37°C. MSC between passages 2 and 6 were used for MP generation.

Generation of MSC Membrane Particles

MSC were collected, counted, washed twice with phosphate-buffered saline (PBS), and centrifuged at 2,000 × g for 5 min. The MSC pellet was incubated in Milli-Q water at 4°C for ∼20 min to induce osmotic lysis and release of cell nuclei. This step was carefully monitored by an optical microscope and stopped when nuclei were released from the cells. Cell extracts were isolated from unbroken cells and nuclei by centrifugation at 2,000 × g for 20 min. Then, the supernatant was transferred to Amicon Ultra-15 filter tubes (100 kDa pore size) and concentrated by centrifugation at 4,000 × g for 45 min. The concentrated pellet consisted of crude membranes and was diluted in filtered PBS. To prepare a small and uniform size of MP, the membranes were extruded three times through polycarbonate membrane filters (Merck, KGaA, Darmstadt, Germany) using LiposoFast LF-50 (AVESTIN Europe, Mannheim, Germany) at 20 psi, first with a pore size filter of 800 nm, then 400 nm, and finally 200 nm. All procedures were performed on ice. To obtain fluorescent MP, MSC were labeled with the red fluorescent PKH-26 dye (PKH-MP), which intercalates into lipid bilayers, according to the manufacturer's instructions (Sigma-Aldrich), before generation of MP.

Nanoparticle Tracking Analysis

Absolute size distribution and concentration of MP was performed using the NTA by NanoSight NS300 (NanoSight Ltd.). NTA automatically tracked and sized particles based on Brownian motion and the diffusion coefficient. First, the samples were diluted to obtain the right number of particles (1×10^8 particles/ml) in accordance with the manufacturer's recommendations. Three measurements per sample (30 s/measurement) were captured under the following conditions: temperature 23.61 ± 0.8°C; viscosity 0.92 ± 0.02 cP, frames per second (25). After capture, the videos were analyzed

to give the mean, mode, median, and estimated concentration for each particle size with a detection threshold 3 (determined with a protein solution).

Cryo-Transmission Electron Microscopy

The morphology of MP was visualized by Cryo-TEM. A thin aqueous film was foMPed by applying a 3 μl droplet of MP suspension to a specimen bare EM grid. For that, glow-discharged holey carbon grids were used. Then, the grid was blotted against filter paper, leaving a thin sample film covering the grid holes. These films were vitrified by immersing the grid into ethane, which was maintained at its melting point by liquid nitrogen using a Vitrobot (Thermo Fisher Scientific Company, Eindhoven, Netherlands) to prevent samples from freezing at 95% humidity. The vitreous sample films were transferred to a Tecnai Arctica microscope (Thermo Fisher Scientific, Eindhoven, Netherlands). Images were taken at 200 Kv with a field emission gun using a direct electron detector Falcon III (Thermo Fisher Scientific).

Culture of Human Monocytic Cell Line THP-1

THP-1 (ATCC: TIB-202) is a human monocytic cell line derived from an acute monocytic leukemia patient. THP-1 cells were cultured in RPMI 1640-GlutaMAX (Gibco, Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS and 1% P/S at 37°C under 5% CO_2. Cells were grown to a density of 1–8 $\times$ 10^5 cells/ml and used for experiments between passage 2 and 10. Differentiation of THP1 cells into macrophage-like cells was induced by stimulation with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma) for 72 h. Following differentiation, PMA-containing media was replaced with fresh media, and cells were rested in culture for 24 h.

Culture of Human Umbilical Vein Endothelial Cells

HUVEC pooled from multiple donors were purchased from Promocell (Promocell, Germany). Cells were cultured in endothelial cell basal medium (EBM, Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA), endothelial cell growth medium supplements (EGM, Cambrex Bio Science), 5% FBS and 1% P/S at 37°C under 5% CO_2. At 90% confluence, HUVEC were collected by incubation in 0.05% trypsin-EDTA at 37°C. HUVEC between passages 2 and 7 were used for the experiments.

Uptake of MP by Macrophages and HUVEC

THP-1 macrophages (2 $\times$ 10^4 cells/ml) and HUVEC (1 $\times$ 10^4 cells/ml) were cultured with PKH-MP at different ratios (Cell: MP; 1:10,000, 1:50,000, 1:100,000) at 37°C, 5% CO_2, and 95% humidity. To determine if the uptake of MP was *via* an active process, cells were alternatively incubated at 4°C. MP uptake by macrophages and HUVEC was analyzed through detection of PKH positive cells by flow cytometry (FACS Canto II, Becton Dickinson) at 1, 6, and 24 h.

For confocal microscopy analysis, THP-1 macrophages, and HUVEC were cultured with PKH-MP (ratio 1:50,000) for 24 h. Cell membranes of macrophages and HUVEC were labeled with CD81-APC (BioLegend, San Diego, CA) and the nuclei with 10 μM Hoechst 33342. Images were performed on a Leica TCS SP5 confocal microscope (Leica Microsystems B.V., Science Park Eindhoven, Netherlands) equipped with Leica Application Suite – Advanced Fluorescence (LAS AF) software, DPSS 561 nm lasers, using a 60 $\times$ (1.4 NA oil) objective. Images were processed using ImageJ 1.48 (National Institutes of Health, Washington, USA).

MP Uptake Inhibitors

Cells were preincubated for 30 min at 37°C in complete medium containing Heparin (0.1–100 μg/ml; H3149; Sigma), Dynasore (20–160 μM; D7693; Sigma), Chlorpromazine (1–50 μM; C8138; Sigma), Nystatin (5–40 μg/ml; N6261; Sigma), Cytochalasin D (0·25–2 μM; C8273; Sigma), or Wortmannin (0.1–10 μM; W1628; Sigma). Cell viability was measured by flow cytometer using BD Via-Probe™ Cell Viability Solution containing 7-AAD. PKH-MP were then added to the cells (ratio 1:100,000) and analyzed after 6 h by flow cytometer. Following the results of these experiments, combination treatment with inhibitors of endocytosis (10 μg/ml Heparin and 80 μM Dynasore) and phagocytosis (5 μM Wortmannin) was also tested. Drug vehicle controls were used for the experiments: 0.1% PBS as a control for Heparin, Chlorpromazine, Nystatin, 0.1% dimethyl sulfoxide (DMSO) for Dynasore, Cytochalasin D, and Wortmannin.

MP Uptake by Macrophages and HUVEC Under Inflammatory Conditions

For simulating inflammatory conditions *in vitro*, macrophages were primed with fresh medium supplemented with 20 ng/ml IFNγ (Gibco, Thermo Fisher Scientific) and/or 100 ng/ml lipopolysaccharides (LPS) (Sigma) to generate pro-inflammatory M1 macrophages. For anti-inflammatory conditions, macrophages were primed with 20 ng/ml IL-4 (PeproTech, London, UK) and/or 20 ng/ml IL-13 (PeproTech) to generate anti-inflammatory M2 macrophages. The incubation time was 24 h for all the conditions. PKH-MP were then added to macrophages (ratio 1:50,000) and incubated for another 24 h after which uptake was analyzed by flow cytometer and confocal microscopy.

HUVEC were primed with fresh medium supplemented with 10 ng/ml TNFα and/or 50 ng/ml IFNγ for 24 h. MP were added to the cells (ratio 1:50,000) and incubated for another 24 h after which uptake was analyzed by flow cytometer. After 48 h, HUVEC were collected for functional analysis by flow cytometer, after staining with HLA-I-PacBlue (BD Biosciences, San Jose, CA), HLA-II-PerCP (BioLegend), CD40-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), CD144-PE (Ebioscience, Thermo Fisher Scientific).

Quantitative RT-PCR Analyses

Macrophages were polarized in M1 and M2 macrophages by LPS, IFNγ, IL-4, and IL-13, respectively. HUVEC were primed with TNFα and IFNγ. After 24 h, MP were added to macrophages or HUVEC (ratio 1:50,000) and incubated for 48 h. Cells were harvested, washed with PBS-diethylpyrocarbonate

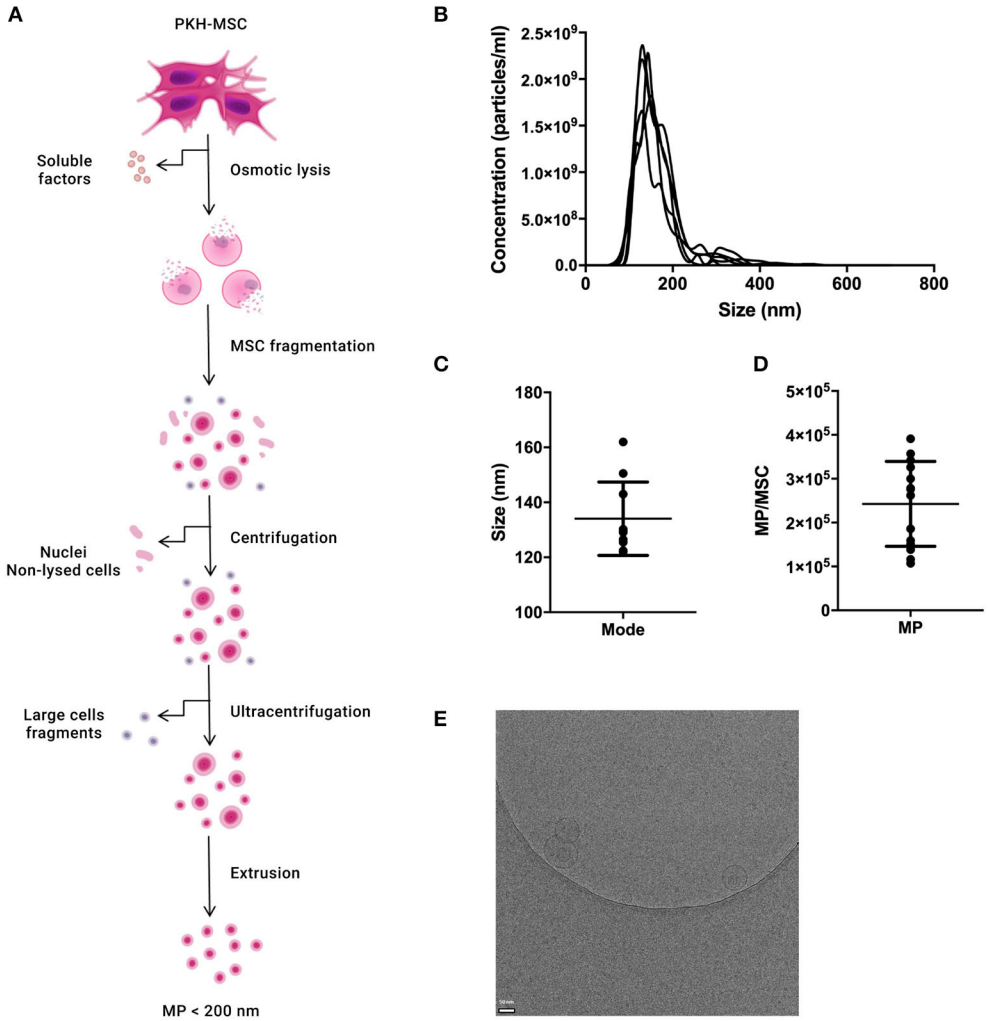

FIGURE 1 | Characterization of reconstructed membranes (MP) generated from MSC. **(A)** Schematic overview of the generation of MP. **(B)** Nanoparticle tracking analysis (NTA) profile of MP. **(C)** MP size distribution. **(D)** Number of MP generated per MSC. **(E)** Cryo-electron microscopy image of MP. MP (arrow) show a spherical shape and a visible lipid bilayer. Data in C and D are presented as mean ± SD from 12 independent preparations of MP.

(DEPC; Sigma-Aldrich), and stored at −80°C. Total RNA was isolated and 500 ng used for complementary DNA (cDNA) synthesis. Gene expression was determined by Quantitative Real-Time PCR (qPCR) using the TaqMan Universal PCR Master Mix (Life Technologies), and the assay-on-demand primer/probes for CXCL10 (Hs00171042), CCR7 (Hs00171054), Interleukine-1β (IL-1β, Hs00174097), Interleukine-10 (IL10, Hs00174086), tumor necrosis factor-α (TNFα, Hs99999043), transforming growth factor- β (TGFβ, Hs00171257), CCL22 (Hs00171080), and CD209 (Hs01588349) for macrophages; and Interleukine-6 (IL-6, Hs00174131), Interleukine-8 (IL-8, Hs00174114), and Endothelin (Hs 00174961) for HUVEC. 18S (Hs99999901) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905) mRNA served as a housekeeping genes for macrophages and HUVEC normalization, respectively.

Statistical Analysis

Data were analyzed for statistical significance by Student's *t*-test and one-way and two-ways ANOVA, and analysis using GraphPad Prism 5 software. $P < 0.05$ was considered significant.

RESULTS

Characterization of MP

MP were generated from culture-expanded MSC and characterized by NTA and Cryo-TEM to determine their concentration, size distribution, and morphology (**Figure 1A**). The percentage of particles with a size larger than 200 nm was lower than 5% (**Figure 1B**). The mode size of MP was 134.1 ± 13.3 nm (**Figure 1C**). The average number of MP generated per MSC was $2.4 \times 10^5 \pm 10.0 \times 10^4$ (**Figure 1D**) There was no significant difference in size distribution or

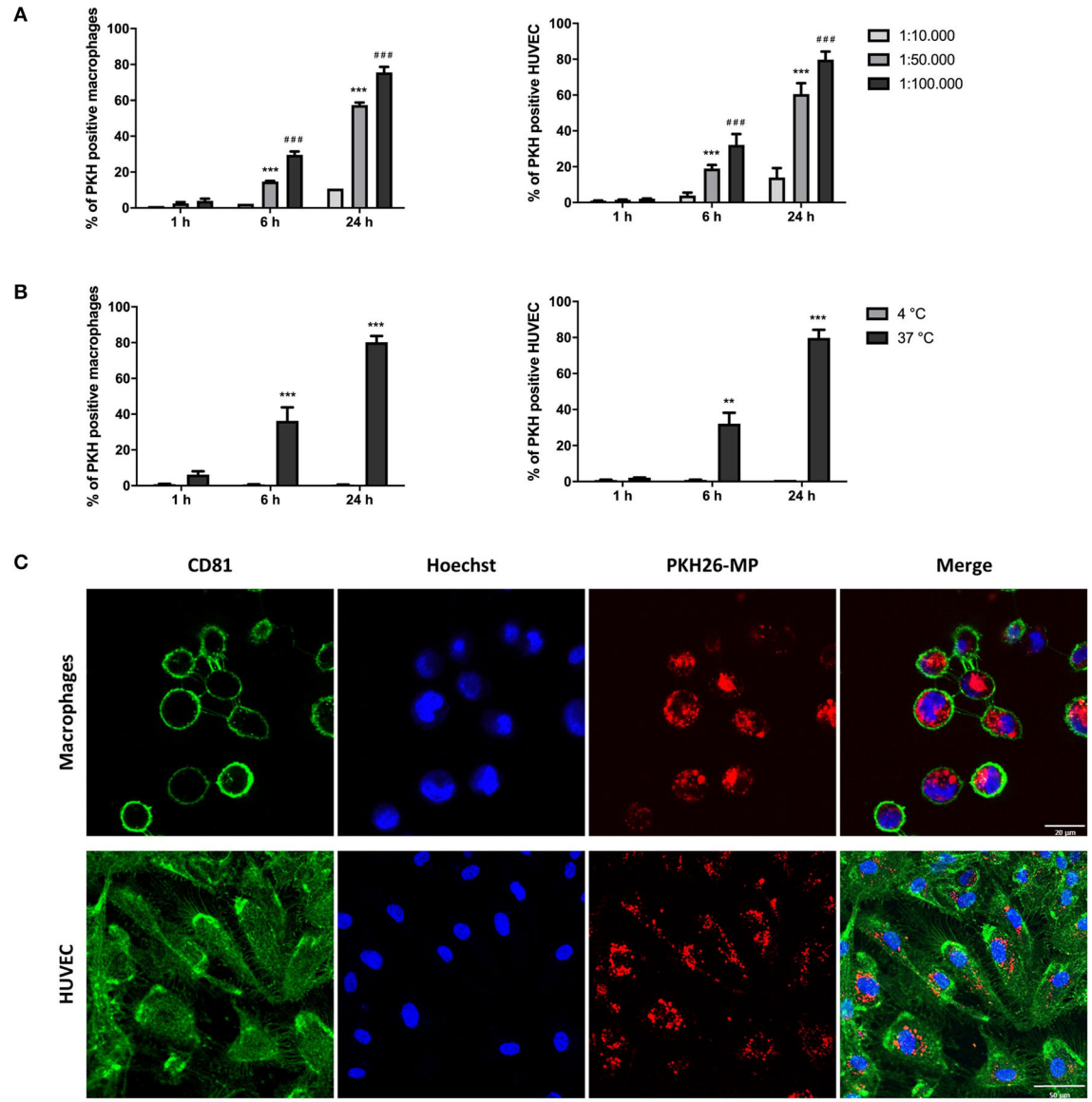

FIGURE 2 | Macrophages and HUVEC internalize MP in a dose-, time-, and temperature-dependent manner. MSC were labeled with PKH-26 before the generation of MP (PKH-MP). PKH-MP were added to macrophages or HUVEC (ratios 1:10,000, 1:50,000, 1:100,000), incubated for 1, 6, and 24 h at 4 or 37°C, and analyzed by flow cytometer or confocal microscopy. **(A)** Percentage of macrophages and HUVEC positive for PKH-MP in different doses of MP over time, and **(B)** at 4 and 37°C over time. **(C)** Representative confocal microscopy analysis of MP uptake by macrophages and HUVEC (ratio 1:50,000) at time point 24 h. Staining for CD81 cell membrane (green), Hoechst 33,342 nucleus (blue), and PKH26-MP (red) demonstrated that MP are internalized by macrophages and HUVEC. Data are presented as mean ± SD from 3 to 4 experiments. ****P < 0.001 vs.* ratio 1:10,000 and *###P < 0.001 vs.* ratio 1:50,000 in **(A)**; ***P < 0.001* and ***P < 0.001 vs.* 4°C in **(B)**. Scale bars: 20 μm (macrophages) and 50 μm (HUVEC).

concentration between MP and fluorescently-labeled MP (PKH-MP) (**Supplementary Figure 1**). Cryo-TEM showed that MP have a spherical shape and a discernible lipid bilayer. Some MP were encapsulated inside larger MP (**Figure 1E**).

Macrophages and HUVEC Internalize MP in a Dose-, Time-, and Temperature-Dependent Manner

THP-1 macrophages and HUVEC were cultured with PKH-MP at different ratios (1:10,000, 1:50,000, 1:100,000) at 4 and 37°C and analyzed by flow cytometer after 1, 6, and 24 h. We found that macrophages and HUVEC uptake MP in a dose and time-dependent manner. MP uptake increased with rising concentration and time of incubation (**Figure 2A**) and was completely inhibited at 4°C, which indicates a temperature-dependent process (**Figure 2B**). The interaction of MP with macrophages and HUVEC was visualized by confocal immunofluorescence microscopy. The confocal microscopy images showed that both macrophages and HUVEC internalized PKH-MP (**Figure 2C**).

MP Internalization Depends on HSPG-, Dynamin- and Clathrin-Mediated Endocytosis, but Does Not Involve Caveolin-Mediated Endocytosis

To elucidate whether specific endocytic processes are responsible for MP internalization, macrophages, and HUVEC were pre-treated with pharmacological inhibitors that interfere in different endocytosis pathways as described in **Figure 3A**. First, the effect of different concentrations of the inhibitors on viability of the recipient cells was evaluated (**Supplementary Figure 2**). For the following experiments, we used the maximum doses that did not affect cell viability.

MP bind to cells through cell-surface receptors, after which they are internalized. Our results showed that heparan sulfate proteoglycans (HSPGs) are involved in binding of MP as the recipient cell-MP interaction is partially inhibited in the presence of heparin (a soluble analog of HSPGs) (21) in a dose-dependent manner (**Figure 3B**). Dynamin is a regulator of the endocytosis processes and is involved in clathrin- and caveolin-dependent routes (26). We blocked dynamin activity by its selective inhibitor Dynasore, which dose-dependently reduced cellular uptake of MP by macrophages and HUVEC (**Figure 3C**). To discriminate endocytotic routes, we used Chlorpromazine and Nystatin to block clathrin- and caveolin-mediated endocytosis, respectively. We observed that Chlorpromazine led to a dose-dependent inhibitory effect on MP uptake, and it was greater in HUVEC than in macrophages (**Figure 3D**). Nystatin had no significant effect on the uptake of MP by macrophages but it slightly increased uptake of MP by HUVEC (**Figure 3E**). No effect was observed with the carrier controls, PBS, or DMSO.

MP Internalization Involves Phagocytosis and Macropinocytosis

Phagocytosis and macropinocytosis involve the polymerization of actin and phagosome formation mediated by PI3K protein (23, 24) as described in **Figure 4A**. Cytochalasin D, an inhibitor of actin polymerization, dose-dependently inhibited MP uptake, and its effect was stronger in macrophages than HUVEC (**Figure 4B**). Wortmannin, an inhibitor of PI3K, led to a dose-dependent blocking of MP uptake by both cell types (**Figure 4C**). Combination treatment with 10 μg/ml Heparin, 80 μM Dynasore, and 5 μM Wortmannin to block MP binding, endocytosis, and phagocytosis resulted in a synergistic reduction of MP uptake of more than 85% in both HUVEC and macrophages (**Figure 4D**). No significant change in cell viability was observed following combined treatment (**Figure 4E**).

MP Modulate Macrophage Function

To investigate the uptake of MP by macrophages under different immunological conditions, we primed macrophages with LPS and IFNγ (pro-inflammatory M1 macrophages) or IL-4 and IL-13 (anti-inflammatory M2 macrophages). Inflammatory conditions led to macrophage elongation and anti-inflammatory conditions resulted in more rounded and loosely attached macrophages within 24 h of stimulation (**Supplementary Figure 3A**). M1 macrophages produced higher gene expression levels of CXCL10, CCR7, IL-10, and TGFβ, whereas M2 macrophages produced higher levels of CCL22 and CD209 within 72 h of stimulation (**Supplementary Figure 3B**). Significant changes in gene expression were not observed for IL-10.

PKH-MP were added to macrophages and assessed by flow cytometry after 24 h. We found that anti-inflammatory M2 macrophages take up more efficiently MP than pro-inflammatory M1 macrophages, which significantly decreased their ability to internalize MP as indicated in **Figure 5A**. Confocal images showed that M1 and M2 macrophages internalized PKH-MP into the cytoplasm (**Figure 5B**). mRNA expression of a number of genes with pro- and anti-inflammatory function was analyzed in macrophages by qPCR after 48 h of stimulation with MP to determine whether MP could affect macrophages gene expression and immune function. MP induced anti- and pro-inflammatory genes in macrophages exposed to LPS and IFNγ treatment. MP decreased the gene expression of pro-inflammatory chemokines CXCL10 and CCR7 and increased anti-inflammatory cytokine IL-10 and TGFβ in M1 macrophages. However, MP also increased the gene expression of pro-inflammatory cytokines IL-1β and TNFα (**Figure 5C**). Following IL-4 and IL-13 treatment, MP increased gene expression of the anti-inflammatory chemokine CCL22 and the immunoregulatory marker CD209 in M2 macrophages (**Figure 5D**).

MP Uptake Modulates HUVEC Function Under Inflammatory Conditions

To investigate the uptake of MP by HUVEC in inflammatory conditions, we primed HUVEC with single or combined TNFα and IFNγ doses. HUVEC exposed to TNFα and IFNγ alone led to cell elongation 24 h after stimulation and this morphological change was more pronounced when TNFα and IFNγ were combined (**Supplementary Figure 4A**). Moreover, HUVEC-surface expression level of HLA-I, HLA-II, and costimulatory molecule CD40 increased after single γ or combined doses of TNFα and IFNγ for 72 h (**Supplementary Figure 4B**). TNFα alone also increased HLA-I level. Significant changes on HUVEC-surface expression were not observed for CD144 (vascular endothelial cadherin), which is involved in the formation of endothelial intercellular junctions. TNFα and IFNγ upregulated the mRNA expression of pro-inflammatory cytokines IL-6 and IL-8 and of the angiogenic mediator endothelin in HUVEC after 72 h (**Supplementary Figure 4C**). TNFα alone also increased IL-8 gene expression.

We found that MP uptake by HUVEC after 24 h was not affected under TNFα and IFNγ stimulation, alone or in combination as indicated in **Figure 6A**. HUVEC were collected for functional analysis by flow cytometry and qPCR after 48 h of stimulation with MP to determine whether MP affected HUVEC protein surface and gene expression levels in quiescent and inflammatory environment (TNFα and IFNγ). MP decreased the surface expression level of HLA-I, HLA-II, and CD40 costimulatory molecule on HUVEC under inflammatory conditions (**Figure 6B**).

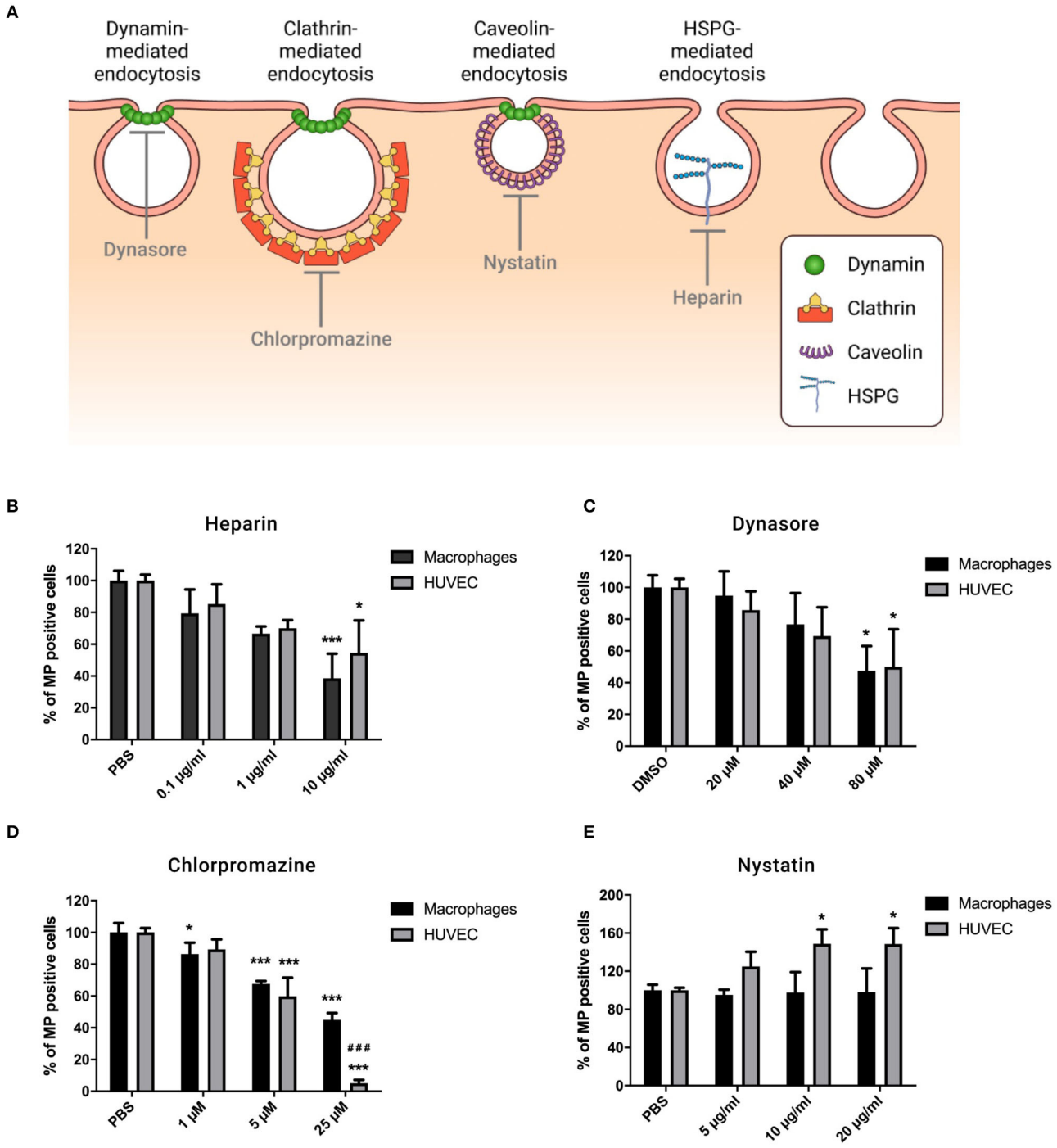

FIGURE 3 | MP uptake by macrophages and HUVEC depends on HSPG-, dynamin-, and clathrin-mediated endocytosis. Cells were preincubated for 30 min at 37°C in complete medium containing Heparin (0.1, 1, and 10 µg/ml), Dynasore (20, 40, and 80 µM), Chlorpromazine (1, 5, and 25 µM), or Nystatin (5, 10, and 20 µg/ml). PKH-MP were then added to cells (ratio 1:100,000) and analyzed after 6 h by flow cytometer. PBS and 0.1% DMSO were used as a control. **(A)** Receptor-mediated endocytosis pathways. **(B)** Percentage of macrophages and HUVEC positive for PKH-MP in the presence of increasing concentrations of Heparin **(B)**, Dynasore **(C)**, Chlorpromazine **(D)**, and Nystatin **(E)**. Data are presented as mean ± SD from 4 to 6 experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs.* vehicle; $^{\#\#\#}P < 0.001$ *vs.* macrophages.

Moreover, MP increased the expression level of CD144 in non-inflammatory and inflammatory conditions. As in macrophages, MP also induced a pro-inflammatory response in HUVEC. MP upregulated gene expression of pro-inflammatory cytokines IL-6 in inflammatory HUVEC and IL-8 in non-inflammatory and inflammatory HUVEC. MP downregulated

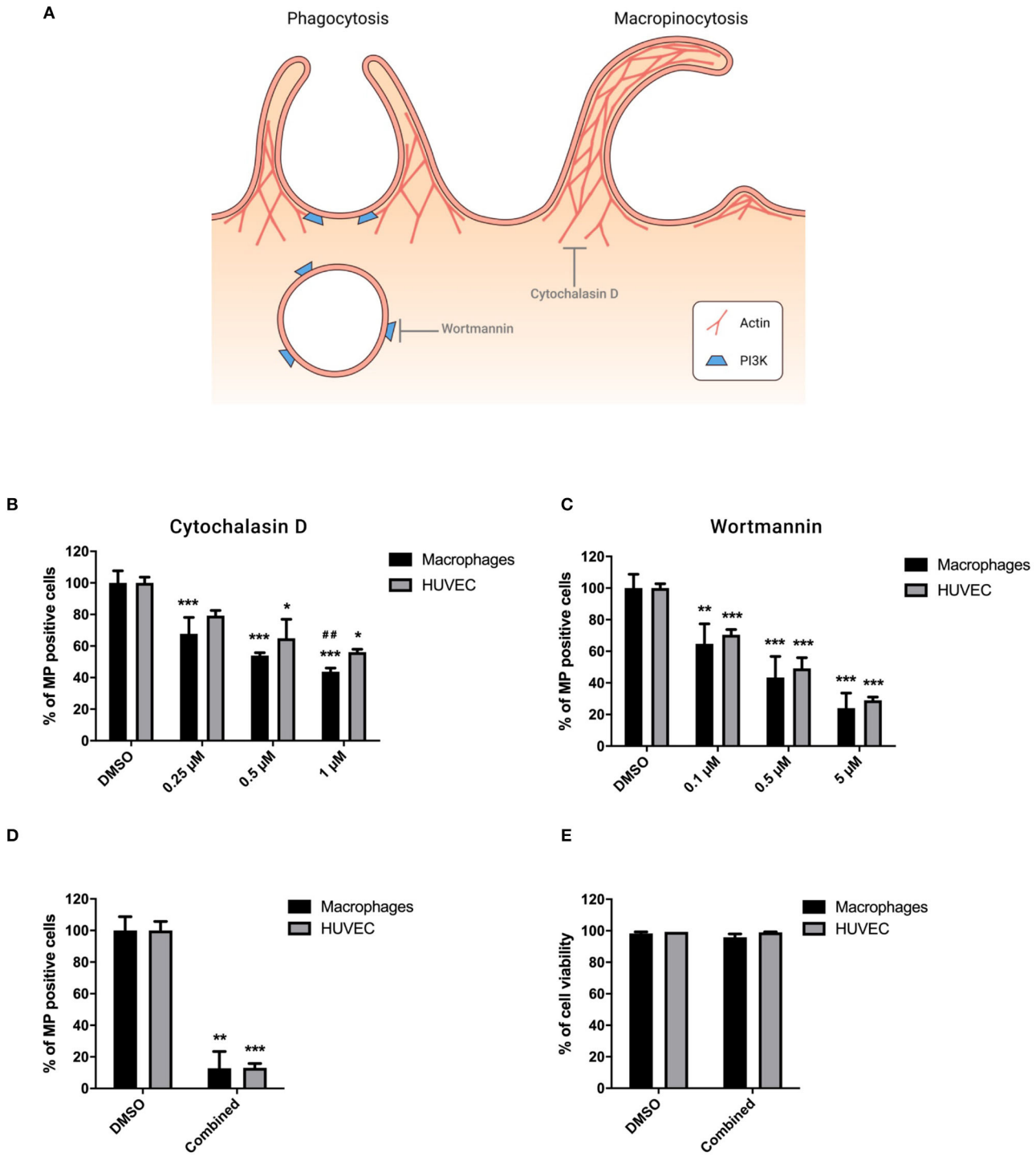

FIGURE 4 | MP internalization involves phagocytosis and macropinocytosis. Cells were preincubated for 30 min at 37°C in complete medium containing Cytochalasin D (0.25, 0.5, and 1 µM), Wortmannin (0.1, 0.5, and 10 µM), or combination treatment with inhibitors of endocytosis (10 µg/ml Heparin and 80 µM Dynasore) and phagocytosis (5 µM Wortmannin). PKH-MP were then added to cells (ratio 1:100,000) and analyzed after 6 h by flow cytometer. PBS and 0.1% DMSO were used as a control. **(A)** Phagocytosis and macropinocytosis mechanisms. Percentage of macrophages and HUVEC positive for PKH-MP in the presence of increasing concentrations of Cytochalasin D **(B)**, Wortmannin **(C)**, and combined treatment **(D)**. **(E)** Percentage of macrophage and HUVEC viability after combined treatment. Data are presented as mean ± SD from 4 to 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* vehicle; $^{\#\#}P < 0.001$ *vs.* HUVEC.

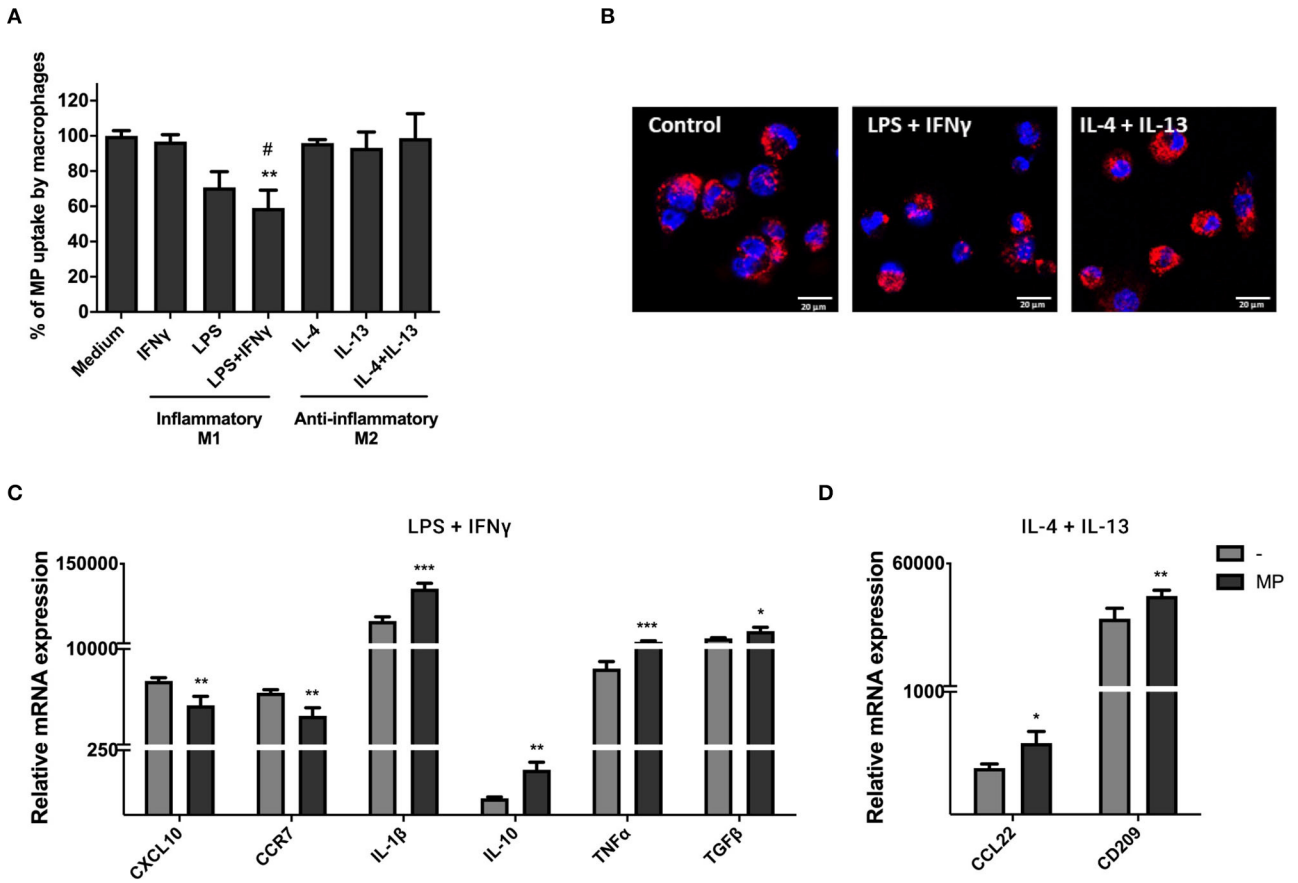

FIGURE 5 | MP uptake under pro- and anti-inflammatory conditions modulates macrophages function. Macrophages were primed with 100 ng/ml LPS and 20 ng/ml IFNγ (M1 macrophages) or with 20 ng/ml IL-4 and 20 ng/ml IL-13 (M2 macrophages). **(A,B)** PKH-MP were added to macrophages (ratio 1:50,000) and incubated for 24 h for uptake analyze by flow cytometer and confocal microscopy. **(A)** Percentage of macrophages positive for PKH-MP in different culture conditions. **(B)** Representative confocal microscopy analysis of MP uptake by macrophages under stimulation of IFNγ + LPS or IL-4 + IL-13. **(C,D)** Macrophages were separated from MP and assessed by real-time RT-PCR after 48 h. mRNA expression of macrophages treated with MP in the presence of **(C)** LPS + IFNγ (CXCL10, CCR7, IL-1, IL-10, TNFα, and TGFβ) and **(D)** IL-4 + IL-13 (CCL22 and CD209). Data are presented as mean ± SD from 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* medium control or *vs.* macrophages without MP and $^{\#}P < 0.05$ vs. macrophages stimulated with IL-4 + Il-13. Scale bar: 20 μm.

gene expression of endothelin under both conditions (**Figure 6C**).

DISCUSSION

In this study, we investigated the mechanisms of MP uptake and its effect on macrophage and HUVEC phenotype. Our findings revealed that MSC-derived MP enter macrophages and HUVEC through various mechanisms, involving receptor-mediated endocytosis, macropinocytosis, and phagocytosis, and modulate macrophage and HUVEC function. Identification of mechanisms involved in MP internalization under different conditions allows specific modulation of MP delivery to target cells.

MSC-derived MP therapy has several advantages over MSC themselves. MP contain the membrane-bound proteins of MSC and the expression of these proteins on MP is not modified by the environment after infusion as in living cells. Since MP are not a living cellular product, there is no risk of transformation after administration. Because of their small size, MP are better capable of crossing the pulmonary barrier than MSC. Unlike the collection of naturally occurring extracellular vesicles, which are a mixture of vesicles budding off from the cell membrane and endoplasmic reticulum, MP generation is a simple, low-cost, and scalable process. The MP production process offers a number of possibilities to modify their activity, which would be more complex for extracellular vesicles. Firstly, as the protein make-up of MP mirrors that of their mother MSC, treatment of MSC with for instance pro-inflammatory cytokines to induce the expression of membrane bound proteins with anti-inflammatory function or lentiviral transfection of MSC to induce the expression of a particular protein of interest would result in the incorporation of such proteins in MP generated from these MSC. Secondly, the closure of membrane fragments into circular membrane particles allows the enclosure of drugs of

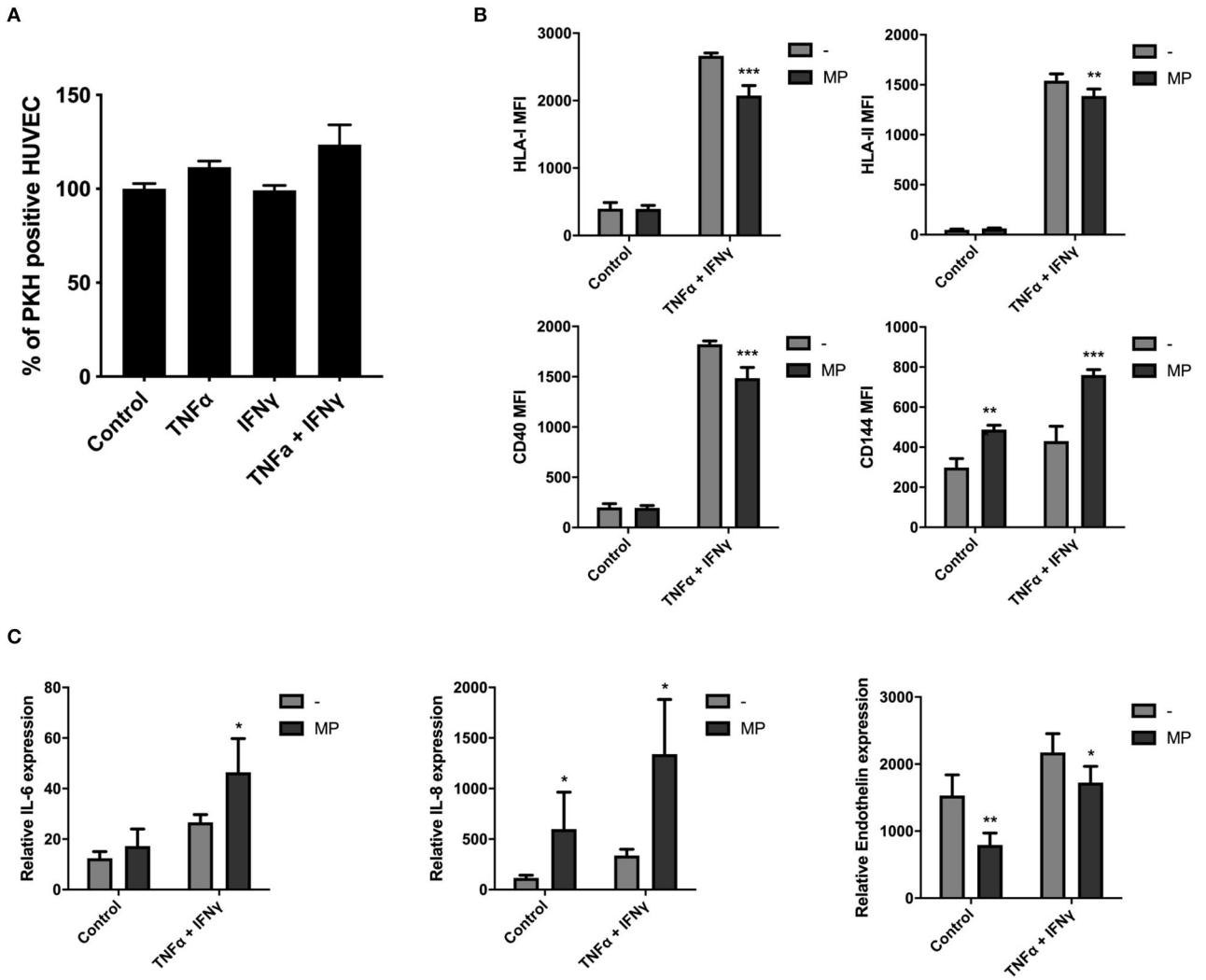

FIGURE 6 | MP uptake modulates HUVEC function under inflammatory conditions. HUVEC were primed with single or combined doses of 10 ng/ml TNFα and 50 ng/ml IFNγ. PKH-MP were added to the cells (ratio 1:50,000) and incubated for 24 h after which uptake was analyzed by flow cytometer. **(A)** Percentage of HUVEC positive for PKH-MP in different culture conditions. **(B,C)** HUVEC were separated from MP and assessed by flow cytometer and real-time RT-PCR after 48 h. **(B)** HUVEC surface levels of HLA-I, HLA-II, CD40, and CD144 and **(C)** mRNA expression of HUVEC for IL-6, IL-8, and endothelin after treated with MP under quiescent or TNFα + IFNγ conditions. Data are presented as mean ± SD from 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* medium control or HUVEC without MP.

interest, which will be delivered to target cells upon MP uptake. In this way target cells can be treated with MP and simultaneously with a drug.

The focus of the present study was on mechanisms of MP uptake and internalization by two cell types with barrier and immune patrolling functions, namely, endothelial cells (represented by HUVEC) and macrophages (represented by THP-1 macrophages). Cellular and extracellular environments, such as temperature, exposure time, inflammatory environment, and the available uptake machinery in target cells can govern the bio-distribution of MP (19, 24). We established that macrophages and HUVEC uptake MP in a dose-, time, and temperature-dependent manner and internalized MP into the cytoplasm. In our previous studies, we showed that MP do not physically interact with T cells, but interact with monocytes and HUVEC by binding to the plasma membrane through fusion and internalization, respectively (16) (unpublished data). This discrepancy might be explained by the fact that MP-cell interaction is strictly dependent on cell contact, but T cell activation requires soluble factors. Therefore, the interaction of MP with the plasma membrane of macrophages and HUVEC supports the idea that MP can be a natural delivery vehicle for macrophage and endothelial cell targeting drugs. These results need to be confirmed in primary cells as it is possible that the cell lines used in the present paper generate different results than primary cells.

In the present study, we choose to explore the mechanisms of MP uptake by the use of a number of inhibitors at

concentrations that did not induce cell death within 6 h. We cannot rule out the possibility that longer incubations would lead to cell death, or that mild cytotoxicity, not resulting in cell death within 6 h, did occur. However the involvement of some key pathways in MP uptake was already observed at concentrations far below necrosis-inducing doses. Six different mechanisms of endocytosis were addressed in this study: HSPG-, dynamin-, clathrin-, caveolin-mediated endocytosis, phagocytosis, and macropinocytosis. Our data revealed that MP bind to cells *via* cell-surface receptors and are later internalized through HSPG-, dynamin-, and clathrin-mediated endocytosis. HUVEC internalized MP *via* clathrin-coated pits more efficiently than macrophages, suggesting that there are differences in the dominant uptake pathways between different cell types (endothelial cells express more clathrin on their cell membrane). In addition, we found that MP uptake is a caveolin-independent process. Several studies report that caveolin-independent, but dynamin-dependent endocytosis is involved in the formation of non-coated vesicles in the plasma membrane (21, 24, 27, 28). In cells without caveolin, the same dynamin-dependent pathway can functionally replace caveolar endocytosis (29). Moreover, studies suggest that the internalization of particles is limited to particles smaller than the size of caveolin (about 50–100 nm) (30, 31). However, inhibition of caveolin pathway slightly increased uptake of MP by HUVEC. We hypothesize that alterations in the caveolin pathway can stimulate other uptake mechanisms as a result of endocytic compensation.

We showed that MP internalization by macrophages and HUVEC also depends on the actin cytoskeleton and PI3K through phagocytosis and macropinocytosis process. Macrophages internalized MP *via* actin-mediated mechanisms more efficiently than HUVEC, indicating that MP are preferentially internalized by phagocytes cells (24). Moreover, combination treatment that interferes with HSPG-, dynamin, and actin-mediated mechanism almost completely blocked MP internalization, suggesting that receptor-mediated endocytosis, and phagocytosis are processes that independently contribute to MP uptake.

Our findings demonstrated that anti-inflammatory M2 macrophages take up more efficiently MP than pro-inflammatory M1 macrophages. Tumor-associated macrophages exhibit the tumor-promoting M2 phenotype rather than the tumor-suppressing M1 phenotype (32). In the early stage of tumor development, pro-inflammatory stimuli recruit monocytes and polarize them into M1 macrophages (33), which inhibit cancer progression and angiogenesis (34–36). However, in later stage, tumor cells induce the differentiation of M1 macrophages into M2 and in this way escape the immune system and support tumor progression (37, 38). The efficient uptake of MP by M2 macrophages accompanied by increase production of CCL22 and CD209, which recruit regulatory T cells and dendritic cells into cancer tissue (39, 40), enhanced the M2 immune activity and can be used to target macrophages involved in cancer progression. Such activity of MP could be further enhanced by loading MP with anti-cancer drugs. Despite the low uptake by M1 macrophages, MP regulated inflammation, and induced the expression of tumor inhibitory cytokines IL1-β and TNFα (34–36), indicating that M1 macrophages may be a potential target for MP therapy in inflammatory diseases and cancer.

Moreover, we demonstrated that MP promoted both pro- and anti-inflammatory effects on macrophages and HUVEC under different stimuli. We speculate that MP contain both pro- and anti-inflammatory proteins from MSC and induce gene expression changes in target cells and, subsequently, support the dynamic immunomodulatory activities of cell repair and regeneration. In one of our previous studies, we detected intact mRNA for VEGF, IL-8, and CD90 from the MSC on MP samples (unpublished data). In the other study, we found CD90 from MSC was upregulated on monocytes after interaction with MP (16). However, further studies are required to clarify the MP components.

Taken together, this study broadens our knowledge on the molecular pathways involved in the uptake of MP by macrophages and HUVEC and on the effects of MP uptake on cellular function. This knowledge can lead to the design of MP with target cell specificity under particular inflammatory conditions. MP thereby become a potential novel tool to modulate inflammatory responses in immune and degenerative diseases and cancer.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FC planned the research, performed experiments, and wrote the manuscript. SK performed experiments. MO, AM, and EL planned the research and reviewed the manuscript. CB and MR reviewed the manuscript. MH planned the research and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.651109/full#supplementary-material

Supplementary Figure 1 | Characterization of MP and PKH-MP. **(A)** Nanoparticle tracking analysis (NTA) profile of MP and PKH-MP. **(B)** The size distribution of MP and PKH-MP.

Supplementary Figure 2 | Macrophages and HUVEC viability after treating with selective inhibitors. Cells were preincubated for 30 min in complete medium containing Heparin (0.1–100 μg/ml), Dynasore (20–160 μM), Chlorpromazine (1–50 μM), Nystatin (5–40 μg/ml), Cytochalasin D (0.25–2 μM), or Wortmannin (0.1–10 μM). PBS and 0.1% DMSO were used as a control. Cell viability was measured by flow cytometer after 6 h using 7-AAD. Percentage of macrophage **(A)** and HUVEC **(B)** viability. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs.* vehicle.

Supplementary Figure 3 | Macrophage profile under different culture conditions. Macrophages were primed with 20 ng/ml IFNγ and 100 ng/ml LPS (inflammatory condition), or 20 ng/ml IL-4 and 20 ng/ml IL-13 (anti-inflammatory condition). **(A)** Photomicrographs showing characteristic morphologies of macrophages exposed to different culture conditions for 24 h (Magnification 100x). **(B)** mRNA expression of macrophages by real-time RT-PCR for CXCL10, CCR7, IL-1β, IL-10, TNFα, TGFβ, CCL22, and CD209 after treated with IFNγ + LPS or IL-4 + IL-13 for 72 h. Data are presented as mean ± SD from 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* medium control.

Supplementary Figure 4 | HUVEC profile under different culture conditions. HUVEC were primed with single or combined doses of 10 ng/ml TNFα and 50 ng/ml IFNγ and assessed by flow cytometer and real-time RT-PCR. **(A)** Photographs showing characteristic morphologies of HUVEC exposed to different culture conditions for 24 h (Magnification 100×). **(B)** HUVEC surface levels of HLA-I, HLA-II, CD40, and CD144 and **(C)** mRNA expression of HUVEC for IL-6, IL-8, and endothelin in the presence of single or combined doses of TNFα and IFNγ after 72 h. Data are presented as mean ± SD from 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* medium control.

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Conflict of Interest: MO and EL were employed by Takeda Madrid. Erasmus MC filed a patent on MSC derived MP (PCT/NL2017/050334).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Small Molecule Analysis of Extracellular Vesicles Produced by *Cryptococcus gattii*: Identification of a Tripeptide Controlling Cryptococcal Infection in an Invertebrate Host Model

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The small molecule (molecular mass <900 Daltons) composition of extracellular vesicles (EVs) produced by the pathogenic fungus *Cryptococcus gattii* is unknown, which limits the understanding of the functions of cryptococcal EVs. In this study, we analyzed the composition of small molecules in samples obtained from solid cultures of *C. gattii* by a combination of chromatographic and spectrometric approaches, and untargeted metabolomics. This analysis revealed previously unknown components of EVs, including small peptides with known biological functions in other models. The peptides found in *C. gattii* EVs had their chemical structure validated by chemical approaches and comparison with authentic standards, and their functions tested in a *Galleria mellonella* model of cryptococcal infection. One of the vesicular peptides (isoleucine-proline-isoleucine, Ile-Pro-Ile) improved the survival of *G. mellonella* lethally infected with *C. gattii* or *C. neoformans*. These results indicate that small molecules exported in EVs are biologically active in *Cryptococcus.* Our study is the first to characterize a fungal EV molecule inducing protection, pointing to an immunological potential of extracellular peptides produced by *C. gattii*.

Keywords: *Cryptococcus gatti*, extracellular vesicles, small molecules, mass spectrometry, *Galleria mellonella*

INTRODUCTION

Cryptococcus gattii is a fungal pathogen that causes disease in immunocompetent individuals. This fungus was responsible for outbreaks in the Pacific Northwest and in the Vancouver Island (1). *C. gattii* virulent strains, which are endemic in Brazil (2), likely emerged from South America (3). *C. gattii* can cause severe lung disease and death without dissemination. In contrast, its sibling species *C. neoformans* disseminates readily to the central nervous system (CNS) and causes death from meningoencephalitis (1). *C. gattii* and *C. neformans* share major virulence determinants, including the ability to produce extracellular vesicles (EVs) (4–6). EVs are membranous structures produced by prokaryotes and eukaryotes, including 14 fungal genera (7). In fungi, they were first

characterized in culture fluids of *C. neoformans* (6). A decade later, *C. gattii* was also demonstrated to produce EVs in liquid matrices (4).

The perception that EVs are essential players in both physiology and pathogenesis of fungi is now consolidated. Much of the knowledge on the functions of fungal EVs has derived from studies of their composition. During the last decade, proteins, lipids, glycans, and nucleic acids were characterized as components of fungal EVs (8, 9). Molecules of low molecular mass, however, have been overlooked. Two recent studies have characterized the small molecule composition of *Histoplasma capsulatum* (10) and *Penicillium digitatum* EVs (11), but the low molecular mass components of other fungal EVs are unknown. Considering the molecular diversity found in both *H. capsulatum* and *P. digitatum*, it is plausible to predict that many still unknown functions of EV components of low molecular mass remain to be characterized. In fact, the metabolome analysis of *P. digitatum* EVs revealed the presence of phytopathogenic molecules that inhibited the germination of the plant host's seeds (11).

We have recently described a protocol for the isolation of cryptococcal EVs through which the vesicles were obtained from solid fungal cultures (5). Although the general properties of fungal EVs obtained from solid cultures resembled those described for vesicles obtained from liquid media, a recent analysis of the protein composition of cryptococcal EVs obtained from solid medium revealed important differences in comparison to those obtained in early studies using liquid cultures (12, 13). This observation and the fact that culture conditions impact the composition of small molecules in *H. capsulatum* EVs (10) reinforce the importance of the compositional characterization of vesicles obtained from solid medium.

In this manuscript, we characterized the low mass components of EVs produced by *C. gattii*. The synthesis of some of the small molecules detected in the EVs revealed a vesicular peptide that protected an invertebrate host against a lethal challenge with *C. gattii* in a dose-dependent fashion. These results indicate the existence of new venues of exploration of the functions of EVs in fungal pathogens, and suggest that small molecules of fungal EVs have immunological potential.

RESULTS

Small Molecule Characterization of *C. gattii* EVs

C. gatti EV samples (**Figures 1A,B**) were prepared as independent triplicates. EV extracts were analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), and the data submitted to molecular networking analysis in the Global Natural Product Social Molecular Networking (GNPS) platform, an interactive online small molecule–focused tandem mass spectrometry data curation and analysis infrastructure (14). Molecular networking using high-resolution MS/MS spectra allows the organization of vesicular compounds in a visual representation (15, 16). In this analysis, each node is labeled by a precursor mass and represents a MS/MS spectrum of a compound, and compounds of the same molecular family are grouped together, connected by arrows, forming clusters of similarity (15–18). Since the molecules can be identified in a database through their fragmentation patterns and are represented in the molecular networking, the benefits of this approach include fast dereplication, identification of similar compounds, and effortless comparisons between different metabolic profiles or conditions (16, 17).

The cluster-based molecular networking analysis revealed secondary metabolites present in the *C. gattii* EVs. The molecules detected in our analysis were classified as EV components if they were detected in the three replicates. Using this criterion, our small molecule analysis identified 13 genuine components of the *C. gattii* EV samples (**Table 1**). This analysis revealed previously unknown components of EVs, including peptides, amino-acids, vitamins, and a carboxylic ester. The metabolites were identified through hits in the GNPS database (**Supplementary Figures 1–13**) and corresponded to Ile-Pro-Ile (*m/z* 342.2384), Phe-Pro (*m/z* 263.1387), pyro Glu-Ile (*m/z* 243.1335), pyro Glu-Pro (*m/z* 227.1022), Leu-Pro (*m/z* 229.1544), pyro Glu-Phe (*m/z* 277.1180), Val-Leu-Pro-Val-Pro (*m/z* 652.4025), cyclo (Trp-Pro) (*m/z* 284.1393), cyclo (Tyr-Pro) (*m/z* 261.1234), tryptophan (*m/z* 205.0972), asperphenamate (*m/z* 507.2278), riboflavin (*m/z* 377.1456), and pantothenic acid (*m/z* 220.1181). The structures and MS data of the detected metabolites are shown in **Figure 1C**, **Table 1**, respectively. The cluster-based molecular networking analysis of the *C. gattii* EV components is detailed in **Figure 2**.

For validation of some key GNPS hits, we performed another round of spectrometric characterization of *C. gattii* small molecules including additional criteria as follows. We classified as authentic EV compounds those whose structure was observed in EV extracts, but not in mock samples (extracted from sterile culture medium). Finally, these compounds obligatorily had chromatographic and spectrometric properties similar to those of synthetic standards. Due to the easiness in chemical synthesis and lack of detailed information in the literature, the linear dipeptides Phe-Pro, pyro-Glu-Ile, pyro-Glu-Pro, Leu-Pro, and pyro-Glu-Phe, and the tripeptide Ile-Pro-Ile were selected for the validation assays. We then searched for their presence in EV and mock extracts. The six peptides selected for chemical synthesis were classified as authentic EV components according to these criteria (**Table 2**). Indeed, this analysis revealed similar fragmentation patterns and retention times for the vesicle peptides and the standard metabolites (**Figure 3**). The peptides exhibited typical fragments of protonated amino acids at *m/z* 70.06, 86.09, 116.07, and 120.08 (**Supplementary Figure 14**). In compounds containing proline, fragments at *m/z* 116.07 and 70.06 corresponded, respectively, to the loss of protonated proline and subsequent loss of H_2O and CO. In peptides composed by isoleucine or leucine, fragments at *m/z* 132.02 and 86.09 corresponded, respectively, to protonated leucine/isoleucine and subsequent loss of H_2O and CO. Finally, the loss of H_2O and CO in protonated phenylalanine formed the major fragment ion at *m/z* 120.08 (19). Assuming that the vesicular components are synthesized within the cells and exported extracellularly, we also analyzed cellular and supernatant extracts. The six peptides listed in **Table 2** were

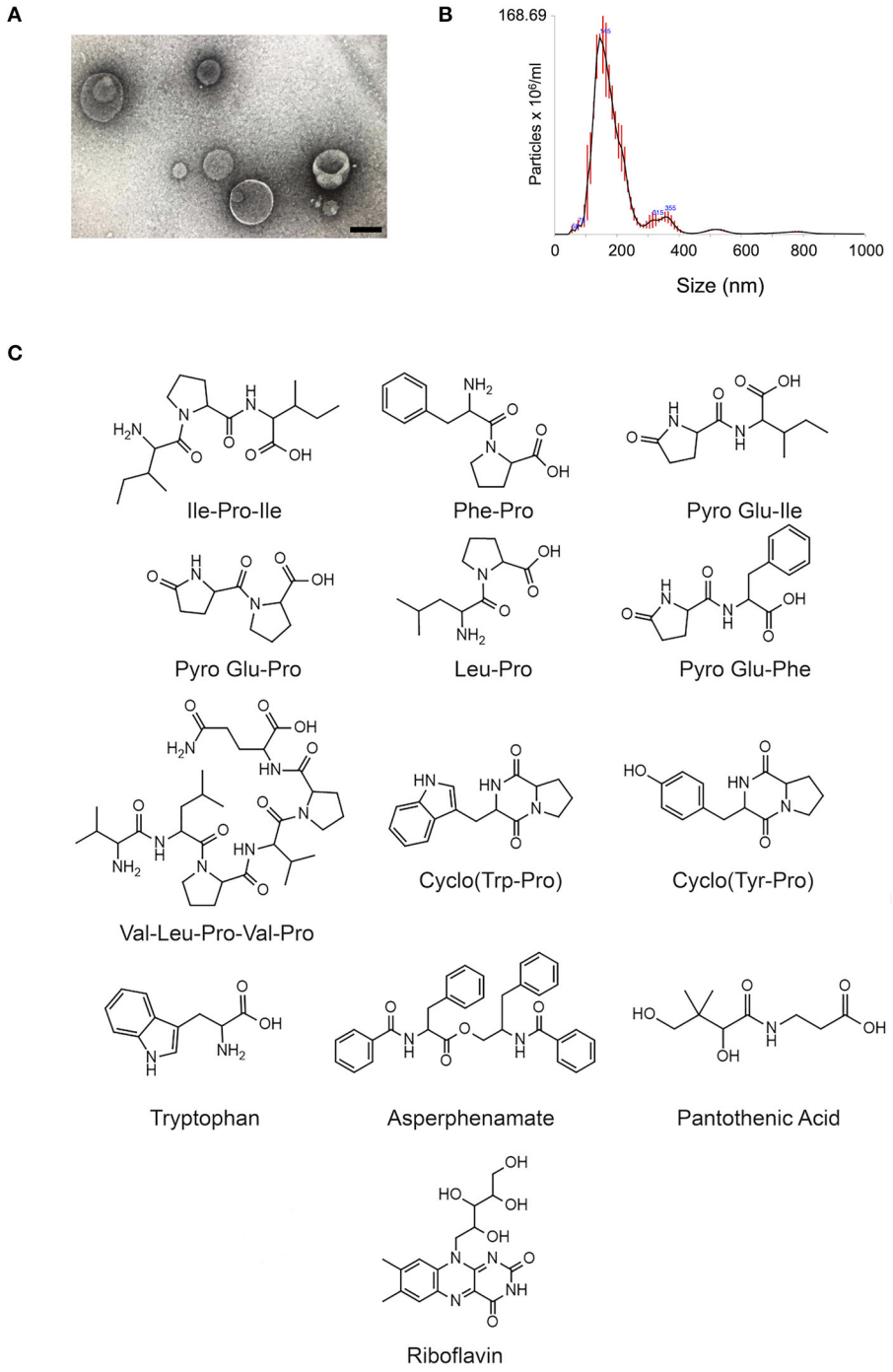

FIGURE 1 | Small molecule characterization in EVs produced by *C. gattii*. **(A)**. Transmission electron microscopy analysis (negative staining) of a *C. gattii* EV sample submitted to chemical characterization by mass spectrometry. Scale bar, 100 nm. **(B)**. Nanoparticle tracking analysis of EVs obtained from *C. gattii* cultures, showing the typical distribution of EVs in the 50–250 nm range, and a minor population in the 300–400 nm range. EVs shown in **(A,B)** illustrate the characteristics found in three independent samples with similar results. **(C)**. Structures of the metabolites identified in *C. gattii* EVs through the GNPS MS/MS database. Amino acid codes represent isoleucine (Ile), proline (Pro), phenylalanine (Phe), glutamic acid (Glu), leucine (Leu), valine (Val), tryptophan (Trp), and tyrosine (Tyr).

also found in these extracts (data not shown). These results were highly reproducible. Of note, the analysis of the presence of the peptides listed in **Table 2** was performed independently by two laboratory members in 12 EV samples produced by three different strains of *C. gattii*. All six peptides were found in all assays (data not shown).

TABLE 1 | MS data obtained for *Cryptococcus gattii* secondary metabolites detected on EVs.

Compound	Ion formula	Calculated *m/z*	Experimental *m/z*	Error (ppm)
Ile-Pro-Ile (Diprotin A)	$C_{17}H_{32}N_3O_4$	342.2392	342.2384	−1.1
Phe-Pro	$C_{14}H_{19}N_2O_3$	263.1395	263.1387	−1.3
Pyro-Glu-Ile	$C_{11}H_{19}N_2O_4$	243.1344	243.1335	−1.8
Pyro-Glu-Pro	$C_{10}H_{15}N_2O_4$	227.1031	227.1022	−1.7
Leu-Pro	$C_{11}H_{21}N_2O_3$	229.1552	229.1544	−1.4
Pyro-Glu-Phe	$C_{14}H_{17}N_2O_4$	277.1188	277.1180	−1.0
Val-Leu-Pro-Val-Pro	$C_{31}H_{54}N_7O_8$	652.4033	652.4025	−1.2
Cyclo(Trp-Pro)	$C_{16}H_{18}N_3O_2$	284.1399	284.1393	−2.1
Cyclo(Tyr-Pro)	$C_{14}H_{17}N_2O_3$	261.1239	261.1234	−1.9
Tryptophan	$C_{11}H_{13}N_2O_2$	205.0977	205.0972	−2.4
Asperphenamate	$C_{32}H_{31}N_2O_4$	507.2283	507.2278	−1.0
Riboflavin	$C_{17}H_{21}N_4O_6$	377.1461	377.1456	−1.3
Pantothenic acid	$C_9H_{18}NO_5$	220.1184	220.1181	−1.3

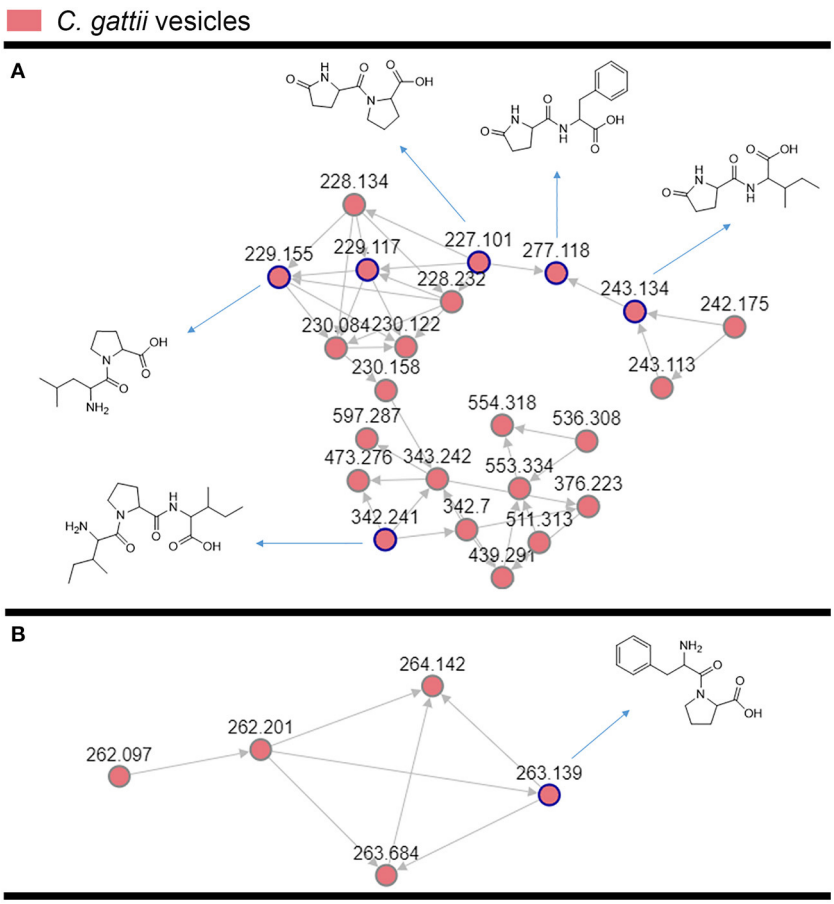

FIGURE 2 | Molecular networking visualization of the peptides identified in the *C. gattii* EV cargo. Clusters **(A,B)** were obtained through molecular networking analysis of the components of *C. gattii* EVs, which were obtained after organic extraction of secondary metabolites. Each node is represented as a compound's MS/MS spectrum. Blue nodes are represented as spectra of identified constituents obtained by comparison with the GNPS platform database. The peptides identified in the GNPS platform had their identity further confirmed by comparison with synthetic standards. The gray nodes represent the spectra of their unknown analogs with similar fragmentation patterns.

TABLE 2 | Chromatographic identification of peptides in cryptococcal EVs*.

Peptide	Control	Mock	EVs	Synthetic standards
Ile-Pro-Ile	NF	NF	4.03	3.76
Phe-Pro	NF	NF	3.18 and 3.64	3.28 and 3.62
Pyro-Glu-Ile	NF	NF	3.55	3.53
Pyro-Glu-Pro	NF	NF	1.8	1.8
Leu-Pro	NF	NF	2.83	2.78
Pyro-Glu-Phe	NF	NF	4	4

Sample (retention time, min).

**Peptide identification was performed in blank samples (control) in addition to preparations obtained from sterile medium (mock) or fungal EVs. The results were compared to those obtained with synthetic peptides. NF, not found.*

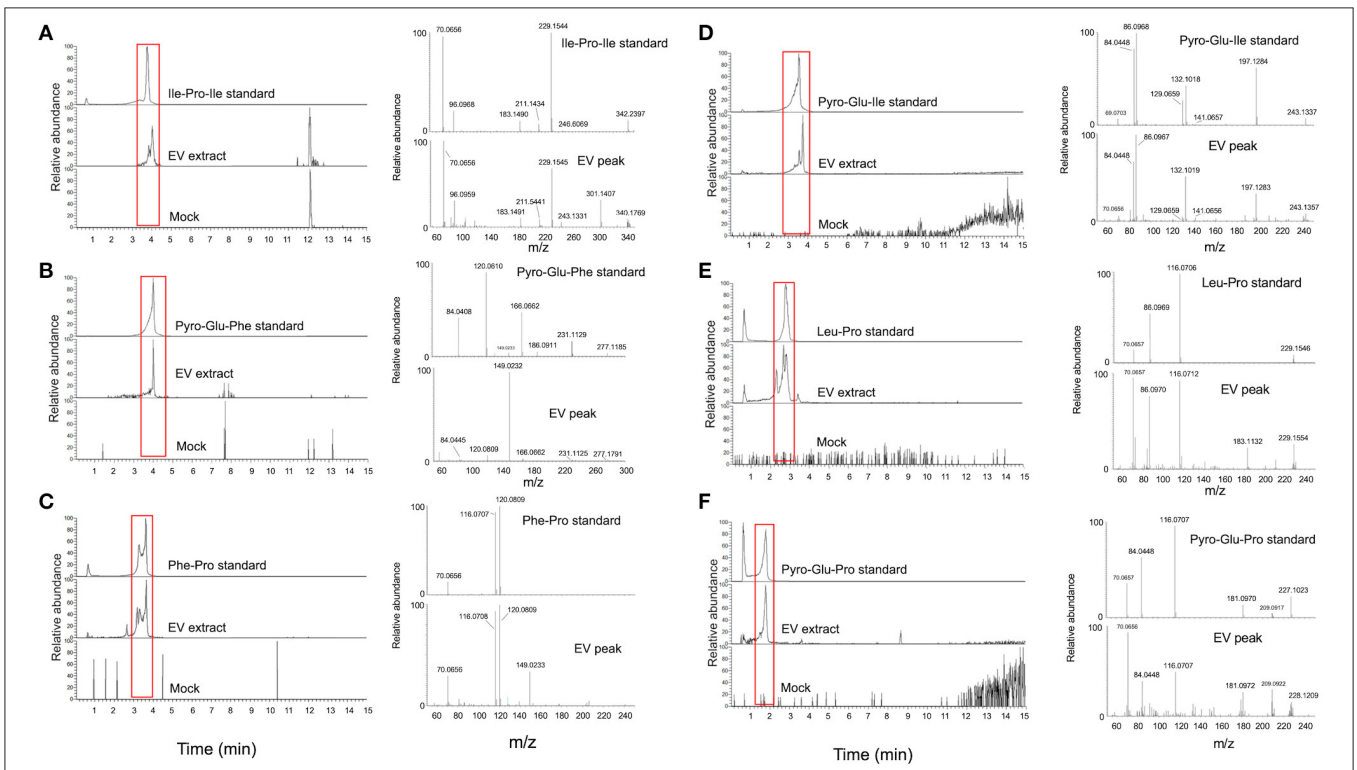

FIGURE 3 | Structural analysis of EV peptides produced by *C. gattii*, including Ile-Pro-Ile **(A)**, pyro-Glu-Phe **(B)**, Phe-Pro **(C)**, pyro-Glu-Ile **(D)**, Leu-Pro **(E)**, and pyro-Glu-Pro **(F)**. For each peptide, the chromatographic separation of synthetic standards, EV extracts, and control (mock) samples is presented on the left side of each panel. The peaks with retention times similar to the corresponding standards (red boxed area) were selected for fragmentation by mass spectrometry (MS). The MS fragmentation profiles are shown on the right side of each panel. These analyses confirmed that the structural match between the EV components and the synthetic standards.

Biological Activity of EV Peptides of *C. gattii*

After characterization of Ile-Pro-Ile, Phe-Pro, pyro-Glu-Ile, pyro-Glu-Pro, Leu-Pro, and pyro-Glu-Phe as authentic EV components of *C. gattii*, we used their synthetic forms to analyze their possible biological activities. On the basis of the previously reported ability of fungal peptides to kill bacteria (20), we initially tested their antibacterial capacity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. None of the peptides had any effect on microbial growth (data not shown). Since cryptococcal EVs regulate intercellular communication (4), we also speculated that the peptides could mediate quorum sensing, Titan cell formation, or capsule growth. Once again, none of the peptides had any apparent effects on these processes in *C. gattii* (data not shown).

It has been recently reported that fungal EVs, including cryptococcal vesicles, protect mice and the invertebrate host *Galleria mellonella* against lethal challenges with pathogenic fungi (12, 21–23). The vesicular molecules responsible for the protection remained unknown. We then asked whether the peptides listed in **Table 2** could protect *G. mellonella* against

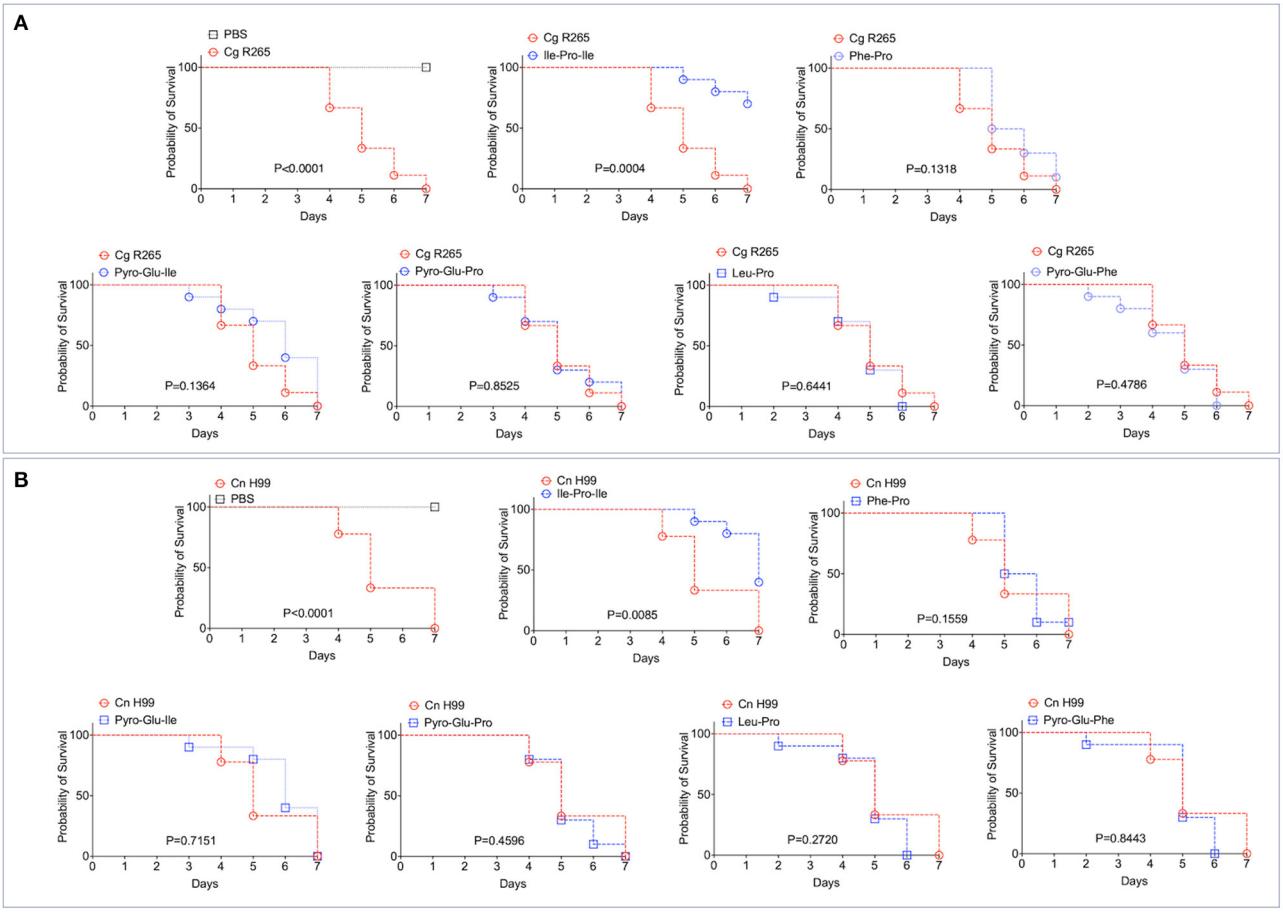

FIGURE 4 | Effects of the EV peptides (1 μg/μl, equivalent to 10 μg per animal) on the survival of *G. mellonella* lethally infected with *C. gattii* R265 (Cg; **A**) or *C. neoformans* H99 (Cn; **B**). A. Ile-Pro-Ile was the only peptide prolonging the survival of *G. mellonella*. The other peptides did not interfere with the host's survival. The experiment illustrated in **(A)** was repeated using *C. neoformans* H99 instead of *C. gattii* R265, producing similar results. *P*-values resulting from the comparison of the survival curves were obtained using Log-rank Mantel-Cox test with the Graphpad Prism software, version 9.0. *P*-values lower than 0.05 represented significant statistical differences. Survival controls were obtained through injection of *G. mellonella* with PBS alone.

a lethal challenge with *C. gattii*. We compared the mortality curves of *G. mellonella* infected with *C. gattii* alone with the mortality of the invertebrate host receiving *C. gattii* and each of the peptides at 1 μg/μl (equivalent to 10 μg per animal; **Figure 4A**). Phe-Pro, pyro-Glu-Ile, pyro-Glu-Pro, Leu-Pro, and pyro-Glu-Phe did not have any effect on the survival curves. In contrast, the tripeptide Ile-Pro-Ile significantly improved the survival of *G. mellonella*. We performed this experiment using *C. neoformans* instead of *C. gattii* and obtained similar results (**Figure 4B**). On the basis of these results, we selected Ile-Pro-Ile for tests at lower concentrations (1, 0.5, and 0.1 μg/μl, equivalent to 10, 5, and 1 μg per animal) in the *C. gattii* infection model. Once again, the peptide was highly efficient in prolonging the survival of lethally infected *G. mellonella* in a dose-dependent fashion (**Figure 5A**). The improved survival of lethally infected *G. mellonella* was accompanied by a significant reduction in the fungal burden, as concluded by counting colony forming units (CFU) in peptide-treated (10 μg per animal) and untreated larvae at 3- and 5-days post infection (**Figure 5B**).

DISCUSSION

The knowledge of the functions of fungal EVs has continuously increased in the recent years (7), but the biological roles of low mass structures exported in EVs are unknown. Small molecules secreted by *Cryptococcus* are immunologically active and affect IL-1β inflammasome-dependent secretion (24), but their association with EVs has not been established. In our study, we aimed at proving the concept that biologically active small molecules are exported in cryptococcal EVs. This idea culminated with the characterization for the first time of a fungal EV molecule inducing protection against pathogenic fungi.

Fungal EVs were demonstrated to mediate intercellular communication (4), prion transmission (25), biofilm formation associated with antifungal resistance (26), immunological responses *in vitro* (23, 27–30), and protection of different hosts against lethal challenges with fungal pathogens (12, 21–23). In any of these examples, these biological effects attributed to the EVs were correlated with the identification of the

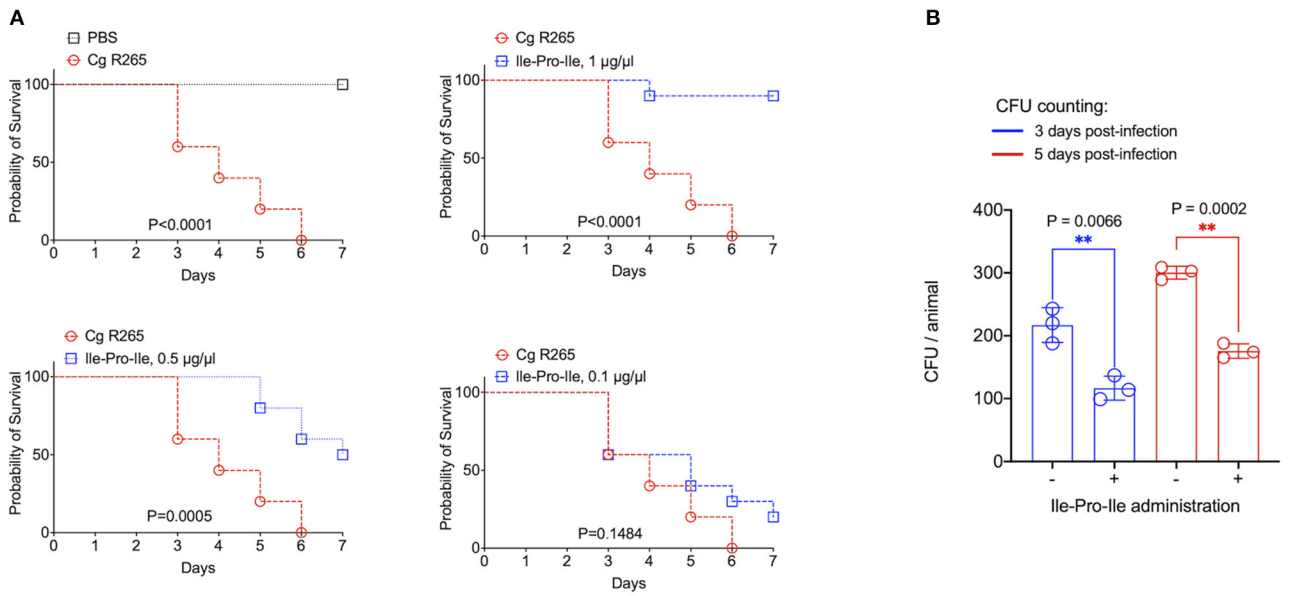

FIGURE 5 | Protection of *G. mellonella* against *C. gattii* (Cg) induced by Ile-Pro-Ile. **(A)**. Survival of *G. mellonella* after injection with PBS alone (survival control) or with *C. gattii* yeast cells (left panel) is shown, in addition to the comparative survival curves of *G. mellonella* after injection with *C. gattii* alone (red curves) or with the fungus in the presence of variable concentrations of Ile-Pro-Ile. *P*-values resulting from the comparison of the survival curves were obtained using Log-rank Mantel-Cox test with the Graphpad Prism software, version 9.0. *P*-values lower than 0.05 represented significant statistical differences. **(B)**. Determination of fungal burden in infected larvae. Untreated and Ile-Pro-Ile-treated (1 μg/μl, equivalent to 10 μg per animal) infected larvae were macerated at days 3- and 5- post infection for CFU determination, which revealed a significantly decreased colonization of *G. mellonella* by *C. gattii* in peptide-treated systems (**). Paired statistical analysis after comparison between untreated and peptide-treated systems were performed using Student's *t*-test with the Graphpad Prism software, version 9.0.

bioactive vesicular molecules. The only known exception was the protection of *G. mellonella* induced by cryptococcal EVs enriched with sterol glycosides and capsular polysaccharides (22). However, it is important to mention that the EVs in this study were produced by genetically engineered cells and, therefore, did not correspond to native vesicles. It remained also unknown if other molecules influenced the protective effects, since compositional studies have not been performed.

The identification of bioactive EV molecules is challenging in multiple aspects. The compositional analysis of fungal EVs in different models include a formidable variability in culture conditions, since each of the fungal pathogens tested so far manifest growth particularities. In this scenario, biomarkers of fungal EVs are still not known, although it has been suggested that mannoproteins and claudin-like Sur7 family proteins are important components of vesicles produced by *C. neoformans* and *C. albicans*, respectively (12, 31). The knowledge of small molecules mediating important biological activities in fungal EVs is even more limited. In *H. capsulatum*, carbohydrate metabolites were abundantly detected in EVs, in addition to L-ornithine and ethanolamine, among other small molecules (10). Noteworthy, in the *H. capsulatum* study, the conditions used for small molecule identification differed from those used in our study. Under similar conditions, we identified a comparable number of molecules in the EVs produced by the plant pathogen *P. digitatum* (11) and in *C. gattii* (this study). Specifically, small peptides were found in the EVs of these two distant species, reinforcing the notion that this molecular class is present in different fungal EVs. As for the possible detection of these molecules as artifacts in the *C. gattii* model, it is noteworthy that all identified peptides manifested high solubility in water, being susceptible to efficient removal by washing if they were not contained within the EVs. On the basis of their short sequences (2-4 amino-acids), the possibility that they will form insoluble, tertiary structures that will co-precipitate with EVs during ultracentrifugation is negligible.

Fungal toxins were also identified in *P. digitatum*, but this class of molecules is not normally produced by members the *Cryptococcus* genus. In *P. digitatum*, EVs were characterized as the carriers of tryptoquialanine A, a toxin that inhibited the germination of orange seeds (11). So far, tryptoquialanine A is the only low mass component of fungal EVs with a reported function. In this model, the mycotoxin fungisporin was also detected (11), but its function in fungal EVs remains to be determined. Together, these findings illustrate the need for an improved knowledge of the composition and functions of EV metabolites in fungi.

The isolation of cryptococcal EVs from solid medium is much more efficient than the similar protocols using liquid cultures (5). RNA and proteins in cryptococcal EVs obtained in liquid cultures were characterized in early studies (8, 32), but their distribution in EVs obtained from solid medium was only recently described in *C. neoformans* (12). Other molecules remained unknown, and the metabolite composition

of cryptococcal EVs has not been investigated so far. In our study, we initially aimed at understanding what are the low molecular weight components exported by *C. gattii* in solid medium. We identified small molecules of different chemical natures as putative components of cryptococcal EVs, but their functions remain widely unknown. However, our chemical and biological methods for structural validation revealed that one tripeptide was capable to protect *G. mellonella* against lethal challenges with *C. gattii* or *C. neoformans*. The mechanisms by which the peptides induced protection against cryptococcal infection remain unknown, but the immune response of *G. mellonella* is innate and relies on the activity of hemocytes in combination with antimicrobial peptides and lytic enzymes, among others (33). Accordingly, immunity to *Cryptococcus* relies on innate immune cells coordinating adaptive responses to stimulate fungal killing (34). Therefore, we hypothesize that the tripeptide identified in our study is an inducer of innate responses, which have a key general role in the control of fungal infections (35). Other possibilities, however, cannot be ruled out, as follows below.

The peptide inducing protection against *Cryptococcus* in *G. mellonella* was demonstrated to have important biological activities in other models. Ile-Pro-Ile, also known as diprotin A, is an inhibitor of dipeptidyl peptidase 4, an enzyme participating in insulin metabolism (36) and chemotaxis of murine embryonic stem cells toward stromal cell-derived factor-1 (37). Its role in fungal physiology and/or pathogenesis was also suggested. In *Aspergillus fumigatus*, a dipeptidyl peptidase 4 was purified from fungal cultures and a role in binding to collagen and activation of CD4+ T cells was speculated (38). It was also reported that *Blastomyces dermatitidis* produces dipeptidyl peptidase 4. In this model, the enzyme was responsible for disabling innate immunity mechanisms and promoting pathogenicity (39). If a similar mechanism is functional in the *Cryptococcus* model, free Ile-Pro-Ile administered in the *G. mellonella* infection model could inhibit the fungal dipeptidyl peptidase 4 with a consequently decreased pathogenicity. Noteworthy, our study did not elucidate any physiological or pathogenic functions. Instead, we present a proof of concept that fungal EVs are the vehicles for exporting biologically active molecules of low molecular mass that may be involved in immunological and/or pathogenic mechanisms. Since fungal EVs have been consistently proposed as vaccine candidates in different models, the potential of these findings can be substantial.

METHODS

Preparation of EVs

The EV-producing isolate used in this study was the standard strain R265 of *C. gattii*. Of note, the R265 strain has been recently reclassified as *C. deuterogattii* (40). In this study, we kept its classification as *C. gattii*, as largely employed in the *Cryptococcus* literature. EV isolation was based on the protocol that we have recently established for *C. gattii* and other fungal species (5). Briefly, One colony of *C. gattii* R265 cultivated in solid Sabouraud's medium was inoculated into yeast extract-peptone-dextrose (YPD) medium (5 ml) and cultivated for 1 day at 30°C with shaking. The cell density was adjusted to of 3.5×10^7 cells/ml in YPD. From this suspension, aliquots of 300 µl were taken for inoculation in YPD agar plates, which were cultivated for 1 day at 30°C. The cells were recovered from the plates with an inoculation loop and transferred to a single centrifuge tube containing 30 ml of PBS filtered through 0.22-µm-pore membranes. The cells were then removed by centrifugation (5,000 × g for 15 min at 4°C), and the supernatants were centrifuged again (15,000 × g for 15 min at 4°C) to remove debris. The resulting supernatants were filtered through 0.45-µm-pore syringe filters and again centrifuged (100,000 × g, 1 h at 4°C). Supernatants were discarded and pellets suspended in 300 µl of sterile PBS. To avoid the characterization of medium components as EV molecules, mock (control) samples were similarly prepared using sterile plates containing YPD. Four petri dishes were used for each EV isolation, and EV isolation was performed independently three times. In all samples, the properties of EVs and their concentration were monitored by nanoparticle tracking analysis (NTA) and transmission electron microscopy as described by our group (5). The samples prepared for mass spectrometry analyses had the typical properties of *C. gattii* EVs, and were in the range of $4–6 \times 10^{10}$ EVs within the triplicate set.

Mass Spectrometry Analyses

C. gattii EVs were vacuum dried and extracted with 1 ml of methanol during 1 h in an ultrasonic bath. The extracts were filtered (0.22 µm), dried under a gentle N_2 flux and stored at −20°C. EV extracts were resuspended in 200 µl of MeOH and transferred into glass vials. Ultra high-performance liquid chromatography-mass spectrometry (UHPLC-MS) analyses were performed using a Thermo Scientific QExactive® hybrid Quadrupole-Orbitrap mass spectrometer with the following parameters: electrospray ionization in positive mode, capillary voltage at 3.5 kV; capillary temperature at 300°C; S-lens of 50 V and *m/z* range of 100.00–1500.00. Tandem Mass spectrometry (MS/MS) was performed using normalized collision energy (NCE) of 20, 30, and 40 eV; maximum 5 precursors per cycle were selected. Stationary phase was a Waters ACQUITY UPLC® BEH C18 1.7 µm (2.1 × 50 mm) column. Mobile phases were 0.1% (v/v) formic acid in water (A) and acetonitrile (B). Eluent profile (A:B) 0–10 min, gradient from 95:5 up to 2:98; held for 5 min; 15–16.2 min gradient up to 95:5; held for 3.8 min. Flow rate was 0.2 mL min^{-1}. Injection volume was 3 µL. UHPLC-MS operation and spectra analyses were performed using Xcalibur software (version 3.0.63).

Molecular Network

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data was filtered by removing all MS/MS fragment ions within ±17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the ±50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine

score above 0.5 and more than 5 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.5 and at least 5 matched peaks (14).

Peptides

The peptides selected for biological tests were synthesized by GenOne Biotechnologies (https://www.genone.com.br, Rio de Janeiro, Brazil). Purity and structural properties of each peptide were confirmed by high-performance liquid chromatography coupled to mass spectrometry. All peptides were water-soluble and had their purity at the 95% range.

Galleria mellonella Infection Model

Groups of 10 larvae (250–350 mg) were used for injection into the last left proleg using a Hamilton micro-syringe. The injection systems (10 μl) consisted of sterile PBS alone, sterile PBS containing 10^6 cells of *C. gattii* or *C. neoformans*, or sterile PBS containing *C. gattii* or *C. neoformans* and Ile-Pro-Ile, Phe-Pro, pyro-Glu-Ile, pyro-Glu-Pro, Leu-Pro, and pyro-Glu-Phe at 1 μg/μl (equivalent to 10 μg per animal). Due to its promising effects, Ile-Pro-Ile was also tested at 0.5, and 0.1 μg/μl (equivalent to 5 μg and 1 per animal) in a *C. gattii* model of infection. Injected larvae were placed in sterile Petri dishes and incubated at 37°C. The survival was monitored daily in a period of 7 days. Larvae were considered dead if they did not respond to physical stimulus. Statistical analysis in the survival curves was performed using the Log-rank Mantel-Cox test with the Graphpad Prism software, version 9.0. Infected larvae were also used for determination of fungal burden. Additional experimental sets were prepared as described for survival curves, but the experiments were interrupted at days 3- and 5- post infection. Ile-Pro-Ile concentration in these assays corresponded to 1 μg/μl. Surviving larvae ($n = 5$ at day 3-post infection; $n = 4$ at day 5-post infection) were macerated in 3 ml PBS and 100 μl were plated onto Sabouraud agar plates supplemented with 1% penicillin and streptomycin. The plates were incubated at 30°C for 48 h for further CFU counting. Paired statistical analysis after comparison between untreated and peptide-treated systems were performed using Student's *t*-test with the Graphpad Prism software, version 9.0.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FR, JC, and LH performed the experiments. LN, TF, and MR raised funds, interpreted the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.654574/full#supplementary-material

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Proteomic Characterization, Biodistribution, and Functional Studies of Immune-Therapeutic Exosomes: Implications for Inflammatory Lung Diseases

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Dendritic cell (DC)-derived exosomes (DC EXO), natural nanoparticles of endosomal origin, are under intense scrutiny in clinical trials for various inflammatory diseases. DC EXO are eobiotic, meaning they are well-tolerated by the host; moreover, they can be custom-tailored for immune-regulatory or -stimulatory functions, thus presenting attractive opportunities for immune therapy. Previously we documented the efficacy of immunoregulatory DCs EXO (regDCs EXO) as immunotherapy for inflammatory bone disease, in an *in-vivo* model. We showed a key role for encapsulated TGFβ1 in promoting a bone sparing immune response. However, the on- and off-target effects of these therapeutic regDC EXO and how target signaling in acceptor cells is activated is unclear. In the present report, therapeutic regDC EXO were analyzed by high throughput proteomics, with non-therapeutic EXO from immature DCs and mature DCs as controls, to identify shared and distinct proteins and potential off-target proteins, as corroborated by immunoblot. The predominant expression in regDC EXO of immunoregulatory proteins as well as proteins involved in trafficking from the circulation to peripheral tissues, cell surface binding, and transmigration, prompted us to investigate how these DC EXO are biodistributed to major organs after intravenous injection. Live animal imaging showed preferential accumulation of regDCs EXO in the lungs, followed by spleen and liver tissue. In addition, TGFβ1 in regDCs EXO sustained downstream signaling in acceptor DCs. Blocking experiments suggested that sustaining TGFβ1 signaling require initial interaction of regDCs EXO with TGFβ1R followed by internalization of regDCs EXO with TGFβ1-TGFβ1R complex. Finally, these regDCs EXO that contain immunoregulatory cargo and

showed biodistribution to lungs could downregulate the main severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) target receptor, ACE2 on recipient lung parenchymal cells *via* TGFβ1 *in-vitro.* In conclusion, these results in mice may have important immunotherapeutic implications for lung inflammatory disorders.

Keywords: dendritic cells, exosomes, lung diseases, immune therapy, COVID-19

INTRODUCTION

Exosomes (EXO) are nanoparticles of endosomal origin secreted by all cells, including dendritic cells (DCs), the most potent antigen presenting cells and "directors" of immune response (1). EXO contain proteins, nucleic acids and lipid cargo that mediate intercellular communication and signaling. They are secreted into tissues and body fluids and can act locally or from at a distance (2). In addition, EXO have desirable traits as a drug delivery system, which includes their small size (30-150 nm), their low clearance from target tissues and cargo preservation (3–6). DC-derived EXO are already being used to deliver molecular cargo to promote cytotoxic T cell responses for cancer (7) or inhibit effector T cell responses and hyperinflammation in autoimmune/inflammatory diseases (8).

We have previously reported important aspects of the immunobiology and functions of DC-derived EXO subtypes, isolated from DCs at distinct stages of maturation. Most notably among these subtypes are immune-regulatory (regDCs EXO), loaded with TGFβ1 and IL10 and deficient in costimulatory molecules. These have been shown to "reprogram" recipient DCs and CD4+ T cells towards an immune-regulatory response *in vitro* and *in vivo*. Immature or immune-null DC exo, (iDCs EXO) and EXO from matured DCs, called immune-stimulatory (stimDCs EXO), were also characterized for immune functions (5). The stability of these EXO, their ability to protect their cargo, and be retained at inflamed mucosal sites and inhibit inflammatory bone loss has also been documented. Other groups have had similar success with DC-derived EXO loaded with immune-regulatory cargo in various disease states such as inflammatory colitis and other inflammatory disease states (9–11). Currently lacking however, is a more in-depth characterization of the proteomic cargo of therapeutic DC EXO and their biodistribution to different body organs, needed to interpret the on-target and off-target effects of such immune regulatory nanoparticles. In addition, how regDCs EXO modulate cytokine signaling in recipient cells (5), needs further investigations.

TGFβ1 is a master regulator of the immune response (12, 13). TGFβ1 is a pleiotropic cytokine that, at high levels activates SMAD2/3, inhibits DC maturation, and suppresses effector Th1 and Th2, and Th17 cells, thereby promoting anti-inflammatory FoxP3+ T-regulatory cells. Moreover, TGFβ1 may have other therapeutic advantages in fatal infectious diseases such as COVID-19, by inhibiting one of the SARS-CoV-2 attachments and point of entry, like the ACE2 receptor (14–16).

SARS-CoV-2 is a coronavirus that gains entry primarily *via* the mucosal respiratory tract and is the etiologic agent of the COVID-19 pandemic (17). Symptoms of SARS-CoV-2 infection can vary greatly, depending on host factors, from asymptomatic infection to a severe and intense hyperinflammatory state creating multiorgan failure, especially in the respiratory tract (18). The severe inflammatory disease called acute respiratory distress syndrome (ARDS) is one of the leading causes of death in COVID-19 patients (19–21). An exaggerated immune/inflammatory response, due to release of pro-inflammatory cytokines, i.e. the "cytokine storm", is a main characteristic of ARDS in COVID-19 patients, and is responsible for producing severe damage to numerous organs including the lung tissue and frequently death (22, 23). One of the main entry points for SARS-CoV-2 invasion is *via* its structural proteins such as spike (S) and others, *via* the ACE2 expressing cells (24), however the ability to regulate ACE2 using DC-derived EXO is unclear. Several immunotherapeutic approaches to regulate the inflammatory process or block ACE2 have been proposed, however the data on efficacy and the associated adverse effects are contradictory (25, 26).

The purpose of the present murine study was 4-fold: 1. To characterize, in-depth, the proteomic cargo of immune therapeutic and non-therapeutic DC EXO subtypes, to validate on-target functions and potential off-target effects; 2. To track, in live mice, the biodistribution patterns of intravenously injected therapeutic regDCs EXO into major organs, including the lungs *in vivo*; 3. To reveal how TGFβ1 in regDCs EXO activates target cell signaling; and 4. To test the ability of putative recipient cells of SARS-CoV-2 to uptake regDC EXO, and thus influence ACE2 expression.

METHODS

Ethics Statement

The Institutional Animal Care and Use Committee (IACUC) of Augusta University (protocol # 2013-0586) approved all experimental procedures on C57BL/6 mice.

Generation of Dendritic Cell Subsets

DC subsets including immature, immune-stimulatory (mature) and immune-regulatory were generated as we previously described (5, 27). Briefly, bone marrow was isolated from tibias and femurs of 6- to 8-week-old mice. Contaminating erythrocytes were lysed by ACK cell lysing buffer (Invitrogen, Thermofisher scientific, and Columbia, SC, USA). Cells were cultured in complete media (RPMI 1640 containing 10% FBS and 100 IU/mL penicillin/streptomycin) containing 20 ng/ml of murine GM-CSF and IL-4 (Peprotech, Rocky Hill, NJ, USA). Culture media was changed

every 2 days and cells were re-incubated on day 6 in EXO depleted complete media to generate iDCs or in the presence of 1ug/ml LPS (Sigma, St. Louis, M.O., USA) to generate mature stimDCs for 48 hours. regDCs were generated by adding TGFβ1/IL10 recombinant cytokines on Day 5, in which 1×10^7 DCs were incubated for 2 hours with 1ug/ml TGFβ1 (R&D Systems, Inc. Minneapolis, MN) and 1 μg/mL of the recombinant murine IL-10 (Cell Sciences, Canton, Massachusetts) in a total volume of 1 mL serum-free media, then diluted 1:10 in fresh complete media for 24 h. This is followed by harvesting, washing and culturing for 48 h in EXO depleted growth media. Culture supernatants were collected for EXO isolation on day 8.

Phenotypic characterization of DCs subsets were defined by expression level of differentiation and maturation markers including CD11c+ (N418) (Invitrogen), MHCII (M5/114.15.2) (Milteny biotech Auburn, CA,USA) and CD86 (GL1) (Invitrogen), (Milteny biotech) using flow cytometry and by expression level of pro/anti-inflammatory cytokine mRNA by PCR, including IL6 (Mm00446190_m1), IL12 (Mm01288989_m1), IL23 (Mm00518984_m1), TGFβ1: Mm01178820_m1 and TNF: Mm00443258_m1, (Thermofisher Scientific). Phenotypic profile was as follows: regDCs : CD11c +, low MHCII+, low CD86+, low CD80 + and low CD40+, iDCs: CD11c+, intermediate MHCII+, intermediate CD86+, intermediate CD80+, intermediate IL6+, intermediate IL12+ and intermediate IL23+ and stimDCs: CD11c+, high MHCII+, high CD86+, high CD80+, high IL6+, high IL12+ and high IL23 (5).

Exosome Isolation and Purification

EXO isolation was performed as previously described (9). Briefly, culture supernatants were subjected to differential centrifugation (successive centrifugations at 500 g for (5 min), 2000g for (20 min), and 10,000 g for (30 min)) to eliminate cells and debris, followed by ultrafiltration 3x with 0.2 um and 3x with 100 kDA filters (to remove free proteins) and ultracentrifugation for 1.5 h at 120,000 g. To further remove excess free proteins, EXO pellets were washed with a large volume of PBS and ultra-centrifuged 2x at 120,000 g for 1.5 h, and finally re-suspended in 100 ul of PBS for further studies.

Cytokine Loading of Immunoregulatory Dendritic Cell Exosomes

To increase the concentration of immunoregulatory factors like TGFβ1 and IL10, 1 $x10^9$ particles of regDC EXO were actively loaded by sonication (3) with 5ug TGFβ1 and 5ug IL10 in 500 ul of PBS then filtered 3x by ultrafiltration with 100KDA filter to remove free proteins and washed 3x with large volume of PBS and ultra-centrifugation at 120,000 g for 1.5 h to further purify EXO from free molecules, and finally re-suspended in 100 ul of PBS. The supernatants of where regDCs EXO were incubated were isolated and checked for any contaminants of free TGFβ1 and IL10 by ELISA. It is important to mention that in our previous we have seen that TGFβ1 and IL10 were naturally loaded in regDCs EXO but their concentrations were very low. Thus, additional artificial loading was performed to achieve the desired immunoregulatory effect (5).

Characterization of Dendritic Cell-Derived Exosomes

DCs EXO subsets were characterized for their size distribution, particle number and shape using nanotracking analysis and TEM respectively, and for exosomal markers using Western blot (WB) as we previously showed. In brief, nanoparticle tracking analysis (NTA) was used to visualize and quantitate size and count of nanoparticles in suspension using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and software (ZetaView 8.02.28). For TEM, EXO samples were loaded onto a copper grid. After precipitation of EXO, the sample liquid was isolated, and counter stained for 10 minutes with 2% phosphotungstic acid solution and then placed under an incandescent lamp for 5 min. EXO sample was then analyzed with TEM. For WB analysis, EXO lysates were isolated to confirm principal EXO proteins using anti-TSG101 (MA1-23296), anti-Alix (MA1-83977), anti-CD63 (10628D) and GRP94 (MA3-016) from (Invitrogen, Thermofisher scientific West Columbia, SC, USA) as we showed previously (5).

Liquid Chromatography–Mass Spectrometry Analysis

Three biological replicates of regDCs, iDCs and stimDCs EXO samples were lyophilized to dryness. 100 ul of freshly prepared 50mM ammonium bicarbonate buffer with 0.1% acid labile detergent RapiGest SF Surfactant (Waters) was added to each sample to resuspend exosomes. This was followed by reduction with dithiothreitol, alkylation using iodoacetamide and digestion overnight using trypsin (Thermo Scientific #90057). Trifluoroacetic acid (TFA) was added to a final concentration of 0.1% to the digested sample, followed by incubation at 37°C for 40 minutes. Peptide digests were analyzed on an Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific) coupled with an Ultimate 3000 nano-UPLC system (Thermo Scientific). Two microliters of reconstituted peptide were first trapped and washed on a Pepmap100 C18 trap (5um, 0.3X5mm) at 20ul/min using 2% acetonitrile in water (with 0.1% formic acid) for 10 minutes and then separated on a Pepman 100 RSLC C18 column (2.0 um, 75-μm × 150-mm) using a gradient of 2 to 40% acetonitrile with 0.1% formic acid over 40 min at a flow rate of 300nl/min and a column temperature of 40°C. Analysis of DCs EXO samples were then performed by data-dependent acquisition in positive mode using Orbitrap MS analyzer for precursor scan at 120,000 FWHM from 300 to 1500 m/z and ion-trap MS analyzer for MS/MS scans in top speed mode. Collision-induced dissociation (CID) was used as fragmentation method. Raw data were processed using Proteome Discoverer (v1.4, Thermo Scientific) and submitted for SequestHT search against database of Uniport. Fixed value Peptide spectrum matching (PSM) validator algorithm was used for peptide spectrum matching validation. SequestHT search parameters were 10 ppm precursor and 0.6 Da product ion tolerance, with static Carbamidomethylation (+57.021 Da).

Bioinformatics Analysis

Clustered heat map of the expression profiles of the differentially expressed overlapped proteins in DCs EXO subtypes was conducted by ClastVist software (28). All proteins that showed a fold-change of at least 1.5 and satisfied $p < 0.05$ were differentially expressed. The database of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were performed using Web Gestalt software (29) to categorize exclusive, shared, differentially, and non-differentially expressed proteins in DCs EXO subsets. Functional categories and pathways with a corrected $p < 0.05$ were considered as significant. Percentage and significant level of DCs EXO subsets proteins that are related to the identified pathways were then identified.

In Vivo Imaging of Immunoregulatory Dendritic Cell Exosomes and Biodistribution

In Vivo Imaging

1.5 to 2 mCi of Indium-111-Oxine (AnazaoHealth Corporation, Tampa, FL, USA) in PBS was incubated with 200 µl of exosomes particles (~2 x 10^9 particles) at 37°C for 20 minutes. Free Indium-111-Oxine was removed by repeated PBS washes through an Amicon ultrafiltration device. Isolated Indium-111-Oxine-labeled EXO were diluted to 200 µCi of radioactivity per dose and injected intravenously *via* tail vein. Control animals received an injection of equivalent activity of free Indium-111-Oxine. Whole body and head single photon emission spectroscopy (SPECT) images were acquired by Mediso's nanoScan microSPECT/CT system (Mediso, USA) at 3 and 24 h after injection. Images were reconstructed and imaging software was used to calculate radioactivity in the major organs and lymph nodes, as a percentage of total radioactivity (whole body). Afterwards, organs were excised and weighed after the last time point and *ex vivo* radioactivity measurements were performed by gamma counter (Perkin-Elmer Packard Cobra II Auto-Gamma) and expressed per mg wet weight (30).

Exosome Uptake *In Vitro*

For EXO uptake study *in vitro*, EXO labeled with Dil (D282, Thermofisher Scientific) were co-cultured with DCs or mouse primary tracheal/bronchial respiratory epithelium cells (PTBECs) (C57-6033, Cell Biologics, USA) for 24 h. Cells were fixed and stained on glass slides with Alex flour 647 phalloidin (A22287) and DAPI (D1306) (Invitrogen, Thermofisher scientific West Columbia, SC, USA). In some experiment's cells were stained for TGFβR1 primary Antibody (PA5-32631) and labeled with Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488 conjugate (A27034) (Thermofisher Scientific, USA). The images were then acquired by scanning confocal fluorescence microscopy.

Cell Culture and Reagents

Immature DCs or mouse primary tracheal/bronchial epithelium cells (PTBECs) were incubated with and without 10^8/ml EXO regDCs EXO, iDCs EXO in the presence or absence of TGFβ1R blocker (SB 431542, R and D, USA, endocytosis inhibitor Cytochalasin D (C8273, Sigma Aldrich, USA) and free TGFβ1 (with dose approximately matching that in regDCs EXO) for 1 h and 24 hrs. Cells were harvested and ACE2 mRNA were measured by polymerase chain reaction (PCR) while ACE2 surface markers levels were measured by flow cytometry. Phosphorylation of TGβ1 transcription factors was assessed by western blot using anti PSMAD2/3 (D6G10), anti SMAD2/3 (D7G7), with anti-GAPDH (D16H11) or anti-Beta-actin (8H10D10) as loading control (Cell Signaling Technology, Danvers, MA, USA).

Flow Cytometry and Antibodies

FACS Staining Buffer (Thermofisher scientific) was used to stain cells on ice. FC receptors (FcR) were blocked using mouse FcR blocking reagent (Miltenyi Biotec) for 15 minutes protected from light. Primary goat anti mouse ACE2 antibody (AF3437) at recommended concentration were added for 30 minutes followed by APC-conjugated Anti-Goat IgG Secondary Antibody (F0108) (R and D). Cells were washed, re-suspended in FACS buffer and data was acquired using Milteny biotech machine and software.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from DCs *in vitro* and from oral mucosal (gingiva) tissue of the experimental groups used for *in vivo* studies using QIAGEN RNeasy mini kit (Qiagen, Inc., Valencia, CA, and USA). RNA purity and concentration were analyzed with Nanodrop (NanoDrop 1000 UV-VIS Spectrophotometer Software Ver.3.8.1, Thermofisher Scientific). Ratio of 260/280 of 2.0 was considered adequate for analysis and was reverse transcribed to cDNA. Amplification by PCR was performed using the High-Capacity cDNA Reverse Transcription Kit and PCR in total reaction of 20 µL. Quantitative real-time PCR was performed using TaqMan gene expression primers specific for ACE 2 (Mm00446190_m1), and Beta Actin (Mm02619580_g1) (Thermofisher Scientific). RT-PCR was run in StepOnePlus Real-Time PCR System. Relative gene expression was determined using delta-delta CT and plotted as relative fold change.

Western Blotting

Cells or EXO lysates were extracted by addition of RIPA buffer supplemented by protease/phosphatase inhibitor cocktail and incubated for 20 minutes on ice. Proteins (10 µg) were separated using 14% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad Laboratories, Hercules, CA), and transferred onto PVDF membranes (Sigma-Aldrich). After blocking with 5% nonfat dry milk in PBS, the membrane was incubated with primary antibodies, washed with TBST, and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were developed by ECL kit and imaged with ChemiDoc MP Imaging Gel (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Data analysis was performed by two-way or one ANOVA followed by Tukey's multiple-comparisons test using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Values are expressed as mean ± standard deviation (SD) and experiments were done in triplicates.

RESULTS

Bona Fide Dendritic Cell Exosome Subtypes for Proteomic, Functional Studies

Phenotypic analysis of bone marrow derived donor DC subtypes from C57BL/6 mice, as well as isolation, purification, and validation procedures of DC EXO from these subtypes are described in methods. Briefly, correct size distribution (30-150 nm) and shape of EXO were confirmed by nanoparticle tracking analysis (**Figure 1A**) and TEM (**Figure 1B**), while surface charge/colloidal stability was measured by the zeta potential (**Figure 1C**). Our published report (5) further validated bona fide EXO, based on their expression of CD63, CD81, Escort related proteins including ALIX and TSG101 and negative expression of GRP94.

Liquid Chromatography–Mass Spectrometry Analysis of Dendritic Cell Exosome Proteins

DC EXO subtypes are complex nano-particles, with on-target functions typically consistent with the phenotype of source DCs. This was previously reported by our group and includes regulation by regDC EXO of inflammatory cytokines, and the reprogramming of DC maturation and Treg-Th17 cell effector differentiation (5). In this study, we focused on in-depth proteomic LC-MS/MS analysis, to identify both on- and off-target proteins that could lead to unintended consequences of DC EXO therapy. We were able to identify 1276 overlapping or shared proteins. Moreover, we identified 859,1054 and 634 proteins unique to regDCs EXO, stimDCs EXO and iDCs EXO, respectively. These are illustrated in a Venn diagram (**Figure 2A**). Details of the unique and overlapping proteins are listed in **Supplementary Tables 1–5**. Of the overlapped proteins, 235 showed significantly different expression levels. A Clustered Heatmap performed using ClustVist software shows the expression patterns of the differentially expressed proteins (DEPs) in DCs EXO subsets (**Figure 2B**). More details of DEPs showing PSM/sum of total PSM values in each DCs EXO subsets are listed in **Supporting Information Table 5**.

By annotating unique and overlapped expressed proteins into the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and identifying the top ten pathways, we discovered that regDC EXO proteins are involved in: metabolic pathways, ubiquitin mediated proteolysis, endocytosis, ABC transporters, Pyrimidine metabolism, protein processing in the endoplasmic reticulum, peroxisome, Wnt signaling pathway, and in cell cycle and mRNA surveillance pathways. iDCs EXO proteins are involved in: neuroactive ligand receptor interactions, metabolic pathways, ether lipid metabolism, lysosome, Toll-like receptor signaling pathways, taste transduction, arachidonic acid metabolism, Fc gamma R-mediated phagocytosis, cytokine-cytokine receptor interaction and in the calcium signaling pathway. StimDCs EXO proteins are involved in: metabolic pathways, ubiquitin mediated proteolysis, MAPK signaling pathway, African trypanosomiasis, cytokine-cytokine receptor interaction, Chagas disease (American trypanosomiasis), pathways in cancer, purine metabolism, pyrimidine metabolism and peroxisome. **Figures 3A–C**. The non-DEPs were related to metabolic pathways, ribosome, phagosome, pentose phosphate pathway, leukocyte trans-endothelial migration, lysosome, protein processing in endoplasmic reticulum, antigen processing and presentation, Leishmaniasis and pathways in cancer (**Figure 4A**). DEPs were involved in ribosome, metabolic pathways, phagosome, proteasome, regulation of actin cytoskeleton, Fc gamma R-mediated phagocytosis, bacterial invasion of epithelial cells, chemokine signaling pathway, endocytosis, and protein processing in endoplasmic reticulum (**Figure 4B**). Collectively, these data showed that DC EXO subsets are mostly involved in pathways related to cellular immune function.

Further validation of high-throughput proteomic analysis was evidenced by expression in all DCs EXO subsets of EXO proteins CD63, CD81, CD82, C9, ALIX and TSG101 (**Figure 5A**), previously identified by WB and immunogold plating (5). DC markers and immunological/inflammatory molecules including CD11c, MHCII, CD205, ICAM1, SHIP1, LT3B, PDL1, PDL2, STAT3, IL1a, IL1β, TNF, IL6, IL10 and TGFβ1 were found in DC EXO. The negative regulators of inflammation included SHIP1, LT3B, and STAT3 and were expressed in both regDC EXO and stimDC EXO. In line with our published WB data, ELISA and immune gold plating (5), IL6, TNF, IL1β, and ILα were detected only in stimDCs EXO, while TGFβ1 and IL10 were exclusively detected in regDCs EXO (**Figure 5B**). In addition, a variety of integrins and chemotactic markers were differentially expressed in DCs EXO subsets (**Figure 6**). RegDCs EXO contain integrin alpha-1, integrin alpha-7, integrin beta-4 and CCR6, while stimDC EXO contain integrin alpha-2, and CCL5 and iDC EXO contain CCL24. DCs EXO subsets were also found to express integrin alpha-2b, integrin alpha-4, integrin alpha-5, integrin alpha-L (LFA-1), integrin alpha-M (CD11B), integrin alpha-V, integrin beta-1, integrin beta-2, integrin beta-3, integrin beta-7 and CD47, as well as other chemotactic factors including CCR7, CCL6, CCL9, and CXCR2. Collectively, these data suggest that DCs EXO subsets differentially express chemotactic and adhesion molecules that may have a role in homing, trafficking, cell adhesion and immune regulation. In addition, regDCs EXO are enriched with naturally as well as artificially loaded immunoregulatory/anti-inflammatory cargo.

We next analyzed the putative protein functions in DCs EXO subtypes according to the biological process and molecular function by Gene Ontology (GO) analysis. These results are shown in detail in **Figures 7** and **8**. Briefly, while unique expression patterns in DCs EXO subtypes were observed, all

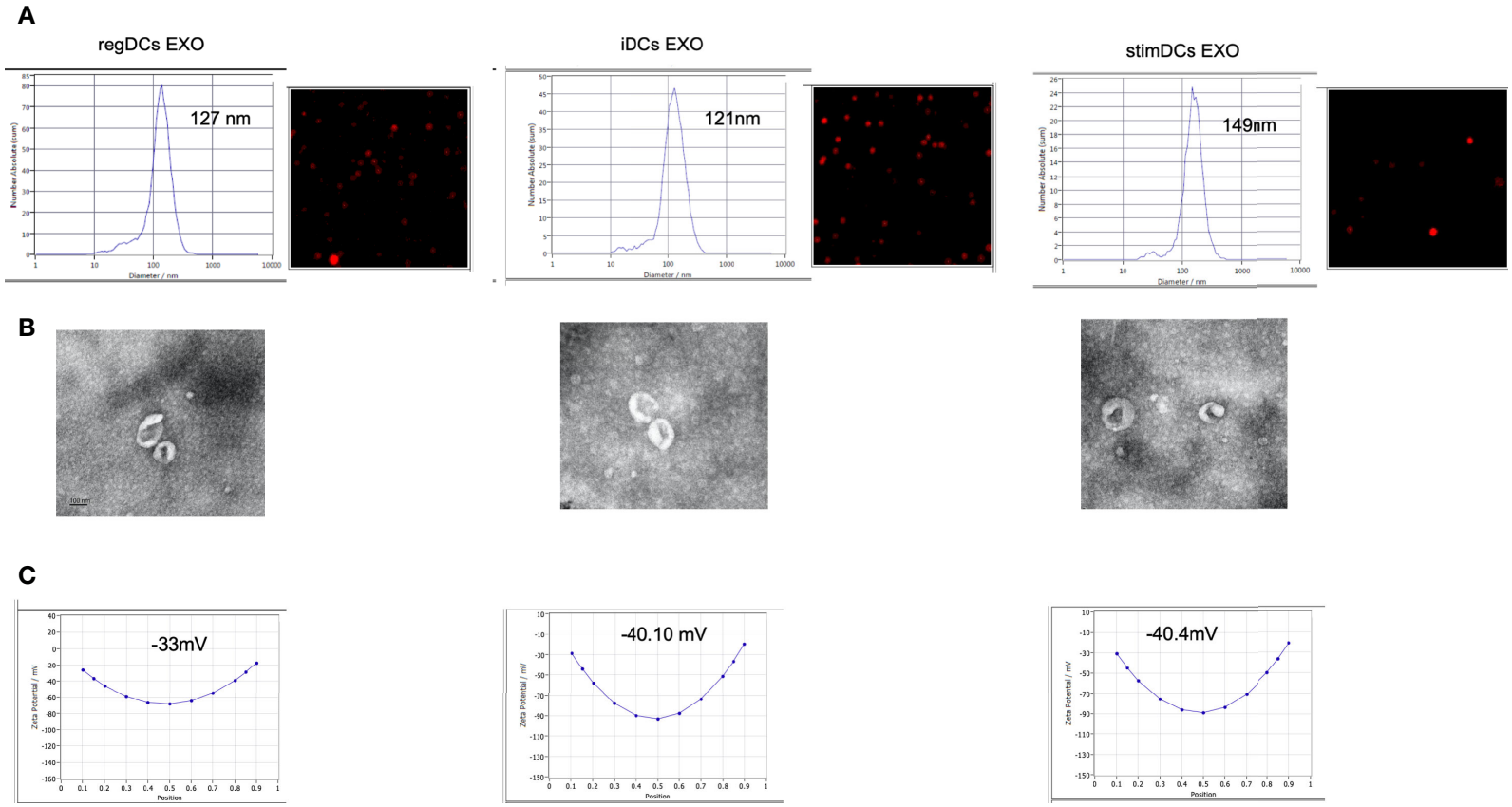

FIGURE 1 | **(A)** Nano-tracking analysis to determine EXO number and size distribution in nm. **(B)** Transmission electron microscopy (TEM) to visualize EXO shape and size. **(C)** Zeta potential analysis to determine EXO surface charge. regDCs EXO (Left), iDCs EXO (middle) and stimDCs EXO (right).

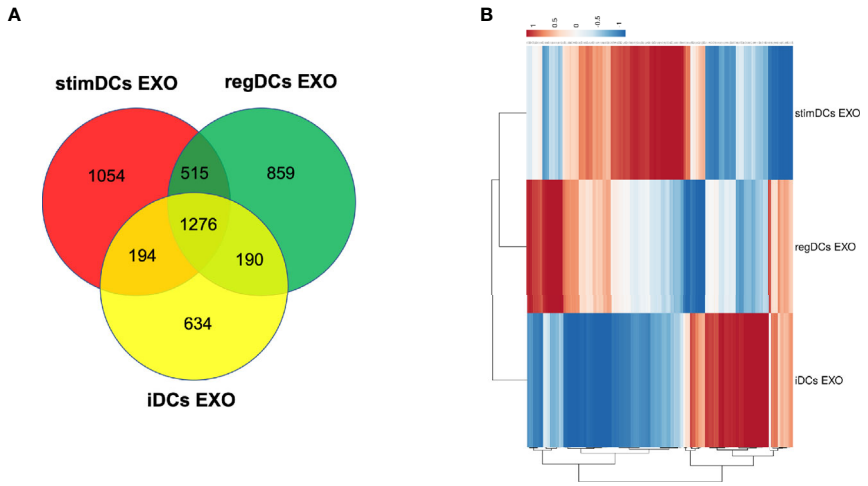

FIGURE 2 | **(A)** Venn diagram showing the overlap of proteins between regDCs EXO, iDCs EXO and StimDCs EXO. 1278 proteins were overlapped, whereas 859, 1,054, and 634 proteins were unique to regDCs EXO, stimDCs EXO, and iDCs EXO, respectively. **(B)** Heat map showing differential expression in the overlapped proteins. The dots are color coded with red and blue indicating upregulation and downregulation, respectively.

are enriched in proteins of cellular localization, metabolism and protein and phospholipid binding.

In Vivo Live Imaging and Anatomic Biodistribution of Radioisotope Indium-111-Oxine-Labeled Dendritic Cell Exosome

Based on proteomic analysis, we predicted that regDCs EXO would be particularly active in cellular trafficking and localization, as well as cell surface receptor binding and immune regulation. We therefore, conducted *in vivo* tracking of regDCs EXO after intravenous injection in live mice. Indium-111-Oxine-labeled-regDCs EXO or free label at equivalent radioactivity was injected *via* tail vein. Three and 24 h after administration, animals were scanned by SPECT/CT and reconstructed images were analyzed and a percentage of biodistribution to the lungs, liver, spleen and lymph nodes was assessed. At the 3hr time point, we observed the highest accumulation of labeled regDC EXO in the lungs (26 ± 2%), followed by the liver (16 ± 2%), spleen (7 ± 1%) and lymph nodes (6 ± .5%), while free label was rapidly dispersed to the liver (**Figures 9A, C**). After 24 h, regDCs EXO were found mainly in the liver, followed by the lungs, spleen and lymph nodes, respectively (**Figures 9B, D**). These data suggest a predilection of regDCs EXO for biodistribution to the lungs shortly after IV injection and they persisted in the lungs for at least 24 hrs. Afterwards, liver clearance appears to predominate.

To confirm these results, animals were euthanized, and organs were harvested and weighed. The emitted gamma activity from the harvested organs was measured to calculate radioactivity per milligram of tissue. Consistent with *in vivo* tracking, *ex-vivo* gamma activity measurements in animals treated with Indium-111-Oxine -labeled regDCs EXO showed gamma activity mostly in the lungs, liver and spleen. The gamma activity in these tissues was significantly higher for Indium-111-Oxine -labeled regDCs EXO in comparison to those treated with free In-111 (**Supplementary Figure 1**).

Immunoregulatory Dendritic Cell Exosome Uptake Is Essential to Maintain Sustained TGFβ1R-Mediated Signaling in Recipient Cells

To further examine mechanisms of action of regDC EXO, we conducted coculture experiments with acceptor DCs using 10^8/ml Dil-labeled regDCs EXO, iDCs EXO, free TGFβ1 (at an equivalent dosage to that contained within regDCs EXO by ELISA) or control culture media, in the presence or absence of TGFβ1R inhibitors or the uptake inhibitor cytochalasin D (CytoD). Blocking the TGFβ1 receptor with the specific inhibitor SB431542 in the recipient DCs prevented the regDCs EXO mediated activation/phosphorylation of TGFβ1 signaling transcription factor SMAD2/3 after 24 h (**Figure 10A**), suggesting its pivotal role in regDCs EXO mediated TGFβ1 signaling. Uptake of Dil-labeled regDC EXO, along with TGFβ1R, by recipient DCs was documented by immunofluorescence confocal microscopy. In the absence of regDCs EXO, TGFβ1R was extracellular (**Figure 10C**), with no phospho-SMAD2/3 evident at 1 or 24 h (**Figure 10B**). By contrast, upon regDC EXO treatment, TGFβ1R and regDCs EXO were internalized (**Figure 10C**). High phosphorylation of SMAD2/3 induced by regDCs EXO was observed at 1 h and was sustained at the 24 h timepoint (**Figure 10B**). CytoD prevented uptake of regDC EXO (**Figure 10C**) and prevented sustained phospho-SMAD2/3 signaling at 24 h, but not 1 h signaling (**Figure 10B**). Free TGFβ1 activated the 1 h signaling but did not sustain the 24h time point (**Figure 10B**). IDC EXO were internalized, but TGFβ1R was extracellular (**Figure 10C**), with almost no phosphorylation of SMAD2/3 (**Figure 10B**). Collectively, these data suggest optimum and sustained intracellular signaling by regDC EXO involves early

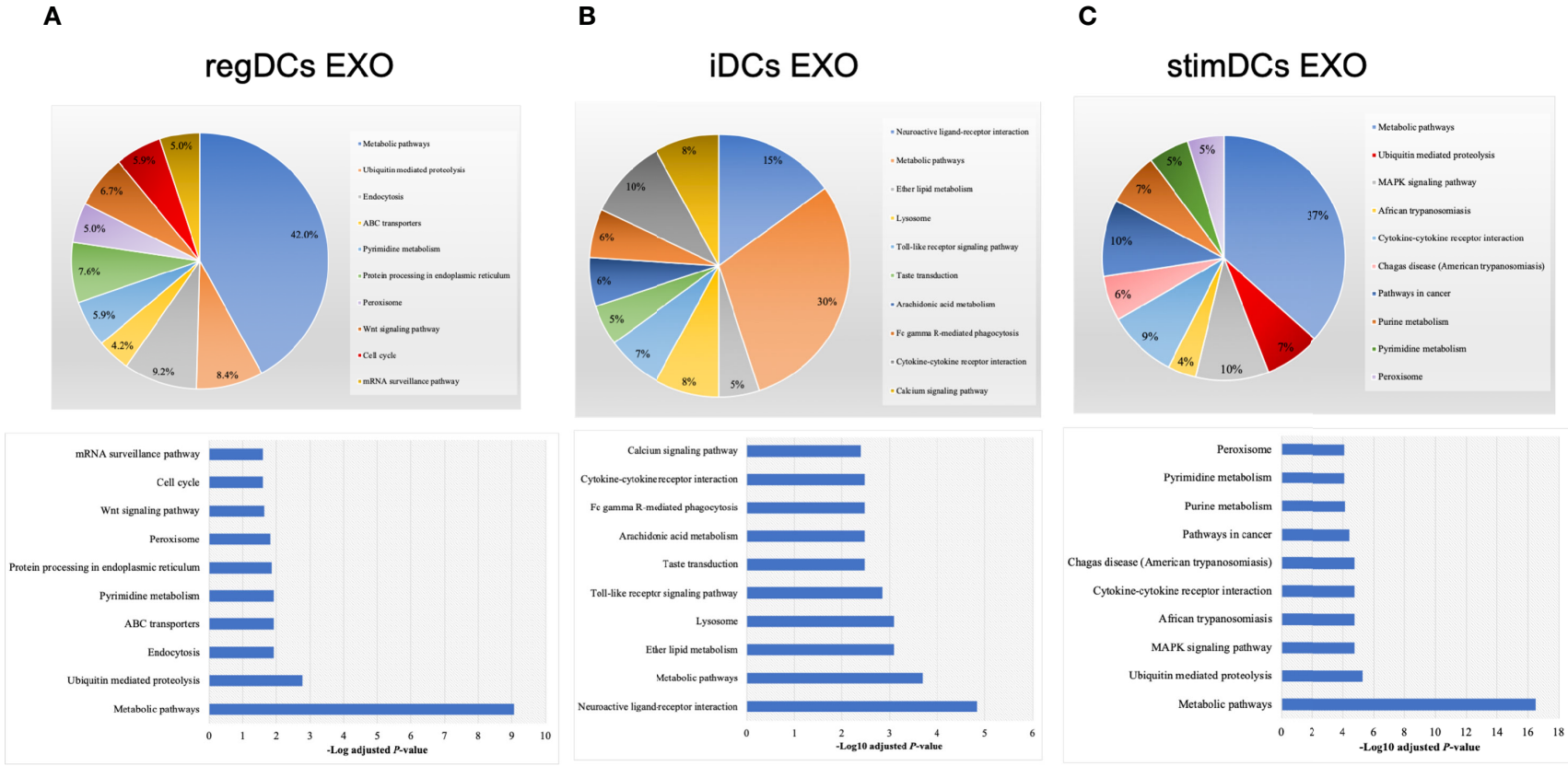

FIGURE 3 | KEGG pathway enrichment analysis of the uniquely expressed proteins listed on their percentage (upper panel) and level of significance (lower panel) in **(A)** regDCs EXO, **(B)** iDCs EXO, and **(C)** stimDCs EXO.

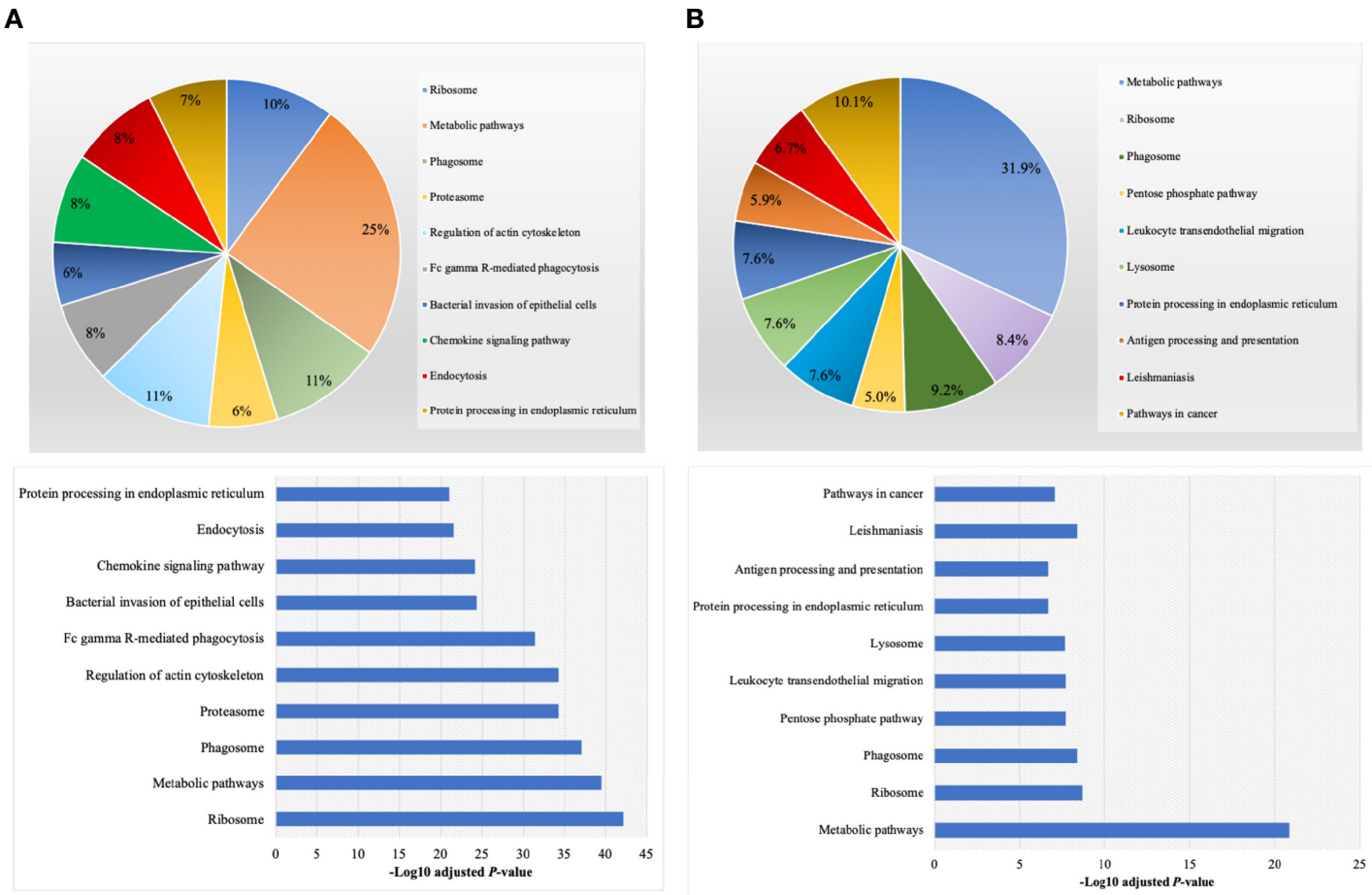

FIGURE 4 | KEGG pathway enrichment analysis of **(A)** non-differentially expressed and **(B)** differentially expressed proteins listed on their percentage (upper panel) and level of significance (lower panel).

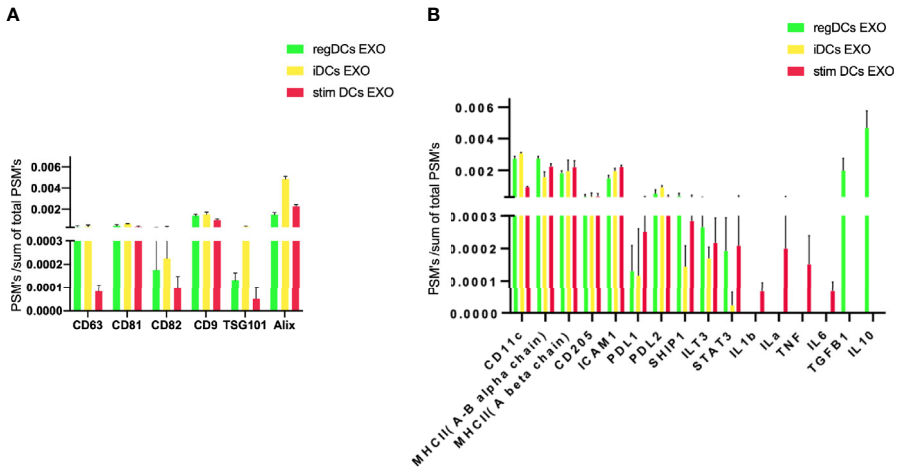

FIGURE 5 | **(A)** Identification of exosomal markers tetraspanins and ESCRT complex related proteins and **(B)** DCs markers, immune-stimulatory/inhibitory molecules and pro/anti-inflammatory cytokines in DCs EXO.

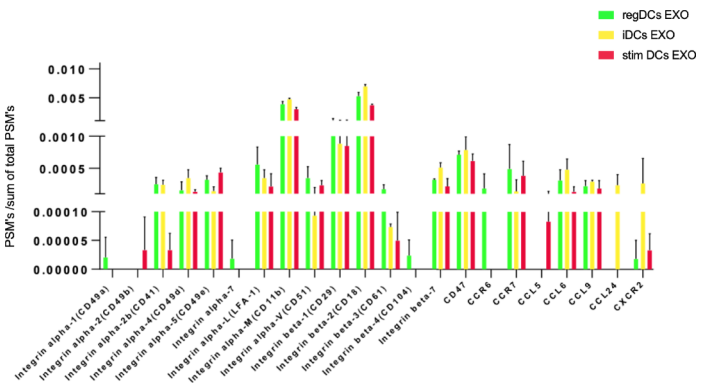

FIGURE 6 | Identification of integrins and chemotactic factors in DCs EXO.

binding to TGFβ1R, followed by internalization of TGFβ1R and sustained intercellular SMAD signaling.

Uptake of Immunoregulatory Dendritic Cell Exosome by Recipient Primary Tracheal/Bronchial Epithelial Cells, Inhibiting ACE2 Expression, via a TGFβ1-Dependent Mechanism

In view of the predilection of regDC EXO for accumulation in lung tissue, and consistent with their protein content, we examined *in vitro* whether regDC EXO are taken up by putative recipient cells of SARS-CoV-2 and how expression of ACE2 (the main SARS-CoV-2 receptor), is influenced. PTBECs were co-cultured with or without 10^8/ml dil-labeled regDCs EXO, in the presence or absence of TGFβ1R inhibitor for 24 h. PTBECs take up regDCs EXO (**Figure 11A**), commensurate with inhibition of ACE 2 expression (**Figures 11B–D**). Moreover, blocking the TGFβ1 receptor using specific inhibitor SB431542, reduced the inhibitory effect of regDCs EXO on ACE2 levels, suggesting a crucial role for TGFβ1 in regDC EXO-mediated inhibition of ACE 2 expression. This is consistent with previous reports of TGFβ1 cytokine induced inhibition of the ACE2 receptor, the SARS-CoV-2 point of entry (14–16).

DISCUSSION

Studies of EXO biology, especially of DC origin and their therapeutic applications have rapidly expanded over the last few years (31), revealing promising approaches to reprogram harmful and excessive immune responses (32, 33). Advancing such approaches into therapeutic applications requires more in-depth knowledge of the proteomic cargo, biodistribution and mechanism of action of DC EXO. This will enable investigators

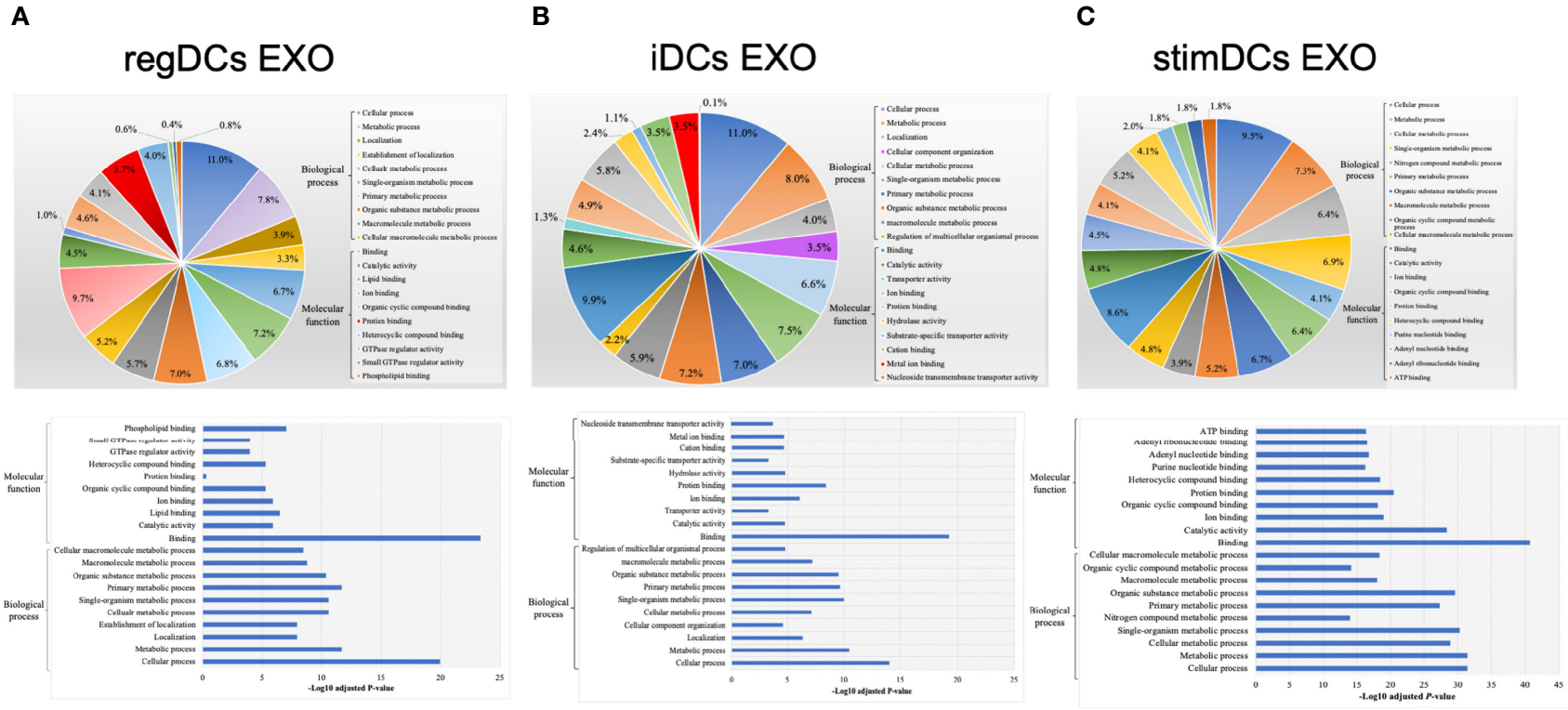

FIGURE 7 | Gene ontology (GO) enrichment analysis of the uniquely expressed proteins listed on their percentage (upper panel) and level of significance (lower panel) in **(A)** regDCs EXO, **(B)** iDCs EXO, and **(C)** stimDCs EXO.

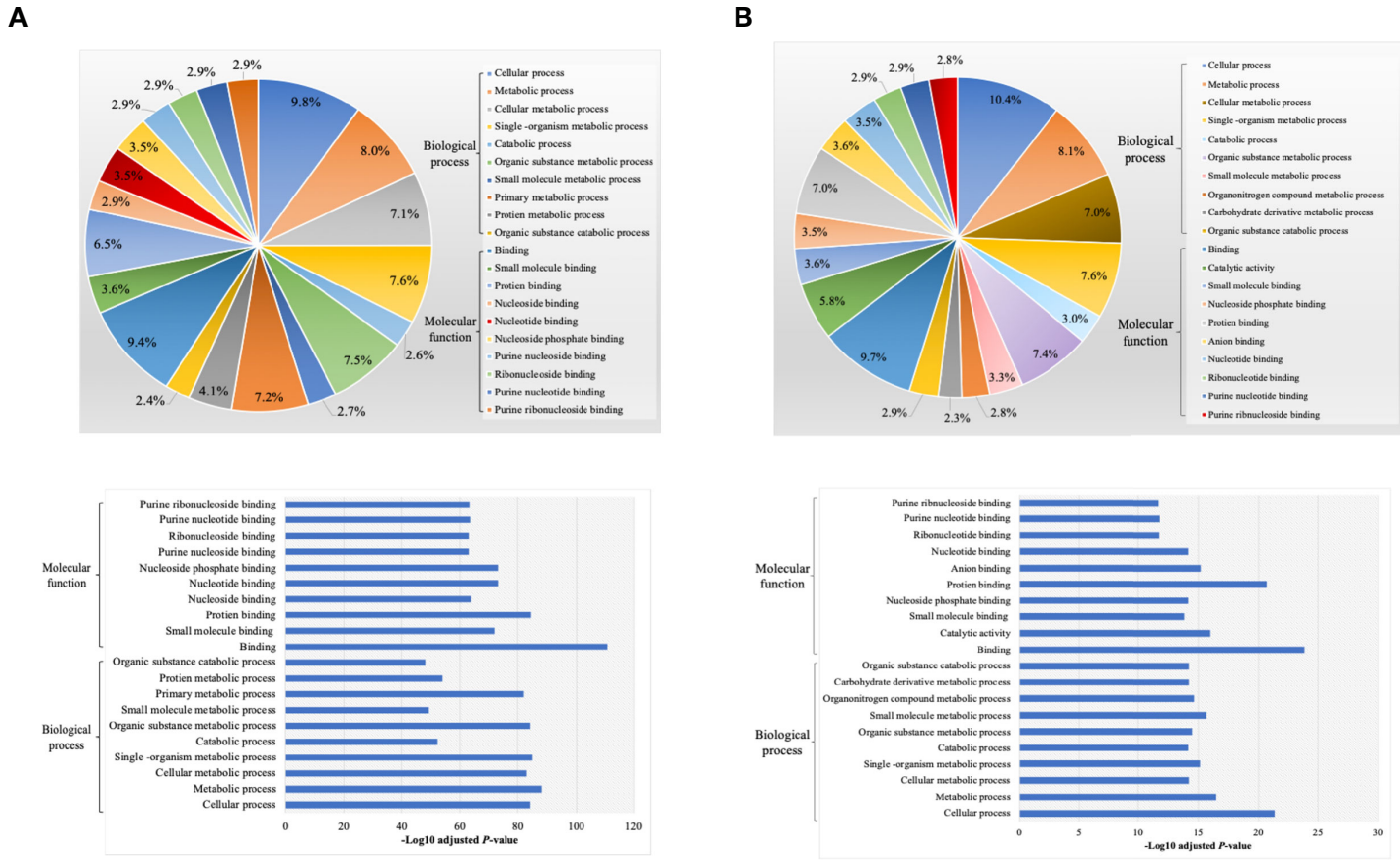

FIGURE 8 | Gene ontology (GO) enrichment of proteins **(A)** non differentially expressed and **(B)** differentially expressed proteins between DCs exosomes subsets, listed on their percentage (upper panel) and level of significance (lower panel).

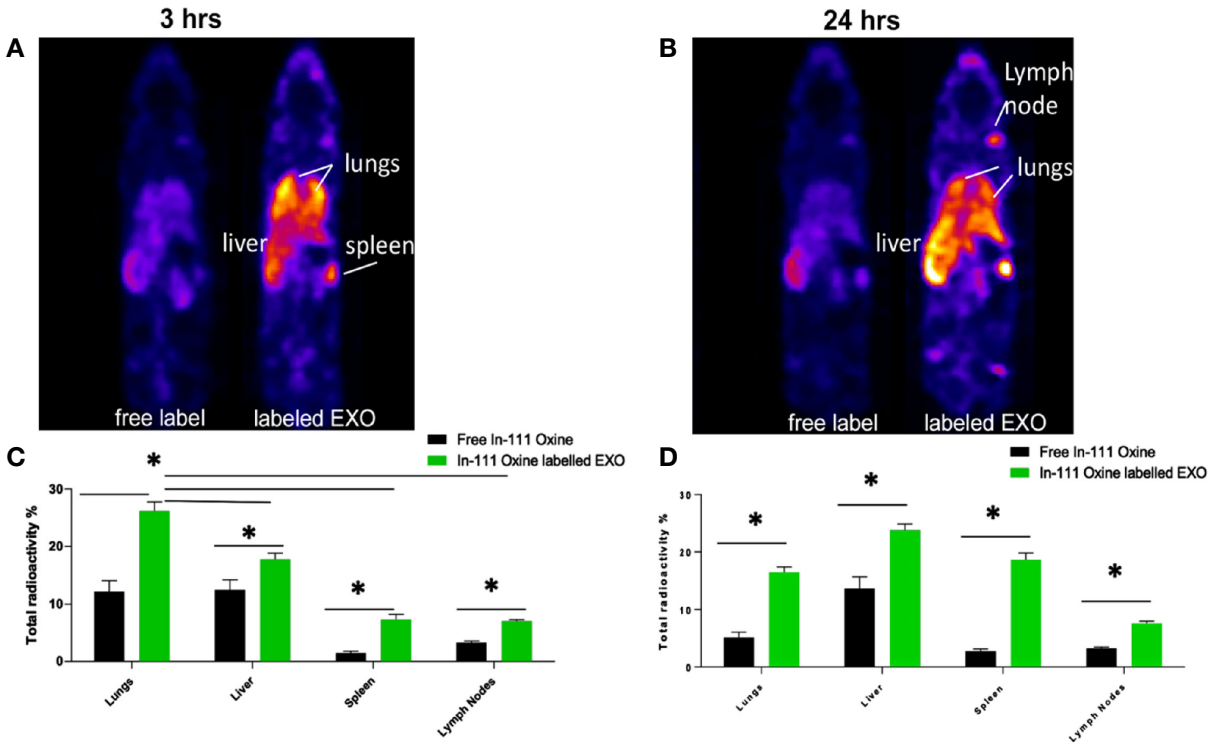

FIGURE 9 | Biodistribution of IV administrated EXO at 3 h and 24 h time points. SPECT CT live animal in vivo imaging of free In-111 (left) or In-111-labeled exosomes (right) in mice after **(A)** 3 h and **(B)** 24 h of IV administration. Radioactivity in lung, liver, spleen, and lymph nodes, relative to total, when free radiolabels or bound to DC EXO, expressed as % determined using SPECT CT images after **(C)** 3 h and **(D)** 24 **h** of EXO IV injection. N = 3; *P < 0.05 by two-way ANOVA, followed by Tukey's multiple comparisons.

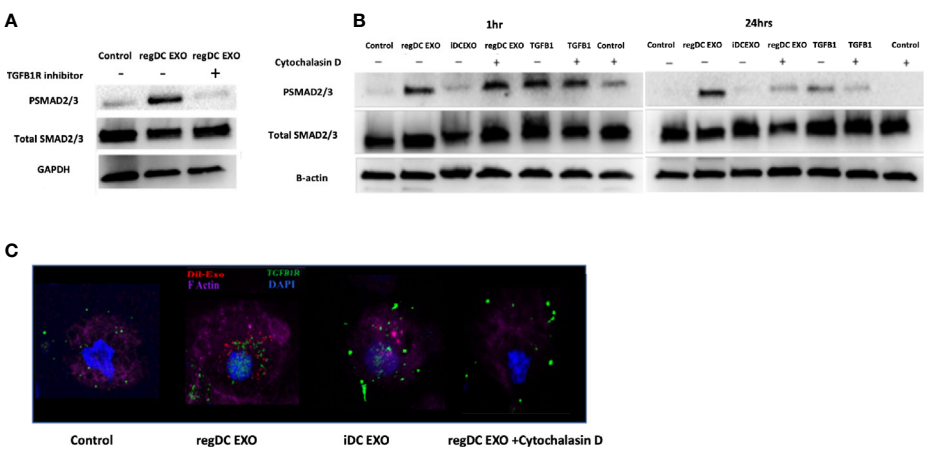

FIGURE 10 | Early and sustained pSMAD2/3 signaling by uptake of regDC EXO with TGFβRI: **(A)** Immunoblot of Psmad2/3 and total smad2/3 in recipient DCs co-cultured for 24 h with reg DCS EXO +/- TGFβ1R inhibitor SB431542. Loading control was GAPDH **(B)** Immunoblot of Psmad2/3 and total smad2/3 in recipient DCs co-cultured for 1 and 24 h with reg DC EXO or iDC EXO +/- cytochalasin D. Loading control was B-actin. **(C)** Uptake of Dil labeled EXO (red) by recipient DCs, DAPI (blue), Alexa Fluor 680 phalloidin (violet) for FActin, Alexa flour 488 (green)-mouse anti-TGFβR1, visualized under confocal microscopy. Dil-DCs EXO or no EXO were added to recipient DCs at a 10:1 EXO : DC ratio (24 h shown). Results shown are representative of three independent experiments.

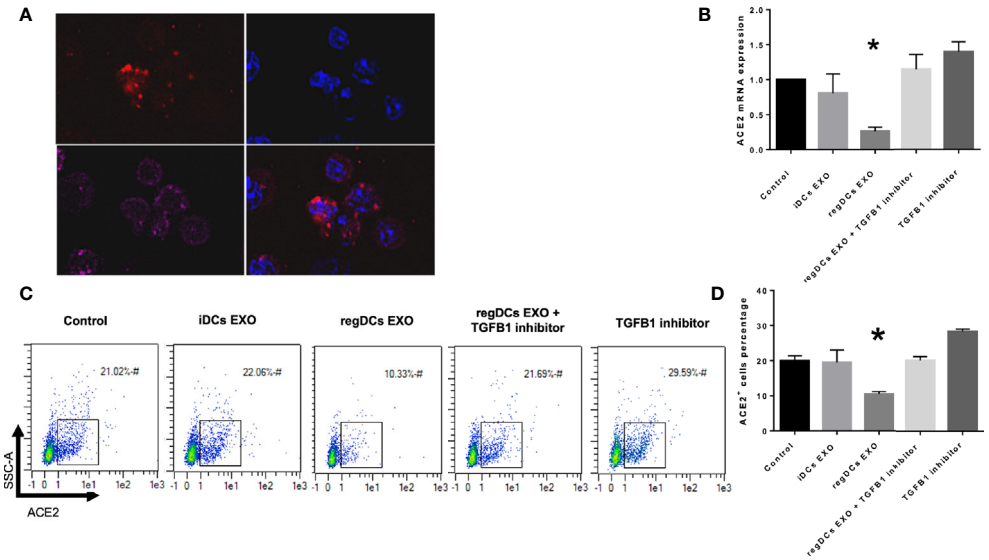

FIGURE 11 | regDCs EXO are taken up by acceptor PBTECs, inhibiting ACE2 expression in vitro. **(A)** Uptake of DiI labeled EXO (red) by PBTECs, counterstained with nuclear stain DAPI (blue), phalloidin (Alex flour 647) for cell membrane and visualized under confocal microscopy. ACE2 mRNA expression **(B)** and flow cytometry scattergrams showing ACE2 positive cells percentage **(C)** in PBTECs treated or not treated with iDCs or regDCs EXO in the presence or absence of TGFβR1 inhibitor SB431542. **(D)** representative bar graph of **(C)**. Results shown are representative of three independent experiments (*P < 0.05 by one-way ANOVA followed by Tukey's multiple comparisons).

to optimize on-target effects and minimize off-target or unintended consequences of such therapy.

We describe the complexity of the protein cargo of DC EXO subtypes, which consist of 1278 shared proteins, and 859,1054 and 634 proteins unique to regDCs EXO, stimDCs EXO and iDCs EXO, respectively (**Figures 2** and **3**). Our study revealed that proteins associated with antigen presentation and processing, phagosome, leukocyte endothelial transmigration and chemokine signaling pathways were common to all DC EXO subtypes, suggesting putative roles in homing to and entry into peripheral tissues from circulation, uptake and modulation of immune responses (**Figure 4**). PDL1 and PDL2, important regulators of the immune response and targets for T cell-based immunotherapy, were identified in all three DCs EXO subtypes. Exosomal proteins including tetraspanins CD63, CD81, CD82, CD9 and those involved in ESCRT complex ALIX and TSG101 were commonly expressed (**Figure 5A**) and further validated by Western blotting and TEM analyses (5). DC markers including CD11c and MHCII, and CD205 were detected in all three EXO subsets, indicative of the parental cells of origin. ICAM1, a positive regulator of leukocyte transmigration across the endothelium and a promotor of naive T cells priming and activation (34) was also found to be expressed in all DC EXO.

Apart from TGFβ1 and IL-10, other proteins unique to regDCs EXO included those in the Wnt signaling pathway; namely, Wnt1 and Wnt9, both of which are involved in the regulation of immune tolerance and bone formation (35). Other negative regulators of the immune response were also identified in regDCs EXO such as SHIP1 (36), ILT3B (37), STAT3 (38), ostensibly resulting from parent DC treatment with TGFβ1 or IL10. These negative regulators were also found in stimDC EXO, possibly upregulated in parent mature DC, counter regulatory to overexpression of IL6, TNF, IL1β and IL1a. EXO proteins involved in Toll-like receptor signaling pathway were elevated in iDC EXO, consistent with antigen recognition functions of parent immature DCs (39). Cytokine-cytokine receptor interaction pathway proteins were common to iDCs EXO and stimDCs EXO (**Figure 3**). All DCs EXO subset proteins are detailed in **Supplementary Tables 1–5**.

The predominant expression in regDC EXO of immunoregulatory proteins, as well as proteins involved in trafficking, cell surface binding, and transmigration, sparked our interest in how these EXO might biodistribute to major organs. Many previous studies used either fluorescence imaging or bioluminescence imaging to track administered EXO. Our nuclear imaging approach, has many advantages over these technologies (5, 40–42), including a superior tissue penetration, a higher resolution, a higher signal to noise ratio and an improved sensitivity for deeper organs, thus enabling more accurate imaging (43–45). Blood levels of EXO after IV administration are a dynamic process, and decrease by greater than 50% within 30 minutes of administration (46), with complete elimination by 4 h (43). An initial phase of distribution to the lungs, spleen and liver within minutes, is followed by an elimination phase through the liver and kidney (42, 46, 47). In our study, a significant accumulation of regDC EXO occurred after 3 h in lung tissue, followed by liver, splenic tissue and lymph nodes (**Figures 9A, B**). Significant levels of regDC EXO persisted in lungs for up to 24 h, with a notable increase in hepatic and splenic tissue (**Figures 9C, D**). Ex-vivo

gamma radiation measurements of postmortem tissue confirmed the *in vivo* SPECT/CT analysis (**SFig.1**).

Chemokine receptors and integrins and their binding partners shape the homing patterns of cells and ostensibly, EXO in the body (48). Of particular note are the chemotactic and adhesion factors identified in reg DCs EXO (**Figure 6**), including CCR6, CCR7, CXCR2, Integrin alpha-M (CD11b), Integrin beta-1 (CD29), CD47, Integrin alpha-1 (CD49a), ICAM-1(CD54), integrin beta-2, integrin alpha-5, and integrin alpha-L (LFA1) (49–60). CCR7 (50) mediates blood-derived lymphocyte trafficking to bronchial associated lymphoid tissue while CXCR2 directs neutrophil recruitment to the lungs (51). CD49a expression promotes selective trafficking and retention of lymphocytes into respiratory tissues (52–54). LFA-1 and ICAM-1 function by binding lymphocytes to bronchial endothelium cells (55, 56), while CCR6 and integrin beta-2 promote group 2 innate lymphoid cell migration to the lung (57, 58). Neutrophil trafficking in the lung is regulated by integrin beta-1 (CD29), CD47, integrin alpha-M (CD11b) and ICAM-1 (CD54) (59). Integrin beta-1 and integrin alpha-5 are involved in mesenchymal stem cell distribution in the lungs (60). Other investigators have observed a high retention of extracellular vesicles in the lungs 4 h after parenteral administration (42, 43). The accumulation of regDCs EXO observed here in splenic tissue and lymph nodes may be attributed to their interaction with abundant immune cells found in lymphoid tissue. EXO can also bind to lymphocytes, DCs and macrophages which circulate in the bloodstream and migrate to the spleen (61). CCR7 regulates cells or exosomes migration and homing to lymphoid organs and splenic tissue (62). CCR7 was found to be highly expressed in both regDCs EXO and stimDCs EXO. The liver is a large organ and contains a large population of macrophages (Kupffer cells) that can uptake a considerable amount of the injected regDCs EXO for clearance. DCs and hepatocytes can also uptake exosomes. Moreover, EXO express phosphatidylserine (PS) on their surfaces that could enable the recognition and uptake by hepatic phagocytes (42, 61).

Our previous work with these DC EXO subtypes, showed efficacy in regulation of the inflammatory bone disease and periodontitis, when administrated locally in gingival tissue. Locally injected EXO persisted at the site of inflammation and the adjacent lymph nodes, but were minimally cleared to distant tissues such as the lung, spleen and liver (5). The route of administration of EXO is also important to their biodistribution, especially to lymph nodes (63), brain (64) and retina (65). The cell source of EXO and their dose may also affect the biodistribution (66). Adhesion molecules like the tetraspanins, integrins, and chemotactic factors can direct EXO to immune cells found in inflammatory sites (10, 67).

Our previous work showed TGFβ1 in regDCs EXO as a key immunoregulatory factor that recruits T-regulatory cells to target inflamed tissue to deactivate the inflammatory process (5). Thus, we were interested to understand how TGFβ1 when in form of EXO activate the target signaling on acceptor cells. Examination of mechanism of action showed that initial binding of regDC EXO to TGFβR1, stimulated early SMAD2/3 phosphorylation, followed by internalization sustained SMAD2/3 phosphorylation in recipient DCs (**Figure 10**). An equivalent dose of free TGFβ1 could not maintain optimum TGFβ1 signaling. Several reports emphasize the role of endosomal translocation of EXO with TGFβ1-TGFβ1 receptor complex, in order to prevent the lysosomal degradation for prolonged internal SMAD2 signaling (68–70). Also, EXO internalization and TGFβ1 release inside the cell, can lead to TGFβ1 recycling to the cell membrane where it can be secreted and act on the cell surface receptor in an autocrine manner (71).

The present study demonstrated preferential biodistribution and retention of regDCs EXO into lung tissue. It is tempting to speculate that this supports a possible therapeutic implication of regDC EXO, or its cargo, in patients with COVID-19 infection. A significant amount of lung damage in COVID-19 patients is due to an exaggerated host immune response (72–74). In severe stages of COVID-19 infection, high levels of IL-1β, and IL-6, TNF along with decreased levels of antiviral factors (interferons-IFNs) are secreted from respiratory epithelial cells, DCs, macrophages and T cells. The resultant "cytokine storm" culminates in ARDS and multiorgan damage and eventually failure (75–78). Thus, reprogramming inflammatory cells such as DCs and T cells, with regDC EXO as we have described (5) may actually reverse the inflammatory response and attenuate the infectious process and thus diminish the severity of the infection (79). ARDS is an acute inflammatory response in lung tissue and frequently leads to severe damage of tissue and ultimately death in COVID-19 patients (19–23). The use of short-term TGFβ1-loaded regDCs EXO to attenuate the acute inflammation in the lung could prevent the severe lung damage seen during the acute phase and could also decrease the chronic consequences of severe pulmonary COVID-19 infection (80). TGFβ1 has been shown to inhibit early and acute responses upon intranasal lipopolysaccharide (LPS) challenge in an acute lung injury model (81). TGFβ1 suppressed neutrophils and induced $Foxp3^{+}$r regulatory T cell responses needed to resolve acute inflammation in the lungs (79). Given the acute inflammatory nature of severe COVID-19 infection, regDCs EXO may therefore represent a natural nano-therapy modality. Furthermore, this may be also alleviate the need to use concurrent anti-inflammatory agents such as hydroxychloroquine and dexamethasone (26). Moreover, this may overcome limitations of immunoregulatory cell-based therapy such as phenotypic instability and low cell numbers (9). There are currently several clinical trials registered in clinicaltrials.gov using extracellular vesicles (EVs) as therapy for COVID-19. Extracellular vesicles from mesenchymal stem cells reportedly retain their ability to inhibit lung inflammation by shifting proinflammatory monocytes/macrophages toward the regulatory phenotype in an ARDS model. This is presumably done through reprograming the lung-infiltrating DCs and T cells toward a regulatory phenotype (82) as has been described in the oral mucosa (5).

In addition to immunoregulatory functions (5) and unique proteins expressed by regDC EXO, we investigated their ability to regulate expression of the SARS-COV-2 receptor, ACE2 (25, 26). This was particularly pertinent in view of recent findings linking TGFβ1 and ACE 2 to SARS-COV-2 internalization (14–16). In

our study, regDCs EXO were shown to inhibit ACE2 expression in respiratory tract epithelial cells (PBTECs), which was abrogated by TGFβ1R blockers (**Figure 11**).

DCs EXO have many therapeutic advantages. These include protection of cargo against proteolytic degradation and damage by complement system, active migration, and localization to target lung tissue, affinity for interaction with immune cells and the capability to be loaded with therapeutic and diagnostic factors (5, 9–11, 83). Mention should be made of other EXO based approaches proposed for COVID-19, including EXO tailored to express decoy ACE2 (84), to encapsulate the S protein of the SARS-CoV-2, and used as a vaccine (85), or to encapsulate antiviral drugs (86).

In conclusion, our study shows that although regDC EXO contain a complex profile of proteins, their functions appear reflective of the dominant proteins in the parent donor DCs. These include proteins that mediate trafficking to inflamed tissue, cell binding and retention in lung tissue. In addition, the impact of regDCs EXO on regulating TGFβ1 cargo signaling and the main SARS-CoV-2 receptors expression such as ACE2 was shown. Overall, the capabilities of regDC EXO may suggest their future utility as an immunotherapeutic modality to improve the treatment of lung inflammatory diseases. Preclinical studies using ARDS animal models and early clinical trials are still necessary to evaluate the safety and efficacy of EXO from DCs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Augusta University (protocol # 2013-0586).

AUTHOR CONTRIBUTIONS

ME and RE conceived and designed the research, performed the experiments, analyzed the data, interpreted the results, prepared the figures, drafted the manuscript, and edited and revised the manuscript. MME, RA, MR, WZ, AE, and AA analyzed the data and interpreted the results. CC analyzed the data, interpreted the results, prepared the figures, drafted the manuscript, and edited and revised the manuscript. MH, YL, JV, and RL edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.636222/full#supplementary-material

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Emerging Role of Exosomes in Tuberculosis: From Immunity Regulations to Vaccine and Immunotherapy

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Exosomes are cell-derived nanovesicles carrying protein, lipid, and nucleic acid for secreting cells, and act as significant signal transport vectors for cell-cell communication and immune modulation. Immune-cell-derived exosomes have been found to contain molecules involved in immunological pathways, such as MHCII, cytokines, and pathogenic antigens. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), remains one of the most fatal infectious diseases. The pathogen for tuberculosis escapes the immune defense and continues to replicate despite rigorous and complicate host cell mechanisms. The infected-cell-derived exosomes under this circumstance are found to trigger different immune responses, such as inflammation, antigen presentation, and activate subsequent pathways, highlighting the critical role of exosomes in anti-MTB immune response. Additionally, as a novel kind of delivery system, exosomes show potential in developing new vaccination and treatment of tuberculosis. We here summarize recent research progress regarding exosomes in the immune environment during MTB infection, and further discuss the potential of exosomes as delivery system for novel anti-MTB vaccines and therapies.

Keywords: *Mycobacterium tuberculosis*, exosomes, extracellular vesicles, innate immunity, immune evasion, vaccine

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INTRODUCTION

By inducing over 1.2 million deaths and an additional 251,000 (Range: 223,000–281,000) human immunodeficiency virus (HIV)-positive deaths in 2019 (1), tuberculosis remains one of the most fatal public health threats in the world. Additionally, an increasing prevalence of drug-resistant and multidrug-resistant *Mycobacterium tuberculosis* (MTB) is seen under current anti-tuberculosis chemotherapy with limited efficacy, especially in underdeveloped and developing countries. Therefore, it is in urgent demand to develop novel vaccines and therapies against tuberculosis based on the in-depth understanding of the relationship between MTB and host immunity.

Innate immune cells, including macrophage and dendritic cells (DCs), are host cells for MTB, and perform most of the antibacterial activities during MTB infection. As the first barrier in MTB infection, alveolar macrophages (AMs) could produce inducible nitric oxide synthase (iNOS) that

participates in the killing of MTB *via* the production of nitric oxide (2). Meanwhile, classical activation of macrophage induces polarization into the M1 antibacterial phenotype, with strong abilities in killing MTB by producing pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (3, 4). DCs, the "sentinels" of the immune system, are responsible for initiation of adaptive immune responses against MTB infection by migrating from infected lungs to local lymph nodes for T cell activation (5). Adaptive immunity is subsequently activated and engages in the host anti-mycobacterial activities, mediated by a range of different T cell and B cell subsets.

During MTB infection, a specific immune environment is formed when host immune cells interact with the pathogen at the infection site. The bactericidal immune responses of host cells are fine-tuned and balanced by multiple signal pathways to regulate immune cell functions. There are three widely accepted signal transduction methods among immune cells: direct immune cell contact (e.g. antigen presentation from DCs to T cells *via* direct MHC and T cell receptor contact); secretion of cytokines by immune cells to induce immune activities [e.g. interferon-γ promotes anti-MTB immunity (6); IL-10, transforming growth factor-β (TGF-β), and IL-35 can regulate immune function by manipulating inflammation (7, 8)]; and extracellular vesicle which include exosomes and microvesicles trafficking among immune cells. It has been demonstrated that exosomes from MTB-infected immune cells can regulate immune functions by transferring signal molecules into recipient cells (9, 10). In order to develop new anti-tuberculosis vaccine or therapy strategy based on exosomes, the underlying mechanisms of exosome in TB immunity need to be clarified. In this review, we discuss and summarize the immune regulator role of exosomes in the immune system in response to MTB which can extend our understanding of exosomes in TB immunity. Then we further discuss the possible role of exosomes in MTB immune evasion, as well as the protective role of exosomes to serve in anti-MTB vaccine.

THE VECTOR ROLE OF EXOSOMES IN EXTRACELLULAR SIGNAL CONDUCTION

Exosomes, a type of nano-sized extracellular vesicle generated from multivesicular bodies (MVBs), contain constituents like protein, lipid, DNA, and RNA. They have been found to have unique physiological mechanisms and functions (11). Although exosomes are considered as waste carriers in autophagy process, more evidence has emerged to support cellular communication roles of exosomes (12). After summarizing several proteomic studies using different types of cells, Suresh Mathivanan has concluded that exosomes contain proteins like MVB biogenesis molecules [e.g. ALG-2 interacting protein X (Alix), and tumor susceptibility 101 (TSG101)], member RAS oncogene family (Rabs, facilitating exosome docking and fusion on the membrane), and annexins (assisting membrane trafficking and fusion events) (13). In recent years, researchers have also discovered various RNA contents in exosomes, including mRNA and miRNA, which can be transferred into recipient cells for cellular function regulations (14). Meanwhile, lncRNA is also found in exosomes that can regulate cellular functions. For example, HIF-1α-stabilizing lncRNA (HISLA) released from tumor-associated macrophages can enhance the aerobic glycolysis and apoptotic resistance of breast cancer cells (15).

After being released into extracellular environment, exosomes can be absorbed by different kinds of cells, in which they will perform cellular signal transduction and communication in two main ways. The first one is the binding of exosomes to specific cell membrane molecules. For example, genetically engineered chimeric antigen receptor T lymphocyte cells (CAR-T cells) can secret exosomes with chimeric antigen receptor (CAR) protein on the surface, which can inhibit tumor growth through binding to specific tumor antigens (16). The second way is that exosomes can be transferred into the target cells through endocytosis. Upon entering, exosomes can release their cargo into the target cells and execute biological functions (17). Therefore, exosome performs important vector role in facilitating cell-cell signaling communication by using its contents to regulate various cellular functions.

DOUBLE ROLES OF EXOSOMES IN ANTI-INFECTION IMMUNITY

When infection occurs, the innate immune system tries to kill the pathogen as well as presents antigens to prime the adaptive immune system for more effective pathogen clearance. Exosomes have been found to contain plenty of immune-regulating molecules with functions such as indirect activation of T cells by DC-derived exosomes to help recipient cells to confer HIV resistance (18, 19). It has also been discovered that DC can release exosomes with MHC-I/peptide complexes for other naïve DCs to uptake, eventually helps to prime CD8+ T lymphocyte cells (20). In tumor environment, DC-derived exosomes containing TNF, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRaIL) could lead to tumor cell apoptosis (21) and activate natural killer cells *via* TNF superfamily ligands for enhanced tumor inhibition (22). Macrophages secrete cytokines to create a pro-inflammatory environment against the pathogen, during which the exosomes play critical roles. It has been found that multiple intracellular pathogens, such as MTB, *Bacillus Calmette-Guerin* (BCG), *Salmonella typhimurium*, and *Toxoplasma gondii*, can induce infected macrophages to secrete exosomes with pathogen-associated molecular patterns (PAMPs). Those exosomes are in turn transferred into uninfected macrophages to be activated through Toll-like receptor (TLR) and myeloid differentiation factor 88-dependent (MyD88) pathway (23, 24). More interestingly, exosomes from T cells could be transferred into DC and induce more resistant DC antiviral responses *via* the cyclic GMP-AMP synthase/stimulator of interferon response cGAMP interactor 1 (cGAS/STING) cytosolic DNA-sensing pathway and *via* the expression of interferon regulatory factor 3 (IRF3)-dependent interferon regulated genes (25).

Immune evasion pathways are found both in MTB infection and other pathogen-induced diseases, where exosomes are closely engaged in immune attack regulation, creating a pro-bacteria or pro-virus environment. In Hepatitis C virus (HCV) infection, hepatocyte-derived exosomes containing TGF-β could promote the expansion of T follicular regulatory (Tfr) cells in healthy subjects' PBMCs, inhibiting the function of T cells and B cells, leading to an environment in favor of HCV survival (26). This means that exosomes can also act negatively during immune regulation in infectious disease and its vector role in the immune system is a double-edged sword. Another study found that Newcastle disease virus (NDV)-infected HeLa cell-derived exosomes can promote NDV replication by three miRNA inside, which were associated with enhancing NDV-induced cytopathic effects and suppressing IFN-β gene expression (27). Therefore, exosomes could have either beneficial or harmful properties during infection, depending on the type of their regulatory molecules.

EXOSOMES FUNCTION AS PROTECTIVE STIMULATORS DURING MTB INFECTION

MTB infection stimulates immune cells to secrete different kinds of exosomes which act as pre-stimulators for immune system even before the activation of DCs, macrophages, T cells, and B cells. Meanwhile, exosomes can activate inflammatory and autophagy signaling pathways in host cells, which can not only enhance the anti-MTB immunity by helping to kill intracellular MTB, but also prepare uninfected immune cells for upcoming MTB infection.

MTB-INFECTED MACROPHAGES-DERIVED EXOSOMES STIMULATE NAÏVE MACROPHAGES AND INDUCE SECRETION OF PRO-INFLAMMATORY CYTOKINES

Macrophages, the first in defense line in face of MTB infection, could trigger intracellular downstream inflammatory signaling pathways for anti-MTB activities, such as the activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling pathways (28). Meanwhile, they can activate the downstream transcription factors to start translation of target genes and initiate inflammatory responses. Pattern recognition receptors (PRRs) and PAMPs are critical in inflammation, especially TLRs, which can induce releasing of pro-inflammatory cytokines, iNOS, and antimicrobial peptides *via* MyD88 (29, 30). Interestingly, it is found that exosomes secreted by MTB-infected macrophages also contain bacterial-derived RNA, providing strong evidence for exosome-induced anti-MTB immune responses. Notably, MTB peptides are also found in serum extracellular vesicles from persons with latent tuberculosis infections (31, 32). These studies strongly suggest that exosomes from MTB-infected cells could act as PAMPs, which induce naïve macrophage activation and would be beneficial for the anti-MTB immunity.

By regulating pro-inflammatory responses, exosomes from infected macrophages is an essential force against MTB infection as they can stimulate a higher level of cytokines and chemokines production from bone marrow-derived macrophages (BMMs) than from resting macrophages (9). Furthermore, Singh et al. tested the ability of exosomes from MTB-infected macrophages to influence the immune cells using Transwell system, and found that exosomes-treated macrophages could induce stronger transmigration ability than the resting cells. The researchers also extracted exosomes from BCG-infected mice serum, and found them to activate macrophages as pro-inflammatory phenotype, and recruit macrophages and $CD11b^+$ cells to the lungs of mice (9). This study provides direct evidence for the hypothesis that exosomes from infected macrophages could activate uninfected macrophages and recruit other immune cells. Additionally, another research revealed that extracellular vesicles (EVs) secreted from MTB-infected macrophages or mice could activate endothelial cells, which indicated that exosomes might play comprehensive roles during immune activation (33).

A subsequent study demonstrated that exosomal RNA from MTB-infected cells could stimulate a higher level of cytokine and chemokine, and induce more significant apoptosis in macrophages than that by exosomal RNA from uninfected cells (31). Moreover, exosomes from BCG-infected macrophages could activate TLR/MyD88-dependent proinflammatory pathways in BMMs, associated with the lipoarabinomannan (LAM) and the 19-kDa lipoprotein contained in the exosomes that are capable of promoting TNF production (23). With the above evidences, it is suggested that MTB-infected macrophages-derived exosomes could serve as naïve macrophages activating components by regulating pro-inflammatory responses.

EXOSOMES PRESENT ANTIGEN TO ACTIVATE ADAPTIVE IMMUNE SYSTEM DURING MTB INFECTION

$CD4^+$ T lymphocyte cells play an important part in activating macrophages against MTB infection and inducing apoptosis in infected cells *via* IFN-γ, which is mainly secreted by T helper type-1 (Th1) cells. To initiate adaptive immunity, antigen-presenting cells (APCs) load exogenous antigens on MHC-II molecules *via* endocytic pathway and increase the surface expression of T cell costimulatory molecules (34). Exosomes from MTB-infected macrophages have been found to contain various mycobacterial proteins such as Antigen 85-C and early-secreted antigenic target of 6-kDa (ESAT-6), all important antigens for T cell activation against MTB infection (35). Exosomes secreted from MTB culture filtrate protein (CFP)-treated macrophages could activate macrophages, DCs, as well as naïve T cells *in vivo* to mediate protective responses with high expression of antigen-specific IFN-γ and IL-2, resulting in lower MTB loads in the lungs of mice (36). Such *in vitro* and *in vivo*

studies demonstrated the role of exosomes in the process of MTB antigen presentation to T cells to activate T cell protective responses against MTB.

DC derived-exosomes have been found to contain MHC and costimulatory molecules, indicating potential ability of exosomes in antigen presentation. Moreover, antigen-bearing exosomes from DCs can activate antigen-specific naïve $CD4^+$ T lymphocyte cells *in vivo*, but cannot induce the $CD4^+$ T lymphocyte cells activation without mature DCs *in vitro*, suggesting DC's ability to present antigens without contacting bacteria by manipulating exosomal antigen-MHC complex (19). The ability of antigen presentation and naïve T cell activation can also be found in exosomes secreted from MTB-infected macrophages, which can induce the activation of DCs and the generation of memory $CD4^+$ T lymphocyte cells and $CD8^+$ T lymphocyte cells (10).

Additionally, some factors are reported to influence the antigen-presenting role of exosomes. Ramachandra et al. reported that MTB synergized with ATP to induce a more potent release of exosomes containing MHC-II molecules, which were capable of antigen presentation (37). In Rab27a-knockout mice, exosome concentration decreased as Rab27a mediated MVB docking to the plasma membrane, which caused further diminution of T cell responses, leading to increased bacterial loads in the lungs of mice. The reduction of exosomes also led to decreased trafficking of antigens (Ag85A) to exosomes, explaining the reduced priming ability of exosomes (38). It has been clearly demonstrated that MTB-infected immune cell-derived exosomes play important roles in both innate and adaptive immunity, and any disruption of exosomal functions would impair part of the anti-tuberculosis immune protection.

EXOSOME MIGHT STIMULATE AUTOPHAGY FOR MTB CLEARANCE

Autophagy, a form of cellular metabolism involved in innate immunity, is a pathway for the clearance of cellular waste substances or organelles. Low energy, malnutrition, and stress could all contribute to the activation of intracellular autophagy signaling pathways, which degrades the phosphorylation of mammalian target of rapamycin complex 1 (mTOR1), followed by the initiation and continual expansion of membrane to form autophagosomes (39). By further fusion with the lysosomes, autophagosomes would turn into autolysosomes, which have strong abilities in eliminating damaged organelles, protein aggregates, and intracellular pathogens. For host cells, autophagy has been recognized as an important innate defense mechanism against intracellular MTB, and in this way, host cells are sacrificed for effective attack on MTB (40). The autophagy-defective mice are found to show excessive inflammation in lungs and increased bacterial burden, indicating the critical roles of autophagy against MTB infection (41).

A growing number of studies have demonstrated that the antibacterial function of EVs is mainly achieved through modulating autophagy. EVs extracted from infected and uninfected macrophages can both significantly reduce the bacterial loads in mice lungs. However, EVs from uninfected macrophages reduce the bacterial load by production of C-C motif chemokine ligand 2 (CXCR2), while EVs from MTB-infected macrophages produce TNF-α (42). These results indicate that the contents of EV could inhibit MTB both *in vitro* and *in vivo*. EVs secreted from MTB-infected human neutrophils can induce the production of pro-inflammatory cytokines such as TNF-α, IL-6, and superoxide anion to inhibit the intracellular MTB in human macrophages (43). Meanwhile, autophagy is significantly upregulated, and higher expression of autophagy-related marker LC3-II is seen in infected macrophages co-localizing with MTB (43). These results further confirm that EVs from infected innate immune cells can upregulate autophagy for intracellular MTB clearance to lower the intracellular bacterial load. MTB RNA is found to be contained in EVs from infected BMMs, which could pass through host nucleic acid-sensing pathways (RIG-1/MAVS/TBK1/IRF3) to further activate autophagy in MTB-infected macrophages with the co-localization of LC3-II and MTB for MTB elimination (44).

As a member of EV family, exosomes have also been found to be in strong association with autophagy. The newly discovered evidence indicates that exosome biogenesis is closely related to autophagy linked by the endolysosomal pathway (45). Exosomes secreted from miR-181-5p-modified adipose-derived mesenchymal stem cells are found to prevent liver fibrosis *via* autophagy activation, showing its capability to activate autophagy (46). These results reveal the potential bidirectional regulation effects between exosomes and autophagy. This regulation is mediated by the unique biogenesis mechanism involved in autophagy, as well as by the MTB components and membrane molecules contained in exosome that might induce autophagy for MTB clearance (47, 48). As shown in **Figure 1**, we summarize that exosomal contents from infected innate immune cells, including RNA, antigens of MTB, and some MTB peptides, would trigger multiple antibacterial immunological functions *via* a series of traditional pathways. Additionally, we consider that exosomes that inherit the functions of their parent cells could perform cargo delivery function to induce immune functions, such as inducing autophagy. However, almost all published works focused on the effects of exosomes on other innate immune responses. Further studies are needed to explore how exosomes act on autophagy of host cells against MTB infection.

EXOSOMES CONTRIBUTE TO IMMUNE EVASION OF MTB

Exosomes secreted from infected immune cells can induce stronger anti-MTB activities in different immune cells, supporting the potential use of exosomes for the development of vaccine or immunotherapy strategies. However, as one of the cleverest bacteria, MTB is continuously altering the environment for immune evasion. Early infection defense can be induced by

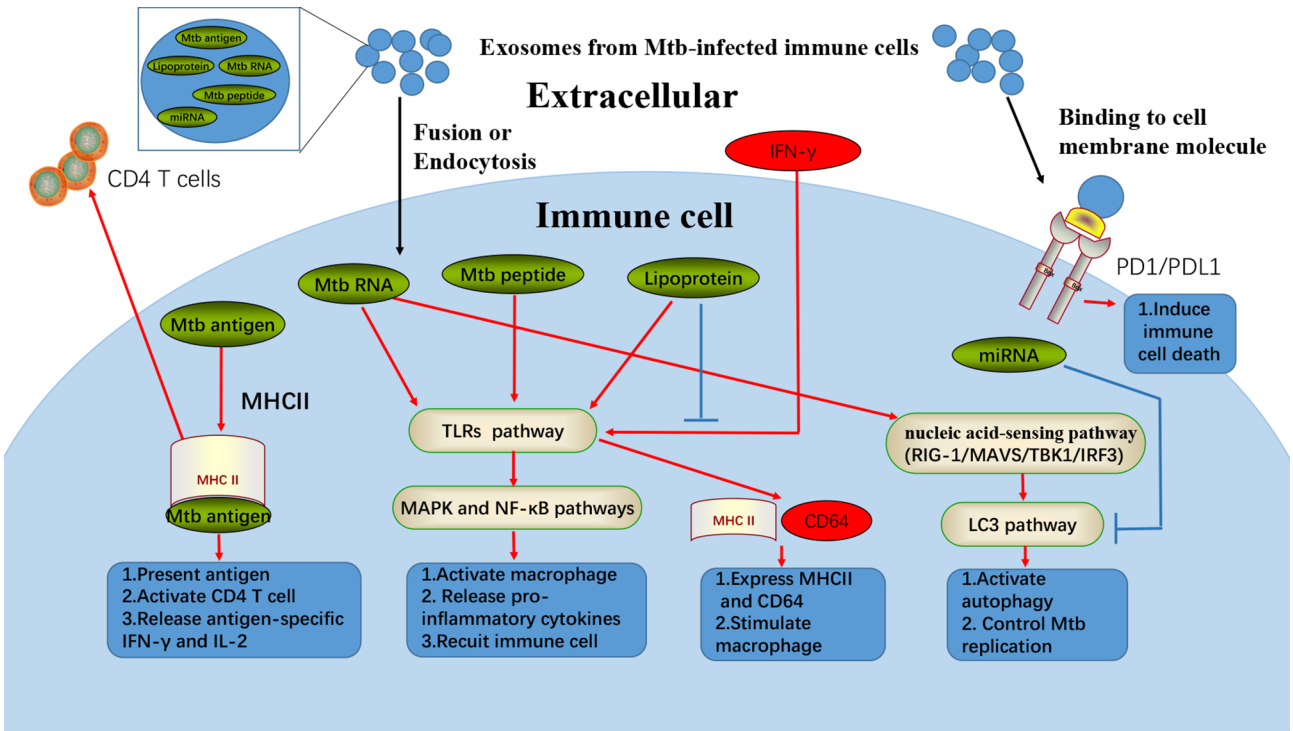

FIGURE 1 | Exosomes from Mtb-infected immune cells could induce multiple cellular responses. Exosomes from Mtb-infected immune cells contain various regulatory materials, such as Mtb antigens, Mtb RNA, Mtb peptide, lipoprotein, and miRNA. In fusion with cell membrane or passing through endocytosis, exosomes can release some Mtb specific contents to induce different anti-Mtb responses in immune cells. Mtb antigens from exosomes can be transferred into uninfected-DC, where the antigens can combine with MHCII for antigen presentation and activating the adaptive immune systems. Mtb RNA, peptide, and lipoprotein from exosomes are found to be responsible for exosomes induced-inflammation, which are in strong association with TLRs, MAPK, and NF-κB pathways. Furthermore, Mtb RNA from exosomes can also stimulate macrophage autophagy through nucleic acid-sensing pathways (RIG-1/MAVS/TBK1/IRF3). However, exosomes from Mtb-infected immune cells can also inhibit anti-Mtb functions. For example, lipoprotein from exosomes can suppress the IFN-γ induced MHCII and CD64 expression. Besides, miRNA from exosomes can inhibit autophagy of immune cells, while Mtb infection as well as PD1/PDL1 from exosomes can also act as immune inhibitor.

constituents from MTB that interact with host protein and interfere with host immune cells. However, in long-term infection, these constituents from MTB could be harmful. Moreover, host proteins and miRNA are changed during MTB infection and can facilitate immune evasion of MTB. These molecules can be transferred to different immune cells by exosomes, creating an environment more prone to MTB immune evasion.

Exosome can induce various biological or immunological effects depending on the cargo inside. Pramod et al. identified 41 mycobacterial proteins present in exosomes released from MTB-infected J774 cells, including some very important MTB antigens. Many of these identified proteins were characterized as highly immunogenic, especially Ag85b, which was widely used for TB vaccine development (35). Small EV proteome isolated from active tuberculosis (ATB) were found to carry host proteins in TB-positive patients, which showed significant deregulation and could be useful in developing alternate host-directed therapeutic interventions (49). Using multiplexed multiple reaction monitoring mass spectrometry (MRM-MS), Nicole A et al. analyzed exosomes isolated from human serum samples obtained from culture-confirmed active TB patients and found 76 peptides representing 33 unique MTB proteins (50). Twenty of the 33 proteins detected were found in the exosomes of TB patients, including several peptides from eight important MTB proteins, which were known to contribute to the intracellular survival of MTB (50). These MTB and host proteins, as well as the molecules that we have summarized above in exosomes, allow exosomes to directly interact with different immune cells with immune regulation effects. Here we summarized the exosome sources, different isolation methods, and culprit cargos from the quoted studies (**Table 1**).

Although specific proteins and peptides from MTB have been identified, exosome-induced immune regulation effects are still under investigation because MTB components can induce complicated effects. For example, LAM from MTB has been found in the urinary extracellular vesicles of tuberculosis patients (51) and also in exosomes isolated from the broncho alveolar lavage fluid (BALF) of BCG-infected mice (23). LAM from MTB or other pathogenic mycobacteria is a high-molecular-mass, amphipathic lipoglycan with a defined critical role in mycobacterial survival during infection (54). It is thought to show both active and passive protection against TB (55). Although LAM shows ability to activate immune cells, Nicole

TABLE 1 | Summary of the exosome sources, different isolation methods, and different culprit cargo from the studies.

Exosome sources	Isolation methods	Identified cargo	Author
MTB-infected macrophages	Sucrose-gradient ultracentrifugation, Ultracentrifugation	MTB components Lipoarabinomannan and the 19-kDa lipoprotein	Bhatnagar et al. (23)
MTB-infected macrophages	Ultracentrifugation	MicroRNA, MRNA	Singh et al. (31)
Serum from persons with latent tuberculosis infection	Exoquick	MTB peptides (Ag85c, DnaK, HspX, Ag85A et al.)	Mehaffy et al. (32)
MTB-infected and CFP-treated macrophages	Sucrose-gradient ultracentrifugation	Mycobacterial proteins (Antigen 85-C, GlnA, 19 kDa Lpqh et al.)	Giri et al. (35)
MTB CFP-treated macrophages	Exoquick, Ultracentrifugation	19 kDa lipoprotein	Cheng et al. (36)
MTB-infected macrophages	Exoquick, Ultracentrifugation	MTB RNA	Cheng et al. (44)
Serum of tuberculosis patients	Exoquick, Sucrose-gradient ultracentrifugation	Host proteins (KYAT3, SERPINA1, HP, and APOC3)	Arya et al. (49)
Serum of tuberculosis patients	Exoquick	MTB peptides (Antigen 85B, Antigen 85C, Apa, BfrB, GlcB, HspX, KatG et al.)	Kruh-Garcia et al. (50)
Urine of tuberculosis patients	Centrifugation	Lipoarabinomannan and CFP-10	Dahiya et al. (51)
MTB-infected macrophages	Sucrose-gradient ultracentrifugation	Lipoproteins	Singh et al. (52)
Serum of tuberculosis patients	Ultracentrifugation	MicroRNA (hsa-miR-1246, hsa-miR-2110, hsa-miR-370-3P, hsa-miR-28-3p, and hsa-miR-193b-5p et al.)	Lyu et al. (53)

P et al. also demonstrated that LAM from MTB could reduce the expression of chemokine receptors CXCR2 by a mechanism that involved the activation of p38 MAPK (56). As CXCR1 and CXCR2 determines the functional properties of granulocytes, the LAM inside exosomes from MTB-infected immune cells suggest the ability of the host to limit inflammation induced by granulocytes after MTB infection. Despite the anti-MTB effect brought by exosomes, MTB has been menacing human health throughout history. With a wide variety of immune evasion mechanisms and its strong pathogen-host interaction ability, MTB can also have exosomes act negatively (57). Several studies have proved that MTB could influence macrophage functions such as apoptosis, autophagy, and MTB-lysosome fusion by some of the MTB components that interfere with anti-MTB effects (58, 59). An affinity tag purification mass spectrometry (AP-MS) study has demonstrated the interaction map between MTB components and host cell proteins, and found that a lot of the components can interact with host proteins by regulating various cell functions (60). More importantly, exosomes from MTB-infected macrophages are found to induce a decline of IFN-γ-induced MHC-II and CD64 expression through TLR2 and Myd88 pathway, partially *via* the lipoproteins in exosomes (52). This study reminds us that exosomes can act negatively in defending MTB infection, which means that the immune regulation roles of exosomes can be either inhibiting MTB infection or promoting MTB infection. Based on these results, we hypothesize that at the early stage, the cargos coming from MTB inside the exosomes could activate the anti-MTB effect. However, MTB can survive in macrophages, and after the early stage, an increasing amount of MTB components are able to interact with host proteins and interfere with the activated immune function. We further speculate that the exosome-induced immune suppression of IFN-γ-induced MHC-II and CD64 expression (52) is like MTB components-induced immune evasion, which mainly happens at the post-infection stage. Therefore, exosomes can suppress the activated macrophages' function instead of acting as an immune stimulator. For our understanding, the cargos inside the exosomes would be a very good anti-TB immune stimulator in inactivated immune cells. However, as exosomes are part of the existing signal conduction pathways of cells during MTB infection, they show two-sided effects on TB immunity as the pathogen might pretend to use these exosomes for their immune escape. Therefore, these exosomes from MTB-infected cells might be anti-MTB vaccine candidates due to their ability to activate anti-MTB immunity in rest of the immune cells. However, they might also block cellular antibacterial immunity by "hijacking" immune cells.

Furthermore, RNA in exosomes, especially miRNA for regulating host cell functions, plays important roles in MTB evasion. RNA sequencing provides a powerful strategy to explore the miRNA in exosomes from MTB-infected macrophages and plasma from tuberculosis patients, which are predicted to be closely linked with the metabolism and energy production-related pathways (53, 61). MiR-18a, a type of miRNA also found in exosomes, is upregulated to promote mycobacterial survival in MTB-infected macrophages by inhibiting the autophagy pathways (62). In MTB infection, individual miRNA alternation might induce cellular function changing as miRNA could regulate gene expression post-transcriptionally. Furthermore, miRNA can influence multiple antibacterial functions of different immune cells (such as macrophages, DCs, and $CD4^+$ T lymphocyte cells) by regulating apoptosis, autophagy, and polarization (63, 64), making miRNA an important host material for immune regulation and a pathway for MTB immune evasion. As miRNA contained in exosomes can be transferred to different cells of the immune system, harmful immune functions can also be shared by MTB-benefiting miRNA.

Moreover, as a star molecule in immune suppression, programmed cell death 1 ligand 1 (PD-L1) is also found in

exosomes, which suppresses the function of $CD8^+$ T lymphocyte cells and facilitates tumor growth (65). Increased programmed cell death protein 1 (PD-1)/PD-L1 expression, which increases the macrophage susceptibility to MTB-specific $CD8^+$ T lymphocyte cells, can lead to cell deaths (66), and this is already proved in MTB infection. This indicates that the exosomal PD-L1 might also contribute to the immune evasion of MTB. Taking all the results into account, exosomes from MTB-infected cells could also act as an accomplice for MTB immune evasion by delivering components benefiting MTB survival, as shown in **Figure 1**. More attention should be paid to the exploration of MTB-benefiting components in the exosomes, which would help develop novel immunotherapy strategies to restrain MTB infection.

EXOSOMES FOR VACCINE AND IMMUNOTHERAPY DEVELOPMENT DURING MTB INFECTION

The full role of exosomes during MTB infection is still to be revealed. We should be exploring how exosomes help MTB escape from the immune attack and also trying to utilize the antibacterial function of exosomes for TB treatments. Exosomes carrying mycobacterial antigens can significantly protect mice against MTB infection, indicating the potential of exosomes in serving as a novel cell-free vaccine targeting MTB infection (36). EVs from MTB-infected BMM can induce autophagy for *in vitro* MTB killing and also decrease mycobacterial burden in the lungs of mice with lower tissue damage (44). These studies strongly suggest that exosomes may be a candidate vector for vaccine or drug delivery.

In theory, exosomes are promising delivery tools for vaccines and treatments thanks to their natural nano-size lipid membrane structures. The vaccine based on exosomes with specific antigens inside can activate multiple immune responses *via* antigen presentation pathways. For example, exosomes loaded with tumor-associated-antigens can activate adaptive immunity as well as improve antitumor efficacy both *in vivo* and *in vitro* (67, 68). In another study, researchers developed a novel vaccine based on Ag85B-ESAT-6 fusion protein expressed in exosomes, which could be further introduced for activating antigen-specific INFγ-secreting T lymphocytes in the lungs and spleen (69). Moreover, the anti-MTB immunity induced by exosomes-based vaccine can also be improved by costimulatory molecules in exosomes. Hao et al. established ovalbumin (OVA)-pulsed exosomes from dendritic cells to target CD4+ T cells as a cancer vaccine, which also successfully stimulated CD8+ cytotoxic T lymphocyte (CTL) responses for enhanced antitumor immunity (70). They found that the exosomal CD80 (70) and exosomal CD40L (71) were crucial in the development of functional memory CTLs. Interestingly, it was also found that exosomes observed in BALF were expressing MHC class I and II, CD54, CD63, and the costimulatory molecule CD86, suggesting that exosomes might have a role in antigen delivery or immune regulation during airway antigen exposure (72). Using MTB-infected antigen-presenting cell-derived exosomes as vaccine not only can realize antigen presentation but also express costimulatory molecules which can stimulate strong anti-MTB immunity.

Additionally, exosomes can act as adjuvants that stimulate immune responses, which can improve vaccine efficiency by mediating the immune environment of adaptive immune system. It has been proven that hepatitis B recombinant antigen (HBsAg), combined with exosomes from LPS-stimulated macrophages, can induce pro-inflammatory cytokine expression and antibody release similar to HBsAg alone (73). However, exosomes could have further immunomodulatory effects on the cellular immune response, highlighted by the enhancement of IFN-γ secretion as Th1 cell responses (73). Another study also demonstrated that exosomes from TGF-β1-silenced leukemia could promote DC maturation and their immune function, backing up the roles of exosomes as adjuvants to establishing enhanced anti-tumor immunity (74). Exosomes have the abilities, inherited from parent cells, of boosting anti-MTB immunity through traditional pathways, including macrophages, DCs, and neutrophil granulocytes. These anti-MTB immune responses can be triggered by some regulatory proteins in exosomes, such as heat-shock protein (75) and PAMP (23). These findings demonstrated the possibility of using exosomes from infected innate immune cells for specific antigen loading, costimulatory molecules stimulating, and immunity boosting, which would be beneficial for vaccine development.

Apart from acting as components in immunological regulation, exosomes can also be applied to direct drug delivery, which help overcome the major challenges of drug treatment, i.e., delivery of cargos across impermeable biological barriers and improvement of the target effects and pharmacokinetics of drugs (76). As a novel drug delivery system, exosomes isolated from M1 macrophages have been proved to enhance the cytotoxicity of Paclitaxel (PTX) in cancer cells and show stronger antitumor efficacy (77). A majority of published studies focused on using exosomes for anticancer drug delivery, inspiring the application of exosomes in anti-MTB drug delivery, which could potentially improve the current TB chemotherapy efficacy. Furthermore, certain components with antibacterial regulation effects, such as non-coding RNA, can also be loaded into exosomes for anti-MTB treatment. Endowed triply for its potentials in novel vaccine, immune therapy, and chemotherapy strategy developments as shown in **Figure 2**, exosomes bring hope for anti-MTB treatments.

Lipids are critical components of exosomal membranes, and it is well-known that some specific lipids are even more enriched in exosomes compared to their parent cells. Therefore, the use of exosomes for delivery can be considered as using "natural lipid nanoparticles." As a "competitor" against the current lipid nanoparticles (nanolipids), it would be crucial to compare the properties of exosomes with normal lipid nanoparticles, which would benefit our understanding of the advantage and limitation of exosomes as delivery systems. As shown in **Table 2**, firstly, exosomes have natural immunogenicity and antigenicity depending on the cargo loaded inside, while normal lipid

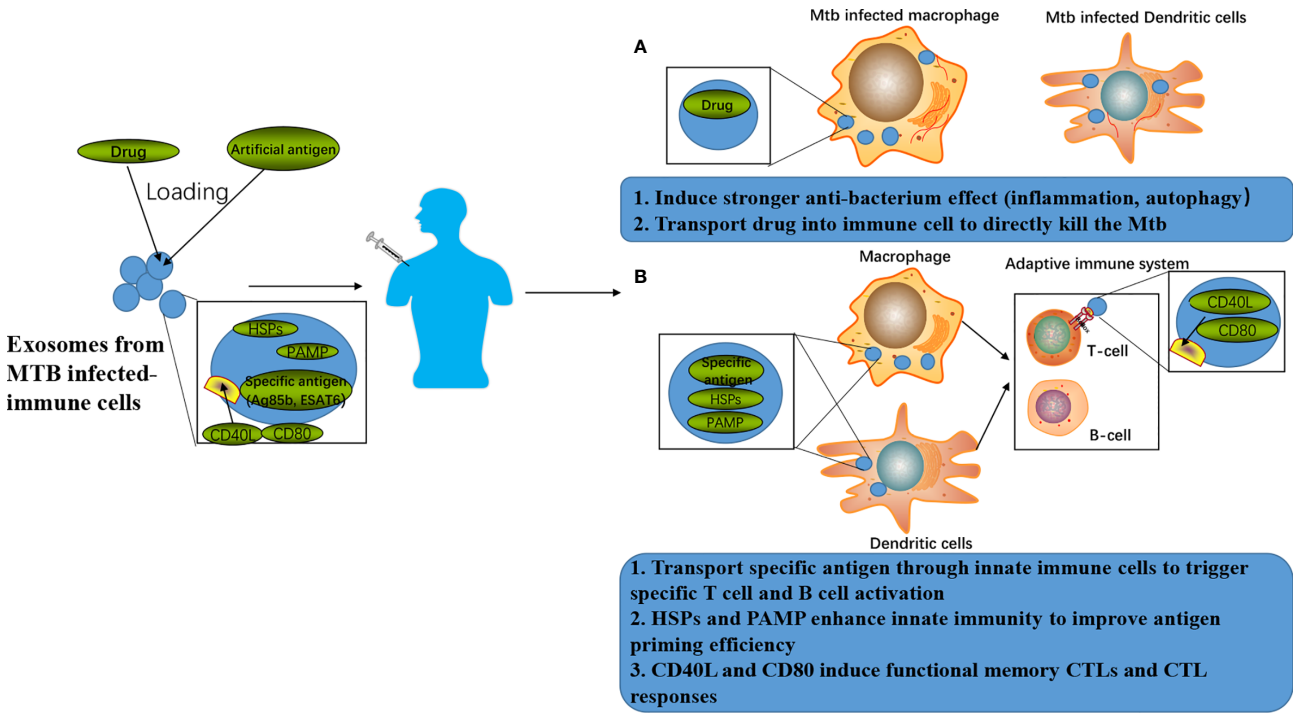

FIGURE 2 | Exosomes-associated anti-TB strategies. **(A)** Exosomes can be loaded with drugs to achieve enhanced anti-TB therapy by combining the improved killing efficiency induced by the drugs and anti-TB responses induced by the exosomes. **(B)** Exosomes can be loaded with specific antigens to achieve enhanced anti-TB immune responses by combing the T cell and B cell activation induced by the specific antigen as well as anti-TB responses induced by the exosomes.

nanoparticles do not have similar abilities. This property can distinguish between lipid nanoparticles and exosomes, and the immune regulatory function of exosomes can be mediated by controlling the parent cells. Based on these properties, exosomes can be used as natural adjuvants, natural vaccines, or natural immunomodulators. Secondly, the cell membrane structure of exosomes can protect the loaded drug, protein, or RNA from the extracellular environment with less rejection than chemosynthetic lipid nanoparticles, providing much safer transportation environment (78). Last but not least, exosomes have the ability to pass through the blood-brain barrier without further modification (79), while lipid nanoparticles can require specific chemical modification (80).

However, we cannot deny that limitation exists in the application of exosomes as delivery vectors. The quality control of exosomes is still a big challenge to be addressed. The contents inside exosomes always change along with the functions and status of parent cells, leading to an unpredictability for both cargo delivery and subsequent effects. Besides, as the structure of exosomes is similar to the cell membrane, some environmental factors such as pH and temperature, as well as preparation procedures such as ultracentrifugation and freezing-thawing, might introduce unexpected damage to exosome structures, subsequently affecting the quality of exosomes (81). However, chemosynthetic lipid nanoparticles can be prepared with constant quality, which is also easy to be further modified and stored. Moreover, the types of nanoparticles-induced responses in recipient cells are constant, while exosome-induced cellular responses are much more complicated, unpredictable, and uncontrollable. For example, exosomes from MTB-infected macrophages can induce multiple anti-MTB effects, but at the same time promote the immune escape of MTB. Furthermore, the detailed mechanism that parent cells sort out MTB components and dock them into exosomes remains unknown,

TABLE 2 | Advantages and limitations of exosomes compared with nanolipids (lipid nanoparticles) for vaccination.

	Advantages	Limitations
Nanolipid-based vaccination	1. Easy to be modified 2. Controllable size and shape 3. Long-term stability 4. Inexpensive expense 5. Easy for quality control	1. No natural antigenicity or immunogenicity 2. Need specific modification to pass blood-brain barrier
Exosome-based vaccination	1. Natural antigenicity and immunogenicity 2. Natural immunomodulators 3. Better protection of inside cargo by cell membrane structures 4. Natural ability to pass blood-brain barrier	1. Difficult in quality control 2. Unpredictable content insides 3. Difficult to store and transportation 4. MTB constitutes docking pathway unclear 5. Modification techniques immaturity 6. Expensive expense

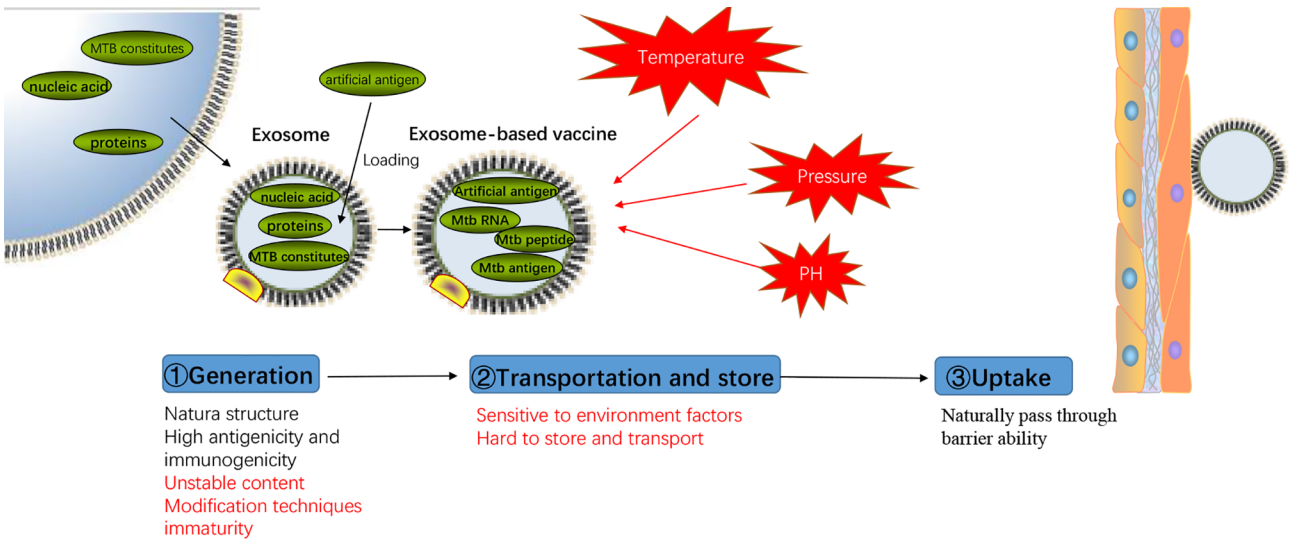

FIGURE 3 | Advantages and disadvantages of exosomes-based vaccine. Representation of the process of exosomes-based vaccine generation, transportation, storage, and uptake with the advantages and disadvantages (mark in red) of each step.

which also introduce unpredictability into their delivery actions. Additionally, the modification techniques of exosomes are still very limited, rendering the surface modification of exosomes difficult. Finally, it is also worth noting that the high cost of exosome preparation would also be considered a critical limitation compared with lipid nanoparticles.

Taking all these considerations into account (as shown in **Figure 3**), although exosomes show multiple limitations for vaccination, their advantages, including their natural antigenicity and immunogenicity to regulate immune responses, provides an attractive blueprint for more powerful vaccine developments. Thus, to extend the application of exosomes as effective drug delivery systems for vaccination and therapy, it would be critical to clarify the underlying mechanisms involved in the formation and cargo loading of exosomes in parent cells. Additionally, more attention should be paid to the quality control, preparation, and modification methods of exosomes to obtain homogeneous, constant, storable, and more functional exosomal products, benefiting the use of exosomes for novel vaccination and therapy strategy development.

CONCLUSION AND REMARKS

In MTB infection, exosomes from the infected immune cells have double inherent immune regulation functions their parent cells with double-edged sword regulation effects on anti-MTB immunity. However, previous studies only focused on exosomes from innate immunity cells, and little is understood about the exosomes secreted from adaptive immune cells, which are of more importance in facing MTB infection. Therefore, exploring potential effects of exosomes from innate immune cells on MTB infection is helpful in developing new vaccination and therapy. Up to now, diagnosis, vaccination, and treatment of TB, especially drug-resistant TB, remains major clinical challenges. Different RNA molecules have been found in exosomes after MTB infection, which shed new light on the potential role of exosomal RNAs as novel TB biomarkers for developing the next generation of TB diagnostic strategy and relevant studies have already begun (82–84). Exosomes have shown strong potential in delivering vaccine components (proteins, peptides, and RNA) in different infectious disease, showing the potential to provide a more effective vaccine strategy for TB. Albeit the limited knowledge regarding the drug delivery roles of exosomes for anti-MTB treatment, the strong ability of macrophages to internalize the nanosized system allows macrophage-targeted drug delivery for anti-MTB treatment. However, careful consideration is still in need when applying exosomes as drug delivery systems as they also have negative roles in immune function. Further surface functionalization of exosomes with specific ligands would increase the targeting effects against specific cell types (85), which reminds us that some ligands with macrophage targeting effects could benefit the anti-MTB therapy by exosomes functionalization and drug delivery. Most importantly, designing cell systems to produce functional anti-MTB exosomes would dramatically expand the application of exosomes in developing vaccine or drug delivery methods.

AUTHOR CONTRIBUTIONS

Y-FS was in charge of research and drafting. JP helped in revision. J-FX was responsible for leading this work and

revising the manuscript. All authors contributed to the article and approved the submitted version.

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Membrane Particles Derived From Adipose Tissue Mesenchymal Stromal Cells Improve Endothelial Cell Barrier Integrity

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Proinflammatory stimuli lead to endothelial injury, which results in pathologies such as cardiovascular diseases, autoimmune diseases, and contributes to alloimmune responses after organ transplantation. Both mesenchymal stromal cells (MSC) and the extracellular vesicles (EV) released by them are widely studied as regenerative therapy for the endothelium. However, for therapeutic application, the manipulation of living MSC and large-scale production of EV are major challenges. Membrane particles (MP) generated from MSC may be an alternative to the use of whole MSC or EV. MP are nanovesicles artificially generated from the membranes of MSC and possess some of the therapeutic properties of MSC. In the present study we investigated whether MP conserve the beneficial MSC effects on endothelial cell repair processes under inflammatory conditions. MP were generated by hypotonic shock and extrusion of MSC membranes. The average size of MP was 120 nm, and they showed a spherical shape. The effects of two ratios of MP (50,000; 100,000 MP per target cell) on human umbilical vein endothelial cells (HUVEC) were tested in a model of inflammation induced by TNFα. Confocal microscopy and flow cytometry showed that within 24 hours >90% of HUVEC had taken up MP. Moreover, MP ended up in the lysosomes of the HUVEC. In a co-culture system of monocytes and TNFα activated HUVEC, MP did not affect monocyte adherence to HUVEC, but reduced the transmigration of monocytes across the endothelial layer from 138 ± 61 monocytes per microscopic field in TNFα activated HUVEC to 61 ± 45 monocytes. TNFα stimulation induced a 2-fold increase in the permeability of the HUVEC monolayer measured by the translocation of FITC-dextran to the lower compartment of a transwell system. At a dose of 1:100,000 MP significantly decreased endothelial permeability (1.5-fold) respect to TNFα Stimulated HUVEC. Finally, MP enhanced the angiogenic potential of HUVEC in an *in vitro* Matrigel assay by stimulating the formation of angiogenic structures, such as percentage of covered area, total tube length, total branching points, total loops. In conclusion, MP show regenerative

effects on endothelial cells, opening a new avenue for treatment of vascular diseases where inflammatory processes damage the endothelium.

Keywords: membrane particles, nanovesicles, mesenchymal stromal cells, endothelial cells, regeneration, immune cell interaction

INTRODUCTION

The endothelium forms an interactive barrier between the circulatory system and the tissues in the body. It plays a pivotal role in the regulation of vascular permeability, hemostasis, and immunological processes (1). Alterations of endothelial cells (EC) play a central role in the pathogenesis of a broad spectrum of the most dreadful of human diseases, such as atherosclerosis (2), stroke (3), heart disease (4), diabetes (5), allograft rejection (6), and chronic kidney failure (7). Inflammatory mediators cause overexpression of cell adhesion molecules (CAM) on EC and together with the secretion of cytokines this permits the attraction and adhesion of circulating immune cells to the endothelium, and consequently, the transmigration of leukocytes into inflammation sites (8). Therapies that protect the endothelium from stress and immune factors or enhance the repair processes may be capable of curing or preventing diseases where the endothelium has a key role.

Mesenchymal stromal cells (MSC) represent such therapy as they have immunomodulatory and regenerative capacities and are known to deliver endothelial protective signals (9). The endothelial protective effects of MSC are due to their anti-inflammatory and repair properties that have shown substantial therapeutic promise in preclinical models, such as for instance in atherosclerosis (10). Moreover, MSC hold great promise for revascularization of tissues as they secrete pro-angiogenic and anti-apoptotic factors in large amounts (11).

The translation of the endothelial protective and reparative effects of MSC found in the *in vitro* setting to an effective therapy is hampered by the poor biodistribution of infused MSC after intravenous administration. It is demonstrated that after intravenous infusion, MSC get trapped in the lungs and have a short survival time (12, 13). This implies that MSC do not reach sites of injury and cannot interact locally with injured tissue. Viable MSC may secrete cytokines and growth factors in the circulation and target distant cells *via* this route, but recent work demonstrated that inactivated MSC, which lost their capacity to secrete factors, maintain their immunomodulatory capacity in an animal model (14), suggesting that cell membrane dependent interactions with target cells play a role in the immune regulatory effects of MSC. Furthermore, MSC-conditioned media have shown to possess similar regenerative properties as MSC on tissue damage and contribute to the modulation of inflammation (15). Conditioned medium is composed of growth factors, cytokines, and extracellular vesicles (EV). EV are spherical membrane fragments heterogeneous in size and composition that carry and transfer proteins, lipids, and RNA from the source cells to resident cells in damaged tissue (16). MSC-derived EV have shown therapeutic effects in several diseases' models including CVD (17) and acute kidney injury (18). Despite EV may be a promising alternative cell-free therapy, clinical translation is hindered by the lack of suitable and scalable technologies for the generation and purification of extracellular vesicles (19, 20). Thus, novel methods are needed to make pharmaceutically controllable and homogeneous membrane vesicles for targeting injured tissues.

We previously reported on the generation of large amounts of membrane particles (MP) from human adipose tissue MSC (AT-MSC) (21). The size of these man-made MP was with on average 120 nm, like naturally occurring EV, and electron microscopy showed they have a spherical shape. MP were shown to be able to modulate immune cells, thereby showing a great potential as a novel cell-free immune therapy, and a good alternative to EV therapy as MP can be produced in large amounts, highly purified, in an easy and economic process.

In the present study, we have investigated the potential therapeutic effects of MP derived from AT-MSC on the barrier integrity of inflamed endothelial cells using a model of TNFα treated human umbilical vein endothelial cells (HUVEC). We further explored whether MP could enhance the angiogenic ability of HUVEC in an inflammatory environment.

MATERIAL AND METHODS

Ethics Statement and Human Tissue Samples

Human MSC were isolated from subcutaneous adipose tissue from healthy kidney donors that became available during kidney donation procedures. The tissues were collected after obtaining written informed consent, as approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam (protocol no. MEC-2006-190).

Isolation and Culture of MSC From Adipose Tissue

AT-MSC were isolated from subcutaneous adipose tissue of five healthy donors (2 females/3 males). The age of the donors was between 34-58 years old. The tissue was mechanically disrupted and enzymatically digested with 0.5 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) in RPMI for 30 min at 37°C under continuous shaking. Thereafter, the cells were resuspended in MEM-α with 10% fetal bovine serum (FBS; Lonza, Verviers, Belgium), 2 mM L-glutamine and 1% P/S, filtered through a 100 μm cell strainer, and transferred to 175 cm^2 culture flasks (Greiner Bio-one, Essen, Germany). At 90% confluence AT-MSC (passage 2-6) were collected to generate MP. The phenotypic characterization of AT-MSC was performed

by flow cytometry using FACSCANTO-II with FACSDIVA Software (BD Biosciences, San Jose, CA). AT-MSC were incubated with mouse-anti-human monoclonal antibodies against CD13-PE-Cy7; HLA-DR-PERCP; HLA-ABC-APC; CD31-FITC; CD73-PE; PD-L1-PE (all BD Biosciences); CD90-APC and CD105-FITC (R&D Systems, Abingdon, UK). All the antibodies were incubated with the cells for 30 min, at room temperature in the absence of light.

Culture of Human Umbilical Vein Endothelial Cells

First-passage cryopreserved HUVEC from pooled donors were obtained from Promocell (Promocell, Germany). HUVEC were grown in 75 cm^2 flasks at 37 °C, 5% CO_2 with endothelial cell basal medium (EBM, Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA), endothelial cell growth medium supplements (EGM, Cambrex Bio Science), 10% FBS, 100 IU/mL penicillin, and 100 mg/mL streptomycin. At 90% confluence, HUVEC were dissociated by 0.05% trypsin-EDTA (Life Technologies, Bleiswijk, The Netherlands). To establish an endothelial cell model of inflammation, HUVEC were incubated with TNFα (25ng/ml) for 4h or 24h depending on the assay. All HUVEC used in the experiments were between passage 2-7. During these passages, HUVEC conserved their morphology, phenotype, and proliferation rate. For the stimulation of HUVEC with TNFα, three concentrations of TNFα were tested (10, 25, 50ng/ml). All the experiments were performed with the concentration 25 ng/ml due to the difference respect to adhesion molecules and monocyte adhesion assay between Control and TNFα treated cells was enough to allow MP play a role, without inducing HUVEC apoptosis.

Generation of Membrane Particles From AT-MSC

AT-MSC were trypsinized and washed twice with PBS. Then, the MSC were incubated in milliQ water at 4°C to induce osmotic lysis and liberation of the cell nuclei (after about 20 min, monitored by microscope). Cell extracts were cleared of unbroken cells and nuclei by centrifugation at 2,000 x *g* for 20 min. The obtained supernatant was transferred to Amicon Ultra-15 filter tubes (100 kDa pore size) and concentrated by centrifugation at 4,000 x *g* at 4°C. The concentrated pellet consisted of crude membrane and was diluted in 0.2 μm filtered PBS. A population of MP, homogeneous in size was obtained by extruding the plasma membranes 3 times through polycarbonate membrane filters (Merck, KGaA, Darmstadt, Germany), first with a pore diameter of 800 nm, secondly with a 400 nm and last with a 200 nm pore size filter. The extrusion process was performed using LiposoFast LF-50 (AVESTIN Europe, Mannheim, Germany) at 20 psi (**Figure 1**). All procedures were performed on ice.

Analysis of Adhesion Markers on HUVEC

HUVEC were incubated with TNFα (25ng/ml) and two ratios of MP (1:50,000, 1:100,000 HUVEC : MP) during 24h. Then, the cells were trypsinized and washed with FACS Flow (BD Biosciences, San Jose, CA). The immunophenotypic characterization of the activation state of endothelial cells was done by incubating HUVEC with mouse-anti-human monoclonal antibodies against CD54-APC, CD106-BV421, CD62e-PE, CD31-FITC, VEGFR2-PE, CD105-FITC and TIE2-Alex647 (all BD Biosciences). All the antibodies were incubated with the cells for 30 min, at room temperature in the absence of light. After two washes with FACS Flow, flow cytometric analysis was performed using FACSCANTO-II with FACSDIVA Software (BD Biosciences).

Characterization of MP Size and Concentration by Nanoparticle Tracking Analysis

Analysis of absolute size distribution and concentration of MP was performed using NanoSight NS300 (NanoSight Ltd.). With NTA, particles are automatically tracked and sized based on Brownian motion and the diffusion coefficient. The NTA measurement conditions were: detection threshold 3 (determined with a protein solution), three measurements per sample (30 s/measurement), temperature 23.61 ± 0.8°C; viscosity 0.92 ± 0.02 cP, frames per second 25. Each video was analyzed to give the

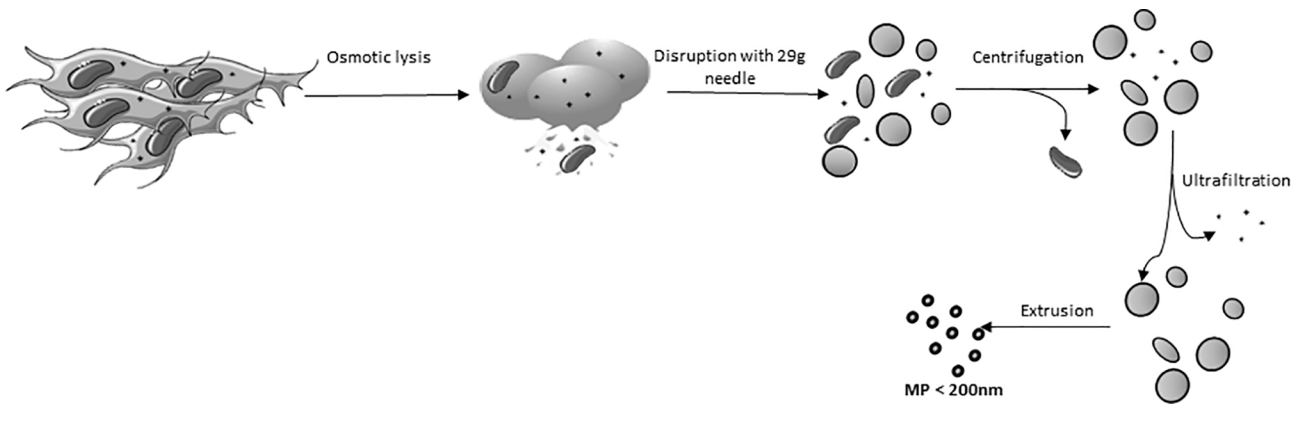

FIGURE 1 | Schematic overview of the generation of Membrane particles.

mean, mode, median and estimated concentration for each particle size. The samples were diluted in 0.2μm filtered PBS, to obtain a measurable concentration of particles (1 x 10^8 particles/ml) in accordance with the manufacturer's recommendations.

Cryo-Transmission Electron Microscopy

The preparations of MP were visualized by the Cryo-TEM method. A thin aqueous film was formed by applying a 3μl droplet of MP suspension to a specimen bare EM grid. Glow-discharged holey carbon grids were used. After the application of the suspension the grid was blotted against filter paper, leaving a thin sample film spanning the grid holes. These films were vitrified by plunging the grid into ethane, which was kept at its melting point by liquid nitrogen, using a Vitrobot (Thermo Fisher Scientific Company, Eindhoven, The Netherlands). The vitreous sample films were transferred to a Tecnai Arctica microscope (Thermo Fisher Scientific, Eindhoven, The Netherlands). Images were taken at 200 Kv with a field emission gun using a Falcon III (Thermo Fisher Scientific) direct electron detector.

Extraction and Identification of DNA/RNA From MP

To examine whether DNA and RNA are present in MP, a High Pure RNA Isolation Kit (Roche Applied Science, Penzberg, Germany) was used to extract DNA/RNA from MP samples following the manufacturer's instructions. After the isolation of the RNA/DNA, the samples were treated with DNase I to quantify the concentration of RNA, whereas for the collection of both DNA and RNA, DNase I treatment was omitted. The concentration and purity of DNA+RNA and RNA in the samples was assessed spectrophotometrically using a NanoDrop ND-1000 (Thermo Fisher Scientific, Bleiswijk, The Netherlands). The quality of the RNA was assessed by assigning an RNA integrity number (RIN) using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative RT-PCR Analysis

MP were stored at −80°C. Total RNA was isolated, and 500 ng used for complementary DNA (cDNA) synthesis. Gene expression was determined by Quantitative Real-Time PCR (qPCR) using the TaqMan Universal PCR Master Mix (Life Technologies ThermoFisher scientific), and the assay-on-demand primer/probes for Thermo Fisher GAPDH (Hs99999905.m1); CD90 (Hs00264235_s1), Vascular endothelial growth factor A (VEGF-A: Hs00173626.m1), Angiopoietin 1 (Hs01586213.m1), IL-8 (Hs00174114.m1). For PCR, cDNA synthesized from 25 ng total RNA was used to perform each amplification.

Assessment of MP Toxicity on HUVEC: Apoptosis Assay

HUVEC were seeded at a density of 2x10^5/well in 12-well plates. Then, unstimulated and TNFα (25ng/ml) stimulated HUVEC were cultured with 2 ratios of MP (HUVEC : MP 1:50,000, 1:100,000) during 24h and 48h. Cell viability was assessed using an Annexin V staining kit (BD Biosciences) according to the manufacturer's recommendations. Briefly, after the incubation time with MP, cells were harvested, washed in PBS, and resuspended in a binding buffer that contained 5 μl Annexin V antibody and 5 μl 7-AAD. Samples were measured by FACSCanto II (BD Biosciences).

Uptake of MP by HUVEC

AT-MSC were labeled with red fluorescent PKH-26 dye, which intercalates into lipid bilayers, according to the manufacturer's instructions (Sigma-Aldrich), enabling the generation of fluorescent MP (PKH-MP). HUVEC were plated at a density of 2x10^5 cells/well on a 12 well plate, treated with/without TNFα (25ng/mL). Two ratios of PKH-MP, (1:50,000 and 1:100,000) were added to the cultures for 4h and 24h and the uptake of MP by HUVEC was quantified by flow cytometry. The data were analyzed using Kaluza Software (Beckman Coulter).

For confocal microscopy analysis, cell membranes of HUVEC were labeled with PKH-67, the nuclei with 10μM Hoechst 33342, and the lysosomes with a LysoSensor dye (Invitrogen Molecular Probes), which changes to yellow fluorescence in acidic environments. PKH-MP uptake by HUVEC was imaged by a Leica TCS SP5 confocal microscope (Leica Microsystems B.V., Science Park Eindhoven, Netherlands), equipped with Leica Application Suite – Advanced Fluorescence (LAS AF) software, DPSS 561 nm lasers, using a 40 X (1.4 NA oil) objective. Microscopic images were processed using ImageJ 1.48 (National Institutes of Health, Washington, USA).

Monocyte Adhesion Assay

HUVEC were seeded at 0.5x10^6 cells/well in a 12 well plate and TNFα added at 25ng/mL for 24h in combination with MP at a ratio of 1:50,000. Peripheral blood mononuclear cells (PBMC) were isolated from a buffy coat of healthy individuals. Monocytes were purified from the buffy coat using auto-MACS Pro by negative-selection (Miltenyi Biotec, Germany). The purified monocytes were labeled with 1 μM of CFSE and kept in suspension (1x10^6 cells/mL) in culture medium consisting of RPMI 1640 medium (Life Technologies), supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin. Monocyte purity was checked using flow cytometry after staining with mouse-anti-human monoclonal antibody against CD14 (BD Biosciences) for 20 min at room temperature. CFSE-labeled monocytes (1x10^5 per condition) were added to the stimulated HUVEC and incubated for 1h at 37 °C, 5% CO_2. The incubation time of 1h was determined as the time where monocytes in suspension were not anymore observed in the TNFα condition (positive control). After a thorough wash with EBM, the cultures were photographed with a Leica TCS SP5 confocal microscope. Microscopic images were processed using ImageJ 1.48. Stained cells were counted in five randomly selected areas using bright field microscopy (× 20).

Transwell Cell Transmigration Assay

HUVEC (1x10^5 cells/transwell) were plated in Transwell®-24 well inserts (Costar, Corning Inc.), consisting of polycarbonate filters (8 μm pore size; 0.33 cm^2 area), and grown to confluence for 24h. HUVEC were then treated with 25 ng/mL TNFα and

50,000 MP per HUVEC for 24h. Then, the supernatant was discarded and the transwells were transferred to a new well containing 500µL of 50 ng/mL Monocyte Chemoattractant Protein-1 (MCP-1, Invitrogen Molecular Probes) in the lower well. Monocytes were isolated, labeled with PKH-26, and plated in the transwell at a ratio of 2:1 (monocyte:HUVEC). Following 2 hours of incubation at 37 °C and 5% CO_2, the supernatant of the transwells was carefully removed together with the non-adhering monocytes. The adherent cells were washed twice with PBS and stained with 10µM Hoechst 33342 for 10 min at 37 °C, 5% CO_2. The inserts were then washed twice with PBS and fixed with 4% formaldehyde dissolved in PBS for 15 min at room temperature. Monocytes that migrated through both the HUVEC monolayer and polycarbonate membrane and adhered to the bottom side of the transwell membrane were visualized by Z-stacks analysis using a Leica TCS SP5 confocal microscope. The number of transmigrated monocytes was determined by counting the number of PKH-26 fluorescent monocytes present in 5 randomly selected fields of view per sample *via* ImageJ 1.48.

Transwell Permeability Assay

To analyze the endothelial cell barrier integrity 50,000 HUVEC were grown on a transwell insert pre-coated with fibronectin (polystyrene, 0.4 um pore size; Greiner Bio-one, The Netherlands) until confluency. The monolayers of HUVEC were then treated with 25 ng/mL TNFα and two ratios of MP (50,000 and 100,000 MP : HUVEC) for 24h. After the incubation time, the supernatant was removed and FITC-dextran (1mg/ml; 70kDa; Bio-connect, The Netherlands) was added to the transwells. After 2h, the FITC-dextran translocated to the lower compartment of the transwell was measured in a microplate reader at excitation/emission wavelength of 490/520nm. As a positive control a transwell without cells was used. By normalizing the fluorescence signals of the treatment group to the control group a measure of endothelial layer leakiness was obtained.

Angiogenesis Assay/Tube Formation Assay

A confluent monolayer of HUVEC was treated with MP at a ratio of 1:50,000 and treated with/without TNFα (25ng/ml) for 24h. After the incubation time, HUVEC were collected by trypsinization and seeded on 50µl polymerized Matrigel (Geltrex, ThermoFisher, USA). The major components of Geltrex™ matrix include laminin, collagen IV, entactin, and heparin sulfate proteoglycans. The protein concentration is 15mg/ml. Each condition was plated in duplicate. After 18h, tube formation was observed and photographed using an inverted light microscope equipped with a digital camera. The percentage of covered area (percentage of tubular structures in the whole area of the image), total tube length (complete length in pixel of the tubular structure), total branching points (a branching point is part of the skeleton where three or more tubes converge), and the number of loop areas (a loop is an area enclosed by tubular structures) were measured by WIMASIS (Onimagin Technologies SCA, Córdoba, Spain).

Sample Size and Statistical Analysis

In the experiments MP from 5 donors were used in duplicate. For the apoptosis, expression of adhesion markers and monocyte adhesion assays, 5 independent experiments were performed where MP from 2 different donors were tested in each experiment. For the monocyte migration assay, barrier integrity and angiogenesis assays, 3 independent experiments were performed where MP from 3 different donors were tested in each experiment. Data were analyzed for normal distribution by Kolmogorov-Smirnov test, and after that T-Test was used to determine the significance between the groups using GraphPad Prism 5 software. $P < 0.05$ was considered significant.

RESULTS

Morphology and Size Distribution of MP Generated From AT-MSC

MP were generated from culture-expanded AT-MSC and characterized by cryo-electron microscopy and NTA to determine their shape, concentration, and size distribution. Cryo-electron microscopy showed that MP have a spherical shape and a discernible lipid bilayer (**Figure 2A**). Some MP were found encapsulated inside larger MP. The size range of MP was between 32 and 345 nm, with an average peak size frequency of 126.5± 22.4 nm. The frequency of particles larger than 200nm (cut-off pore size) was lower than 0.5 ± 0.3% (**Figure 2B**).

Presence of RNA in MP

To examine whether MP preparations contained DNA and RNA, DNA and RNA concentrations were determined by Nanodrop. MP preparations contained 35.2 ± 3.9 ng/ul DNA/RNA (**Figure 2C**). After DNase treatment, the concentration of DNA/RNA did not change (**Figure 2C**), suggesting MP contain RNA, but no DNA. To determine whether the RNA could be detected by RT-PCR, several genes expressed by MSC were analyzed. PCR product was obtained for GAPDH, the angiogenic genes VEGFA, angiopoietin 1 and FGF-2, IL-8, and for the MSC cell surface marker CD90 (**Figure 2D**) suggesting that MP preparations contained RNA from the cell source (**Figure 2D**).

HUVEC Internalize Membrane Particles in a Time Dependent Manner

Fluorescent MP were generated by labeling the cell membranes of MSC with PKH-26 (PKH-MP). HUVEC were incubated with or without TNFα and with two ratios of PKH-MP (ratio: 1:50,000 or 1:100,000) for 4h or 24h. Non-Stimulated HUVEC showed a significant increase in the internalization of the PKH-MP with increasing MP dose and over time. However, there was not statistical difference in the internalization of PKH-MP in TNFα Stimulated HUVEC between the two tested MP doses, but there was a significant increase over time (**Figure 3A**). For the ratios 1:50,000 and 1:100,000 the percentage of Non-Stimulated HUVEC positive for PKH-MP was 75.2 ± 6.3%, and 86.8 ± 7.4% respectively after 24h of incubation, and for TNFα Stimulated

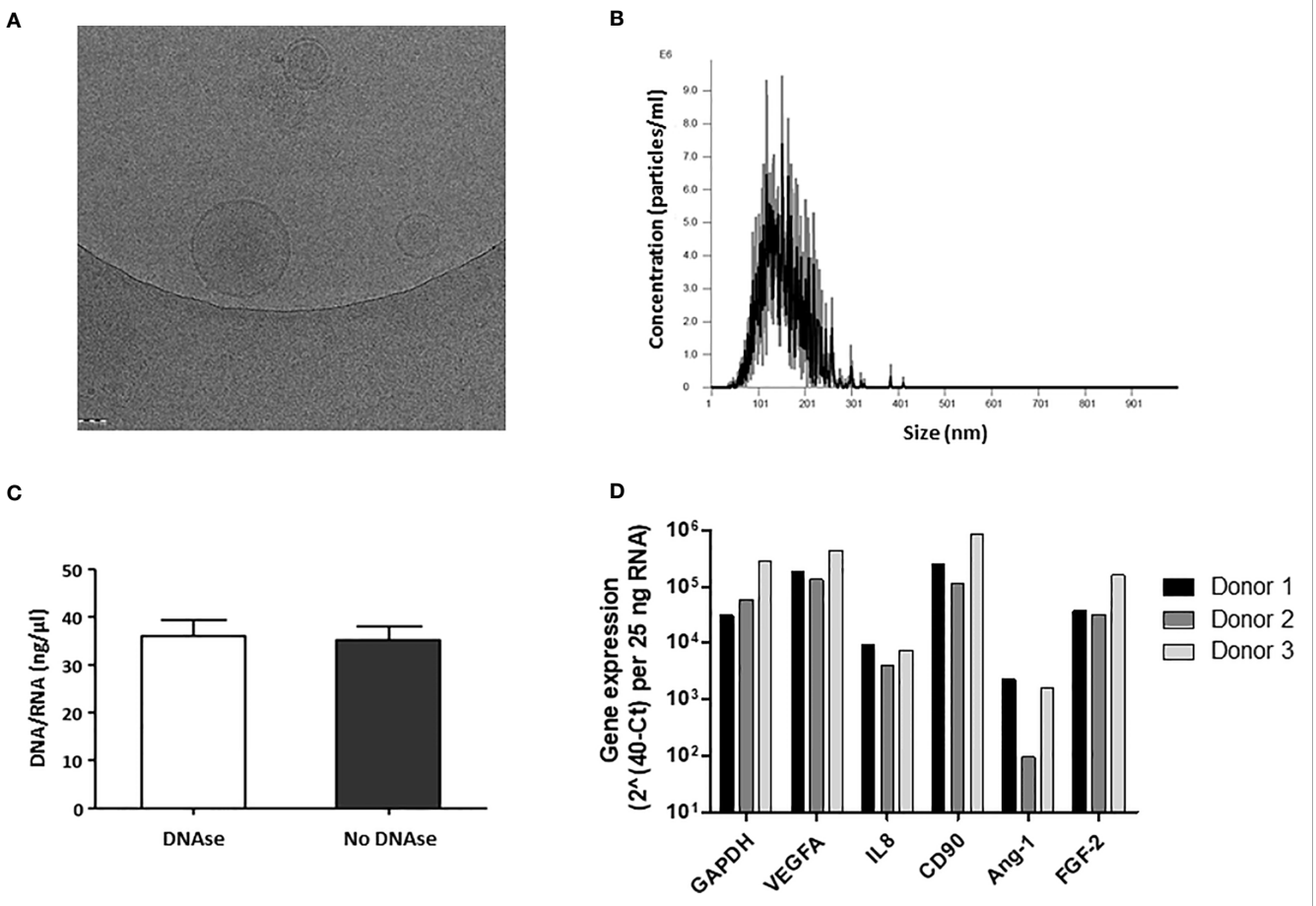

FIGURE 2 | Characterization of physical properties and DNA/RNA composition of membrane particles from MSC. **(A)** Cryo-electron microscopy images of MP. MP show a spherical shape and a discernible lipid bilayer. **(B)** A representative profile of the nanoparticle tracking analysis (NTA) of MP. A graph was generated which plots the distribution in size of the MP against the concentration of MP per ml. **(C)** RNA/DNA concentration (ng/µl) in MP samples before and after DNAse treatment. The error bars represent standard deviation of the mean (SD). **(D)** Relative gene expression of RNA present in MP samples from three different MSC donors.

HUVEC were 82.1 ± 7.1%, and 82.4 ± 11.2%. There was not statistical difference between TNFα and Non-Stimulated HUVEC.

The interaction of PKH-MP with HUVEC was visualized using confocal immunofluorescence microscopy. The analysis showed that PKH-MP were internalized and localized in the cytoplasm of HUVEC (**Figure 3B**). Subsequently, a LysoSensor staining was used to examine whether PKH-MP end up in lysosomes. The LysoSensor staining fluorescently labels endosomes and turns yellow when the pH in the endosomes is acidic, indicative for lysosomes. After incubating HUVEC with PKH-MP for 24 h, fluorescently labeled MP co-localized with lysosomes (**Figure 3C**).

Membrane Particles Do Not Induce Apoptosis or Affect the Expression of Adhesion Molecules in HUVEC

HUVEC were stimulated with TNFα and cultured with two concentrations of MP (1:50,000 and 1:100,000) for 24h and 48h to determine whether MP induce apoptosis. No increase in apoptosis was observed in non-stimulated and TNFα stimulated HUVEC treated with MP at 24h (**Figure 4A**) or 48h (**Figure 4B**).

HUVEC were cultured with MP for 24h to determine whether MP could influence adhesion molecules expression (CD54, CD106, CD62e, CD31, CD105) and molecules involved in angiogenesis (VEGFR2, TIE-2) in non-stimulated and TNFα stimulated HUVEC. MP did not modify the expression of ICAM-1 in non-stimulated or TNFα-stimulated HUVEC (**Figure 4C**) or VEGFR2 (**Figure 4D**). In addition, no changes were observed for the rest of molecules (data not shown).

MP Do Not Affect the Adhesion of Monocytes to HUVEC

HUVEC were treated with or without TNFα for 24h and 1:50,000 MP per HUVEC. Subsequently, the HUVEC were co-cultured for 1h with CFSE-labeled monocytes to examine the adhesion of monocytes to HUVEC. After washing away non-adherent cells, monocyte adhesion was quantified by analysis of

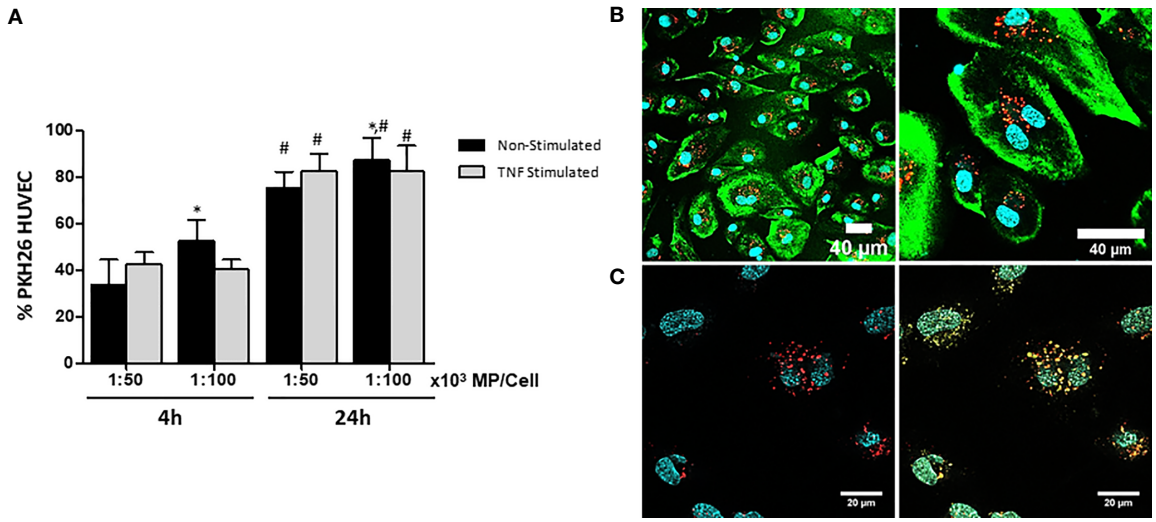

FIGURE 3 | Characterization and quantification of uptake of MP by unstimulated and TNFα-stimulated HUVEC. MSC were labeled with PKH-26 before generation of MP (PKH-MP). PKH-MP were added to HUVEC (ratio 1:50,000) and incubated for 4 and 24h at 37°C. **(A)** Uptake of PKH-MP by unstimulated and TNFα-stimulated HUVEC (ratio 1:50,000 and 1:100,000) was quantified using flow cytometry. Uptake is indicated by PKH-MP positive HUVEC (PKH+ HUVEC). **(B)** Representative confocal microscopy analysis of PKH-MP uptake by HUVEC at time point 24h. Staining for PKH26-MP (red), PKH-67 cell membrane (green), and Hoechst 33342 nucleus (blue) showed that PKH-MP are internalized by HUVEC. Scale bars: 40 µm **(C)** Staining for PKH-MP (red), lysosomes (yellow) and nucleus (blue) showed that PKH-MP (ratio 1:50,000) are co-localized with lysosomes in HUVEC after 24h of incubation. Scale bars: 20 µm.

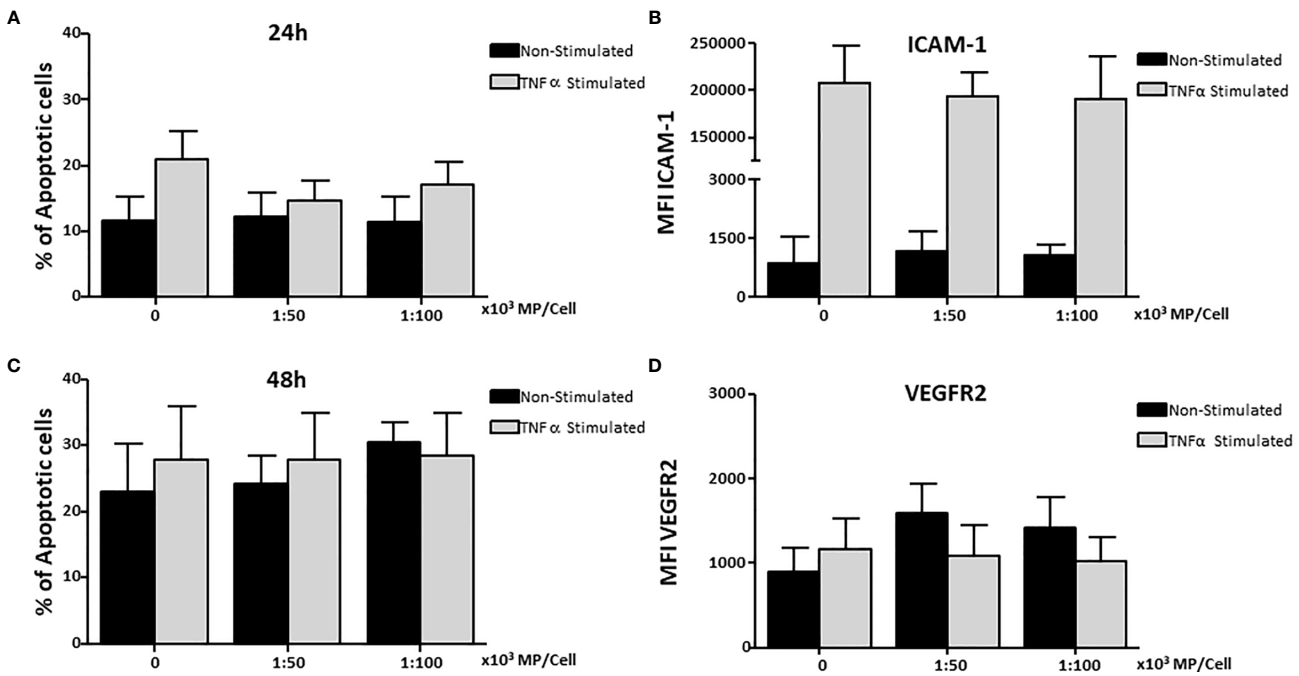

FIGURE 4 | Effect of Membrane Particles on HUVEC apoptosis and adhesion molecules. HUVEC were stimulated with TNFα and treated with two concentration of MP (1:50,000, 1:100,000) and incubated at **(A)** 24h, and **(B)** 48h for the analysis of apoptosis. Surface expression of adhesion molecules on HUVEC was measured at the time point of 24h **(C)** ICAM-1, and **(D)** VEGFR2. Values are means ± SD of the mean fluorescent intensity of the receptors of 5 independent experiments each testing MP from 2 donors in each experiment.

confocal microscopy images. Digital images were captured at ×20 magnification and analyzed by ImageJ software. **Figure 5A** shows representative images of the studied groups. Activating the HUVEC using TNFα significantly increased the number of monocytes adhering to the HUVEC compared to non-stimulated HUVEC (**Figure 5B**). There was no effect of MP on the adhesion of monocytes to Non-Stimulated and TNFα Stimulated HUVEC.

Inhibition of Monocyte Transendothelial Migration by Membrane Particles

To examine the effect of MP on the transendothelial migration potential of monocytes, a HUVEC monolayer on a transwell membrane was treated with TNFα and/or MP and after 24h fluorescent monocytes were added (**Figure 6A**). Monocyte transmigration across the endothelial layer was observed at 2h. Representative confocal microscopy pictures of the assay are shown in **Figure 6B**. Addition of the chemo-attractant (MCP-1) to the lower well significantly increased the number of migrated monocytes 2.3-fold compared to PBS (PBS: 59.7 ± 24.3; MCP-1: 138.2 ± 61 migrated monocytes per microscopic field). MP were able to significantly reduce the number of monocytes that migrated through the TNFα activated HUVEC monolayer (61.3 ± 44.6 migrated monocytes per microscopic field) compared to the MCP-1 (**Figure 6C**). Non TNFα activated HUVEC treated with MP were used to examine whether MP could induce monocyte transmigration under non-inflammatory conditions. The addition of MCP-1 did not induce an increase in transmigrated monocytes (18.2 ± 3,4 migrated monocytes per microscopic field) compared to PBS (11 ± 5.2 migrated monocytes per microscopic field). The number of transmigrated monocytes in the MP treated HUVEC was similar to the Non-treated HUVEC (15.3 ± 7.1 migrated monocytes per microscopic field). The number of monocytes that migrated through the Non-Stimulated HUVEC monolayer was very low compared to TNFα-Stimulated HUVEC and the number of monocytes that adhered to the Non-Stimulated HUVEC was also very low (**Figure 6C**).

MP Increase Endothelial Monolayer Integrity

To analyze whether MP induce a decrease in endothelial intercellular permeability, HUVEC were cultivated as tight monolayers in a transwell system and were treated or not with TNFα, and two ratios of MP (1:50,000; 1:100,000) during 24h. Thereafter, permeability was determined by measuring the passage of FITC-Dextran (molecular mass: 70 kDa) across HUVEC monolayers (**Figure 7A**). Results were normalized to the Non-Stimulated HUVEC control group. The analysis showed that both doses of MP decreased the endothelial permeability in Non-Stimulated HUVEC. TNFα stimulation induced a 2-fold increase in the permeability of the monolayer compared to the Non-Stimulated control. At a dose of 1:100,000 MP significantly decreased endothelial permeability (**Figure 7B**).

MP Have Pro-Angiogenic Properties

The pro-angiogenic potential of MP on non-stimulated and TNFα stimulated HUVEC was determined by measuring four parameters (total tube length, total branching points, total loops, and covered area) (**Figure 8A**) using the tube formation assay. The experiment was performed in both groups of HUVEC after 24h of incubation with and without TNFα and with and without MP. MP enhanced the process of angiogenesis in Non-

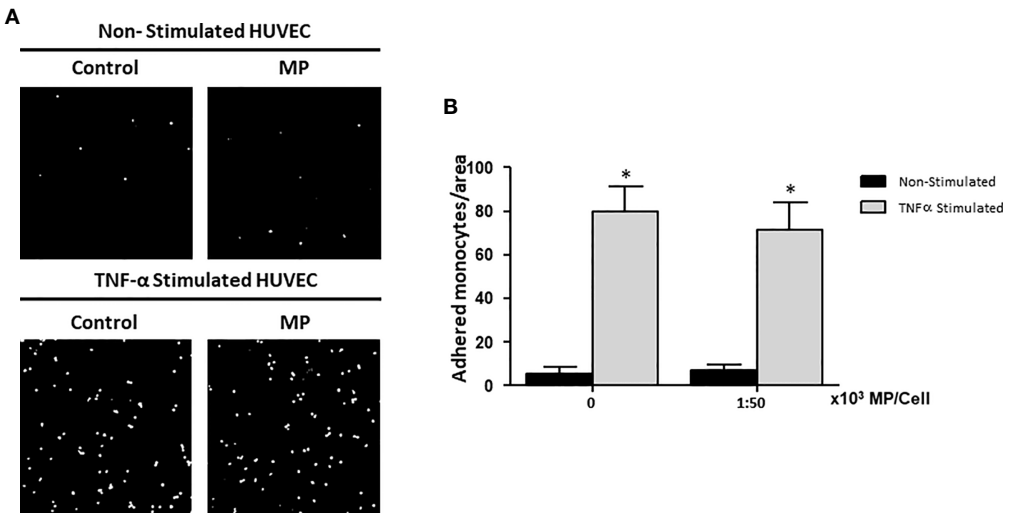

FIGURE 5 | Effects of Membrane Particles on monocyte adhesion to TNFα-activated HUVEC. HUVEC were stimulated with TNFα and treated with MP at ratio 1:50,000. Subsequently, CFSE-labeled monocytes were added during 1h. **(A)** Representative fluorescent microscopy pictures show the adhered monocytes (white dots) to the HUVEC monolayer in non-stimulated and TNFα stimulated conditions. **(B)** Quantitative results of the monocyte adhesion assay analyzed by imageJ. No significance difference respect to the respective control (Non-Stimulated, and TNFα Stimulated HUVEC) was observed when MP were added *$p < 0.05$ compared to Non-Stimulated HUVEC.

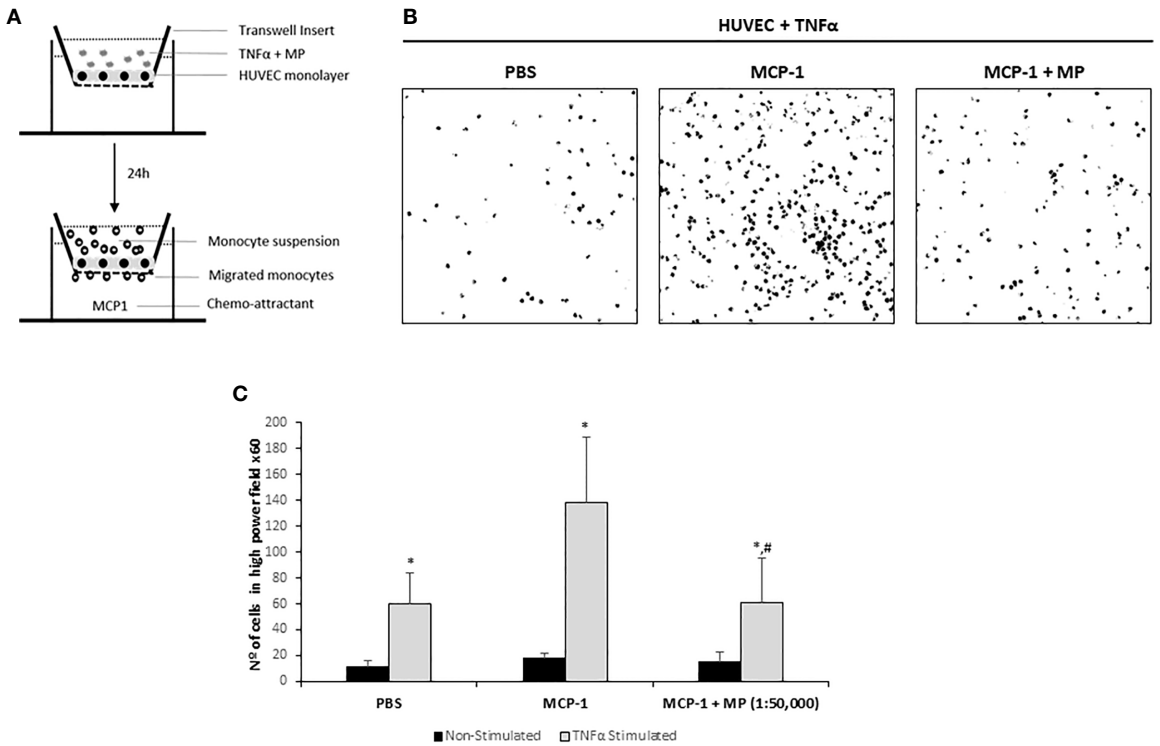

FIGURE 6 | Effect of Membrane Particles on migration of monocytes through a monolayer of TNFα-activated HUVEC. **(A)** Schematic representation of the transmigration assay. HUVEC were seeded on transwell inserts until confluency. The monolayer of cells was treated with TNFα and 1:50,000 MP for 24h. Then, $1x10^5$ isolated monocytes were added during 2h with addition of the chemo-attractant MCP-1 in the bottom well. Three pictures from randomly selected areas of the transwell were taken for the quantification. **(B)** Representative confocal microscopy images of the negative control (no MCP-1), positive control (MCP-1) and the MP treated group analyzed by ImageJ. **(C)** Quantitative results of the transmigration assay. Data represent means ± SD of the number of transmigrated monocytes. *$p < 0.05$ respect to Non-Stimulated HUVEC. #$p < 0.05$ respect to TNFα stimulated HUVEC non treated with MP in the MCP-1 group.

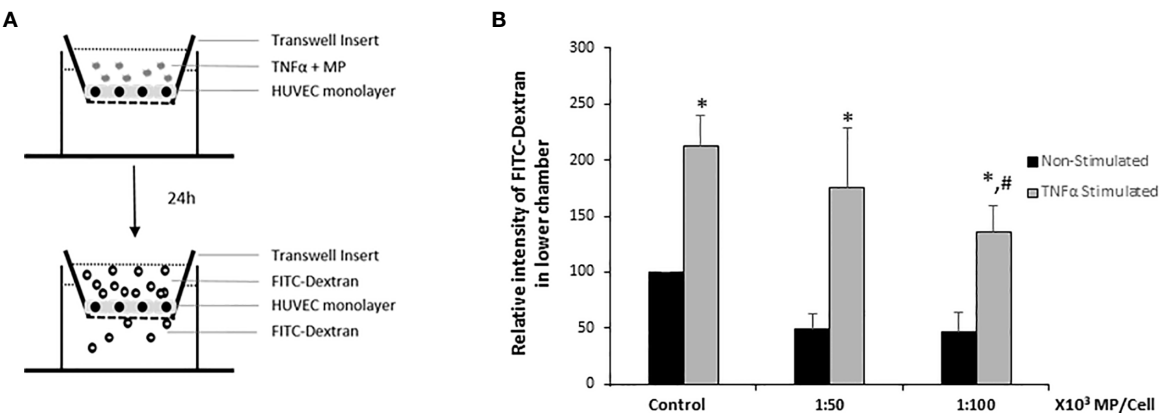

FIGURE 7 | HUVEC barrier integrity. **(A)** Schematic representation of the endothelial barrier model used in the study. HUVEC were seeded on transwell inserts until confluency and then treated with TNFα and MP (1:50,000 and 1:100,000) during 24h. FITC-dextran was added during 2h, after which the fluorescence intensity in the lower chamber of the transwell system was quantified. **(B)** Quantitative results of the HUVEC barrier integrity assay. Data represent means ± SD of 3 experiments using MP from 5 different donors. *$p < 0.05$ compared with the respective control (Non-Stimulated HUVEC). #$p < 0.05$ respect to TNFα stimulated HUVEC (Control).

Stimulated and TNFα Stimulated HUVEC with respect to their control groups (**Figure 8B**). The quantification of the angiogenesis parameters revealed a significant increase in total tube length, total branching points, total loops, and covered area for MP in Non-Stimulated HUVEC, and TNFα Stimulated HUVEC (**Figure 8C**).

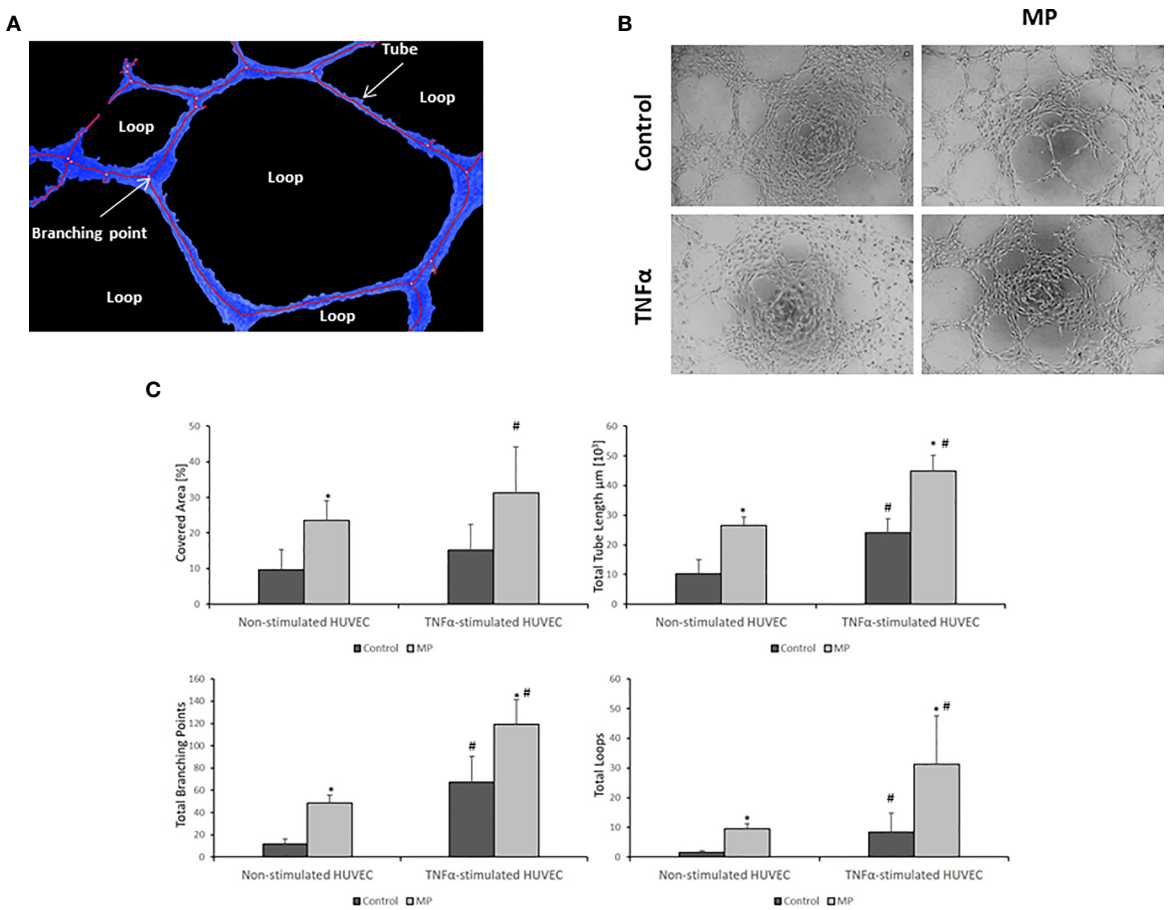

FIGURE 8 | MP induce angiogenesis in Non- and TNFα Stimulated conditions. **(A)** Analysis and identification of angiogenic features. **(B)** Angiogenesis assay images of Non-Stimulated and TNFα stimulated HUVEC under MP treatment. Quantitative image analysis of four angiogenic features (covered area (Blue lines), total tube length, total branching points, and the number of loops) in **(C)** Non-stimulated HUVEC, and TNFα stimulated HUVEC. The analysis was performed by the company WIMASIS. *$p < 0.05$ compared with the control (no MP). #$p < 0.05$ respect to Non-Stimulated HUVEC.

DISCUSSION

The present study demonstrates that small circular fragments of cell membranes from AT-MSC can ameliorate TNFα induced endothelial injury by improving endothelial cell monolayer integrity and enhancing their angiogenic capacity. MP encompass the surface molecules of MSC plasma membranes and contain RNA present in the mother cells, thereby exploiting some of the natural immunomodulatory and regenerative properties of MSC.

The generation of MP as a cell free cell-therapy emerged after our study to heat inactivated MSC (HI-MSC) where we observed that HI-MSC possessed immunomodulatory properties *in vitro and in vivo*, even being dead (14). HI-MSC lost the capacity to secrete factors, or any another function related to the living cells such as proliferation, while keeping the cell membrane intact. This suggests that MSC membranes with their associated proteins can govern at least some of the effects of MSC. The size of HI-MSC is similar as MSC and HI-MSC get trapped in the lung capillary system after their administration (14). To retain the biological properties of MSC and concomitantly overcome the problems of living cells, the generation of MSC membranes in the nano-range devoid of cytoplasm and nucleus represent a new promising approach in the cell therapy field and is supported by the EV studies (22, 23).

MP and EV derived from MSC provide several advantages over MSC. Both types of nanoparticles cannot be modified by the molecular environment after their administration as they are a fixed representation of MSC. Similar to naturally occurring EV, the small size of MP (<200nm) makes them more suitable for crossing the lung barrier than MSC (24, 25) and due to a better biodistribution can exert broadly their effects in the organism (26).

Interestingly, mRNA for factors such as VEGF, IL-8, and CD90 from the MSC were detected in MP. It is assumable that these mRNAs are on the inside of the MP as RNAases would

likely degrade free floating RNA. The relative gene expression of these factors in MP samples was different between donors and may be related to the inherent donor variation or to differences in the grade of RNA degradation during the process of MP generation. To minimize the impact on the results due the different amount of mRNA between samples, several batches of MP per donor were used to perform the experiments. One of the mechanisms proposed for explaining the action of EV is the transfer of RNA to the target cells (27, 28). Whether this mechanism is also occurring with MP deserves further studies.

To evaluate whether MP is a potential treatment to repair inflamed endothelium, several aspects of endothelial repair were studied. We showed that MP were efficiently taken up by HUVEC, and that their last destination are the lysosomes of the cells. Recently, we have studied the mechanisms of MP internalization (29). Specific inhibitors for endocytic pathways revealed that MP internalization depends on heparan sulfate proteoglycan-, dynamin-, and clathrin-mediated endocytosis but does not involve caveolin-mediated endocytosis. MP uptake also involved the actin cytoskeleton and phosphoinositide 3-kinase, which are implicated in macropinocytosis and phagocytosis. Due to the different pathways involved in the uptake of MP, the mechanisms involved in their actions may be very different. Several authors described that endocytosis is the most common pathway used by cells to incorporate natural vesicles such as exosomes, and microvesicles to their cytoplasma (30, 31). Bhagyashree S. Joshi et al. (32) demonstrated that EV are internalized by endocytosis and phagocytosis as MP, and the internalized EV fuse with the limiting membrane of endosomes and lysosomes in an acidification-dependent manner, which results in EV cargo exposure to the cell cytosol. MP may be processed by the cells in a similar manner, but future studies should address this question.

Potential adverse effects such as cytotoxicity and upregulation of adhesion molecules on HUVEC by MP were analyzed. No increase in apoptotic HUVEC was observed even with the highest concentration of MP in the inflammatory condition. MP did not have any role on the modulation of the surface adhesion molecules of HUVEC. It is important to highlight that MP did not induce the activation of the HUVEC under normal conditions, which makes them a safe treatment for EC. Because MP did not downregulate the expression of surface adhesion molecules on HUVEC under inflammatory conditions, the adhesion of the monocytes to the activated EC could not be suppressed. Several studies have described the relation of EC adhesion markers and monocyte adhesion. Blocking ICAM-1 receptors (33) in EC, or downregulating the expression of adhesion receptors in EC with molecules such as L-Arginine (34), and Eicosapentaenoic Acid (35) was correlated with a decrease of monocyte adhesion. Although MP did not decrease the number of monocytes adhered to EC, MP were able to prevent the migration of monocytes through a monolayer of HUVEC. Several authors described that MSC inhibit the recruitment of leukocytes (9, 36), but there are some doubts about the mechanisms of action. MSC could physically obstruct the transmigration of leukocytes (37), or immune cells could interact adhesively with MSC thereby reducing the number of cells available to bind to EC (38). MP cannot physically block the migration of monocytes through the barrier of EC, and furthermore in our experiments, MP were removed before the addition of the leukocytes, so they could not interact with the leukocytes themselves. The most likely mechanism explaining the impeding of monocyte transmigration by MP is that MP restore the HUVEC barrier integrity from TNFα-induced leakiness by stimulating a more compact HUVEC monolayer structure. This characteristic of MP is shared with MSC and EV derived from MSC (39, 40).

Additionally, we showed that MP stimulate the angiogenic potential of HUVEC in normal and inflammatory conditions. This effect has also been reported for EV derived from MSC (41), and the described mechanism is through the transfer of miRNAs from EV to the recipient cells (42). It is possible that MP share this mechanism of action with EV as MP also contain mRNAs involved in angiogenesis such as VEGF, angiopoietin 1.

These features of MP, blocking of transmigration, restoring endothelium integrity, and stimulation of angiogenesis could be used in the treatment of different vascular complications such as atherosclerosis, infiltration of immune cells in organ rejection, in the joins in rheumatoid arthritis, or for injured endothelium after organ ischemia. In comparison with similar treatments such as MSC or EV derived from MSC, MP offer the advantage of their small size, purity and excellent safety profile and the possibility for upscaling production in a controlled manner.

In conclusion, MP show a promising medicinal potential, opening a new avenue for treatment of vascular diseases where the inflammatory process is involved in the damage of the endothelium.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The tissues were collected after obtaining written informed consent, as approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam (protocol no. MEC-2006-190).

AUTHOR CONTRIBUTIONS

AM and MH designed the study. AM, SK, and MS performed the research. AM, MH, CL-I, and CB participated in the interpretation of the data. AM wrote the paper. MO-V, CB, EL, and MH wrote the manuscript (review and editing). All authors contributed to the article and approved the submitted version.

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Conflict of Interest: Erasmus MC filed a patent on the use of MP for immunomodulatory purposes. Authors MO-V and EL were employed by Takeda Madrid.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

frontiers in Immunology

REVIEW
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Placenta-Derived Exosomes as a Modulator in Maternal Immune Tolerance During Pregnancy

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Exosomes are a subset of extracellular vesicles with an average diameter of ~100nm. Exosomes are released by all cells through an endosome-dependent pathway and carry nucleic acids, proteins, lipids, cytokines and metabolites, mirroring the state of the originating cells. The function of exosomes has been implicated in various reproduction processes, such as embryo development, implantation, decidualization and placentation. Placenta-derived exosomes (pEXO) can be detected in the maternal blood as early as 6 weeks after conception and their levels increase with gestational age. Importantly, alternations in the molecular signatures of pEXO are observed in pregnancy-related complications. Thus, these differentially expressed molecules could be the potential biomarkers for diagnosis of the pregnancy-associated diseases. Recent studies have demonstrated that pEXO play a key role in the establishment of maternal immune tolerance, which is critical for a successful pregnancy. To gain a better understanding of the underlying mechanism, we highlighted the advanced studies of pEXO on immune cells in pregnancy.

Keywords: placenta, exosomes, maternal immune tolerance, preeclampsia, gestational diabetes mellitus, preterm

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INTRODUCTION

Pregnancy is a complex process associated with numerous biological changes in the maternal body and our understanding of the complicated relationship between the mother and its semi-allograft fetus is still limited (1). An immune tolerant environment is a prerequisite to a successful pregnancy. However, the understanding of how the fetus avoids maternal immune rejection is an enigma. During pregnancy, the mother needs to have a competent immune system against infection but is tolerant to the developing fetus. Any disruption of the immune tolerance would lead to adverse pregnancy outcomes such as recurrent pregnancy loss (2), miscarriage (3) and preeclampsia (4).

The maternal immune system undergoes a wide variety of biological changes during pregnancy. These include decidual immune cell mobilization, re-distribution and polarization at a local level (5–7) and a universal immunosuppressive state at a systemic level (8, 9). In humans, the trophectoderm of blastocyst protects the growing embryo at implantation (10). After implantation, the syncytiotrophoblast (STB) derived from the trophectoderm, surrounds most of the chorionic villi, and prevents the fetus from a direct contract with the maternal blood. The trophoblasts have a unique human leukocyte antigen (HLA) profile (11). For example, the STBs are

HLA null and are considered as immunologically neutral, while the extravillous trophoblast cells (EVTs) express an unusual repertoire of HLA-I molecules including HLA-G, HLA-C and HLA-E (12, 13). Furthermore, the STBs produce various immunoregulatory factors such as interleukin 10 (IL10) (14), macrophage colony-stimulating factor (M-CSF) (15) and IL-35 (16), which contribute to maternal immune tolerance as well.

Exosomes, firstly regarded as cell burden, are involved in the process of antigen presentation, signal transduction and immune responses. Placenta STB has been demonstrated to continuously releases extracellular vesicles (EVs), microvesicles and exosomes, to the maternal circulation (17, 18). The study on pEXO can date back to 1999 (19) and our understanding of pEXO are significantly increased due to advances in technologies of exosome purification in the last decade. Beyond that, exosomes from other sources-such as stem cells and tumor-have a critical role in growth, metabolism and development. The function of pEXO has been implicated in conferring viral resistance to non-placenta cells, inhibiting T cells recognition and activation, and promoting macrophage differentiation and polarization during pregnancy. Here, we summarize the current knowledge of pEXO in the establishment of maternal immune tolerance and outlined an overview role of its application in disease diagnosis.

PLACENTA-DERIVED EXTRACELLULAR VESICLES

Extracellular Vesicles

Communication among our body cells is traditionally considered to be through autocrine, paracrine, endocrine and direct cell-cell contact. Other than that, EVs are another means of cell-cell communication. According to the guidelines of the International Society for Extracellular Vesicles (ISEV), EVs are lipid-bound vesicles with a diameter ranging from 30 nm to 2 μm released from all kinds of cells (20, 21). Based on the biogenesis process, EVs generally fall into two categories, ectosomes and exosomes (**Table 1**) (20). Ectosomes are vesicles produced by cells *via* direct outward budding. They can be further divided into microvesicles (MVs, 200 nm ~ 1 μm in diameter) and apoptotic bodies (APs, 1 μm ~ 5 μm in diameter) (22). By contrast, exosomes are nano-sized particles with a size ranging from 30 nm to 200 nm in diameter (100 nm on average) generated by inward budding of the plasma membrane *via* a multi-vesicular system (21).

Initially, EVs are considered as cell debris for the purpose of maintaining cellular homeostasis (23, 24). EVs carry various molecular cargoes, such as proteins, microRNAs (miRNAs), mRNAs, lipids and metabolites, which endow the EVs with capacity as a natural vehicle for intercellular communication (25). The function of exosomes has been well documented in tumorigenesis, metastasis, regeneration, mammalian reproduction and development (26). Certain miRNAs are enriched in exosomes compared to those in the cells of origin, indicating that the process of exosomes biogenesis is not random, but in a pre-primed manner (27, 28). However, the mechanism underlying the exosomal cargo incorporation is still unclear.

Placenta Syncytial Nuclear Aggregates, Microvesicles and Exosomes

During pregnancy, the placenta actively releases EVs into the bloodstream of the mother. The STB is the major source of placenta-derived EVs in the maternal blood (29, 30). Unlike those EVs that originate from other tissues, placenta-derived EVs are divided into three categories based on their sizes: syncytial nuclear aggregates (SNAs), microvesicles (MVs) and exosomes (**Figure 1**) (31).

Placenta-derived SNAs, also known as syncytial knot, are the clusters of multinucleated aggregate of syncytial nuclei (20 μm~200 μm in diameter, averaged 60 nuclei per knot) extruded from STB (32). The formation of placenta-derived SNAs is generally considered as a degenerative process, an aging change and an indicator of trophoblastic state when exposed to ischemia or hypoxia (18, 31, 33). The history of placenta-derived SNAs can be dated back to 120 years ago when they were first found in the lungs of post-mortem women (34). However, the origin and formation of SNAs are far from clear. Nuclei within SNAs exhibited condensed morphology compared to the STB and showed little evidence of apoptosis, indicating that SNAs are not fragmented STB (31). SNAs could be used as an alternative source of fetal DNA for prenatal diagnosis (35). The levels of SNAs increase as gestation proceeds and are found to be correlated in pregnancy complications such as preeclampsia (36).

The biological function of placenta-derived MVs is broad, encompassing immune cell activation, proliferation, and endothelial hemostasis (37). MVs collected from normal placenta perfusion have a pro-inflammatory effect *via* activating monocytes and B cells (38). Inhibition of MV internalization cannot block placenta-derived MV-mediated activation of monocytes and B cells indicating that membrane-bound proteins are the key players of the

TABLE 1 | Summary of different subtypes of placenta-derived extracellular vesicles.

	Exosomes	Microvesicles	Apoptotic bodies	Syncytial nuclear aggregates (SNA)
Size	30nm ~ 200nm	200nm ~ 1μm	1μm ~ 5μm	20μ-200μm
Origin	Endocytic pathway	Plasma membrane	Plasma membrane	Syncytiotrophoblast
Function	Intercellular communication	Intercellular communication	Facilitate phagocytosis	Unclear
Contents	Proteins, miRNA, mRNA, lipid and metabolites	Proteins, miRNA, mRNA, lipid and metabolites	Nuclear fractions, cellular organelles	Nucleus, proteins, miRNA, mRNA, lipid, metabolites
Markers	Alix, CD81, CD63, CD9	Integrins, selectins, CD40	Annexin V, phosphatidylserine	Nucleus cluster

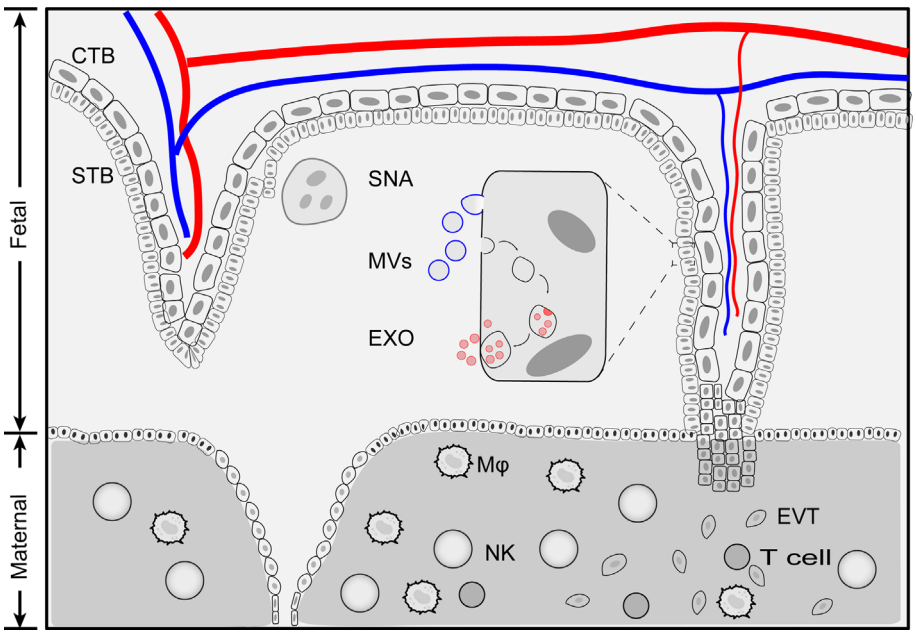

FIGURE 1 | Schematic illustration of placenta extracellular vesicles. Placenta derived extracellular vesicles can be divided into four categories: exosomes, microvesicles, apoptotic bodies and syncytial nuclear aggregates based on size and biogenesis pathway. Exosomes are generated by multivesiculuar body (MVB)-intraluminal vesicles (ILVs) system. first MVBs are generated by plasma membrane inward budding. Further, invagination of the late endosomes forms intraluminal vesicles (exosomes) within multivesiculuar body (MVB). Exosomes release to extracellular space when MVB fuse with membrane plasma. During this processes, membrane components and cytosolic materials are loaded into exosomes. Microvesicles and apoptotic bodies are produced by outward budding of plasma membrane and the size range of 200 nm - 5 μm. Syncytial nuclear aggregates (SNA) are clusters of syncntiotrophoblast with multiple nuclei per SNA. CTB, cytotrophoblast; Exo, exosomes; EVT, extravillous trophoblast; MVs, microvesicles; Mφ, macrophage; NK, Natural killer cells; STB, syncytiotrophoblast; SNA, syncytial nuclear aggregates.

phenomenon. Proteomic analysis revealed that the differential expressed proteins between MVs from normal pregnancy and preeclampsia patients are related to mitochondria, transmembrane transport and membrane transporter activity (39).

pEXO can interact with various target cells including endothelium, T cells, monocytes, natural killer (NK) cells and macrophages. pEXO are found to protect endothelial cells from viral infection (40), inhibit NK cytotoxicity (41), constrain T cell proliferation (42) and promote monocyte differentiation and macrophage polarization (43). During pregnancy, pEXO can be detected as early as 6 weeks (44) and their number increases gradually and finally peaks at term. Pathologically, the levels of exosomes have been correlated with pregnancy-associated complications such as preeclampsia (45), gestational diabetes mellitus (46) and preterm birth (47), which will be described later in this review. Interestingly, all these complications have been demonstrated to associated with alteration of immune system during pregnancy. However, the detailed roles and mechanisms of pEXO in maternal immune adaption and placental development are still obscure.

pEXO PREPARATION AND ISOLATION

To date, pEXO are mainly purified from four types of sources: maternal blood, placental perfusate, placental explant culture, and primary trophoblast culture. However, pEXO isolated by different methods have distinct effects on endothelial cells, T cells and other cells [Reviewed in (48–50)]. Generally, the yield of placenta exosomes in the maternal blood is relatively low. On the other hand, the yields of exosomes from placental perfusion and explant culture are relatively high but the purity of the isolated exosomes is a concern. Since differences in content and immunoregulatory activities of exosome from primary cell and its established cell lines have been reported (51–53). Primary cells are currently the best source of exosomes preparation when sample availability is adequate. However, exosomes from trophoblast cell lines with gene manipulation could also provide valuable information regarding trophoblast-specific gene expression and function (54, 55).

Immuno-capture, centrifugation, precipitation, and size exclusion chromatography are commonly used to isolate exosomes from the biological fluid or culture medium (21, 56–59) (**Figure 2**). The immuno-capture method is commonly used to isolate pEXO in plasma (60). Magnetic beads coated with monoclonal anti-PLAP (placental alkaline phosphatase) antibodies capture placenta-specific exosomes through antigen-antibody interaction. Ultracentrifugation and gradient ultracentrifugation are the most widely used methods in exosome studies. In these methods, EVs are isolated by differential centrifugal forces. Dead cells and cell debris are pelleted with a relatively low centrifugal force (300g for dead cells and 2000g for cellular debris). Higher

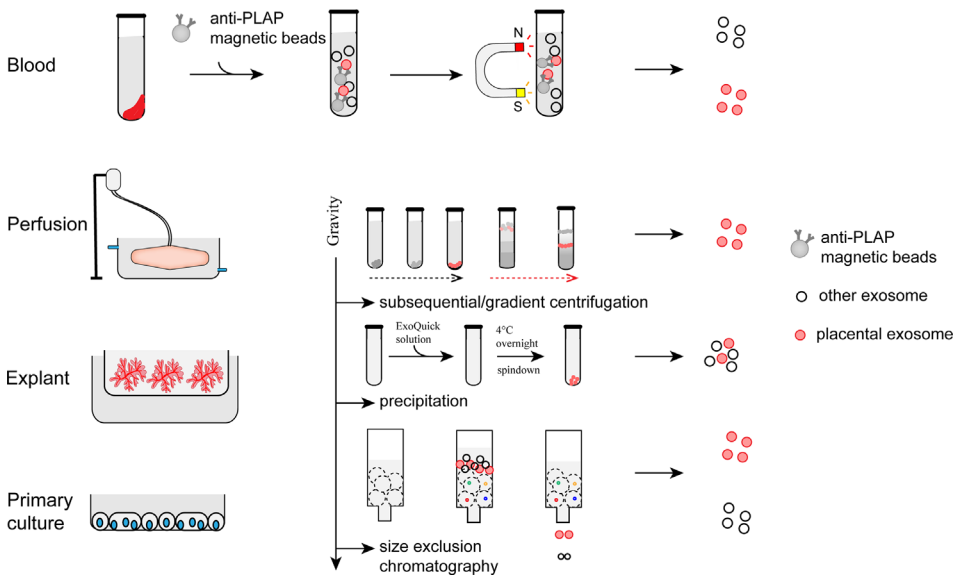

FIGURE 2 | Preparation and isolation of pEXO. Exosomes extruded from placenta can be purified from blood plasma, medium of placental perfusion, explant culture and primary trophoblast culture through immune-capture, ultracentrifugation, gradient centrifugation, or size exclusion chromatography.

centrifugal force at 16,500g is then applied to separate the MVs. Exosomes can be harvested by ultracentrifugation at >100,000g, for 60 minutes. To enhance the purity of exosomes, gradient ultracentrifugation is employed to separate different subtypes of exosomes (59). Precipitation is another method for exosome purification (61, 62). Polyethylene glycol (PEG) functions as a water-excluding molecule that precipitates the exosomes out of the aqueous phase. Usually, exosomes are isolated by a low-speed centrifugation after incubating the sample with a precipitation solution containing PEG. However, proteins may also be precipitated by PEG which could result in a lower purity than those generated by ultracentrifugation. Size exclusion chromatography (SEC) has also been used for exosome purification (63, 64). In this method, exosomes and soluble proteins are separated by a porous matrix. Exosomes that are larger than the size cutoff of the matrix are eluted faster than the soluble proteins. Compared to other methods, exosomes isolated by SEC have a higher purity but lower yields. However, all the methods have their limitation in terms of efficiency and purity. To bridge this gap, new technologies and standardization of protocols for pEXO isolation are needed in future studies.

MATERNAL ADAPTATION OF IMMUNE SYSTEM RESPONSE AT EARLY PREGNANCY

Placenta-driven immune tolerance is a hallmark of a successful pregnancy when exposed to fetal antigens (65–69). Paternal antigens encounter the maternal immune system when the placenta villi are in contact with the maternal blood and when the EVTs interact with the human decidua. Strikingly, the maternal immune cells are abundant in the human decidua in early pregnancy accounting for 40% of the total decidual cells. Among them, NK cells (70%) and macrophages (20%) are the two largest subpopulations, with the rest constituted by T cells. Dendritic cells and B cells are almost absent in the human decidua (70). Interestingly, endometrium exhibits a sharp increase in NK cells and macrophages and a steep decline in T cells during the secretory phase of menstrual cycle, indicating that hormones may influence immune cell population and functions. Although the total cell numbers of the decidual immune cells in the peri-implantation and post-implantation periods are similar, their phenotypes and functions are dramatically different (71, 72).

It is generally accepted that a T-helper type-2 (Th2) cytokine prevailing environment is important in pregnancy (73). The proportion of Th2 cytokines-secreting cells in the endometrium are significantly higher in pregnant women in the first trimester than in non-pregnant women (73–75). On the other hand, Th1 cytokine-dominated immune responses are associated with implantation failures (76), abortion (77) and preeclampsia (78). The excessive Th1 cytokines are also associated with an elevated number of activated $CD8^+$ T cells (79), M1 macrophages (80), Th-17 cells (81) in the decidua. However, several Th1 cytokines such as interferon (IFN)-γ and tumor necrosis factor-α are important in uterine vascular remodeling (82) and implantation (83), suggesting that the Th-1/Th-2 paradigm for pregnancy may be too simplistic. Recently, the concept of Th-1/Th-2 paradigm was gradually expanded to Th1/Th2/Th17/Treg paradigm due to the discovery of new Th cell subsets at the maternal-fetal interface (84, 85).

Systemic changes in peripheral immune cells are also essential for a successful pregnancy. It is supported by the observations in

immunodeficient mice (**Table 2**). In general, adaptive immune cell-deficient female mice are fertile, whereas innate immune cell-deficient female mice are often accompanied by a compromised reproductive performance. However, the mechanism responsible for this observation is still unknown. The significance of peripheral immune cells in pregnancy is also well manifested in pregnant mothers with rheumatoid arthritis, an autoimmune disease which was partially subsided during pregnancy (97). Peripheral Treg cells, granulocytes and monocytic myeloid-derived suppressor cells (M-MDSC) are significantly increased when compared to non-pregnant women (98–100). In contrast, the number of T cells and B cells remain stable (8, 101). Moreover, the cytotoxicity of peripheral NK cells from pregnant women is well constrained when compared to non-pregnant individuals (102, 103).

pEXO AS A MODULATOR OF MATERNAL IMMUNE TOLERANCE

Given that exosomes, but not other EVs, are generated through the endosomal pathway, biological molecules encompassed by the exosomes are believed to have specific functions in cell-cell crosstalk. The placenta secretes a large number of exosomes into the maternal circulation. The NK cells, macrophages and T-cells are the three largest cell types making up >90% of the immune cells at the fetal-maternal interface in the post-implantation period. Thus, this review focuses on the effect of pEXO on these three immune cell populations. Yet it should be emphasized that exosomes are also involved in mediating the bi-direction communications between endometrium and embryo during peri-implantation and implantation phase (104–106). For example, endometrial epithelial cell-derived exosomes promote embryo attachment during implantation *via* miR-30d-dependent upregulation of integrins or through activation of focal adhesion kinase (FAK) signaling pathway (107, 108). Another study shows that diapausing endometrial epithelial cell-derived exosomes enriched with miR-let-7 can protect the embryo from collapsing (109). Conversely, embryo-derived exosomes have been detected in spent embryo culture medium. These exosomes can be internalized by endometrial epithelial/stromal cells (110, 111) and promote endometrial receptivity (112–114).

Natural Killer Cells

Peripheral Blood Natural Killer Cells

NK cells in peripheral blood are divided into two groups: over 90% of peripheral NK cells (pNK) are $CD56^{dim}$ $CD16^{+}$ which are cytotoxic cells; the rest are $CD56^{+}$ $CD16^{-}$ NK cells which are less cytotoxic and can migrate into peripheral tissues. Compare to non-pregnant, pNK of pregnant women have a higher expression of Tim-3 (115), galectin-1 (102) and lower secretion of IFN-γ (103). The cytotoxic activities of pNK at early pregnancy are controversial (101, 116). Moreover, overactivated pNK are associated with repeated implantation and unexplained spontaneous abortion (117). Currently, there is no report on the effect of pEXO on pNK.

Endometrial NK Cells (eNK) and Decidual NK Cells (dNK)

NK cells represent the largest fraction of lymphocytes in the endometrium during the late-secretory phase and early pregnancy. Unlike pNK, majority of the endometrium NK cells (eNK) are $CD56^{+}$ $CD16^{-}$ with a minority being cytotoxic $CD56^{dim}$ $CD16^{+}$. The transformation from eNK to decidual NK cells (dNK) occurs upon implantation, resulting in two cell subsets with distinct transcriptional profiles. Strikingly, the eNK are more active than the dNK as 70% of differentially expressed genes are highly expressed in the eNK (118). On the other hand, the eNK have no expression of NKp30 and cannot produce VEGF and placental growth factor (119). The phenotype and KIR repertoire are also different between the two type NKs; the dNK have a higher expression of KIR2D, the killer immunoglobulin-like receptor for HLA-C recognition than the circulating NK and the non-pregnant eNK (72, 120).

Decidual NK cells (dNK) are abundant in the maternal-fetal interface at early pregnancy- accounting for up to 70% of total lymphocytes in decidua (121). The number of dNK gradually increases upon embryo implantation, peaks at 8-10 weeks of gestation, and returns to the original level at term. In general, dNK have low cytotoxicity and are prone to produce more growth factors with immunomodulatory activities (122). Elevated dNK cytotoxicity is associated with recurrent spontaneous abortion due to increased lysis activity (123). This was, in part, mediated by the

TABLE 2 | Reproductive performance of immunodeficient mouse model.

	Immune cell deficiency							Innate immune cell depletion	
Cell types	Nude	SCID	Nod-SCID	Rag-/-	Rag-/- γc-/-	Treg-/-	Csf1-null	CD11b+	MDSC
Mature B cells	Present	Absent	Absent	Absent	Absent	Present	Present	Present	Present
Mature T cells	Absent	Absent	Absent	Absent	Absent	Present	Present	Present	Present
Dendritic cells	Present	Present	Defective	Present	Present	Present	Present	Present	Present
NK cells	Present	Present	Defective	Present	Present	Present	Present	Present	Present
Treg	N/A	N/A	N/A	N/A	N/A	Absent	Present	Present	Present
Macrophages	Present	Present	Present	Present	Present	Present	Absent	Absent	Absent
Monocytes	Present	Present	Present	Present	Present	Present	Defective	Absent	Defective
MDSC	N/A	N/A	N/A	N/A	N/A	Present	N/A	N/A	Absent
Reproductivity	Fertile	Fertile	Impaired	Fertile	Fertile	Impaired	Infertile	Infertile	Impaired
Reference	(86)	(87)	(88, 89)	(90)	(91)	(92)	(93, 94)	(95)	(96)

MDSC, myeloid-derived suppressor cells.

up-regulation of NKp44 and NKp46 cytotoxicity receptors on $CD56^{bright}$ $CD16^{-}$ and $CD56^{dim}CD16^{+}$ cells (124). In fetal/neonatal alloimmune thrombocytopenia (FNAIT), activated dNK with increased cytotoxicity induce trophoblast apoptosis (125). A recent single-cell study (126) classified the dNK into three subsets: dNK1 ($CD39^{+}KIR2DL^{+}ITGB2^{-}CD103^{-}$), dNK2 ($CD39^{-}KIR2DL^{+}ITGB2^{+}CD103^{-}$) and dNK3 ($CD39^{-}KIR2DL^{-}ITGB2^{+}$). The origin of the dNK remains uncertain, though they are thought to be derived from the NK precursor in the endometrium, or recruited from the circulating NK (126) and/or renewed by the $CD34^{+}$ progenitor cells (127).

dNK are localized closely to EVT and spiral artery (128). They are vital to various processes of pregnancy including embryo implantation, immunomodulation, trophoblast differentiation and invasion, and endothelial cell remodeling. dNK also express unique NK receptors (e.g. 2B4, KIR2DL, ILT2) for interaction with their corresponding ligands (e.g. HLA-C, -E, -G) on EVT to fine-tune their cytolytic activity (129) within the maternal-fetal interface during the first trimester of pregnancy. Recent studies also suggest novel properties of dNK such as providing osteoglycin (OGN) and osteopontin (OPT) for fetal development (130) and selectively killing the pathogenic bacteria inside the trophoblast by injection of granulysin through nanotubes (131).

Effect of pEXO on NK Cells

pEXO can be internalized by NK cells *in vivo* (132) and *in vitro* (**Supplementary Table 1**) (133), which mediate the crosstalk between the placenta and the maternal immune system. The cytotoxic activity of NK cells mainly attributes to its activating receptors on the plasma membrane. NK group 2 member D (NKG2D) is widely expressed on the NK cells, activated $CD8^{+}$ T cells and macrophages for removal of infected cells or foreign pathogens. NKG2D is remarkably downregulated in NK cells by NKG2D ligands expressed on pEXO (**Supplementary Table 1**) (134).

NKp30 is another activating receptor on the NK cells responsible for eliminating cancer cells and inducing dendritic cells maturation by secretion of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), perforins and granzymes (135). B7H6, one of NKp30 endogenous ligands, is widely expressed on cancer cells and trophoblasts, while soluble B7H6 (sB7H6) was a decoy agent for ligand-receptor interaction and compromising NK cytotoxicity. High levels of exosome-packed sB7H6 or soluble B7H6 are correlated with poor tumor prognosis, likely due to inhibition of the NK cytotoxicity against the tumor cells (136). During pregnancy, both exosome-packed B7H6 and sB7H6 are present in the serum of pregnant women (**Supplementary Table 1**), indicating its potential contribution *via* a similar mechanism to inhibit NK cells in the establishment of maternal immune tolerance (137).

In addition to reducing the cytotoxicity of NK cells, exosomes from the serum of pregnant women can selectively increase the caspase-3 activity in $CD56^{dim}$ NK cells, pointing to an alternative way of exosome-mediated immune tolerance by inducing apoptosis of the $CD56^{dim}$ NK cells (**Supplementary Table 1**) (133). pEXO proteomic study showed that glycodelin A (GdA), a glycoprotein with immunosuppressive activities, is abundantly expressed in human decidua and pEXO (138). We demonstrated that decidua-derived GdA stimulated the conversion of peripheral $CD56^{bright}$ $CD16^{-}$ NK cells to cells with a decidual NK cell-like phenotype *via* upregulation of CD9, CD49a and production of VEGF (139). Together, this evidence indicated that the pEXO contribute to maternal immune tolerance through modulating NK cytotoxicity, inducing $CD56^{dim}$ NK cells apoptosis and promoting the development of decidual NK cell-like phenotype.

Monocytes and Macrophages

Peripheral Blood Monocytes

Circulating monocytes are the primary phagocytic cells and the major APCs in blood (140). Notably, monocytes are able to differentiate into dendritic cells and macrophages for antigen presentation and removal of foreign pathogens, respectively. In humans, peripheral monocytes can be divided into three main subtypes based on the expression of CD14 and CD16 (141): classical monocytes ($CD14^{++}CD16^{-}$); intermediate monocytes ($CD14^{+}CD16^{+}$) and non-classical monocytes ($CD14^{-}CD16^{++}$). Approximately 80% of the total monocytes are classical monocytes, while the non-classical monocytes comprise about 2-11%. Non-classical monocytes retain a highly inflammatory characteristic and their number is elevated in both chronic and acute inflammation. The population of the intermediate monocytes (2-8%) with both inflammatory and phagocytic capacities expands during ZIKA viral infection and is the main target for ZIKA infection during pregnancy (142). Despite the conflicting results on the proportion of classical monocytes in peripheral blood between pregnant and non-pregnant women, classical monocyte number is lower in pregnancy complications such as preeclampsia (143, 144), indicating a possible regulatory role of monocyte in pregnancy.

Decidual Macrophages

Decidual macrophages are the second most abundant type of lymphocytes (~20%) and the major antigen-presenting cells (APC) in human decidua during early pregnancy (70). They contribute to maternal-fetal immune homeostasis, spiral artery remodeling and trophoblast functions (145). Decidual macrophages display the transcriptional profile of both classically activated macrophages (M1 macrophages) for immune activation and alternatively activated macrophages (M2 macrophages) with anti-inflammatory and immunosuppressive functions (146, 147). Thus, the decidual macrophages do not fit into the conventional M1/M2 classification of macrophages. Indeed, decidual macrophages show dynamic changes throughout pregnancy (13). For instance, seminal plasma-induced M1 macrophage infiltration contributes to embryo implantation in mice (5) and early placentation (5, 67, 148). As pregnancy proceeds, the M2-dominated microenvironment protects the fetus from rejection. At the time of parturition, M1 macrophage accumulation facilitates uterine contraction (149). The driving forces underlying the phenotype changes remain unclear, yet it is generally believed that the surrounding micro-environment is essential for macrophage transformation and maturation.

Tissue-resident macrophages arise from three subsets of precursors: early yolk sac macrophages, fetal liver monocytes and bone marrow-derived monocytes (150, 151). In other words, the tissue-resident macrophages can be generated by self-renewable macrophages or replenished from circulating monocytes. However, the origin of human decidual macrophages remains uncertain. Kammerer et al. reported a unique $CD209^{+}CD14^{+}CD68^{+}$ $HLA\text{-}DR^{+}$ $CD83^{-}$ proliferating APCs in the decidual of early human pregnancy, suggesting that the human decidual macrophages maintain themselves through self-renewal (152). On the other hand, a gene knockout mice study indicates that the decidual macrophages are replenished by peripheral monocytes expressing circulating lymphocyte antigen 6 complex $(Ly6C)^{hi}$ *via* a Chemokine (C-C Motif) Ligand 2 (CCL2) - CC chemokine receptor-like 2 (CCR2) dependent pathway driven by CSF-1 (153).

Effect of pEXO on Circulating Monocytes and Decidual Macrophages

Early pregnancy is in a pro-inflammatory state. Monocytes in the maternal blood are progressively activated in pregnant women compared to non-pregnant women (144). Placenta-derived EVs can transform the phagocytic classical monocytes ($CD14^{++}CD16^{-}$) to the intermediate monocytes ($CD14^{+}CD16^{+}$) (143) with enhanced migratory capacity and secretion of pro-inflammatory factors such as IL-1β, IL-6, serpinE1, granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF and TNF-α (**Supplementary Table 1**) (154, 155). On the other hand, the number of $CD14^{+}HLA\text{-}DR^{low}$ monocytes is elevated in the maternal blood of the first trimester of pregnancy, and displays an immunosuppressive phenotype when compared with non-pregnant controls (99). Downregulation of HLA-DR endows monocytes with a tolerogenic ability (156). Similarly, tumor-derived exosomes contribute to a systemic immune tolerance *via* modulating the monocyte phenotype. Exosomes from chronic lymphocytic leukemia induce a high expression of PD-L1 in monocytes in a Toll-like receptors 7 (TLR7)-dependent manner (157). Head and neck squamous cell carcinoma-derived exosomes promote monocytes differentiation into an M2 macrophage-like phenotype *via* activation of miR-21 (158).

Studies of pEXO on decidual macrophage are sparse. On the other hand, Nguyen et al. demonstrated that pEXO from pregnant mice are specifically targeted to the lungs and liver, and are taken up by lung interstitial macrophages (97). However, the physiological implications of this observation are unclear. Interestingly, tumor-derived exosomes play a critical role in modulating the differentiation of tumor-associated macrophages (TAMs) *via* exosomal miRNAs, proteins and metabolites (26, 159–161). Similarly, exosomes from the trophoblastic cell line (Swan 71) induce monocyte recruitment and differentiation (**Supplementary table 1**) (155). Another study found that exosome-carrying fibronectin stimulates the production of IL-1β from macrophages (**Supplementary table 1**) (162). Of note, pEXO contain molecules known to promote the induction of decidual macrophages. For example, programmed death-ligand 1 (PD-L1), a factor mainly released by trophoblast in early pregnancy, is identified in trophoblast-derived exosomes, and trophoblast-derived soluble PD-L1 promotes decidual macrophages polarization (163–165). Taken together, pEXO favor pregnancy maintenance by inducing monocyte activation, differentiation and decidual macrophage polarization.

T Cells

Decidual and Peripheral Blood T Cells

T cells are the main cell types responsible for immune surveillance, pathogen recognition and elimination. $CD3^{+}$ T cells constitute ~10% of the decidual lymphocytes in the first trimester. Among them, the $CD4^{+}$ and the $CD8^{+}$ T cells are the two largest groups of T cells accounting for 30-45% and 45-75% of the population respectively (3, 70). During pregnancy, these T cells are immunologically tolerant to the fetus and remain in a constrained cytotoxic phenotype (166). Compared to the circulating $CD8^{+}$ cells, the decidual $CD8^{+}$ T cells are unable to differentiate into the $CD8^{+}$ effector cells as validated by low production of perforin and granzyme B. Moreover, the decidual $CD8^{+}$ T cells show exhausted T cell phenotype with high expression of PD-1, lymphocyte-activation gene 3 (LAG3), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and T cell immunoglobulin and mucin domain 3 (Tim3) (167). Recent studies further reveal that the $CD8^{+}$ cells are expandable in the decidua with upregulated expression of cell activation markers such as CD25, CD38, CD69 and HLA-DR, as well as enhanced expression of IFN-γ and IL-17A. These partially activated decidual $CD8^{+}$ T cells may be associated with trophoblast invasion and spiral artery remodeling after endothelial monolayer destabilization (126, 168).

Other than the $CD8^{+}$ T cells, $CD4^{+}$ T helper cells (Th) are critical in modulating the immune tolerance to fetal antigens as well. The Th1/Th2 paradigm has been demonstrated to be essential for a successful pregnancy. Furthermore, recent reports have shown that a Th17/Treg balance is well maintained during pregnancy. The number of regulatory Treg cells in both the human decidua and circulation is increased during pregnancy (169–171). Decreased level of $CD25^{+}Foxp3^{+}$ Treg is associated with spontaneous abortion (172), preeclampsia (173), and spontaneous preterm birth (174). Furthermore, acute Treg depletion after conception causes embryo resorption along with maternal systemic inflammation and poor endothelial function (92).

Th17 cells are a subset of $CD4^{+}$ T cells presenting a pro-inflammatory phenotype. Although accounting for only ~2% of $CD4^{+}$ T cells, elevated frequency of Th17 cells is related to spontaneous abortion and chorioamnionitis (85, 175–177). Interestingly, the study of Wu et al., showed that Th17 cell numbers in both peripheral blood and decidua are elevated in the first trimester of pregnancy and IL17 could promote trophoblast migration and invasion (82). An inverse relationship of Treg cells and Th17 cells are observed in a wide range of pregnancy complications (81, 85). Thus, the new Th1/Th2/Th17/Treg paradigm indicates that T cell homeostasis is an indispensable factor in pregnancy.

Effect of pEXO on T Cells

The roles of pEXO in T cell response have been widely documented. Recent progress suggests that the pEXO mediate immunosuppression *via* transfer of exosomal proteins to the T cells, leading to T cell

apoptosis, inhibition of T cell proliferation, induction of Treg differentiation and reduction of T cell cytotoxicity.

T Cell Apoptosis

It has long been known that T cell apoptosis in human decidua is a characteristic of early pregnancy. Fas ligand/receptor triggered apoptosis is instrumental in the establishment of immune privilege of the fetus and safeguards its development. pEXO with surface Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) can induce apoptosis in the Jurkat T cells and activate peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner *in vitro* (42). Moreover, pEXO from maternal blood inhibit T cell activation by down-regulation of CD3 ζ and JAK3, with a more notable effect on $CD8^+$ T cells than on $CD4^+$ T cells (**Supplementary Table 1**) (178).

Treg Differentiation

The role of exosomes in the differentiation of Treg cells has been implicated in tumor immunology (179–181). Tumor-derived exosomes inhibit T cell proliferation, cytotoxic activities and macrophage polarization (179, 182, 183). Exosomes isolated from the normal placenta *via* perfusion also inhibit lymphocyte proliferation and induce Treg/memory T cells differentiation (**Supplementary Table 1**) (184–186). Placental mesenchymal stromal cells (PMSC)-derived exosomes alleviate tubulointerstitial fibrosis by increasing infiltration of the $Foxp3^+/IL17^+$ cells in kidneys of the unilateral ureteral obstruction animal model, indicating the involvement of PMSC-exosomes in Treg differentiation (187). Together, these findings indicate that the pEXO are one of the modulators in Treg differentiation during pregnancy.

Cytotoxicity Activity of T Cell

NKG2D ligands such as MHC class I chain-related (MIC) and UL-16 binding protein (ULBP) are expressed on pEXO. Interestingly, the levels of the soluble forms of the MIC protein A and B are negatively correlated with the survival time of cancer patients. The soluble MIC supports tumor escape *via* binding to NKG2D and downregulating its expression on cytotoxic T cells and NK cells. Similarly, pEXO carrying MIC and ULBP down-regulates the expression of NKG2D receptor on $CD8^+$ T cells and cytotoxic activities of the $CD8^+$ and gamma delta T (γς T) cells (**Supplementary Table 1**) (134). The expression of syncytin-2, an endogenous retroviral protein exclusively expressed on the human placenta, on the pEXO is down-regulated in preeclampsia patients. Lokossou et al. reported that the pEXO bearing syncytin-2 are immunosuppressive *via* reducing Th1 cytokine production in activated PBMCs (**Supplementary Table 1**) (188). Together, these findings indicate that exosomes contribute to immune tolerance through the presentation of MHC molecules or other surface ligands.

EXOSOMES IN PREGNANCY COMPLICATIONS

Pregnancy-associated complications such as preeclampsia, gestational diabetes mellitus and preterm birth, are the major threats to human reproductive health. Despite advances in technology and understanding of pregnancy, the rates of pregnancy-related morbidity and mortality increased slightly over the last two decades (189). The current preventive and prognostic approaches for these complications are limited. Thus, a comprehensive understanding of pregnancy-related complications is much needed for better diagnosis and treatment.

Peripheral blood represents the most widely used biological sample for clinical diagnosis. Circulating fetal DNA in maternal plasma and serum has been used for non-invasive prenatal diagnosis (190). Alterations in pEXO have been demonstrated in pregnancy complications. Thus, pEXO might be a promising alternative for screening the following disorders in pregnancy.

Preeclampsia

Preeclampsia, characterized by new onset of hypertension and proteinuria, is one of the most severe complications in pregnancy affecting 5% of pregnant women globally (45). Nonetheless, the most effective treatment for preeclampsia is delivery. For decades, its pathogenesis has largely been attributed to 1) compromised trophoblast invasion (191); 2) dysregulated maternal immune tolerance (192) and 3) endothelial dysfunction (193). However, preeclampsia patients often have more than one defect. It is not clear, to what extent, how each of these causes contributed to preeclampsia as a whole. Considering the growing body of evidence that pEXO are key in modulating maternal homeostasis, we hereby summarize these studies to provide new insight on preeclampsia treatment.

pEXO levels in maternal blood of preeclamptic patients are remarkably increased compared to those of normal pregnancy. Moreover, omics data found that the molecular signatures of pEXO are largely different between preeclampsia and normal pregnancy. For example, proteomic analysis of exosomes isolated from maternal plasma by cholera toxin B chain and annexin V binding show that exosomes from preeclamptic patients have a higher expression of serpin peptidase inhibitor (PAI)-1, porphyria cutanea tarda (PCT), S100 calcium-binding protein B (S100b), TGF-β, VEGFR1, natriuretic peptide B (BNP), placental growth factor (PGF) (194, 195). Non-coding RNA-seq of plasma exosomes reveals that miR-486-1-5p and miR-486-2-5p are significant enriched in the preeclampsia group and could be used as potential diagnosis biomarkers (196). More importantly, exosomes from preeclamptic patients elicit preeclamptic symptoms (hypertension and proteinuria) in mice when injected *via* tail veins (197), indicating their indispensable role in preeclampsia occurrence.

Trophoblast invasion and migration are critical in spiral artery remodeling and placentation. In preeclamptic patients, miR-210 is highly enriched in the plasma exosomes compared with that in normal pregnancy, and this in turn contributes to preeclampsia by inhibition of trophoblast invasion through downregulating the potassium channel modulatory factor 1 (198). In addition to the comprised trophoblast function, the miRNA profile is disrupted in preeclampsia exosomes as well. For instance, high levels of miR-517-5p, miR-518b and miR-520h are associated with late-onset preeclampsia (199). Controversially, another study observed that down-regulation

of miR-517-5p, miR-520a-5p and miR-525-5p in patients are related to late-onset of preeclampsia (200). The discrepancy might be due to differences in sample preparation, donor ethnicity and gestational age. Given that pEXO only accounts for a small proportion (15-20%) of the total circulating exosomes and remarkable differences in miRNA profiles between pEXO and total plasma exosomes, the data should be interpreted with caution.

Endothelial function is fundamental in modulating blood pressure. Nitric oxide (NO) mediates vasorelaxation *via* an endothelium-dependent pathway. While the diminished activity of endothelial nitric oxide synthase (eNOS), a key enzyme for NO production, is observed in endothelial cells after treatment with preeclamptic pEXO (201). Moreover, preeclamptic patients have a higher level of miR-155 in plasma compared to healthy control and further study showed that it can inhibit eNOS expression in human umbilical vein endothelial cells (HUVEC) (55). An *in vitro* study showed that the macro-EVs from normal pregnancy but not preeclampsia could protect endothelial cells from activation (138). Moreover, an animal study found that human pEXO could relax mesenteric arteries after injection into pregnant mice (202). Another study showed that trophoblast-derived exosomes could promote vascular smooth muscle cell migration (203).

Disrupted maternal immune tolerance is another hallmark of preeclampsia. Syncytin-1/2, which can inhibit T cell activation and proliferation, is reduced in exosomes from preeclamptic patients (204). PD-L1, involved in decidual macrophage polarization and Treg cell differentiation, was found to be remarkably reduced in the placenta and pEXO of preeclamptic patients. Although the proteomic data on pEXO is rare, tissue proteomic results could be an alternative for the exosome study. For example, the expression of neprilysin (NEP), a membrane-bound metalloprotease associated with hypertension, is increased in the preeclamptic placenta at delivery. Interestingly, Manjot et al. recently demonstrated that exosomes from preeclamptic placenta have a higher expression of active NEP when compared to that of in normal placenta (60).

Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is one of the most common metabolic disorders during pregnancy. It affects ~13.2% of the pregnant mothers in developed countries (205). Without treatment, it may lead to preterm birth, fetal death and other pregnancy complications due to poor placentation induced by hyperglycemia. Although GDM is usually preventable and manageable, infants of mothers with GDM are at increased risk for heart disease, obesity or type 2 diabetes (205–207).

GDM patients have a relatively higher level of total exosomes and pEXO level in maternal plasma (46). Moreover, an *in vitro* study showed that exosomes from GDM patients induced endothelial activation, indicating the importance of pEXO in modulating maternal vascular homeostasis. miRNA compositions in urine-derived exosome and explant culture are different. Exosomes isolated from the urine of GDM patients in the 3rd trimester of gestation have a low level of miR-516-5p, miR-517-3p, miR-518-5p, miR-222-3p and miR-16-5p (208). Those from placental explant culture express another group of miRNAs (miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p and miR-224-5p) (209). Dipeptidyl peptidase IV (DDPIV) modulates glucose hemostasis by cleavage of glucagon-like peptide 1 (GLP-1) and DDPIV inhibitors are used for type 2 diabetes treatment. Manu Vatish *et al.* found that exosomes isolated from GDM placenta through perfusion had an upregulation of DDPIV by 8-fold (210). Moreover, exosomes from GDM pregnancy remarkably reduce migration and glucose uptake of skeletal muscle cells (209). Similarly, plasma exosomes from GDM patients also induce glucose intolerance, reduce glucose-induced insulin secretion and cause poor insulin responsiveness in mouse model (211).

GDM may arise from metabolic dysregulation of adipose tissue, which is critical in the modulation of insulin sensitivity (212). In general, normal pregnancy is accompanied by increased total adipose mass. Maternal body mass index (BMI) has a strong association with the risk of GDM, indicating excessive adipocytes are a potential stressor for placentation (213). Adiponectin and leptin, mainly produced by the placenta during pregnancy, have a wide range of functions in adipose tissue such as vascularization, adipocyte enlargement and expansion (207). Exosomes from adipose tissue of GDM patients altered placental glucose metabolism by increasing gene expression of the glycolysis and gluconeogenesis pathways (214). Thus, pEXO may participate in maternal metabolism *via* modulating the activity of adipose tissue.

Preterm Birth

Preterm birth, also known as premature birth, generally refers to birth at less than 37 weeks of gestational age (215). Nowadays, preterm birth is the leading cause of perinatal morbidity and mortality and has a long-term effect on the health of the fetus (216). For instance, premature infants are vulnerable to heart defects, cognitive disabilities, and respiratory illnesses (217). Nonetheless, the cause of preterm birth is still unclear.

Studies on pEXO of preterm birth are rare. Unlike preeclampsia and GDM, the level of pEXO in preterm birth is significantly decreased compared to full-term pregnancy (218). Placenta senescence and fetal membrane inflammation are generally believed to be the causes of preterm birth. Proteomic study of exosomes from preterm plasma indicates that alterations in protein composition are associated with inflammatory and metabolic signals (219). A similar result was found in amniotic fluid-derived exosomes of preterm patients (220). In animals, Plasma exosomes of CD-1 mice from late-gestation (E18), not early-gestation (E9), induce preterm labor in mice, indicating that exosomes might function as one trigger in labor initiation (221). Moreover, the exosomal miRNA profile of maternal plasma is different between mothers delivering at term and preterm (222, 223). A comprehensive analysis of the exosomal miRNAs reveals that the miRNAs target genes are associated with TGF-β signaling, p53, and glucocorticoid receptor signaling (222). Despite the inconsistency and irreproducibility of miRNA sequencing results, exosomal miRNAs are still suggested as an alternative approach for the diagnosis of preterm birth. Together, these studies indicate that pEXO participate in the processes of labor and delivery.

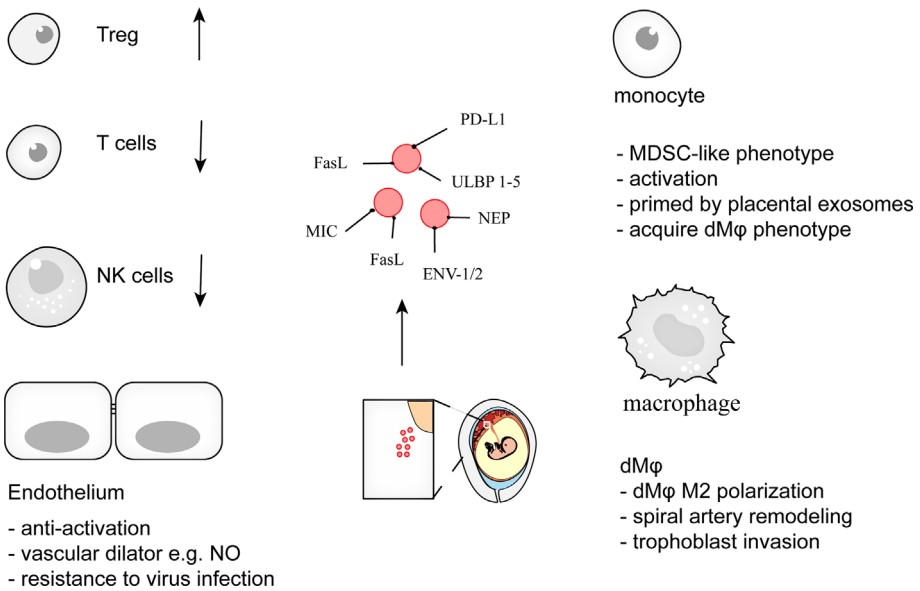

FIGURE 3 | pEXO contribute to maternal tolerance toward the fetus during pregnancy. Exosomes from placenta, syncytiotrophoblast (STB) in particular, support pregnancy *via* induction of Treg differentiation, restraint of cytotoxic activities of T cells and NK cells, promotion of decidual macrophage polarization, and endowing endothelial cells with viral resistance. Disruption of maternal immune tolerance is associated with adverse pregnancy complications such as miscarriage, preeclampsia. The specific cargoes within the pEXO represent the potential target for prenatal diagnosis and pregnancy-related disease screening.

DISCUSSION

Studies of exosome had been tremendously increased in the last two decades and exosomes are gradually demonstrated to be a perfect tool for drug delivery. However, our understanding of exosome biogenesis and the underlying forces that navigate them to their destination is still lacking.

Currently, most studies on pEXO are conducted *in vitro* due to ethical constraints in regard to manipulation of the maternal-feto-placental unit and lack of proper animal models. pEXO isolated from placenta tissue at mid (Abortion)- or term (Delivery)-gestation, may not represent it's *in vivo* functions at early pregnancy. Therefore, the content and biological activities of pEXO at different gestation periods should be investigated. Furthermore, the alternation of pEXO signatures observed from late gestational samples in clinical studies would possibly be the consequence rather than the cause of the pregnancy complications. A large prospective study of the first trimester pEXO isolated from plasma/placenta tissue from pregnant women who develop pregnancy complications at late gestation should be carried out. Apart from that, *in vitro* manipulations (Such as perfusion and explant culture) during exosome isolation may disrupt the molecular signature of pEXO. Thus, pEXO isolated by different isolation methods should be compared in order to establish a standard isolation technique and to set a standard parameter for diagnostic purposes.

In summary, pregnancy is a complex physiological process with a wide range of systemic adaptations in the mother's body. The placenta, the frontline of the maternal-fetal interface, makes these happened in a coordinated way. Exosome, as a signal carrier, links the mother and the fetus and is a key player in immune cell activation, differentiation, maturation and endovascular homeostasis (**Figure 3**). Thus, advances in pEXO research will deepen our understanding of pregnancy and may provide new insight on the prevention and treatment of pregnancy-related complications.

AUTHOR CONTRIBUTIONS

Conceptualization – PC, C-LL and KB. Writing – original draft preparation, KB. Writing – review and editing, XL, JZ, C-LL, PC, EN and WY. Illustration – XL and KB. Supervision – C-LL and PC. Funding acquisition – C-LL and EN. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Exosome-Based Vaccines: History, Current State, and Clinical Trials

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Extracellular vesicles (EVs) are released by most cell types as part of an intracellular communication system in crucial processes such as inflammation, cell proliferation, and immune response. However, EVs have also been implicated in the pathogenesis of several diseases, such as cancer and numerous infectious diseases. An important feature of EVs is their ability to deliver a wide range of molecules to nearby targets or over long distances, which allows the mediation of different biological functions. This delivery mechanism can be utilized for the development of therapeutic strategies, such as vaccination. Here, we have highlighted several studies from a historical perspective, with respect to current investigations on EV-based vaccines. For example, vaccines based on exosomes derived from dendritic cells proved to be simpler in terms of management and cost-effectiveness than dendritic cell vaccines. Recent evidence suggests that EVs derived from cancer cells can be leveraged for therapeutics to induce strong anti-tumor immune responses. Moreover, EV-based vaccines have shown exciting and promising results against different types of infectious diseases. We have also summarized the results obtained from completed clinical trials conducted on the usage of exosome-based vaccines in the treatment of cancer, and more recently, coronavirus disease.

Keywords: extracellular vesicles (EV), immunization, infectious diseases, cancer, exosomes

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EXOSOME FUNCTIONS: BIOGENESIS AND CARGO

Extracellular vesicles (EVs) are a group of biological, nano-sized, bilayered membrane vesicles produced by almost all cells. EVs can be found naturally in body fluids, such as blood, saliva, and breast milk (1–4). Classically, EVs are classified by size, molecular cargo, and the biogenesis pathway (5). However, there was a debate in literature regarding the definition of EVs due to inconsistencies in EV purification and characterization (6, 7). Fortunately, significant progress has been achieved regarding the establishment of criteria for a standardized nomenclature of EVs, and minimal requirements are set for experimental controls during EV separation, concentration and, characterization endorsed by the International Society of Extracellular Vesicles (ISEV) (8). In terms of biogenesis, EVs can be broadly divided into two dominant classes, namely exosomes and microvesicles (MVs). Exosomes are 30-150 nm EVs that initially demonstrate formation as intraluminal vesicles inside multivesicular bodies (MVBs) and are released after fusion of MVBs

with the plasma membrane (3, 6, 9, 10). Microvesicles are formed by the outward budding of the plasma membrane, a process regulated by the translocation of phospholipids (9, 11). However, according to ISEV, there is no consensus on specific markers of each EV subtype, therefore assigning an EV to a specific biogenesis remains a challenging process (8).

In the extracellular space, exosomes can undergo fusion with the plasma membrane of recipient cells and deliver their packaged cargo into the cytosol. Exosomes are highly heterogeneous vehicles that can transport a wide variety of molecules, including lipids, proteins, and nucleic acids, such as mRNAs and miRNAs. The transport of these molecules can occur within the exosome itself or *via* attachment with the surface of recipient molecules, as evidenced in the case of major histocompatibility complex (MHC) molecules (12). Healthy cells release exosomes under normal physiological conditions that play a role in several cellular processes, for example, intercellular communication by facilitating the carriage and delivery of multiple molecules that can modulate crucial processes, such as growth, differentiation, and stress response (13, 14). Thus, considerable research attention is focused on the biology of EVs. However, according to Edgar (15), emerging interest in exosome biology is attributable to the association of exosomes with disease development. Indeed, infectious, inflammatory, and neurodegenerative diseases, as well as cancer, exhibit specific biomarkers that are carried by their respective exosomes (16–18).

HISTORY OF EXOSOME-BASED VACCINES

EV release was initially thought to be a random process; however, in 1983, two independent studies using different animal models discovered that reticulocytes released transferrin receptors inside EVs (19, 20). Barz et al. demonstrated that different lymphoma variants could produce EVs with distinct profiles of proteins and lipids that could be associated with tumor immune escape and cancer invasion (21). A year later, Schirrmacher and Barz observed that tumor-derived exosomes (TDEs) displayed antigens similar to their corresponding tumor cells (22). The same study was the first to show the anti-tumor effects of exosomes on cytotoxic lymphocytes (CTLs). In 1987, Johnstone et al. coined the term exosomes as a reference for EVs carrying transferrin receptors (23). Raposo et al. demonstrated the role of exosomes in antigen presentation by revealing MHC class II molecules in exosomes derived from B lymphocytes, which induced specific MHC class II T cell responses (24). These findings reveal that exosomes can be exploited as biomarkers and can be used in immunotherapeutic strategies for vaccine development.

The concept of a cancer vaccine is not new; it dates back to the early 70s. However, the feasibility of a vaccine against cancer is challenged by several issues, such as transplant rejection (25, 26). Tumor peptides have generated promising results and have shown potential applicability as a cancer therapeutic agent; however, peptide-based vaccines exhibit poor immunogenicity (27–29). In 1998, Zitvogel et al. (30) published a study in which they found that DEXs (exosomes derived from dendritic cells) express functional MHC class I and II molecules. They observed that tumor peptide-pulsed dendritic cells (DCs) released DEXs presenting tumor antigens on the membrane, which induced *in vivo* CTL priming and consequent tumor growth suppression. This study was the first to support the development of a novel cell-free vaccine using exosomes, representing a milestone in exosome-based vaccine research.

In the new millennium, Wolfers and colleagues have reported that TDEs represent a source of T-cell cross-priming which is realized *via* transfer of antigens to DCs, and this induces CTL anti-tumor responses *in vitro* and *in vivo* (31). During *in vitro* stimulations, TDEs were more effective in eliciting protection against autologous tumors than other cancer immunization strategies, such as irradiated tumor cells, apoptotic bodies, or tumor lysates (31, 32). In 2004, the Zitvogel group published two articles that comprehensively described the transfer of MHC class I molecules from DEXs to naïve DCs for efficient CTL activation, and the role of toll-like receptors in combination with DEXs in triggering an MHC-restricted response in $CD8^+$ T cells using *in vitro* stimulations and HLA-A2 transgenic mice (33, 34). In the same year, exosomes released by plasmacytoma cells were successfully used as a cancer vaccine; in this case, plasmacytoma exosomes conferred protection to the animals through reduction in tumor growth by 80% after a single vaccination (35). The use of exosome-based vaccines has since spread to different research areas outside cancer therapy. Exosomes derived from DCs previously co-cultivated with *Toxoplasma gondii* generated a strong and specific immune response to induced acute and chronic toxoplasmosis (36). Further investigation using *Toxoplasma gondii* has been detailed in section 5. Exosomes derived from an antigenic extract of *Salmonella enteritidis* strain were isolated and cultivated with serum samples obtained from naturally infected and healthy chickens (37). After completion of the exosome treatment, surface structures from *Salmonella*, such as flagellin and porins, were found to be immunogenic in serum samples collected from infected chickens but did not exhibit immunogenicity in healthy ones. These results represented a concrete evidence highlighting that *Salmonella*-derived exosomes could be used in the preparation of vaccines (37). Recently, a vaccine was designed by using a plasmid to generate *Salmonella* exosomes containing highly immunogenic membrane antigens and it showed satisfactory immune responses against several *Salmonella* strains (38).

From this point onward, exosome research increased due to the development of more sophisticated techniques, such as exosome engineering for drug delivery systems and artificial antigen presentation models (**Figure 1**). In the mid-2000s, the first results from clinical trials on exosome-based vaccines were reported (39–43). Clinical trials using exosome-based vaccines have been detailed in section 6. Currently, several exosome-based vaccine candidates are under development for diseases such as cancer, AIDS, hepatitis B, and other infectious diseases (44–48). The vaccines have been discussed in further detail in the subsequent sections.

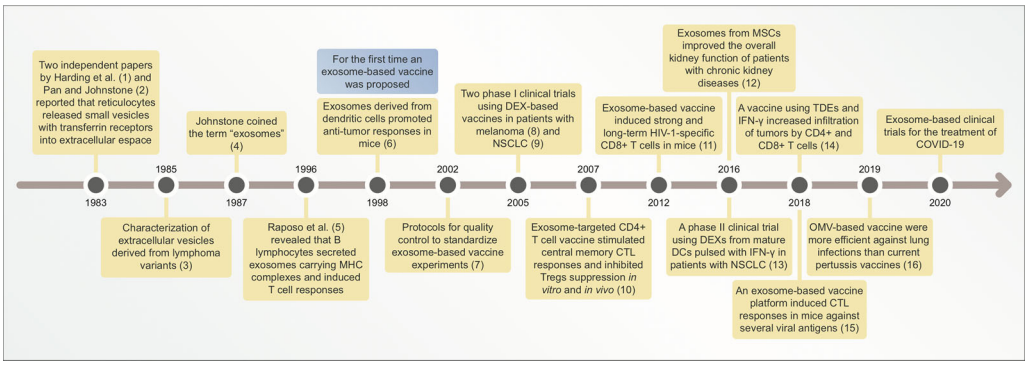

FIGURE 1 | Timeline illustrating main discoveries related to exosome-based vaccines. CTL, cytotoxic lymphocyte; DEXs, exosomes derived from dendritic cells (DCs); NSCLC, non-small cell lung cancer; MSCs, mesenchymal stem cells; TDEs, tumor-derived exosomes; OMV, outer membrane vesicle; COVID-19, coronavirus disease-19.

EXOSOME-BASED VACCINES AS A CANCER THERAPEUTIC STRATEGY

Tumor cells can evade immune surveillance through several regulatory mechanisms, such as reduced immune recognition or the establishment of an immunosuppressive tumor microenvironment (49). In this scenario, cancer cells can undergo proliferation and facilitate the recruitment of immune and stromal cells to favor tumor progression, which can lead to metastasis (50). Cancer immunotherapy has emerged as a clinical strategy for controlling the immune system and for reactivating anti-tumor immune responses (51). Immunotherapy approaches include targeting of immune tolerance *via* co-inhibitory checkpoints, adoptive T-cell therapy, and cancer vaccination (52).

Cancer vaccines differ from traditionally engineered vaccine for infectious diseases in the intervention approach. Traditional vaccines are preventive, on the other hand, cancer vaccines are focused on the therapeutic aspect. However, there are prophylactic interventions to reduce cancer incidence, morbidity, and mortality for virus-related cancers, such as hepatitis B (HBV) and human papilloma virus (HPV) (53). Therapeutic cancer vaccines can target a wide variety of antigens expressed by cancer cells, including antigens that are exclusively expressed in cancer cells, also known as tumor-specific antigens (TSAs), for example, mutated P53 and RAS. Cancer vaccines can also target antigens that have low levels in normal but highly expressed in tumor cells, the tumor-associated antigens (TAAs), such as MAGE-1, HER2, and HPV (54–56). There are also different platforms available, such as peptide-based, DNA-based, protein-based, viral-based, whole cancer cells, recombinant factors, and pulsed DCs (53, 56–58). Currently, only three cancer vaccines are approved for clinical use by FDA to treat early-stage bladder cancer (TheraCys®), metastatic castration-resistant prostate cancer (PROVENGE), and metastatic melanoma (IMLYGIC®). These vaccines have produced slightly improved overall survival of patients with early-stage disease (58). For patients with advanced or metastatic tumors, cancer vaccines are likely to have a therapeutic role in a combination therapy approach (59).

Despite suboptimal results, recent cancer vaccine interventions are clinically promising and have shown potential applicability, especially with respect to overall patient survival (60). According to Melief et al., a robust cancer vaccine design must enable the induction of potent effector $CD4^+$ and $CD8^+$ T-cell responses (60). Target antigen selection is challenging; selection is based on overexpressed antigens in tumors relative to normal tissue (61). Owing to the immunosuppressive tumor microenvironment, cancer vaccines should be administered in combination with adjuvants to overcome immunosuppression (62). Adjuvants are key components of several successful vaccines that boost the vaccine's immune response, quality, and efficacy (63). An interesting strategy for vaccines based on TAAs is the use of a combination of adjuvants and immunomodulatory antibodies (62). Exosomes exhibit features for application as adjuvant carriers, such as optimal size, biocompatibility, stability in systemic circulation, and target-specific delivery (64). Recently, an exosome-based adjuvant delivery system was developed using genetically modified murine melanoma B16BL6 cells, in which the exosomes derived from these cells containing CpG DNA were injected three times with a 3-days intervals and successfully induced immunostimulatory signals in mice 7 days after the last immunization (65). These results shed light on the novel use of exosomes as adjuvant carriers for future cancer vaccine development. Adjuvant strategies to increase cancer vaccine efficacy have been thoroughly reviewed by Bowen et al. (62).

To design a successful cancer vaccine, researchers must also consider administration routes and optimal delivery vehicles. DC injection is a common delivery system that triggers initiation and controls the direction of antigen-specific immune responses (64). However, DC-based immunotherapy has shown inconclusive results in clinical trials. Moreover, DC vaccines are an expensive therapeutic strategy for implementation in large populations, and they are difficult to ensure standardized production and lose efficacy over long periods of storage (49, 66). DEXs have emerged

as a viable option for cancer vaccination because they possess higher stability for a longer period than DCs because of their lipid composition. DEXs also possess more peptide-MHC I and -MHC II complexes than DCs, thereby rendering the use of DEXs a less time- and space-consuming strategy (**Figure 2A**) (66–68). Additionally, DEXs are more resistant to immunosuppressive mechanisms in the tumor microenvironment than DCs (69). Exosomes are reportedly more capable of inducing immunocompetence in DCs than in microvesicles. An *in vivo* comparison of immunostimulatory potential between microvesicles and exosomes derived from ovalbumin (OVA)-pulsed DCs showed that only exosomes induced antigen-specific $CD8^+$ T cells and increased the proportion of germinal center B cells. Exosomes were also superior in terms of OVA levels, while microvesicle-associated OVA was barely detectable; however, microvesicles and exosomes both induced higher OVA-specific IgG production relative to controls (70).

Several studies revealed that DEXs can activate $CD4^+$ and $CD8^+$ T cells, indicating the ability of DEXs successfully carry antigen-MHCI/II complexes *in vivo* and *in vitro* (34, 71–73). Once activated, $CD8^+$ T cells can become memory T cells. Wang et al. using a melanoma mice model, induced the $CD8^+$ T cells differentiation to CTLs *via* DEXs from mature DCs. Three months after the immunization, the immunized mice group was boosted with DEXs and the number of CD8+ T cells expressing antigen-specific T cell receptor (TCR) was expanded six- to seven-fold in immunized mice. Another experiment in this study was to challenge immunized mice and control groups with melanoma cells three months after immunization protocol. Immunized mice were tumor free and control mice died of lung metastases. Moreover, these antigen-specific CD8+ T cells express CD44, a marker for memory T cells (74). The immunological memory induced by DEXs was also observed in CD4+ T cells of mice treated with OVA-pulsed DEXs, which induced an immune response towards to Th1 type. Interestingly, in this study, an efficient long term memory response of OVA-specific Th1 cells after a boost was dependent of prior B cell activation (75). $CD4^+$ cells after uptake OVA-pulsed DEXs could stimulate efficient antigen-specific CTL responses and long-term T $CD8^+$ cell memory in immunized C57BL/6 mice against OVA-transfected melanoma cells expressing OVA challenge after three months of complete immunization (76). On the other hand, DEX vaccines failed to induce antigen-specific T cell responses in clinical trials (further discussed in section 6). Preclinical results showed that DEXs released by DCs treated with interferon-γ (IFN-γ) express high levels of molecules capable to induce a strong $CD8^+$ T cell activation, such as CD40, CD80, CD86, and CD54 (77). However, this enhancing DC strategy did not translate into results in a phase II clinical trial, which the peptide-specific T cell responses were not detectable (43).

Recently, a combination of cancer vaccination and checkpoint blockade strategies was designed to induce anti-tumor responses *in vitro* and *in vivo*. Exosomes released by modified anti-CTLA-4 antibody and OVA-pulsed DCs ($DEXs_{OVA\text{-}CTLA\text{-}4}$) were enriched in MHC I/II molecules and were found to exert strong T-cell activation and proliferation *in vitro*. Vaccination with $DEXs_{OVA\text{-}CTLA\text{-}4}$ increased the migration of $CD4^+$ and $CD8^+$ T cells to the tumor site and elevated the ratio of CTLs/Tregs in the microenvironment of B16 melanoma tumor model after 12 days (78). Hao et al. demonstrated that exosomes derived from OVA-pulsed DCs and their uptake by

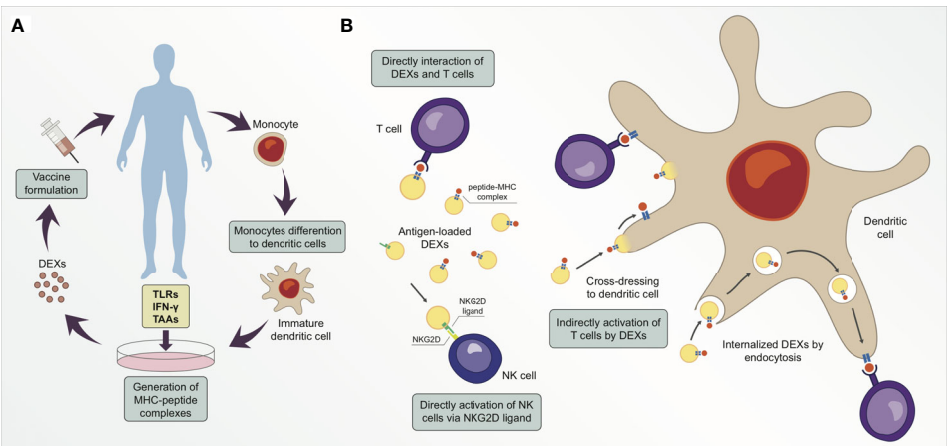

FIGURE 2 | Exosomes derived from dendritic cells (DEXs) are potential targets for cancer therapeutic strategy. **(A)** Simplified illustration of a personalized vaccine using DEXs. **(B)** DEXs can directly catalyze the transfer of peptide-MHC complexes from their membrane surface to T cell membrane surface (cross-dressing). Moreover, DEXs can stimulate T cell responses in an indirect manner *via* cross-dressing with dendritic cells or *via* exosome uptake and processing, following the peptide-MHC complex presentation to T cells. DEXs can also induce activation and proliferation of NK cells by establishing interaction of the NKG2D ligand on DEXs with NKG2D receptors on the NK cell membrane.

CD4+ T cells stimulated the proliferation and differentiation of central memory CTLs and inhibited Treg suppression *in vitro* using BL6 melanoma cells. Also, in this study, C57BL/6 mice immunized with OVA-pulsed DEXs showed an elevated number of OVA-specific $CD8^+$ $CD44^+$ T cells three months after the immunization in comparison to control group (42). Long-term functional CTL memory was observed in animals injected with OVA-pulsed DCs and was then challenged with OVA-expressing B16 melanoma cells (79). Different mechanisms of antigen presentation by DEXs have been proposed (**Figure 2B**) (80, 81). Recipient DCs may establish interaction with antigen-loaded DEXs *via* the endosomal pathway, followed by the transfer of the peptide-MHC complex to the DC surface membrane for antigen presentation to T cells (82). Furthermore, a second indirect antigen presentation mechanism called cross-dressing occurs when an acceptor DC captures DEXs by facilitating the merging of membranes and retains the peptide-MHC complex on the DC surface without processing (80, 83). The direct interaction of DEXs with T cells seems to demonstrate poor efficiency in stimulating T cell responses, therefore DEXs have less T cell stimulation potential than their parent DCs (66, 84). Some authors suggest that exosomes are not able to interact directly with effector cells, thus prior capturing and processing the exosomes by DCs is a superior pathway of priming specific T cells *via* DEXs (75, 81, 85, 86). A study using the direct interaction of DEXs with T cells showed that DEXs from mature DCs are better at stimulating T cells than DEXs from immature DCs (87). Robbins and Morelli suggest that the low ability of exosomes to stimulate T cells *in vitro* is probably due to the small size and dispersion of exosomes caused by Brownian motion (88). These authors also suggest that T cell stimulation by exosomes can be enhanced when exosomes are immobilized and at high concentration (88).

Damage-associated molecular patterns (DAMPs) are signaling molecules released by dying cells that trigger immune cells to activate defensive mechanisms (89). For example, tumor-derived DAMPs establish interaction with Toll-like receptors (TLRs), which directly lead to the activation of T cells and indirectly result in the induction of the release of inflammatory cytokines (90). Damo and colleagues developed different exosome vaccines derived from OVA and TLR ligand-pulsed bone marrow DCs (91). Their results showed that the TLR-3 ligand-DEXs vaccine (OVA + poly I:C) stimulated higher antigen-specific $CD8^+$ T-cell proliferation and effector functions and increased the population of $TNF\alpha^+CD4^+$ T cells in the lymph nodes of vaccinated mice with melanoma compared to other vaccine formulations 19 days after priming. Additionally, this group showed that purified DEXs successfully carried melanoma epitopes and induced potent anti-tumor immune responses, thereby slowing tumor progression. Recently, a DEX-based vaccine combined with microwave ablation was reported to inhibit tumor growth in hepatocellular carcinoma (HCC) mouse models compared to microwave ablation (a common therapy for HCC patients) alone, in this case, the tumor disappeared 10 days after microwave ablation in combination with DEX injection (92). Additionally, HCC features a high expression level of α-fetoprotein (AFP), which has been used as an HCC antigen for monitoring and diagnosis (93). DEXs from AFP-enriched DCs generated strong antigen-specific immune responses *in vitro* tumor suppression after 26 days in HCC mice under a vaccination regimen of a weekly injection for three weeks (71).

In addition to carrying MHC complexes on their surface, DEXs carry proteins that can stimulate cells of the innate immune system. For example, a study showed that DEXs expressing BAT3 on the surface, which is a protein responsible for engaging natural killer (NK) cell activation, induced NK cell-mediated cytokine release *in vitro* (94). DEXs induced a strong NK cell activation and stimulated the release of IFN-γ in a dose-dependent manner *via* TNF in mice (95). DEXs also express several other ligands on their surface that can mediate innate immune functions, such as TNF, FasL, and TRAIL (95). Moreover, the DEX membrane contains the activating receptor NKG2D ligand, which is responsible for the activation and proliferation of NK cells (96).

Although DCs have been pulsed with TLRs, biomarkers, and tumor antigens derived from lysates, TDE-pulsed DCs were reported to generate the most remarkable results as a potential anti-tumor vaccination. As mentioned earlier, TDEs provide a broad range of TAAs for antigen presentation. TDEs also transfer mRNAs and non-coding RNAs, such as miRNAs and long non-coding RNAs (lncRNAs) (97, 98). Recent data suggests that mRNAs packaged inside TDEs are responsible for stimulating the immune response by MHC I cross-presentation to DCs (99–101). For example, TDEs derived from CD40L/4-1BBL-expressing Mel526 melanoma cells induce potent DC activation *in vitro* (100). The interaction of 4-1BBL with its receptor 4-1BB results in the formation of a complex that induces CD8+ T cell activation and expansion (102). Interestingly, peptides derived from introns and exons of mRNAs derived from mouse melanoma cells act as tumor-associated peptides that can be delivered to DCs and result in the promotion of CD8+ T cell activation and proliferation (99). A recent study using sequencing technology showed that exosomes derived from plasma of 150 patient with cancer contained abundant levels of lncRNAs that could act as potential biomarkers for cancer diagnosis, specially 5 lncRNAs that can serve as HCC biomarkers diagnosis (103). Exploitation of lncRNAs derived from TDEs seems promising as a vaccination approach. For example, *LINC02195* is an lncRNA capable of regulating MHC I molecules during antigen processing and presentation (104). Furthermore, a signature was identified as a prognostic predictor of laryngeal cancer using the lncRNAs of TDEs (105).

A vaccine designed using TDE-loaded DCs showed superior immune response induction compared to tumor lysate-loaded DCs as evidenced by results obtained in mouse myeloid leukemia and renal cell carcinoma models (106). Recently, the same effect was observed in lung cancer, in which TDE-pulsed DCs induced a reduction in the population of regulatory T cells (Tregs) *in vitro*, while they suppressed tumor growth and increased animal survivability *in vivo* (107). DCs pulsed with TDEs derived from different types of cancers (such as leukemia, renal carcinoma,

glioblastoma, and pancreatic cancer) elicit anti-tumor immune responses (108–112). DC activation and maturation can be induced by the high-mobility group nucleosome-binding protein 1 (HMGN1), a well-known Th1-polarizing alarmin (113, 114). TDEs bound to the N-terminal portion of HMGN were found to induce persistent anti-tumor immunity in orthotopic HCC mice (115).

In most studies, TDEs derived from patient sera have been found to be biocompatible and exhibit low immunogenicity. However, it is relevant that TDEs play roles in all steps of cancer progression, including metastasis and they can be immunosuppressive in certain types of cancer (115–117). The immune-suppressive potential of TDEs has been reported to inhibit the effector activity of CD4+ and CD8+ T cells and NK cells (118). Recently was demonstrated that TDEs can carry the programmed death ligand (PD-L1), which is responsible for T cell exhaustion (119). Moreover, TDEs can block the differentiation of DCs, induce apoptosis, and diminish the overall T cell responses in different types of cancer (120–122). In addition, several studies show that TDEs have potential to suppress the effects of therapeutic agents (123, 124), for example, TDEs are associated to acquired chemoresistance (125).

TDEs may also exert a dual effect, improving DC vaccine efficiency *in vitro*, while favoring tumor progression *in vivo* (117). Immunomodulatory molecules combined with TDEs may induce enhanced anti-tumor immune responses. For example, a vaccine designed with TDEs released by mouse cancer cell lines subjected to treatment with IFN-γ and interferon receptor factor-1 (IRF-1) was found to increase the number of infiltrated CD4+ and CD8+ T cells and reduce tumor size in C57BL/6J female mice transfected with Hepa 1-6 hepatoma cells or MC-38 colon carcinoma cells after 21 days of the exosome injection (46). Additionally, in a recent study reported by Shi et al., a vaccine with exosomes derived from IFN-γ-modified RM-1 prostate cancer cells under a vaccination regimen of 4 injections (on days 0, 4, 8, and 12), decreased the number of Tregs and reduced the tumor metastatic rate in C57BL male mice with lung metastasis (126). These findings indicate that pulsing DCs with a wide variety of molecules can help produce exosomes capable of generating a robust anti-tumor immune response (**Table 1**). These methods represent promising and potentially individualized TDE- and DEX-based vaccine strategies for cancer immunization.

EXOSOME-BASED VACCINES FOR TREATMENT OF VIRAL INFECTIOUS DISEASES

Similar to cancer, exosomes act as a double-edged sword because of their ability to carry and deliver molecules to target cells in infectious diseases. Exosomes play a crucial role in the pathogenesis of infection, but also trigger immune responses to confer protection against pathogens (134). This effect can be observed in the context of viral infections, where exosomes derived from infected cells can deliver viral content to surrounding cells, but can also induce antiviral immune responses (135). The "Trojan exosome" hypothesis proposed by Gould et al., describes the evolutionary similarities of viruses and exosomes with regard to their biogenesis and transmission pathways, suggesting exosomes as a potential tool for

TABLE 1 | Different experimental models and design using exosomes to induce anti-tumor immune responses against several types of cancer.

Experimental model	Cancer type	Experimental design	Clinical outcome	Reference
C57BL/6 mice; Hepa1-6, 4T1, Hela, and EL4 cell lines	HCC	Intravenous injection of DCs pulsed with TDE-N1ND	Generation of long-term memory T cells and robust anti-tumor immunity	(115)
C57BL/6 and IRF3-KO mice; E0771 cell line	Breast	Cancer cells treated with topotecan	TDEs from treated cells contain immunostimulatory DNA	(127)
C57BL/6 mice; A549 and LLC cell lines	Lung	Vaccination with 3 doses of DCs pulsed with TDEs	TDEs promoted DC maturation, which increased tumor-infiltrating $CD8^+$ T cells in mice	(107)
Zipras/myc-9-infected C57BL/6	Prostate	Vaccination with 4 doses of TDEs pulsed with IFN-γ	Prolonged survival time, attenuated expression of PD-L1, reduced tumor metastasis rate	(126)
C57BL/6 and CD45.1 mice	–	Antigen transfer from DEXs released by plasmacytoid DCs to conventional DCs	Cross-priming of naïve $CD8^+$ T cells	(128)
C57BL/6 and BALB/c mice; Hepa1-6, RAW264.7, LLC, and 4T1 cell lines	Lung and liver	Vaccination with a single dose of exosomes from cancer-bearing mice after photothermal therapy	Promoted infiltration of T cells into the tumor tissue	(129)
Transgenic HLA-A2/HER2 mice; 4T1 and BT474 cell lines	Breast	Vaccination with a single dose of DEXs from HuRt-specific DCs transfected with an adenoviral vector	Activation of $CD8^+$ T cell cytolytic functions against breast cancer cells in *vitro* and reduced tumor growth in *vivo*	(130)
BALB/c and C57BL/6 mice; H22, B16, and CT26 cell lines	Melanoma, liver, and colon	Vaccination with 3 doses of TDEs released by different cancer cell lines	Promoted DC maturation and elicited T cell anti-tumor responses	(131)
HepG2 and K562 cell lines	HCC	Isolation of exosomes released by cancer cells treated with anti-cancer drugs	TDEs exhibited heat shock proteins in their surface that activated NK anti-tumor response	(132)
4T1	Breast	Modified TDEs with microRNAs to enhance their immune stimulation function	Modified TDEs induced DC maturation *in vitro*	(133)

vaccination against human immunodeficiency virus (HIV) (136). The exosomal biogenesis pathway that is hijacked by HIV for viral spread can be exploited as a potential therapeutic approach (137).

Efforts are ongoing to evaluate the potential of exosome-based vaccines against HIV. Dr. Jim Xiang's research group pioneered this research area and developed a vaccine termed as Gp120-Texo. This vaccine was designed with DEXs derived from DCs transfected with an adenoviral vector, AdV_{Gp120}, which expressed the HIV-specific envelope glycoprotein Gp120 (138). Gp120-Texo induced strong and long-term HIV-specific $CD8^+$ T-cell responses independent of $CD4^+$ T cells and DCs in mice (44, 138). Later, the Xiang group designed a vaccine to induce a specific immune response against Gag (Gag-Texo) (139), a group of proteins responsible for HIV maturation and infection (140). Gag-Texo induced Gag-specific immunity in animal models of chronic infection, suggesting that this vaccine might induce CTL responses to attack HIV-infected cells (139). Nef is an HIV protein associated with multiple cellular functions, such as the survival of infected cells and vesicular trafficking (141). An exosome-based vaccine was engineered by incorporating a Nef mutant (Nef^{mut}) into exosomes. In this case, Nef^{mut}-exosomes were absorbed by DCs, which then presented the antigens, thereby eliciting CTL immune responses in mice against several viral antigens, such as those for HIV, Ebola, influenza, HBV, and hepatitis C virus (HCV) (47, 142, 143).

Even with current diagnostics and therapeutics that enable viral suppression, HBV continues to represent a major healthcare concern worldwide (144). HBV is frequently associated with the development of chronic liver diseases, such as HCC (145). Exosomes released by HBV-infected cells contain several proteins encoded by the HBV genome, as well as miRNAs that regulate gene expression in host cells (146–148). This sheds light on the potential use of exosomes to understand HBV transmission and HBV-host interactions. However, there is a lack of literature on exosome-based HBV vaccination. Few studies have investigated the potential of a general exosome-based vaccine platform for multiple viral antigens, including HBV. Additionally, a vaccine formulation designed with unmodified exosomes as adjuvants for the recombinant HBV antigen showed promising results, in which exosomes induced a Th1 immune response, thereby enhancing the levels of IFN-γ in mice (149). These studies are in the early phase, and further investigations are warranted to identify therapeutic targets for consideration as vaccine candidates against HBV using exosomes as delivery systems or adjuvants.

Influenza virus infection is another example of a healthcare concern that causes significant morbidity and mortality worldwide (150). Despite the wide variety of vaccine types available for influenza infection, studies have shown that exosomes can be used as a new platform for designing influenza vaccines, with exhibition of advantages over classical vaccines (151, 152). For example, airway exosomes released during influenza virus infection can carry host proteins with anti-influenza properties and can help trigger immune responses (153). A study using LC-MS/MS showed that exosomes derived from infected cells also carried similar proteins as those reported in the influenza virions, representing an alternative pathway for the infection of new host cells (154). Lung and serum-derived exosomes from mice infected with influenza virus exhibit high levels of miR-483-3p, and this is associated with the induction of pro-inflammatory cytokine release (155, 156). According to the authors, further studies are warranted to determine whether the transfer of miR-483-3p is involved in the activation of innate immune responses or in the inflammatory pathogenesis of influenza virus infection. Another exosome-based vaccination approach to combat the influenza virus includes EVs released by gram-negative bacteria, which are referred to as outer membrane vesicles (OMVs) (157). Several recent studies have reported that OMV-derived vaccines can induce strong immune protection against the influenza virus *in vivo* (158–161).

EXOSOME-BASED IMMUNIZATION STRATEGY FOR NON-VIRAL INFECTIOUS DISEASES

The release of exosomes by non-viral pathogens such as bacteria and parasites, plays an important role in pathogenesis by establishing interactions with the host immune system and by transferring resistance factors (162). However, exosomes and OMVs derived from bacteria have been reported to be potent immune modulators, rather than aiding pathogenesis (163). The potential of OMVs as immune activators has been investigated using models of different infectious diseases such as pertussis (whooping cough), which is caused by *Bordetella pertussis*, a gram-negative bacterium (164). Currently available vaccines aid the successful reduction of the morbidity and mortality caused by pertussis, but they are also associated with severe adverse effects and weak immune protection (165). According to the World Health Organization (WHO), there is no consensus regarding the antigenic composition of an optimal pertussis vaccine (https://www.who.int/biologicals/areas/vaccines/apertussis/en/). Several studies have now shown that a *B. pertussis* OMV-based vaccine can overcome this composition issue, representing an attractive vaccination model for pertussis (166–168). A recent OMV-based vaccine conferred protection to mice against lung infection more effectively than the current commercial pertussis vaccines (48). Although overshadowed by gram-negative bacteria, EVs derived from gram-positive bacteria have also recently gained attention as a potential vaccine platform for several infectious diseases. EVs released by *Staphylococcus aureus* were modified to possess no toxicity and to serve as vaccine candidates. Genetically engineered EVs showed immunogenic effects and protected mice against lethal sepsis caused by *S. aureus* (169). Additionally, EVs derived from *Streptococcus pneumoniae* incubated with murine DCs were rapidly internalized and enhanced the release of tumor necrosis factor (TNF)-α, which constitutes the inflammatory response (170).

Investigations of exosome-based vaccines for infectious diseases are not limited to viruses and bacteria. Toxoplasmosis is a globally occurring infectious disease caused by the coccidian protozoan *Toxoplasma gondii* (171). Vaccines with live and attenuated tachyzoites are available for animals; however, these vaccines are not effective and safe for humans (172). Therefore, the development of a toxoplasmosis vaccine for humans is of considerable interest for public health. However, few studies have reported the effects of DEXs derived from DCs pulsed with *T. gondii* or *Toxoplasma*-specific antigens (36, 173). In a recent study, DEXs released by DCs stimulated with *T. gondii* lysate were inoculated intranasally and ocularly in mice, which subsequently triggered humoral and mucosal immune responses against *Toxoplasma* infection (174).

Schistosomiasis is a major parasitic disease caused by *Schistosoma mansoni*, affecting a myriad individuals and causing over 280,000 deaths annually worldwide (175). Thus far, there is no vaccine available for schistosomiasis, which underscores the need for the development of vaccines against this disease. Few authors have suggested the use of exosomes as a cell-free vaccination platform against *S. mansoni* infection (176–178). Exosomes released by *S. mansoni* adult worms contain miRNAs and proteins involved in host-parasite interactions, such as invasion, nutrient acquisition, and immunomodulation (178). A study showed that *S. mansoni*-derived exosomes harbored several potential vaccine candidates, including proteins involved in multiple life cycle stages, underlining their potential utility in different stages of the parasite's life cycle (176). These findings represent a promising avenue for further investigation of the potential applicability of exosomes in the development of vaccines against infectious diseases.

CLINICAL TRIALS USING EXOSOME-BASED VACCINES

Clinical trials using exosomes can be divided into three categories with different approaches. First, exosomes can be used as carriers to deliver drugs to specific targets. Second, exosomes derived from mesenchymal stem cells. And last, incorporating specific mRNAs and miRNAs into exosomes elicit responses in patients (179). In 2005, results from two phase I clinical trials using DEX vaccines were obtained. The first trial reported the use of DEXs loaded with HLA-restricted melanoma-associated antigen (MAGE) peptides, which were infused into patients with HLA A2+ non-small cell lung cancer (NSCLC) (41). After the administration of four weekly doses, the vaccine was well tolerated by all patients. However, only one-third of the patients presented with MAGE-specific T-cell responses, while two of the four analyzed patients showed an increase in NK cell activity (41). The second trial reported the use of DEXs derived from DCs pulsed with MAGE and inoculated them to conduct immunization of melanoma patients. No major toxicity event was reported by any patient, except for the occurrence of a grade I fever (five patients out of fifteen); however, no MAGE-specific response of $CD4^+$ and $CD8^+$ cells was observed in peripheral blood. Interestingly, NK cell effector functions were also induced by the DEX vaccine, where eight of the thirteen patients presented with an increased number of NK cells infiltrating the tumor site (40).

According to Fu and colleagues, the lack of an immune response to these vaccines can be associated with the DC type selected by researchers in these clinical trials (69). They used immature DCs, while other studies showed that exosomes derived from mature DCs induced more potent T-cell priming. A phase II clinical trial reported the use of DEXs derived from mature DCs pulsed with IFN-γ in patients with NSCLC, and no toxicity was observed, except for the occurrence of grade III hepatotoxicity in one patient. In this case, the DEX vaccine did not induce a cancer-specific T-cell immune response but resulted in the induction of NK cell functions (43). According to the authors, IFN-γ may lead to an upregulated expression of PD-1 ligands on DEXs, a well-known immune checkpoint that suppresses T-cell activity. Although these vaccines were designed to activate specific MHC-restricted T-cell responses, DEXs proved to be effective in activating NK cells in an MHC-independent manner. Interestingly, DEX-based vaccines have focused on direct CTL activation as an independent process in other immune cells. However, Näslund et al. showed that $CD4^+$ T cells and B cells were necessary for the DEX activation of CTL anti-tumor response (85).

Recently, a non-randomized phase I/II clinical trial showed promising results with a vaccine designed using exosomes derived from DCs pulsed with SART1, a biomarker of squamous cell carcinoma of the esophagus. Pulsed DCs obtained from patients could generate exosomes that were well tolerated and induced antigen-specific CTLs in seven patients (180). One patient of this study remained stable for 20 months after DEXs therapy, although he developed lung metastasis after the stable period. The other six patients had progressive disease and died in a period up to 10 months after vaccination. These findings indicate that the development of a personalized exosome-based immunotherapy is feasible, although incredibly challenging. Patient indication criteria and the preparation of highly competent DCs for vaccine formulation are keystones of a successful exosome-based treatment (180). According to Xu and colleagues, it is important to investigate the anti-tumor immunity induced by DEXs-based vaccines to confirm whether DEXs can be used as tumor antigens for an exosome-based vaccine (52).

In addition to DEX vaccines, other clinical trials using different exosome-based vaccines have been reported. One phase I clinical trial reported the use of exosomes derived from ascites (AEXs) in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) as immunotherapy for colorectal cancer. Injection of AEXs for colorectal cancer was safe and well tolerated by all patients during the four weekly doses administered. Patients with advanced colorectal cancer subjected to treatment with AEXs plus GM-CSF demonstrated a strong anti-tumor cytotoxic T-lymphocyte response against the carcinoembryonic antigen (181), a colorectal cancer biomarker

(182). Exosome-based vaccines have also been developed for the treatment of chronic diseases other than cancer. A phase II/III clinical trial was conducted using exosomes derived from umbilical cord MSCs in patients with chronic kidney diseases, such as type 1 diabetes and interstitial nephritis (45). The participants in the study reported no significant adverse effects during or after the treatment. The use of exosomes derived from MSCs improved overall kidney function and inflammatory immune activity. Currently, tests involving the safety and tolerance of aerosol inhalation of exosomes derived from MSCs are part of a clinical trial comprising healthy volunteers (NCT04313647). Another clinical trial involving he investigation of the use of exosomes derived from MSCs as a therapeutic strategy is underway against macular holes (NCT03437759).

Clinical Trials Using Exosomes as a Potential Vaccine Against Coronavirus Disease (COVID-19)

More recently, due to the coronavirus pandemic, clinical trials for exosome-based therapy have shifted from cancer to COVID-19 treatment for future vaccine development (183). To this date, there are in total, 12 active clinical trials using exosome interventions at ClinicalTrials.gov. A phase I (NCT04747574) and a phase II (NCT04902183) independent clinical trials are recruiting patients with moderate or severe COVID-19 infection to evaluate the safety and efficacy of exosomes overexpressing CD24 of two doses with a patient follow-up for 23 days. CD24 is a costimulatory molecule expressed on several hematopoietic cells, especially progenitor cells, such as B cell progenitors (184). However, CD24 is also associated with autoimmune diseases (185, 186). Two phase I and II clinical trials are being conducted to investigate the safety and efficiency within 28 days after the first treatment of aerosol inhalation of bone marrow MSC-derived exosomes in severe patients hospitalized with SARS-CoV-2 pneumonia and COVID-19 (NCT04602442 and NCT04276987). And another phase I/II clinical trial (NCT04798716) is investigating the safety and efficiency of an intravenous infection of MSC-derived exosomes every other day on an escalating dose of 2:4:8 in the treatment of severe patients with COVID-19. According to these clinical trials description, MSC-derived exosomes may reduce lung inflammation and pathological impairment. Thus far, only one trial has reported results (NCT04491240), and no adverse events have been reported in patients after inhalation of 3 ml of MSC-derived exosomes twice a day for 10 days. However, there is no information about the source of MSCs used to generate exosomes and other relevant information concerning the aerosol formulation in this clinical trial. Additionally, another ongoing phase I/II clinical trial (NCT04389385) is investigating the safety and efficiency of inhaled exosomes derived from COVID-19 specific T cells that were activated and expanded *in vitro via* viral peptide exposure.

However, to this date, the clinical trials do not offer much information concerning the usage of exosomes to induce immunogenic properties and/or long-term memory response. The actual scenario of clinical trials using exosomes against COVID-19 is still evaluating safety and efficacy of exosome treatments. When completed, the ongoing clinical trials can provide the foundation for the conduction of future studies using MSC-derived exosomes in healthy patients. With their ability to elicit anti-inflammatory effects and modulate immune responses (187), MSC-derived exosomes may be important for the future design and development of COVID-19 vaccines.

Recently, a statement published by the ISEV and the International Society for Cell and Gene Therapy (ISCT) encouraged the conduction of further research and clinical trials using exosomes as a therapeutic strategy against COVID-19 (188). However, this statement also underscores the need for good clinical practice and rational clinical trial design.

CONCLUSION

Initially, EVs were considered to demonstrate the sole function of cellular waste elimination; however, EVs are now recognized as crucial mediators of intercellular communication because of their capacity to deliver different molecules and transfer signals over long distances to modulate several physiological mechanisms. The immunomodulatory properties of EVs provide insights into their use as a cell-free therapeutic strategy for different diseases. Several studies have reported promising results on EV-based vaccines against different types of diseases, including cancer and numerous infectious diseases. However, exosomes from cancer cells modulate many aspects of intercellular communication, which they can play a crucial role in tumor progression and suppress anti-tumor activities. Understanding the dual effects of exosomes represent a major challenge for future therapies using exosome-based vaccines. Clinical trials showed modest results, with no antigen-specific response induced by exosome vaccines, i.e., MHC I/II-restricted TAAs did not stimulated anti-tumor properties in effector T cells. Further studies are needed to understand the pharmacokinetic of exosome-based vaccines. On the other hand, clinical trials revealed the ability of exosome-based vaccines in recruitment and activation of innate immunity. Further investigation is warranted for the development of new techniques for loading EVs with specific antigens or drugs, and for engineering EVs to display more efficiency in cargo delivery. When in combination with other therapies, exosome-based vaccines are more promising, for example, different studies showed that PD1/PDL1 blocking therapy combined with DEXs resulted in effective T cell activation (189). However, difficulties such as lack of quality control and standards for EV characterization and purification must be overcome. Also, logistical issues, such as manufacturing, storage, and administration of exosome-based vaccines need to be addressed (190). Additionally, exosome-based vaccination encompasses various issues on exosome biocompatibility for broad clinical usage and for the establishment of large-scale immunization programs. There are several challenges, including the development of an effective cell-free vaccine platform to use exosomes for the treatment of various diseases. A focus on such

aspects and challenges is necessary for future exosome-based vaccine investigations.

AUTHOR CONTRIBUTIONS

FA conceived the idea. PS and FA designed, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Flood Control: How Milk-Derived Extracellular Vesicles Can Help to Improve the Intestinal Barrier Function and Break the Gut–Joint Axis in Rheumatoid Arthritis

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Many studies provided compelling evidence that extracellular vesicles (EVs) are involved in the regulation of the immune response, acting as both enhancers and dampeners of the immune system, depending on the source and type of vesicle. Research, including ours, has shown anti-inflammatory effects of milk-derived EVs, using human breast milk as well as bovine colostrum and store-bought pasteurized cow milk, in *in vitro* systems as well as therapeutically in animal models. Although it is not completely elucidated which proteins and miRNAs within the milk-derived EVs contribute to these immunosuppressive capacities, one proposed mechanism of action of the EVs is *via* the modulation of the crosstalk between the (intestinal) microbiome and their host health. There is increasing awareness that the gut plays an important role in many inflammatory diseases. Enhanced intestinal leakiness, dysbiosis of the gut microbiome, and bowel inflammation are not only associated with intestinal diseases like colitis and Crohn's disease, but also characteristic for systemic inflammatory diseases such as lupus, multiple sclerosis, and rheumatoid arthritis (RA). Strategies to target the gut, and especially its microbiome, are under investigation and hold a promise as a therapeutic intervention for these diseases. The use of milk-derived EVs, either as stand-alone drug or as a drug carrier, is often suggested in recent years. Several research groups have studied the tolerance and safety of using milk-derived EVs in animal models. Due to its composition, milk-derived EVs are highly biocompatible and have limited immunogenicity even cross species. Furthermore, it has been demonstrated that milk-derived EVs, when taken up in the gastro-intestinal tract, stay intact after absorption, indicating excellent stability. These characteristics make milk-derived EVs very suitable as drug carriers, but also by themselves, these EVs already have a substantial immunoregulatory function, and even without loading, these vesicles can act

as therapeutics. In this review, we will address the immunomodulating capacity of milk-derived EVs and discuss their potential as therapy for RA patients.

Review criteria: The search terms "extracellular vesicles", "exosomes", "microvesicles", "rheumatoid arthritis", "gut-joint axis", "milk", and "experimental arthritis" were used. English-language full text papers (published between 1980 and 2021) were identified from PubMed and Google Scholar databases. The reference list for each paper was further searched to identify additional relevant articles.

Keywords: rheumatoid arthritis, intestine, microbiome, immunomodulation, extracellular vesicles, bovine milk

INTRODUCTION

There is increasing awareness that the gut plays an important role in many inflammatory diseases. The intestinal epithelial cell layer is a selectively permeable barrier permitting the absorption of nutrients, but at the same time preventing the entry of microorganisms (gut flora/microbiome) (**Box 1**). The gut also has an active immune surveillance system to actually cope with these microbes and is the largest immune organ of the body (11). Enhanced gut leakiness, dysbiosis, and intestinal inflammation are associated with the pathogenesis of many inflammatory and autoimmune diseases, such as Crohn's disease and rheumatoid arthritis (RA) (12–14). Patients with these diseases also frequently report enhanced disease activity after food intake (15). The relationship between food intake and enhanced disease activity is further supported by antibodies against food components in the blood of these patients (15).

The pathogenesis of most autoimmune diseases is poorly understood, but environmental factors, including the microbiome, and genetic background are known to play a role in the development of these disorders (16). Autoimmunity is breaking self-tolerance and one of the proposed mechanisms is epitope mimicry, a cross reactive immune recognition of self and viral or bacterial epitopes (17). Some bacteria are capable of post-translational modification of body's own proteins by citrullination creating altered self-epitopes (18). Citrullination is catalyzed by host's own but also bacterial peptidylarginine deiminase (PAD) enzymes (18). There is compelling preclinical evidence that the gut microbiome is causally related to this break in self-tolerance and clinically a leaky gut is linked with a higher risk of autoimmune diseases (12). The microbiome consists of all living microorganisms of a defined region, such as the gastro-intestinal tract. Multiple lines of evidence support the potential pathogenic role of microbial gut dysbiosis in inflammatory disorders of the intestine, but also in autoimmune disorders such as RA, indicating an important role for the gut–joint axis in the development of this disease (19). For instance, in experimental arthritis, RA disease is strongly attenuated in germ-free (GF) mice compared to conventionally colonized mice, as was also reported for experimental autoimmune encephalomyelitis (20, 21). Both systemic and intestinal T-helper 17 (Th17) cell differentiation was strongly reduced in these GF mice (20–22), indicating an important role of the microbiome in breaking immune tolerance. Also, targeting intestinal barrier dysfunction before arthritis onset attenuates development of collagen-induced arthritis (23). This makes the gut and its microbiota promising targets for drug- and dietary intervention (24). A way of doing this is to optimize the micromilieu for hosting favorable microorganisms and at the same time increase the barrier function and direct the immune surveillance to target the putative pathogens and prevent their entry. In this sense, antibiotics are like a sledgehammer, and although promising results are obtained in animal models (25, 26), the use of antibiotics is also linked to microbiome dysbiosis and consequently the development of autoimmune disease. Probiotics and prebiotics to modulate the microbiome and thereby the gut–joint axis are currently under investigation (27); also, immune-regulatory components from food are promising options. Milk is a complex biological fluid with unique bioactive components that influence gut immunity, intestinal flora, and growth and development of infants (28, 29). Breastfeeding is associated with a decreased risk of asthma and allergic disease during childhood [reviewed in (30)]. However, a protective effect of breastfeeding against atopy, eczema, and food allergies is not convincingly proven yet (30, 31). On the other hand, several studies indicate a protective effect of raw cow milk consumption early in life against the development of asthma and respiratory tract infections during childhood (32–37). However, in some studies, the effects were not always independent of other farm-related exposures, *e.g.* exposure to straw, silage, or cows (32, 33, 36). The underlying mechanisms for this protection are therefore not always clear, but a potential contributor could be extracellular vesicles found in milk. Many proteins present in milk, such as lactoferrin, lactadherin, and immunoglobulins, are implied in mediating these effects.

Compared to milk protein, fat, and hormones, milk-derived exosomes or extracellular vesicles (mEVs) are less frequently studied components of milk (**Box 2**). Our lab has been on the forefront of researching the functional effects of milk EVs on bone and joint-related diseases. Our initial study revealed that milk-derived EVs could attenuate experimental arthritis in mice (14). Oral gavage with milk EVs, or milk EVs in the drinking

Abbreviations: EV, extracellular vesicle; RA, rheumatoid arthritis, GF, germ-free; TLR, toll-like receptor; MSC, mesenchymal stem cell; IEC, intestinal epithelial cell; NEC, necrotizing enterocolitis; SEC, size-exclusion chromatography; MVE, multivesicular endosome.

BOX 1 | Gut microbiome and gastro-intestinal function.
The gut microbiome consists of bacteria, bacteriophages, yeasts, protozoa, and viruses and can be seen as an external organ. The biggest component of the gut microbiome are bacteria. Colonization with gut microbes starts after birth and depends on many external factors, such as the delivery mode, type of feeding (breast versus formula), maternal factors, and other early life exposures such as infections or use of antibiotics (1, 2). The gut microbiota in early life is important for the maturation of the immune system, and it produces vitamins, minerals, and energy from our diet (3). During childhood, a complex relation between the host and its microbiome develops that stabilizes over time (4, 5). The developed host–microbiome symbiosis is essential for health throughout life. After coevolution of the immune system with the microbiome, keeping the balance is of utmost importance to sustain health. Microbiome disruptions can therefore lead to changes in barrier function and immune responses that contribute to disease development or progression (6). In this respect, a highly diverse microbiome is considered healthy, as it helps to free essential nutrients and energy, helps detoxification of toxic substances such as primary bile acids, and provides colonization resistance against pathogens. Contrarily, a low diversity is linked to microbial dysbiosis and associated with many diseases, including autoimmune disorders such as RA (7, 8). However, there is still debate whether higher diversity is always a good thing (9). Keeping a balanced microbiome is therefore essential for the function of our gut and maintaining health. A diverse diet rich in fibers, polyphenols, and fermented food helps to maintain a healthy microbiome that provides short-chain fatty acids and essential vitamins that are important energy sources for the gut epithelium (10). It is clear that a delicate balance between the host and its microbiome exists that reflects our health and is influenced by many external factors of which lifestyle is the most important.

BOX 2 | Milk processing and milk EV characteristics.
Bovine milk is part of the human diet. Next to the main milk proteins, *i.e.* caseins and whey proteins, milk contains 3.5% fat present in the milk fat globules, and milk EVs as one of the minor milk components. The structure of milk EVs differs from milk fat globules in the fact that they are membrane vesicles that are structured in a bilayered cell membrane, while the fat globules are surrounded by a trilayered membrane. Milk EVs can be characterized by their size, density, and surface markers like flotillin 1 and tetraspanins CD9 and CD81 (38).

Milk EVs can survive digestion (39, 40), allowing the functional transfer of the bovine milk EVs (including membrane components or EV content) into the human body after consumption (41, 42). However, because raw milk is not sterile and may contain pathogens, processing of milk by heat treatment is required to make bovine milk safe for human consumption. There are several heating methods, from which pasteurization and ultra-heat treatment (UHT) are the processes that are applied most frequently. These processing steps can impact the biological activity of the milk EVs. Pasteurization conditions result in preservation of the milk EVs to a large extent, while UHT is detrimental for the milk EVs and its miRNA (43–46). During milk processing, homogenization is also performed to stabilize the milk fat globules in a uniform way in the milk by decreasing their size. Part of the milk fat globules after homogenization have similar sizes as the milk EVs, and are therefore difficult to differentiate from EVs on the basis of size alone. More pure EVs can be isolated with sucrose gradient centrifugation; however, for the scalability of the milk EVs, this is not the best method (47). To remove protein content and thereby create more pure EVs, acidification is also an option (48, 49).

water of mice resulted in reduced severity of experimental arthritis in two different animal models (14). IL1rn−/− mice developed spontaneous arthritis associated with loss of intestinal microbial diversity and specific taxonomic alterations in the microbiota (50). Furthermore, arthritis in these IL1rn−/− mice was diminished under germ-free conditions and was shown to be dependent on the activation of toll-like receptor 4 (TLR4) and subsequent enhanced Th17 differentiation (22). Interestingly, these mice showed reduced cartilage proteoglycan depletion and bone marrow cellularity after treatment with mEVs by oral gavage. Similarly, in a collagen-induced model for arthritis, where one week before immunization with collagen the mice received milk EVs *via* drinking water, the mEV-treated group showed less severe arthritis. This was accompanied by reduced inflammatory markers in the serum (MCP-1 and IL-6), as well as lower Tbet (Th1) and RORyT (Th17) expression in splenocytes, suggesting reduced T cell activation (14).

In this review, we summarize and discuss the current knowledge on the therapeutic potential of bovine milk EVs in inflammatory disorders, in particular in the context of the gut–joint axis in RA.

EXTRACELLULAR VESICLES

EVs is the collective term for vesicles secreted by a variety of cells throughout the body and can be found in all body fluids, such as blood, urine, synovial fluid, and milk (51) (**Box 3**). EVs are small cell membrane-derived phospholipid bilayer structures that range in size from 30 to 2,000 nm in diameter (60). Previously, they were considered to be cellular waste products, but compelling evidence has indicated that EVs transport their cargo, consisting of bioactive proteins, enzymes and lipids, and deliver them to recipient cells. This makes EVs important mediators in cell–cell communication.

Milk is a rich source of EVs, and EVs obtained from human breast milk as well as from raw and pasteurized cow milk have been characterized in great detail, including their microRNA and

BOX 3 | Biogenesis of EVs.
Extracellular vesicle is the collective term for vesicles secreted by a variety of cells throughout the body. This heterogeneous population of vesicles is found in body fluids, such as plasma, urine, synovial fluid, milks, saliva, and cerebrospinal fluid (52). A distinction can be made between three different subtypes of vesicles: microvesicles (MVs), apoptotic bodies, and exosomes (53). The nomenclature of these vesicles is still under debate, and ongoing efforts are made to better distinguish vesicle subtypes [see positional paper ISEV (54)]. Within this review, we will use terminology from the original papers. MV size varies from 50 to 1,000 nm (55), making them overlap slightly with exosomes which are 30–150 nm in diameter. Apoptotic bodies are the largest vesicles, ranging from 500 to 2,000 nm. MVs and apoptotic vesicles arise through direct outward budding and fission of the plasma membrane, a process also known as vesicle shedding (56), and by blebbing of the cell membrane during apoptosis (57), respectively. Exosomes, on the other hand, derive from the multivesicular endosome (MVE). The generation of MVEs involves the lateral segregation of cargo at the membrane of an endosome, followed by inward budding and release of vesicles into the endosomal lumen (58). A comprehensive review on the cell biology of EVs was recently published by van Niel et al. (59).

protein cargo (48, 61). A large part of highly abundant microRNAs in milk-derived EVs are evolutionary conserved and are present in milk of all mammals (62). Numerous microRNAs have been identified in milk-derived EVs, of which a large number have been described as having an immune-modulatory function. In **Table 1**, a list of these commonly identified microRNAs can be found.

Milk-derived EVs have a particularly resilient lipid bilayer membrane, which serves to protect miRNAs from degradation caused by low pH and rich enzymatic environments, as seen in the gastro-intestinal tract. Minimal loss of RNA was observed after exposing milk EVs to digestive juices such as saliva and gastric, pancreatic, and bile juice (39). Also, there are some studies showing that miRNA from milk EVs can be found in blood and organs from humans and mice (41, 76). Additionally, using the *in vitro* TNO intestinal model-1, representing the gastro-intestinal tract from stomach to small intestine, it was shown that 2 h of 'digestion' resulted only in a minor loss of the abundant miR-223 and miR-125b (40). These findings indicate that mEVs can reach the small intestine without losing their integrity. Besides their resilience to low pH and enzymatic degradation, milk EVs can also withstand high temperatures, as milk EVs isolated from store-bought pasteurized milk are still bioactive (77). We will further discuss the bioactivity and effects of milk EVs on various cell types below.

IMMUNOMODULATORY PROPERTIES OF EXTRACELLULAR VESICLES

Milk EVs, and EVs in general, have interesting immunomodulatory properties. Many studies have shown involvement of EVs in the regulation of the immune response, acting as both enhancers and dampeners of the immune system, depending on the source and type of vesicle and the receiving cell type. Immunosuppressive EVs are naturally present in the body, including T cell-derived EVs, which have been shown to downregulate antigen presentation by antigen-presenting cells (78). Additionally, stem cell-derived EVs are vastly investigated for their immune-modulatory properties [reviewed in (79)] Both embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) are producers of EVs with strong immunosuppressive capacities, similar to that found using stem cells as therapeutics themselves. Finally, research, including our own at the Radboudumc, has shown the anti-inflammatory effects of milk-derived EVs, using human breast milk as well as bovine colostrum and store-bought pasteurized milk. Although it is not completely elucidated which factors within the EVs contribute to these immunosuppressive capacities, a number of proteins and miRNAs are likely candidates.

Despite their immunosuppressive role, in many diseases EVs have been found to enhance inflammation as well (80). For example, EVs derived from synovial fluid of RA patients contain high levels of TNFα and have been shown to delay activated T cell-mediated cell death, possibly contributing to the pathogenesis in RA (81). Similarly, sarcoidosis patients have EVs in their bronchoalveolar fluid, which show pro-inflammatory properties (82). Macrophage-derived EVs can also carry alarmins and contribute to bone homeostasis (83). It is noteworthy that the membrane receptor composition, cellular metabolism, and role in the disease process of the recipient cell may also determine the net outcome of the EV response.

T CELL ACTIVATION AND DIFFERENTIATION BY EXTRACELLULAR VESICLES

Activated CD4+ T cells are found in inflammatory infiltrates of the rheumatoid synovium (84), and the hallmark cytokine for Th17 cells, IL-17, is spontaneously produced in synovial explant cultures of RA donors (85). In experimental animal models for RA, such as collagen-induced arthritis and adjuvant arthritis, the disease can be transferred by autoreactive T cells (86). Collagen-induced arthritis is clearly attenuated in IL-17 deficient mice

TABLE 1 | Commonly identified microRNAs in milk-derived EVs.

MicroRNA present in bovine milk EVs	Expected function
Let7	Protection against bacterial infection (63)
miR-21	Linked to regulation of TLR signaling (64) Clearance of apoptotic cells (65) Clearance of bacterial infection (63)
miR-146	Linked to regulation of TLR signaling (66) Clearance of bacterial infection (63)
miR-148	Inhibition of demethylation Foxp3 (43, 67) Suppression of TGFβ signaling *via* SMAD (68) Regulation of DNMT1 and DNMT3, epigenetic homeostasis of DNA methylation (69)
miR-155	Anti-inflammatory effects (70) Regulation of TLR signaling (66) Induction of Tregs (71)
miR-181	Anti-inflammatory effects (72) NFkB signaling (73)
miR-223	Linked to infection and inflammation (74) Eosinophil function (75)

(87), and in IL1rn-deficient mice, spontaneous arthritis is completely prevented in the absence of IL-17 (88). Another important cytokine in the pathophysiology of RA and key in Th17 differentiation is IL-23, which is detectable in RA synovial joints (89, 90). In patients with RA, the Th17 and regulatory T cell (Treg) balance is skewed in favor of Th17 development, contributing to a break in tolerance and autoimmunity (91).

A strong candidate to modulate T cell function, especially Th17 and Treg cells, is transforming growth factor-beta (TGFβ). TGFβ has been found on the surface of EVs from a number of different origins, including mast cells (92), tumor cells (93, 94), but also milk-derived EVs (77) and intestinal epithelial cells (IECs) (95). Most notable is a study by Cai et al. who used TGF-β1 gene-modified dendritic cells (DCs) to produce immunosuppressive EVs, which were able to attenuate inflammatory bowel disease *in vivo*. A significant prevention of weight loss, decreased disease activity scores, as well as reduced intestinal bleeding was observed after the administration of TGF-β1-EVs (96).

Ogino et al. speculate the underlying mechanism could be *via* the induction of Tregs, which are known to downregulate Th17 cells and thereby suppress colonic inflammation (97). Interestingly, milk EVs from both human (98) and bovine milk (14) have been shown to promote Treg differentiation. Admyre et al. (98) were among the first to show Treg differentiation induced by EVs isolated from colostrum and mature breast milk. Their functional analyses showed that milk EVs can inhibit anti-CD3-induced IL-2 and IFN-γ production by T cells and simultaneously increase the number of Treg cells *in vitro*. A potential link to the prevention of asthma by Tregs suppressing Th2 responses was later suggested (99). Additionally, Zonneveld et al. have recently reported that human milk EVs can directly inhibit CD4+ T helper cell activation without inducing tolerance (100). In experimental arthritis studies, our research group at the Radboudumc found circumstantial evidence for this effect on T cells, as mice treated with bovine mEVs showed a marked reduction in Tbet (Th1) and RORyT (Th17) expression in splenocytes. Although no changes were observed in the Treg subset *in vivo*, we were able to confirm that EVs from pasteurized bovine milk enhanced Treg differentiation *in vitro.* Further research is needed to elucidate if the route of EV administration, as well as the timing in the developing immune response, determines the net outcome of the EVs, as has been demonstrated for therapeutic viral vectors and stem cells (101).

MICROBIOME AND BARRIER FUNCTION IN RA

Several studies in RA patients and animal models showed that dysbiosis of the gut microbiota induces an inflammatory response and is associated with disease progression of RA (102). For instance, new onset rheumatoid arthritis (NORA) patients have enriched levels of *Prevotella copri* in their gut, and this correlates with enhanced susceptibility to RA (8). Interestingly, germ-free mice inoculated with *P. copri*-dominated fecal samples from RA patients developed arthritis in a Th17-dependent manner (103). Of great interest, our group showed that these alterations in intestinal microbiome may precede the development of arthritis, as our study showed that the intestinal microbiome undergoes marked changes in the preclinical phase of collagen-induced arthritis (26). It is also known that the intestinal barrier is changed before the onset of RA. Ileal mucosal biopsies from treatment-naïve NORA patients and active RA patients showed a reduced expression of tight junction proteins claudin-1 and occludin compared to healthy controls on mRNA level and histology (23). Also, increased levels of CD3+ T cells, macrophages, and B cells were found in the lamina propria of NORA patients (23). Unfortunately, RA patients are often treated with methotrexate, but this DMARD is known to increase intestinal permeability (104, 105). Interestingly, patients with RA successfully treated with DMARDs show partial restoration of eubiotic gut microbiome, suggesting a crucial role of microbiota in treatment efficacy (106).

MILK EVs PROMOTE GUT BARRIER INTEGRITY

In RA, the gut–joint axis is in part related to the observation of leaky guts in some of these patients as cause of the elevated levels of bacterial cell wall fragments as well as bacterial DNA in the joints of these patients (107–111). The mucosal barrier is an important line of defense against invasion, infection, and bacterial dissemination. Underneath the epithelial cells lies the lamina propria, where T cells, macrophages, B cells, and plasma cells are present, and dendritic cells promote the differentiation of Th17 and Treg cells (112). The intestinal epithelial barrier prevents the entry of microbes into this lamina propria (112). Milk components have a protective effect on the intestine by improving its barrier function and microbiome diversity and limiting inflammatory processes. Milk EVs, from different species, show a similar tendency (113–115). Most milk EV studies focusing on barrier function study the functional effects on the epithelial cells, often using cell lines or animal models for necrotizing enterocolitis (NEC). Porcine milk EVs have been shown to promote cell proliferation of intestinal epithelial cells from newborn (unsuckled) piglets (IPEC-J2 cells), as well as, promote intestinal tract development *in vivo*, as shown by increased villus height, crypt depth, and higher expression of CDX2, PCNA, and IGF-1R (116). Similarly, milk EVs also promote epithelial cell growth, potentially *via* activation of the MAPK pathway (117). Additionally, milk EVs were able to protect mice from intestinal injuries caused by NEC (118). Reduced intestinal inflammation (myeloperoxidase expression) was observed, as well as an increase in goblet cell activity (MUC2+ and GRP94+ cells), highlighting the potential novel application of milk-derived EVs in the prevention of NEC development. Several studies using human milk EVs show comparable results. Martin et al. found that human breast milk-derived EVs had a protective effect on intestinal epithelial cells, reducing oxidative stress-induced cell apoptosis (induced by H_20_2) (119). The factors from EVs that

promote the intestinal barrier function have not been identified, but the expression of *e.g.* polymeric immunoglobulin receptor on EVs could be of importance. This receptor mediates the transcytosis of dimeric IgA and polymeric IgM through the intestinal epithelial layer and by this, protects against bacterial overgrowth and invasion causing leakage. Interestingly, two cow milk EV subsets [isolated by ultracentrifugation 35,000 g (P35K) or isolated at 100,000 g (P100K)] were administered orally by gavage to healthy and DSS (dextran sodium sulfate)-treated mice. P35K EVs and P100K EVs (to a lesser extent) improved several outcomes associated with DSS-induced colitis; they restored intestinal impermeability, replenished mucin secretion, and modulated the gut microbiota (13).

THERAPEUTIC USE OF MILK EVs

The use of milk EVs, either as stand-alone drug, drug carrier, or functional dietary component, is often suggested in recent years. Several research groups have studied the tolerance and safety of milk-derived EVs in animal models, administered either intravenously or by oral gavage, and the consensus is that they are well tolerated with no significant changes or slightly induced cytokine levels systemically (48, 76). Due to its composition, milk-derived EVs are highly biocompatible and have enhanced stability and limited immunogenicity, which gives them many advantages over traditional synthetic delivery vehicles, such as liposomes, indicating that they might be well tolerated. Furthermore, it has been demonstrated that milk-derived EVs are taken up in the gastro-intestinal tract after oral delivery *via* the neonatal Fc receptor, and they stay intact after absorption (120). This receptor mediates bidirectional transcytosis of IgG in epithelial cells and rescues albumin from intracellular degradation, thereby increasing plasma half-lives of these proteins.

As previously mentioned, milk-derived EVs have two important characteristics that make them very suitable as drug carriers; first of all, their lipid bilayer functions as a protective shell for drugs inside, and second, the efficient uptake of EVs results in improved bioavailability (**Box 4**) of the drug. Among one of the first studies is a large study undertaken by the group of Gupta, who developed a scalable isolation method for bulk production of milk-derived EVs that can act as carriers for chemotherapeutic agents (76). They used a number of different chemotherapeutics and chemoprotective compounds, including withaferin A, to test loading efficiency which varied between 10 and 40% depending on the agent. After confirming tumor growth inhibition by drug-loaded EVs *in vitro*, they compared efficacy of drug-loaded EVs to free drug in a long tumor xenograft model *in vivo* and found a significantly greater tumor inhibitory effect with drug-loaded EVs (76). A follow-up study, this time using paclitaxel-loaded EVs, demonstrated oral delivery also resulted in significant tumor growth inhibition in a tumor xenograft model (124). Additionally, the study confirmed the stability of paclitaxel-loaded EVs for storage up to four weeks at −80°C (124). Milk-derived vesicles have also been used as a novel delivery system for small interfering RNA (siRNA) in a therapeutic application against cancer (125, 126). Furthermore, when encapsulated in milk EVs, curcumin showed increased stability, solubility, and bioavailability (127). Of note, as discussed in the previous paragraphs, milk-derived EVs themselves already have a substantial immunoregulatory function, and even without loading, these vesicles can act as therapeutics. Additionally, the characterization of EVs to monitor potential differences is very important, and this is still a field of ongoing research.

FUTURE RESEARCH

There is increasing awareness that the gut plays a vital role in our overall health. The gut represents the largest surface area being exposed to our environment and is also the largest immune organ in our body. An enhanced intestinal leakiness, dysbiosis of the gut microbiome, and bowel inflammation are not only associated with diseases of the gut such as colitis and Crohn's disease, but are also characteristic of many other systemic inflammatory diseases such as lupus, multiple sclerosis, psoriatic arthritis, systemic sclerosis, and RA (128–131). Strategies to target the gut, and especially its microbiome, using pro- and prebiotics (27) are under investigation and hold a promise as a therapeutic intervention for these diseases.

We hypothesize that milk-derived EVs could be a potential therapeutic strategy (**Figure 1**) in modulating the gut–joint axis in RA. Since the net effect of the total dairy matrix on human health is dependent on the health status of the individual, the product type of dairy, and individual preferences towards dairy products, several aspects need to be considered before such application could be implemented. The isolation of pure extracellular vesicles without other milk constituents like fat globules, milk proteins, lactose, and feed-derived milk components, would provide a widely applicable format of

BOX 4 | Bioavailability and safety of milk EVs.

Research has shown that milk EVs are easily taken up by several different cell types. Intestinal cells are particularly quick to take up milk EVs when exposed. Wolf et al. (121) showed that both Caco-2 and IEC-6, intestinal cell lines, are able to take up milk EVs as fast as within 15 min. Intestinal uptake of EVs is likely *via* receptor-mediated endocytosis by intestinal epithelial cells (transcellular transport) or paracellular transport *via* tight junctions. Interestingly, not all cells can take up milk EVs; for example undifferentiated THP-1 cells (monocytes) do not show uptake, whereas their differentiated counterpart (macrophages) do take up EVs (122), indicating there is a cell type or cell differentiation state specific mechanism at work. Besides *in vitro* uptake, several animal studies have shown uptake and biodistribution of milk EVs in mice (48, 123). Both oral intake and intravenous injection (i.v.) resulted in peak uptake in the liver and spleen of mice, after 24 and 3 h, respectively. Interestingly, miRNAs transfected into the milk EVs were found in several organs 6 and 12 h after oral gavage (123), confirming uptake *in vivo*. In the intestine, EVs could exert other additional effects due to their ability to spread, cross the mucus layer, and directly migrate to other tissues and/or interact with different cells of the immune system of the host. In healthy animals, the biocompatibility and safety have been tested, and extensive analysis confirmed that there were no systemic changes upon i.v. injection of milk EVs into mice (48). Blood levels of markers for liver damage (aspartate transaminase, alanine transaminase, and total bilirubin), kidney damage (blood urea nitrogen and creatinine) and hematological parameters were all unchanged (48).

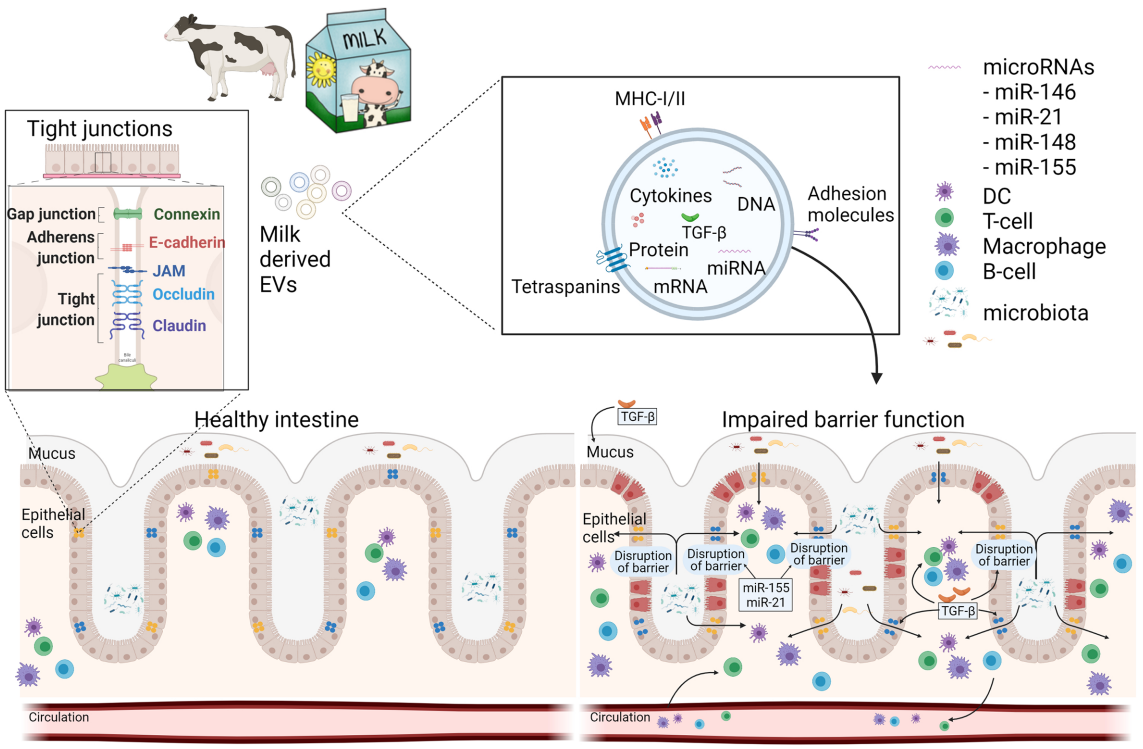

FIGURE 1 | Schematic overview how milk EVs can modulate intestinal barrier function and immunity. A healthy intestine has an intact barrier of various intestinal cells and mucus. Milk-derived EVs may contribute to the restoration of an impaired barrier function during disease by increasing mucus production and expression of tight junctions *via* miRNAs and TGF-β. Furthermore, mEVs can act on immune cells, locally in the gut, or systemically *via* the circulation. This figure was in part created with BioRender and was licenced for use in publication (created with BioRender.com).

milk-derived EVs for therapeutic application. Pure mEVs would be preferred over more complete milk products, since lactose intolerance is prevalent in a large part of the world, and RA patients for example can have increased antibodies against food antigens including milk proteins of cows (132). The isolation procedure is important and should conform GMP guidelines.

One of the important aspects to tackle is the reproducibility of the efficacy of the milk EV product used. Another challenge is the translation of studies performed *in vitro* or in animals into humans.

More research is required to figure out what the active components of the milk-derived EVs are. Whether these are miRNAs, growth-factors, or other proteins, or a combination of these factors is important to understand. Whether further separation, based on size or content, into subpopulations of the heterogeneous population of EVs is required, needs attention. Another parameter determining the content of milk-EVs is the origin of milk, *i.e.* species (cow, camel, horse, goat or sheep), changes during lactation period, food intake, seasonal effects, and animal breeds used.

Additionally, we need to know if these vesicles are actively taken up *via* oral intake in humans and show similar effects to the mouse and *in vitro* models described here. Finally, standard practices for the isolation, especially on a larger scale, are required.

Over the years, many different isolation protocols have been developed for the isolation of milk-derived EVs. Each isolation protocol comes with its own strengths and pitfalls, which are

BOX 5 | Organoids.

In the human body, the intestinal epithelial layer is exposed to the microbiome. Although the microbiome is separated from the enterocytes by a mucus layer, bacterial-derived metabolites can penetrate this mucus layer and affect growth, differentiation, and intestinal health. To mimic these interactions *in vitro*, there are several factors to take into consideration. For instance, the intestine consists of different cell types such as, stem, Paneth, goblet, enteroendocrine cells, and enterocytes. The recent development of intestinal organoid cultures in 3D and 2D, however, allows the use of more sophisticated cultures with all cell types present.

A second hurdle to take, and maybe the most difficult one, is the difference in growth (conditions) between human cells and bacteria. When bacteria are co-cultured with human cells, they will rapidly overgrow the culture and kill the human intestinal cells within hours. In addition, human intestinal cells require high oxygen levels, whereas most intestinal bacteria grow anaerobic. One way to solve these problems is by micro-injecting bacteria into the lumen of organoids/spheroids (139). Williamson et al. injected human fecal microbiota and showed that even oxygen-sensitive anaerobic taxa are maintained for at least 96 h. However, when longer studies are required, the group of Donald E. Ingber has developed an anaerobic human intestine and microbiome-on-a-chip system (140). Although they used Caco2 cells and endothelial cells instead of organoids, they nicely demonstrated that it is possible to create an oxygen gradient that allows the growth of human intestinal cells combined with anaerobic bacteria. The next step would be to apply 2D grown human intestinal organoids, replacing Caco2 cells in this system.

nicely compared in a recent article by Maburutse et al. (133) Ultracentrifugation is the most used isolation method, either as a stand-alone procedure or in combination with further purification using density gradients, isoelectric precipitation, or size-exclusion chromatography (SEC). Several methods to lose the casein and whey proteins, followed by purification of the milk EV *via* ultracentrifugation, size exclusion chromatography, membrane affinity columns, or solid phase extraction have been reviewed (134–136). Which process is most applicable for upscaling, with the preservation of biological functionality of the milk EVs, needs to be validated.

Upfront milk testing and quality control will be an essential component in the milk processing and downstream EV isolation. Furthermore, the milk EV isolation methods that are used can influence the composition of the EV sample. As described by Provost, different subsets of milk EVs are present in commercial milk (137). They found that a milk EV subset, which pellets at low ultracentrifugation speeds, contains and protects the bulk of milk microRNAs from degradation. In addition, sample collection methods as well as storage conditions influence the quality of the EVs. Zonneveld et al. have shown that prolonged storage at 4°C and −80°C can lead to cell death which results in contamination of the EV population in human breast milk. Interestingly, the cow breed and even the diet of the cow can also influence the milk EV composition (137, 138). These are all important considerations in moving forward to establish a standardized, large-scale isolation protocol for milk EVs, ready to be used as potential therapeutics.

FUTURE PERSPECTIVE AND FINAL CONSIDERATION

Altogether, this review highlights the therapeutic potential of milk EVs to treat arthritis and inflammatory gut diseases. Once a suitable large-scale isolation method is established and it is confirmed that the vesicles retained their therapeutic potential *in vitro* and in mouse models of disease, we propose testing the bioavailability and safety in both human organoids (**Box 5**) and humans. It will not replace the current standards of care (DMARDs, biologicals) but will be a sophisticated supportive treatment by disrupting the pathogenic gut–joint axis.

AUTHOR CONTRIBUTIONS

JA and BP wrote the first draft of the manuscript and share first authorship. AF, AB, and JK wrote a part of the text boxes. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Non-Immune Cell-Derived Extracellular Vesicles in Allergy

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Extracellular vesicles (EVs), and especially exosomes, have been shown to mediate information exchange between distant cells; this process directly affects the biological characteristics and functionality of the recipient cell. As such, EVs significantly contribute to the shaping of immune responses in both physiology and disease states. While vesicles secreted by immune cells are often implicated in the allergic process, growing evidence indicates that EVs from non-immune cells, produced in the stroma or epithelia of the organs directly affected by inflammation may also play a significant role. In this review, we provide an overview of the mechanisms of allergy to which those EVs contribute, with a particular focus on small EVs (sEVs). Finally, we also give a clinical perspective regarding the utilization of the EV-mediated communication route for the benefit of allergic patients.

Keywords: extracellular vesicles, exosomes, cellular communication, immune responses, allergy, asthma, atopic dermatitis, allergic rhinitis

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INTRODUCTION

During evolution multicellular organisms developed diverse methods of communication including a direct cell-to-cell contact, which allows for receptor-ligand interactions as well as the release of active mediators providing intercellular information transfer between donor to recipient cells. These include both soluble molecules and extracellular vesicles (EVs) capable of travelling long distances within the body. EVs which comprise apoptotic bodies (AP; 100-5000 nm), ectosomes or shedding microvesicles (MV; 100–1000 nm), secreted mid-body remnants (sMB-Rs; 200-600 nm) and exosomes (50–150 nm) are a group of heterogeneous structures (1, 2) surrounded by a lipid bilayer. EVs are released from practically all cell types including epithelial cells, fibroblasts, mesenchymal cells, dendritic cells (DCs), B cells, T cells, mast cells and tumor cells, among others. The presence of EVs has also been shown in multiple body fluids, including saliva (3), plasma (4, 5), breast milk (6), urine (7), bronchoalveolar lavage (8, 9) and malignant effusions (10–12). The complete biological effects of EVs are not yet well understood, but it is known that MVs and exosomes can bind to cells through several mechanisms, including receptor-mediated endocytosis, direct fusion, phagocytosis, and caveolae- or clathrin-mediated endocytosis and transfer their content to the recipient cell (1, 13). It has also been shown that alveolar epithelial cells internalize MVs *via* fluid-phase endocytosis but not *via* the well-known receptor-mediated EV endocytosis (14); MV uptake has endocytic basis which is energy-consuming and requires

cytoskeletal rearrangement (15); receptor-mediated MV uptake has also been reported (16). Because of their morphological characteristics, exosomes are considered the EVs most pronouncely involved in the information exchange process. The uptake results in functional effects in recipients; hence EVs contribute to the complexity of communication stream between distant cells. Besides the size and density, EV heterogeneity also derives from their diverse cargo, making it arduous for researchers to determine their exact functions (17).

Given their ability to regulate physiological and pathological processes (18, 19) there is growing interest focused on the potential of EVs to serve as novel targets for the development of therapeutic and diagnostic strategies. The role of different EV subtypes largely depends on the type and activation state of a cell producing them (20). Exosomes have been found useful in diagnostics as possible biomarkers, e.g. in oncology and nephropathies (21, 22) and as novel therapeutic approach for treating various diseases, including those with a clear immunological pathomechanism, e.g. atopic dermatitis, asthma, arthritis (23–25). In those, EVs produced by the immune cells are the main focus of the EV field. However, multiple kinds of non-immune cells, often overlooked, have been shown as efficient EV sources; these are often significant contributors to the ongoing immune response. This review, therefore, discusses the role of non-immune cell-derived EVs in immune processes in allergy in contrast to the immune cell-derived EVs.

EXTRACELLULAR VESICLES: TYPES AND BIOGENESIS

EVs are most frequently categorized based on their biogenesis, and sub-grouped into three major types: exosomes, **microvesicles and apoptotic bodies** (**Figure 1**). Recently, a novel type of EVs, namely **secreted midbody remnants (sMB-Rs)** have also been described, along with yet another type of secreted nanoparticles, i.e. "exomeres". While the former appear to be membranous structures and are likely true vesicles, a debate on the latter is ongoing (due to the lack of consensus we did not include exomers in **Figure 1** and **Table 1**). The differences in the origin are directly reflected in the variations in the size, morphology, cargo and surface content of those EV populations (**Table 1**); however, they all likely play a role in cell-to-cell communication, transferring a variety of

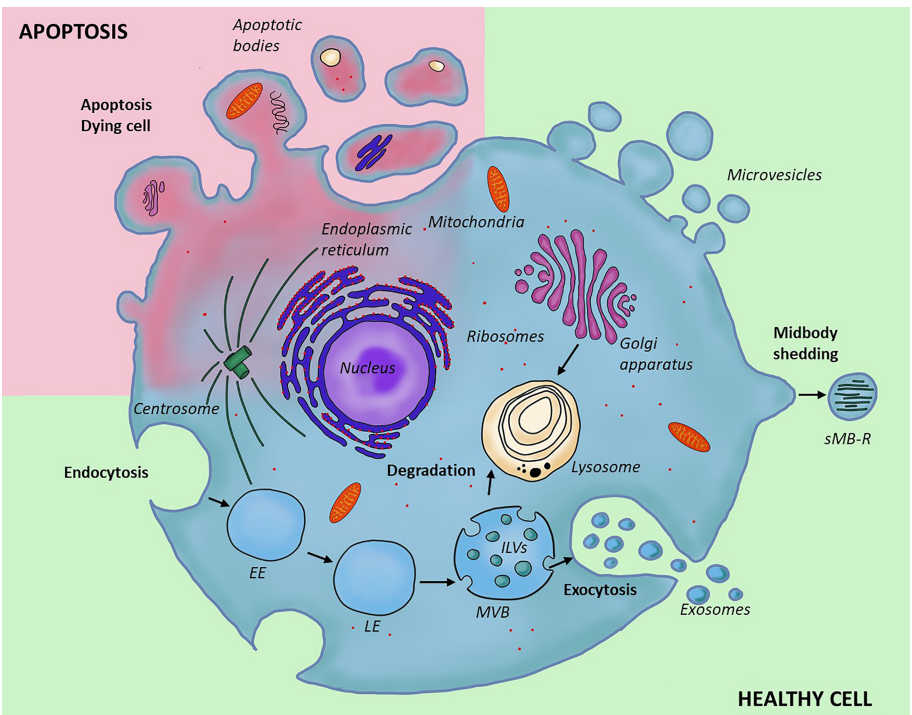

FIGURE 1 | Different types and biogenesis of extracellular vesicles. Two types of EVs form through outward invagination of the plasma membrane; microvesicles and apoptotic bodies. The apoptotic bodies are larger and form in the context of programmed cell death; they enclose organelles removed from the cell during degradation, while microvesicles are produced by a healthy cell; their content is similar to that of the cytoplasm. Secreted midbody remnant is also secreted from the plasma membrane, but contain residual secreted midbody remnants are following cell division. In contrast to this, exosomes form through a distinct cellular pathway and within the endocytic system where inward budding of late endosome leads to the formation of a multivesicular body containing multiple intraluminal vesicles. The content of multivesicular bodies is either digested after fusion with lysosome (degradative pathway) or released into the extracellular space (secretory pathway). EE, early endosome; LE, late endosome; MVB, multivesicular body; ILVs, intraluminal vesicles; sMB-R, secreted midbody remnant.

TABLE 1 | Common markers and cargo found in EVs.

EV type	Markers	EV Cargo
APs (100-5000 nm)	Phosphatidylserine (26)	DNA (30, 31)
	TSP (27)	RNA (32)
	C3b (28)	Peptides (31)
	Calreticulin (29)	Phospholipids (31)
		Annexin V (31)
		Lipids (33)
MVs (100-1000 nm)	Actinin-4 (34)	DNA (42)
	Integrins (35)	RNA (32, 43)
	Selectins (36)	Poteins (44)
	Flotillin-2 (37)	Receptors (45–48)
	CD40 ligand (36)	Lipids (49)
	Metalloproteinase (38)	
	ARF6 (39)	
	VCAMP3 (40)	
	KIF23 (41)	
sMB-Rs (200-600 nm)	KIF23 (2, 50, 51)	Proteins (2)
	Prominin-1 (52)	Centraspindlin (2)
Exosomes (50-150 nm)	CD81 (53)	Receptors (62, 63)
	CD82 (53)	Cytoplasmic proteins (64, 65)
	CD9 (54)	Tetraspanins (66)
	CD63 (55, 56)	DNA (67)
	Alix (54, 57)	RNA (68, 69)
	TSG101 (57)	Lipids (70)
	Flotillin-1 (58, 59)	MHC complex (71, 72)
	Syntenin (34)	Integrins (73)
	Hsp70 (60)	Cytoskeletal components (74)
	CD24 (61)	

biological molecules, i.e. proteins, lipids, nucleic acids and small molecular mediators (18, 19, 75–80) to the recipient cell.

Exosomes compose a population of small EVs (50-150 nm) (1). Due to their size and composition mainly consisting of lipids, these vesicles can squeeze between cells without damage and enter the circulation; this facilitates transfer of their cargo between cells at the longest distances (55, 81, 82). The exosomal wall composition reflects the biogenesis of those vesicles which have unique endocytic origin. Specifically, exosomes form at the level of late endosomes (LEs) which later progress into multivesicular bodies (MVBs) by accumulation of intraluminal vesicles (ILVs) generated through inward budding of the LE membrane (83). The formation of MVBs is mediated by two separate pathways; one involving a multimolecular machinery called *endosomal sorting complex required for transport* (ESCRT) and the other, dependent on a specific lipid composition of the endosomal membrane (84). ESCRT is a protein cascade consisting of approximately 30 proteins which are integrated into four subunits, namely ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III (83, 85, 86). The role of ESCRT-0 is to recognize and sequester ubiquitinated transmembrane proteins in the endosomal membrane which allows the ESCRT-I to bind to these ubiquitinated proteins and activate ESCRT-II to start oligomerization and generation of ESCRT-III. ESCRT-I and ESCRT-II complexes are implicated in the process of membrane deformation which leads to the membrane budding, and ESCRT-III components accomplish vesicle scission (1, 44, 87–89); the ESCRT pathway is ATP-dependent. To disassemble ESCRT subcomplexes from the endosomal membrane, the AAA (ATPases Associated with diverse cellular Activities); ATPase VPS4 (Vacuolar Protein Sorting 4), is required, which enzymatically accomplishes the membrane abscission (90–92). During MVB sorting an accessory factor, ALIX, is required for exosome secretion at the endosome to help sort membrane proteins into vesicles which later bud into MVBs (93, 94). Larios et al. have shown that ALIX- and ESCRT-III–dependent pathway promotes sorting and delivery of exosomal proteins (95). In contrast, the ESCRT-independent pathway relies on the process of converting membrane sphingolipids to ceramides by sphingomyelinase which is necessary for the inward budding and formation of ILVs (57, 96–98). Following the budding, MVBs which accumulate ILVs either fuse with the plasma membrane to release exosomes into the extracellular space *via* exocytosis (secretory pathway) or fuse with lysosomes and their content is digested by the lysosomal enzymes (degradative pathway) (99–101). The ESCRT-independent formation of ILVs in MVBs has been shown to be regulated by CD63 tetraspanin, which is particularly enriched intracellularly and is mostly localized in the endosomes and lysosomes, although in specialized cells it is also associated with lysosome-related organelles and their endosomal precursors (102, 103). Edgar et al. have shown that the formation of small ILVs requires CD63 (104).

Microvesicles (MVs) are vesicles generally larger than exosomes, with sizes in the 100-1000 nm range but some smaller MVs may be difficult to distinguish from exosomes purely based on the size. However, their biogenesis is completely unrelated; they originate through the processes of direct outward budding and fission of the plasma membrane into the extracellular space (105, 106); this explains why the MV surface markers largely depend on the composition of the plasma membrane (107). Based on the way of how the plasma membrane has emerged during the MV formation, MVs may contain various cell surface proteins, such as ARRDC1 (arrestin domain-containing protein 1) (108, 109), Bin-1 (ampiphysin) (110), EGFR (epidermal growth factor receptor), etc. (111). Released MVs may be taken up *via* receptor-mediated uptake (16, 112, 113) to transfer their cargo (surface receptors, lipids, proteins, mRNA, miRNA, infectious particles e.g. prions) to the target cells.

Apoptotic bodies (APs) are the largest subfraction of extracellular vesicles (100-5000 nm), formed and released when the cell undergoes programmed cell death, i.e. apoptosis (114, 115). Many changes occur to the cell during this process, including pronounced changes to the plasma membrane. Specifically, the blebbing generates various types of protrusions and APs form and may be released from those (116, 117). APs carry antigens and a variety of biomolecules, intracellular fragments, disrupted and degraded cellular organelles, membranes, released nucleic acids and cytosolic contents (75). APs have been shown to transfer their cargo and content

between various cells (30, 118). Interestingly, the communication with the immune cells specifically is commonly mediated by vesicle-associated cytokines or damage-associated molecular patterns (DAMPs) (119). This includes mitochondria-derived N-formylated peptides (120), the nuclear protein High Mobility Group Box 1 (HMGB1) (121), histones (122), calcium-binding S100 proteins (123), heat shock proteins (HSPs) (124), ATP (125), uric acid (126), DNA/RNA and actin among many others (127). This richness of the cargo is perhaps not surprising, given the context of the cell death resulting in AP formation. Immune cells recognize these molecules *via* pathogen recognition receptors (PRRs) and drive inflammatory responses (117, 128, 129). APs act locally and are removed from the extracellular environment during phagocytosis by macrophages (117, 130, 131).

Very recent developments in the EV field brought identification of two new types of nanoparticles, i.e. exomeres and secreted midbody remnants. Given their novel nature, these are not yet well described, both in relation to their structure and function, however certain aspects are already known which positions these nanoparticles in the interest of the EV field.

Secreted midbody remnants (sMB-Rs), with sizes in the 200-600 nm range have been described as particles generated during the cell division. Specifically, these are generated at the time when daughter cells are still connected with intercellular cytoplasmic bridge; this bridge is cut during the cytokinesis by a transient organelle called *midbody* which anchors SNARE and exocyst complexes (50, 132). As a consequence, one of the nascent cells retains these midbody remnants and discards them either by autophagy (133) or releases them in the form of secreted vesicles; sMB-Rs. It has been documented that these nanoparticles are distinct from exosomes and shed microvesicles (51, 52). While generated as a byproduct during the cell division, sMB-Rs may also convey messages when internalized, as shown for fibroblasts, in which sMB-Rs promote cellular transformation into an invasive phenotype (2).

Exomeres, with their size at the ≤50 nm mark are the smallest secreted nanoparticles described so far. They also have very distinct characteristics; of all, the lack of a limiting membrane is the most evident differential feature. Exomeres seem to be involved in cargo transport and have been shown to contain proteins, lipids and nucleic acids, which provide functional outcome by receiving cells. Currently, however there is a debate whether exomers should be classified as "vesicles" and the EV field is awaiting specific recommendations in this regard (134–136).

It should be noted that while distinct types of EVs can be described by their origin pathway, as well as a set of specific characteristics including the size, marker profile and cargo content, the technical caveats and lack of very unique markers available to unambiguously define every EV population, it is virtually impossible to specify the origin of the EVs, unless these are imaged during secretion. Therefore, following the recommendation of the International Society for Extracellular Vesicles (ISEV) for the purpose of this review we have used the terms "small" and medium/large EVs" (sEVs and m/lEVs) throughout instead of the original description published in the referenced papers unless the populations are very well defined according to the ISEV guidelines (137).

IMMUNE CELL-DERIVED EVs IN IMMUNITY

Recent progress in the EV field determined that thorough understanding of the EV biology and function is pivotal for our comprehension of immune-driven diseases, including the pathogenesis of allergy. Here, immune cell-derived EVs emerge as important contributors to immune responses, in both the innate and adaptive immunity arms and it may be useful to explore their potential as diagnostic and therapeutic tools. In the innate immunity pathways EVs provided by NK cells, macrophages and neutrophils mediate early host recognition and elimination of invading pathogens. In the adaptive arm, EVs are capable of activating B cells for antibody responses as well as providing both the direct and indirect antigen-specific stimulation to T cells. For the former, class I and class II MHC molecule-enriched EVs from antigen-pulsed DC are able to act as a display system for antigen presentation to cytotoxic and helper T cells (138). Moreover, it has been shown recently that the responses induced by exosomes (defined as tetraspanin and syntenin-positive sEVs) are by far superior in comparison to those obtained from MVs (distinguished as actinin-1-positive, syntenin-negative) (34), further highlighting the distinctive features resulting from the unique exosomal biogenesis pathway, encompassing the MHC-reach cellular compartments. As far as the indirect presentation is concerned, antigen or antigen/MHC complex transfer is also engaged, as well as cross-priming and cross-dressing presentation pathways. These topics have been extensively covered already in excellent publications (138–144). Hence, since the focus of this review is the EVs secreted by non-immune cells which are often overlooked but also extensively participate in immune responses, their contribution will be presented next.

NON-IMMUNE CELL-DERIVED EVs IN IMMUNITY

While not as potent, in some respects, as the EVs secreted from the immune cells, the EVs that are produced by the non-immune cell types have also been shown to exert many distinct roles in the immune system. These non-immune EV-mediated pathways include contribution to both innate and adaptive immunity, ranging from the activating to inhibitory roles (**Figure 2**). As with any cells, the relative impact depends on the type and the activation state of the donor cell, in parallel to the functionality observed at the cellular level. Next section will discuss the ways in which those non-immune cell-derived EVs participate in the mechanisms of the innate and adaptive immunity.

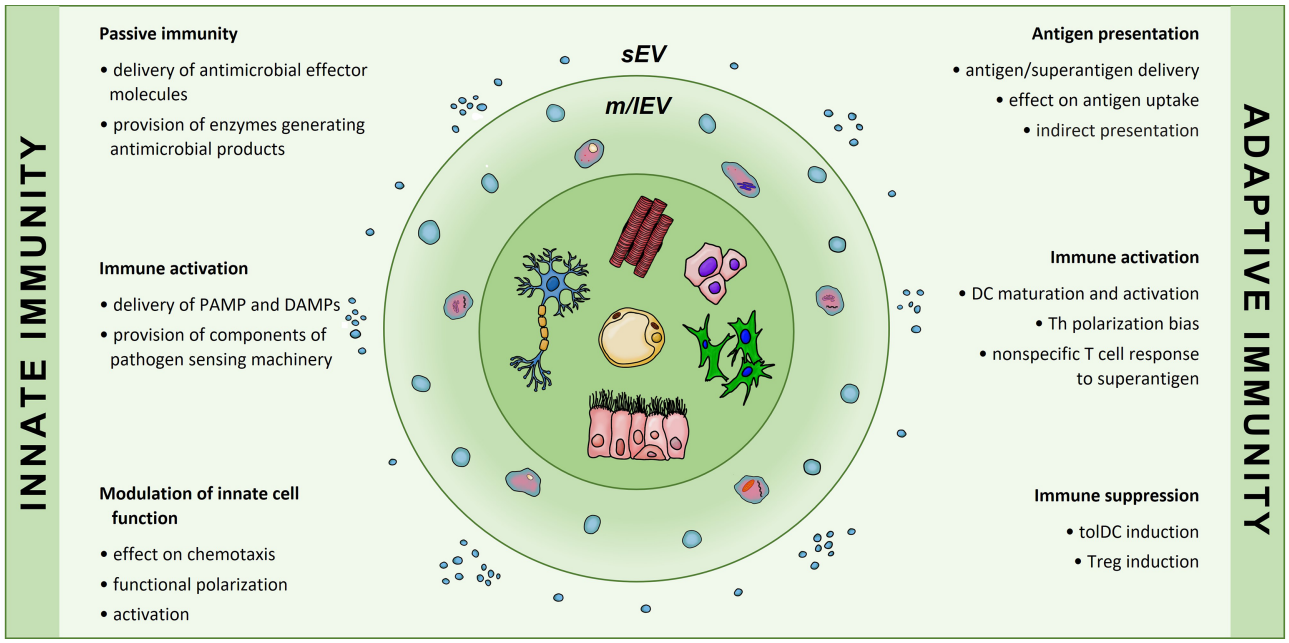

FIGURE 2 | Involvement of non-immune cell-secreted extracellular vesicles in immunological processes of innate and adaptive immunity. Extracellular vesicles produced by cells of non-immune origin participate in exchange of information that contributes to immune responses. In the innate arm EVs enable passive immunity and may both induce activation and modulate innate cell function. In the adaptive arm EVs may influence antigen presentation, affect dendritic cell differentiation and phenotype; they have also been implicated in T cell polarization into Th or Treg subsets. sEVs, small EVs; m/lEVs, medium/large EVs.

Non-Immune Cell-Derived EVs in Innate Immunity

EVs secreted by non-immune cells provide mechanisms of innate control and consist a link between innate immunity and allergic diseases (145). For example, Hu et al. have shown that the activation of the TLR4 signaling results in enhanced luminal sEV production and shuttling of the epithelial antimicrobial peptides (cathelicidin-37 and β-defensin 2) from the gastrointestinal epithelium (146). Nasal mucosa-derived sEVs were also shown to carry proteins involved in the innate immune responses, including inducible nitric oxide synthase (NOS2) which exerts antimicrobial function (147). Similarly to this, Nocera et al. have shown that the secretion of basal nasal mucosa-derived sEVs and the expression of exosomal NO is increased after TLR4-stimulation by lipopolysaccharide. Interestingly, mucosa-derived sEVs had microbiocidal properties and were capable of transferring their immunoprotective cargo to naive epithelial cells to confer passive immunity to recipient cells in the setting of chronic rhinosinusitis (148).

Non-immune cell-derived sEVs can also interfere with the NOD-dependent signaling. Specifically, Vaccari et al. have shown that the expression of the components of the nucleotide-binding-and-oligomerization domain (NOD)-like receptor protein-1 (NLRP-1) inflammasome are increased in the spinal cord motor neurons and cortical neurons after trauma. Interestingly, NLPR-1 inflammasome proteins were found in cerebrospinal fluid-derived sEVs after spinal cord injury and traumatic brain-injured patients. The authors have shown that sEVs derived from neurons loaded with short-interfering RNA against caspase recruitment domain (CARD) can deliver their cargo and reduce inflammasome activation following spinal cord injury in rodents (149). Following this, Li et al. have demonstrated the ability of hepatocyte-derived sEVs (expressing exosome-associated tetraspanins) to induce acute liver injury in severe heat stress by activating the NOD-like receptor signaling pathway in hepatocytes (150). This pathway seems to provide a link between visceral organs and the central nervous system (CNS) as shown in a hepatic ischemia-reperfusion injury model. Liver transplantation may result in neuronal injury and cognitive dysfunction (151); Zhang et al. have demonstrated that circulating sEVs play critical role in hippocampal and cortical injury through regulating neuronal pyroptosis in rats. The authors have shown that neuronal pyroptotic cell death may be caused by sEVs through TLR4 activation of NLRP3 inflammasome (152).

Exosomal transfer of pathogen recognition pathway components may convey the message to the immune cells, such as monocytes and macrophages. Specifically, Mills et al. have shown that poly(I:C) stimulation induces the release of tenascin C-rich sEV from airway epithelial cells; these may potentiate airway inflammation by promoting cytokine production in macrophages (153). Furthermore, airway epithelial cell-derived sEVs have been shown to induce proliferation and infiltration of undifferentiated macrophages into the lungs under the influence of IL-13 in a murine model (20). In contrast, mesenchymal stem cells (MSC)-derived sEVs

are capable of inhibiting macrophage chemotaxis (154), altering the M1/M2 balance (155), inhibiting M1 *via* miR-147 (156) and stimulating the M2 polarization in monocytes (157).

Non-Immune Cell Derived-EVs in Adaptive Immunity

Multiple studies indicate that non-immune cells, in the steady state, secrete EVs that execute immunoregulatory roles in adaptive immunity. For example, bone marrow MSC-derived EVs have been shown to suppress the Th2/Th17-mediated airway hyperresponsiveness and lung inflammation in a model of *Aspergillus hyphal* extract-induced allergic airway inflammation (158). Indeed, MSC-derived sEVs have shown immunosuppressive effects on several types of immune cells (159); including inhibition of B cell and DC proliferation (25), B cell maturation (160), and induction of T regulatory cells (Treg) (161–163). More specifically, Gomzikova et al. have demonstrated that MSC-derived EVs alter DC maturation and functional state (164); the antigen uptake by immature DCs was attenuated and the stimulation rendered DCs with a semi-mature phenotype after LPS exposure (165). These phenotypic changes were accompanied by a functional shift in the cytokine production profile from inflammatory to immunoregulatory (164), suggesting that those sEVs could promote tolerogenic DC (tolDC) induction. MSC-derived EVs have been also shown to reduce inflammatory cytokine (IL-23 and IL-22) production (166), enhancing the anti-inflammatory phenotype and regulatory lymphocyte proliferation, and the ability to produce IL-10 and TGF-β (167). Proliferation of T cells has also been shown to decrease after MSC-derived EV treatment *in vitro*, accompanied by a downregulation in IFN-γ and TNF-α (168). The study by Shigemoto-Kuroda et al. also confirmed that MSC-derived EVs have the ability to suppress Th1 and Th17 development, inhibit antigen presenting cell activation and increase expression of the immunosuppressive cytokine IL-10 (169). In a limited model, murine epidermal keratinocyte-derived sEVs (flotillin and Alix-positive) failed to induce T cell immune response despite some phenotypic effects on DC (170). However, when the donor cells are subjected to IFN-γ activation, keratinocyte-derived sEVs (of exosomal marker characteristics) may act as a transfer vehicle for T cell stimulation by *Staphylococcal aureus* enterotoxin B. Specifically, in this context HaCaT keratinocytes were shown to produce sEVs that contain MHC class I and class II and were able to drive nonspecific proliferation of $CD4^+$ and $CD8^+$ T cells *in vitro* (171). This suggests that the relative contribution of non-immune cell-derived sEVs (and potentially other EVs) to the adaptive immunity and T cell reactivity may change depending on the stimulation received by the donor cell; further evidence supports this (172).

Interesting are the results by Admyre et al. who have demonstrated that human breast milk contains sEVs which reveal immunomodulatory features inhibiting T cell cytokine production from PBMC and increasing the number of $Foxp3^+CD4^+CD25^+$ Tregs in this semi-allogenic system (173, 174). Based on the content of surface molecules, in comparison to the DC-derived sEVs, these sEVs originate from either macrophages and lymphocytes in the breast milk or rather breast epithelial cells (173, 175). To support this, Herwijnen et al. have also shown that human milk-derived EVs contain novel EV-associated bioactive proteins that have distinct functions from other milk proteins; this suggests a novel mechanism of cellular communication between the mother and newborn (176).

THE ROLE OF NON-IMMUNE CELL-DERIVED EVs IN ALLERGIC CONDITIONS

Many studies have been performed to investigate the involvement of non-immune cell-derived EVs content/cargo with different clinical manifestations of allergy; in this section current research regarding the role and function of non-immune cell-derived EVs in allergic conditions is reviewed. Certainly, for the outcome in allergic inflammation much depends on the source of EVs as summarized in **Figure 3**.

Asthma

EVs contribute to the asthma pathogenesis *via* various mechanisms, related to both inflammation and pathological remodeling (177) and there are interesting interdependencies that can be observed. Specifically, it has been shown that fibroblasts-derived EVs secreted by cells obtained from severe asthmatics increase proliferation of bronchial epithelial cells (HBECs) in comparison to those in healthy individuals, due to a decrease in the TGF-β2 content (178). Vice versa, vesicular transfer between epithelial cells and fibroblasts which includes inositol polyphosphate 4-phosphatase type I A (INPP4A) cargo, may regulate inflammation and airway remodeling (179). Further to that, Gupta et al. have shown that sEV transfer between airway epithelial cells (AECs) and human tracheobronchial cells (HTBEs) promotes expression several proteins which may contribute to allergic inflammation and exacerbation of asthma symptoms, i.e. gel-forming mucins (180), complement component C3, SERPIN3. The addition of an allergen source (house dust mite; HDM) to the AEC culture resulted in DC activation by secreted sEVs *in vitro* and increased airway inflammation in a murine model (181); the role for contactin-1 has been demonstrated. Furthermore, it has been also demonstrated that sEVs may be a vehicle of secretion for an important Th2-promoting cytokine, interleukin 33 (IL-33); the cytokine seams to decorate the EV surface rather than be included within the intraluminal cargo (182). At the same time, however, it has been also shown that CD83/OVA-carrying sEVs derived from those cells may promote Treg differentiation (183). Ax et al. have documented that HBECs increase the number of EVs released upon treatment mimicking asthma *milieu* which may contribute to establishing of the neutrophilic airway inflammation associated with Th17-driven asthma (184). Kulshreshtha et al. have shown that IL-13-treated epithelial cells secrete sEVs which stimulate proliferation and chemotaxis of monocytes; suppressing secretion of those sEVs in the lungs alleviates asthmatic inflammation in a murine model of bronchial asthma (20). Similarly, Lee et al. have shown that

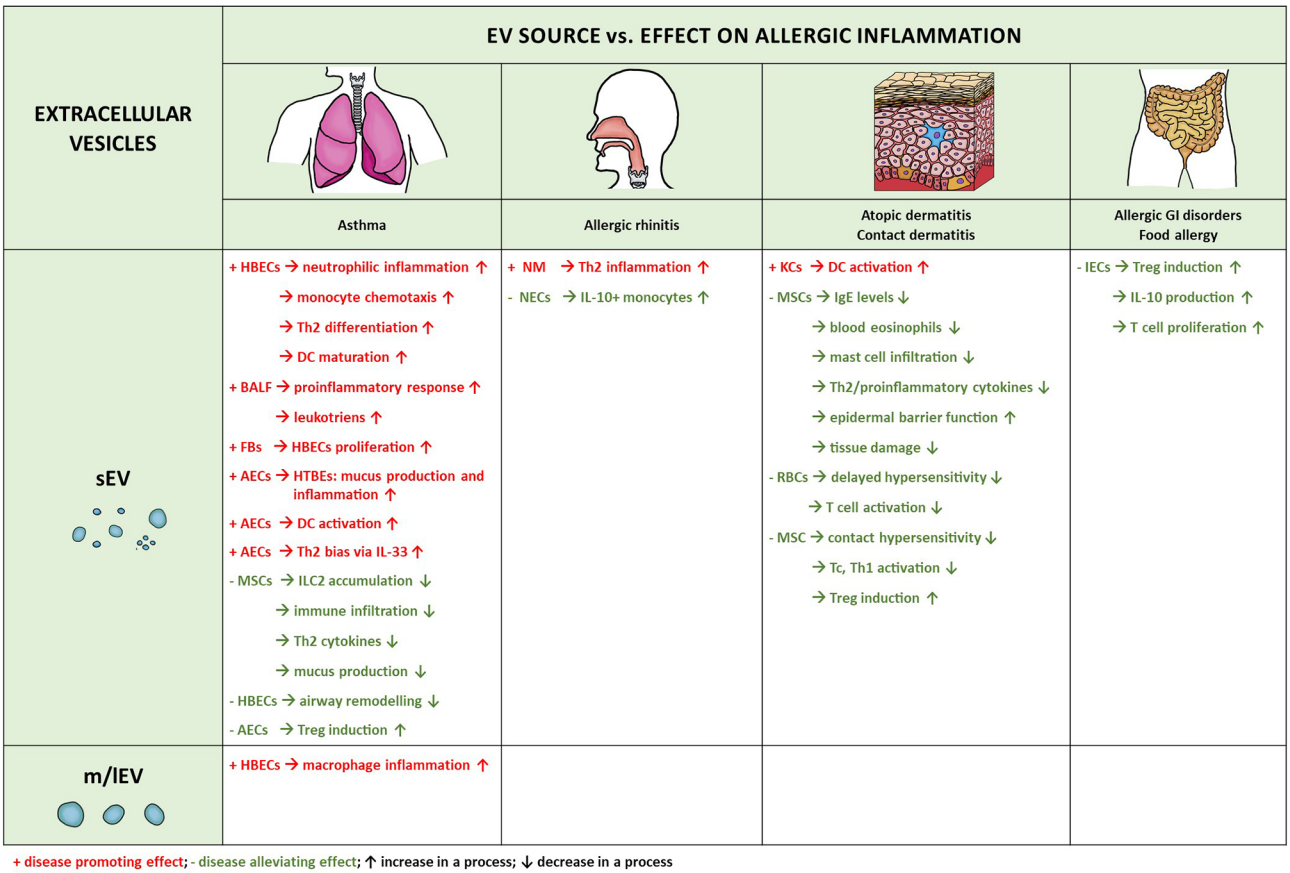

FIGURE 3 | Extracellular vesicles produced by non-immune cells and their involvement in allergic diseases. Microvesicles and exosomes are the two types of extracellular vesicles which have been implicated in the pathogenesis of allergic inflammation. There is significant predominance of the exosomal involvement, likely due to the phenotypic characteristics and physical properties of these vesicles, enabling more without damage and entering the circulation for long-distance delivery. HBECs, human bronchial epithelial cells; BALF, bronchoalveolar lavage fluid; NM, nasal mucus; NECs, nasal epithelial cells; AECs, airway epithelial cells; HTBEs, human tracheobronchial cells; RBCs, red blood cells; IECs, intestinal epithelial cells; KCs, keratinocytes; FBs, fibroblasts; MSCs, mesenchymal stem cells. ↑ increase in a process; ↓ decrease in a process; + disease promoting effect; - disease alleviating effect.

HBEC-derived m/lEVs may promote macrophage-mediated inflammation upon hyperoxia-mediated lung injury *via* miR-221 and/or miR-320a (185). Moreover, three additional miRNAs, i.e. miR-92b, miR-34a and miR-210 found in the sEVs secreted by HBECs have been suggested to have possible roles in regulating Th2 differentiation and DCs maturation in asthma, indicating that airway epithelial miRNA secretion *via* sEVs might be even more implicated in the development of the disease (186). In agreement with this, bronchoalveolar lavage fluid (BALF)-derived EVs isolated from LPS-treated mice drive a mixed Th1/Th17 cell response and enhance production of the Th1/Th17-polarizing cytokines (IL-12p70 and IL-6) by lung DCs in an asthmatic mouse model but are more tolerogenic if the animals are devoid of the LPS stimulation (70). Specifically, Paredes et al. have shown that asthmatic BALF-derived sEVs which carry tetraspanins and MHC class II molecules might reflect increased levels of antigen-presenting capacity and suggest that these sEVs might contribute to the inflammation by increasing cytokine and leukotriene production in AECs (187). Asthmatic patients also have altered sEV proteomic characteristics and eicosanoid profile which is shown to exert pro-inflammatory functions *in vitro*. Specifically, Hough et al. have shown that BALF-derived EVs contain lipids, such as ceramides, sphingosines, prostaglandins and leukotrienes which have been previously identified to drive inflammation in asthma (188). In asthmatic conditions, BALF-derived EVs also exhibit particular miRNA profiles (189) and carry the biosynthetic machinery for the leukotriene biosynthesis pathway (187, 188). In agreement with this, in a human study, EVs isolated from the nasal secretions of children with asthma and chronic rhinitis promoted trafficking of primary monocytes, NK cells and neutrophils thanks to the changes in the exosomal proteome contributing to the alterations in the immune-related functions (147). These effects can be contrasted with a healthy lungs, as demonstrated in an animal model by Wan et al., who have shown that EVs isolated from the lungs of healthy mice contain immunosuppressive cytokines TGF-β1 and IL-10 which inhibit T helper cell proliferation and relieve asthmatic

symptoms in mice (190). The immunomodulatory effect of EVs was also demonstrated by Prado et al. who have shown that intranasal administration of sEVs isolated from BALF of mice tolerized against major pollen allergen in the murine airway inflammation model (Ole e1) induces tolerance and protects naïve mice against allergic sensitization (191).

Finally, innate lymphoid cells type 2 (ILC2s) which are large contributors to the Th2-dominated allergic inflammation in the airways (192) can also be targeted by EV-mediated suppression. Specifically, systemic administration of MSC-derived sEVs resulted in the reduction in the ILC2 accumulation, inflammatory cell infiltration and mucus production in the lung, a reduction in the levels of Th2 cytokines, and alleviation of airway hyperresponsiveness in a mouse model of asthma. It seems that this sEV-mediated preventive effect was conveyed by the transfer of miR-146a-5p (193).

Allergic Rhinitis

Allergic rhinitis (AR) is a disease manifesting as type I allergic hypersensitivity within the nasal mucosa (194), and is characterized by chronic inflammation (195). The imbalance between the Th1 and Th2 differentiation is involved in the development of AR which is suggested to be partly regulated by sEVs. Zhu et al. have reported expression of a long-noncoding RNA (Lnc) GAS5 in the nasal mucus-derived sEVs in AR and in the ovalbumin-stimulated nasal epithelial cell (NEC)-derived sEVs. Here, this Lnc RNA promoted suppression of Th1 cell differentiation and induced Th2 differentiation upon treatment with nasal mucus (NM)-derived sEVs. A potential mechanism seems to involve the regulation of Enhancer of Zeste Homolog 2 (regulating proliferation and differentiation processes, including mediating proliferation and apoptosis of allogeneic T cells), and inhibition of T-bet expression by long-noncoding RNA GAS5 (196).

NEC-derived exosomal miR-146a induces the expression of IL-10 in monocytes in the murine model which seems to suppress allergic reactions downstream. Specifically, IL-10^+ monocytes have an immune suppressor effect on the $CD4^+$ effector T cells and the Th2 polarization in this model of AR (197). Interestingly, the alterations in the miRNA profile obtained from NM-derived EVs of AR patients showed intrinsic dysregulation of EV miRNA content in the disease. Wu et al. have demonstrated significant enrichment of certain biological and cellular processes within these differentially expressed miRNA signatures, namely B-cell receptor signaling pathway, natural killer cell-mediated cytotoxicity and T-cell receptor signaling, among others, implying that vesicular miRNAs exert regulatory function in AR. When investigated in more detail, B cell receptor signaling pathway-related miR-30-5p and miR-199b-3p were significantly increased, also miR-874 and miR-28-3p were significantly down-regulated in EVs from nasal mucus in AR (198).

Atopic Dermatitis and Contact Allergy

Atopic dermatitis (AD) is a chronic inflammatory skin disorder associated with the epidermal barrier disruption, eczematous cutaneous lesions and severe pruritus. AD pathogenesis is complex and characterized by cytokine production predominantly mediated by Th2 cells and ILC2 (199), but also involving innate and Th17 and Th22 components (200).

The importance of keratinocytes of the skin in the disease pathogenesis has been highlighted by the findings demonstrating that insufficiency in the epidermal barrier is key component (201). However, only one study so far has investigated the impact of EVs secreted by keratinocytes in the context of allergic inflammation (170). Here, using a murine allergy model, the authors noticed some signs of DC activation upon exposure to an antigen (OVA peptide) transferred by sEV from secreting keratinocytes. At the same time, however, they failed to detect any changes in the T cell reactivity to this peptide antigen.

Besides that, little is known about EV secretion from other cells in the skin with relation to AD, with more focus directed towards potential new therapies. In this regard, it has been reported that intravenous/subcutaneous administration of human adipose tissue-derived MSC-derived sEVs (showing exosomal characteristics) ameliorate AD symptoms *in vivo* (in a mouse model); the levels of serum IgE, the number of eosinophils in the blood, and the infiltration of mast cells were also shown to be reduced after the treatment. Such sEVs also reduced mRNA levels of IL-4, IL-31, IL-23, and TNF-α in the skin lesions demonstrating that their systemic administration may ameliorate AD-like symptoms through the regulation of inflammatory responses and expression of inflammatory cytokines in the tissue (25). Shin et al. have shown that exosomes-resembling sEVs derived from human adipose tissue-derived MSCs may significantly restore the epidermal barrier function in AD by inducing *de novo* synthesis of ceramides and modulating multiple gene expression programme, including the effects on differentiation of keratinocytes, lipid metabolism, cell cycle, and immune response (202). MSC-derived sEVs were shown to inhibit local inflammatory reaction and reduce tissue damage in atopic eczema (203). Hence, the evidence suggests that MSC-derived sEVs could potentially offer a promising cell-free therapeutic option for AD patients.

Contact allergy and contact sensitization is a common form of a delayed type hypersensitivity to small contact allergens. Contact allergy often develops after repeated or prolonged topical exposure to a particular sensitizing agent (204–206). Nazimek et al. have shown that intravenous administration of syngeneic mouse red blood cells leads to the EV generation that suppresses directed delayed type hypersensitivity in a miRNA-150-dependent manner; specifically, the syngeneic mouse red blood cell-derived EVs decreased T cell activation and enhanced their apoptosis (207). Similarly, human umbilical cord MSC-derived EVs were demonstrated to ameliorate and prevent the pathology of contact hypersensitivity in mice. Specifically, these EVs had a suppressive effect on both $CD8^+$ cytotoxic cells and $CD4^+$ Th1 cells, including the effect on TNF-α and IFN-γ production, induction of Tregs and the level of secreted IL-10 (208).

TABLE 2 | Preclinical models using EVs for allergy treatment in animals.

Study Title	Conditions	Outcomes	Reference
Exosomes from Bronchoalveolar Fluid of Tolerized Mice Prevent Allergic Reaction	Allergy	BALF-derived exosomes induce tolerance and protection against allergic sensitization in mice.	Prado et al, 2008 (191)
Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation	Asthmatic inflammation	IL-13 treated epithelial cell-derived exosomes induce enhanced proliferation and chemotaxis of undifferentiated macrophages in the lungs during asthmatic inflammatory conditions.	Kulshreshtha et al, 2013 (20)
Selective release of miRNAs via extracellular vesicles is associated with house dust mite allergen-induced airway inflammation	Allergic airway inflammation	Selective sorting of Th2 inhibitory miRNAs into airway secreted EVs and increase release to the airway is involved in the pathogenesis of allergic airway inflammation.	Gon et al, 2017 (218)
Exosomes derived from human adipose tissue-derived mesenchymal stem cells alleviate atopic dermatitis	Atopic dermatitis	Intravenously or subcutaneously injected human adipose tissue-derived MSC-derived exosomes ameliorate AD in an *in vivo* mouse model.	Cho et al, 2018 (25)
Extracellular vesicles from mesenchymal stem cells prevent contact hypersensitivity through the suppression of Tc1 and Th1 cells and expansion of regulatory T cells	Allergic contact dermatitis	Human umbilical cord-derived MSC-EVs prevent the pathology of contact hypersensitivity by inhibiting Tc1 and Th1 immune responses and inducing the Tregs phenotype *in vivo* and *in vitro*.	Guo et al, 2019 (208)
Small extracellular vesicles derived from human mesenchymal stromal cells prevent group 2 innate lymphoid cell-dominant allergic airway inflammation through delivery of miR-146a-5p	Allergic rhinitis (patients) ILC2-dominant asthma (mouse model)	MSC-sEVs prevent ILC2-dominant allergic airway inflammation through miR-146a-5p.	Fang et al, 2020 (193)
Exosomes from Human Adipose Tissue-Derived Mesenchymal Stem Cells Promote Epidermal Barrier Repair by Inducing de Novo Synthesis of Ceramides in Atopic Dermatitis	Atopic dermatitis	Human adipose tissue-derived MSC-exosomes effectively repair defective epidermal barrier functions in atopic dermatitis.	Shin et al, 2020 (202)
Syngeneic red blood cell-induced extracellular vesicles suppress delayed-type hypersensitivity to self-antigens in mice	Delayed-type hypersensitivity Contact hypersensitivity	Intravenous delivery of syngeneic mouse red blood cells that is mediated by EVs in a miRNA-150-dependent manner suppresses delayed-type hypersensitivity.	Nazimek et al, 2020 (207)
Intranasal delivery of MSC-derived exosomes attenuates allergic asthma *via* expanding IL-10 producing lung interstitial macrophages in mice	Allergic asthma	Intranasally delivered MSC-derived exosomes inhibit allergic asthma in mice.	Ren et al, 2020 (219)
Epithelial exosomal contactin-1 promotes monocyte-derived dendritic cell–dominant T-cell responses in asthma	Airway allergic models Asthma	Epithelial contactin-1 in exosomes is a critical player in asthma pathology.	Zhang et al, 2021 (181)

TABLE 3 | Registered clinical trial investigating the feasibility of using EVs in allergic patients.

Study Title	Conditions	Interventions	Locations	Identifier
Non-coding RNAs Analysis of Eosinophil Subtypes in Asthma	Allergic Asthma, Severe Eosinophilic Asthma	Biological: Dermatophagoides pteronyssinus allergen Procedure: Blood sampling, Procedure: Bronchial challenge with allergen	Lithuanian University of Health Sciences, Pulmonology Department Kaunas, Lithuania	NCT04542902
Effectiveness of Qufeng Shengshi Fang on Treatment of Allergic Rhinitis	Rhinitis, Allergic, Perennial	Drug: Qufeng Shengshi Fang and Loratadine, Drug: Loratadine	Peking Union Medical College Hospital traditional Chinese medicine department Beijing, Beijing, China	NCT02653339
Cohort Study of the Patterns of Microvesicles in the Serum of Participants With Atopic and Non-atopic Asthma	Asthma, Allergies	Biological: tumor derived microparticles, Drug: cisplatin	The Ohio State University Medical Center Columbus, Ohio, United States	NCT00700726
Influence on Human Bronchial Epithelial Cells Smoker Extracellular Vesicles Influence on Human Bronchial Epithelial Cells	Smokers Human Bronchial Epithelial Cells Lung Pathogenesis Biomarkers	Diagnostic Test: Broncho Alveolar Lavages	HôpitalSaint-Philibert, Lomme, France	NCT03608293
Phase I/IIa Study on Chitin Microparticles in Subjects Suffering From Allergic Rhinitis	Seasonal Allergic Rhinitis	Drug: Chitin microparticles by nasal route	Hammersmith Medicines Research, London, United Kingdom	NCT00443495
Exploratory Study of the Cutaneous Penetration of Biodegradable Polymeric Microparticles in Atopic Dermatitis (MicroIskin)	Atopic Dermatitis	Drug: Biodegradable and biocompatible polymeric microparticles containing a fluorochrome applied to the skin followed by a skin biopsy	Regional University Hospital Besançon, France	NCT02369432
Impact of Narrowband UVB Phototherapy on Systemic Inflammation in Patients With Atopic Dermatitis	Atopic Dermatitis	Other: Narrow band UVB treatment, (NB-UVB)	The Rockefeller University New York, New York, United States	NCT03083730
Trial on Vascular Inflammation in Atopic Dermatitis	Atopic Dermatitis Vascular Inflammation Coronary Atherosclerosis	Other: FDG-PET Scan Other: MDCT, Other: biopsy and blood collection	Innovaderm Research Inc Montreal, Quebec, Canada	NCT02926807
Role of Macrophage in immune-modulation by mesenchymal stem cell derived exosome in asthma	Respiratory diseases	Primary indicator: PD-L1, Immuno-suppression capacity of regulatory T cell	Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University	ChiCTR2000031122

Food Allergy and Allergic Inflammation in the Gastrointestinal Tract

Food allergy is a manifestation of an abnormal immune response to food or food additives (209) which is a complex process involving multiple cellular and molecular mechanisms. It has been shown that early exposure to allergens *via* the gastrointestinal route promotes tolerance (210, 211). It is not clear how much EVs are involved in this process, however, animal models suggest that there could be some contribution. Specifically, intestinal epithelial cells (IECs) subjected to OVA release sEVs that carry IL-10 and OVA/MHC class II complexes recognized by OVA-specific TCR-bearing $CD4^+$ T cells. Here, OVA-specific $CD4^+$ T cells represent type 1 Tregs, produce IL-10 and show immune suppressive effects on effector T cell proliferation. The proposed mechanism involved the role of vasoactive intestinal peptides, which seemed to be required for this effect (212, 213). Furthermore, Treg bias has been also observed following a sEV-mediated transfer of food allergens into the mesenteric lymph nodes (MLNs) of mice, in contrast to a direct transfer of those allergens, which promoted Th2 responses (214); the results also highlighted the role of exosomal integrin αvβ6 as a protective molecule. Finally, given that the diverse composition of the gut microbiome has been shown to be critical in food allergy prevention (215), antigen and mediator transfer *via* EVs secreted by IECs may be also involved in the elimination of pathogenic bacteria to prevent intestinal dysbiosis (146).

CLINICAL PERSPECTIVES

Growing attention has been given to EVs as mediators in both physiological conditions and pathology, including the role in allergic diseases. Extensive research has been carried out showing the capacity of EVs to regulate homeostasis and immune functions in the allergic microenvironment. Alterations in exosomal content in allergic conditions have been shown to distinguish between physiological and diseased states suggesting the potential use of sEVs as biomarkers in the search of diagnostic tools for allergic diseases, for example in asthma phenotype subgrouping (216). Naturally-occurring sEVs can be also potentially used as drugs themselves, supporting healing process, e.g. MSC-derived sEVs participating in wound healing and regeneration of the lung tissue; this highlights the possible use of these sEVs in allergic airway remodeling (158, 202, 217). Several studies have proposed treatment strategies in animal models of allergic disease as summarized in **Table 2**. There are also examples of the use of sEVs as compound carriers are now being investigated as a naturally derived drug delivery systems (DDSs) with a favorable biocompatibility profile, but sEVs can be also potentially used to deliver non-drug anti-inflammatory agents including miRNAs (e.g. let-7-miRNAs). Indeed, there have been already several clinical trials in the past and more are now ongoing which investigate a potential of using EVs for the benefit of allergic patients (**Table 3**).

In summary, non-immune cell-derived EVs contribute to allergic inflammation in the tissue location and potentially systemically; they have a great potential to become a valuable diagnostic option as well as a novel target for allergy therapy. Such EVs are slowly introduced into the clinic within the setting of clinical trials which investigate the feasibility of such an approach.

AUTHOR CONTRIBUTIONS

LH and DG-O wrote the paper. EC, DG-O, and LH prepared the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Effects of Mesenchymal Stem Cell-Derived Exosomes on Autoimmune Diseases

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Recent years, the immunosuppressive properties of mesenchymal stem cells (MSCs) have been demonstrated in preclinical studies and trials of inflammatory and autoimmune diseases. Emerging evidence indicates that the immunomodulatory effect of MSCs is primarily attributed to the paracrine pathway. As one of the key paracrine effectors, mesenchymal stem cell-derived exosomes (MSC-EXOs) are small vesicles 30-200 nm in diameter that play an important role in cell-to-cell communication by carrying bioactive substances from parental cells. Recent studies support the finding that MSC-EXOs have an obvious inhibitory effect toward different effector cells involved in the innate and adaptive immune response. Moreover, substantial progress has been made in the treatment of autoimmune diseases, including multiple sclerosis (MS), systemic lupus erythematosus (SLE), type-1 diabetes (T1DM), uveitis, rheumatoid arthritis (RA), and inflammatory bowel disease (IBD). MSC-EXOs are capable of reproducing MSC function and overcoming the limitations of traditional cell therapy. Therefore, using MSC-EXOs instead of MSCs to treat autoimmune diseases appears to be a promising cell-free treatment strategy. In this review, we review the current understanding of MSC-EXOs and discuss the regulatory role of MSC-EXOs on immune cells and its potential application in autoimmune diseases.

Keywords: mesenchymal stem cells, exosomes, immunoregulation, therapy, autoimmune diseases

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1 INTRODUCTION

Mesenchymal stem cells (MSCs) are pluripotent stem cells with the capacity for self-renewal and multidirectional differentiation into osteoblasts, chondrocytes, adipocytes, and other types of cells (1, 2). MSCs are widely distributed in the body and have been isolated from a variety of tissues, among which the bone marrow and subcutaneous fat are common cellular sources (3). The International Committee established the recognition characteristics of human MSCs, including under standard culture conditions to maintain adhesion appearance, expression of CD105, CD73, and CD90 molecules, no expression of CD45, CD34, CD14, CD45, CD11b, CD79a, CD19, and HLA-DR, with the ability to differentiate into osteoblasts, adipocytes, chondrocytes *in vitro* (4). In addition to its strong differentiation capacity, MSCs also have immunomodulatory potential to modulate innate and adaptive immune cells (5). Abundant evidence indicates that MSCs can act on natural killer (NK) cells, dendritic cells (DCs), macrophages, B lymphocytes, and T lymphocytes *via*

inhibiting the activation, proliferation and differentiation into effector cells (6–9). Following stimulation with inflammatory factors, MSCs exhibit the properties of reducing the inflammatory response, improving tissue repair, and avoiding infection by secreting various immune regulatory factors (10). At present, substantial evidence suggests that MSCs exert their immunomodulatory function through paracrine pathway, especially *via* exosomes (10, 11).

Extracellular vesicles(EVs) are a family of particles/vesicles found in blood and body fluids. They are composed of phospholipid bilayer and carry a variety of molecules that serve as mediators for intercellular communication (12). In 1946, Chargaffaf et al. suspected the existence of EVs and published the first study of EVs (13). It was not until 1967 that Wolf et al. confirmed the existence of EVs with electron microscopy (EM) (14). The term exosome (" exo "= external," some "= body) was introduced in the 1970s, and it wasn't until 1981 that the term" exosome "was first used by Trams to refer to Evs (15). Shortly after Johnstone et al. described multivesicular bodies (MVBs) and their 40-80nm exosomes in 1985 (16), differential centrifugation and ultracentrifugation above 100,000 g were used to separate and distinguish the smallest vesicles (17). With the increase in EVs (especially exosomes) publications, most studies do not clearly distinguish exosomes from other vesicles. To address this problem, in 2013, the International Extracellular Vesicle Society (ISEV) proposed a set of criteria for EV Science (18). In order to ensure normative research, the Minimal Information of Studies of Extracellular Vesicles (MISEV) was revised in 2018 to update knowledge in the field (19).

Exosomes are spherical vesicles composed of lipid bilayer membranes with a diameter of 40-200 nm, which contain complex and abundant active substances such as proteins and nucleic acids. Expression of exosome markers, proteins, nucleic acids, and other bioactive molecules is related to the cell of origin (20). There is increasing evidence that MSC-derived exosomes (MSC-EXOs) play an important role in immune regulation. MSC-EXOs are spherical vesicles secreted by MSCs that contain many anti-inflammatory compounds and modulate the immune response by interacting with immune effector cells (12). In the treatment of autoimmune diseases, MSC-EXOs as a carrier of cell-free therapy have attracted extensive attention, because they not only carry most of the therapeutic effects of MSC itself, but also reduce the concerns about the safety of injecting live cells. MSC-EXOs has significant advantages over MSCs in clinical treatment and may completely replace MSC therapy in the future.

2 CHARACTERISTICS OF MESENCHYMAL STEM CELL-DERIVED EXOSOMES

Both eukaryotic and prokaryotic cells release EVs, which are regarded as a part of their normal physiology and acquired abnormalities (20). EVs represent an important substance for intercellular communication. They participate in normal physiological processes, as well as play a critical role in disease occurrence and progression. Numerous experimental and clinical studies have shown that the immunomodulatory effects of MSCs can be primarily attributed to MSC-derived extracellular vesicles (MSC-EVs) (21). Although the classification of EVs is constantly evolving, it is generally accepted that EVs are classified into three main categories according to their size and biogenesis: apoptotic bodies (ABs), microvesicles, and exosomes (22–24). ABs (greater than 1,000 nm in diameter) are comprised of relatively large fragments of cells containing organelles derived from the cells undergoing apoptosis, which are transferred to phagocytes (25, 26). Microvesicles (100–1,000 nm in diameter) and exosomes (40–200 nm in diameter) belong to EVs at the nano level. Microvesicles, also known as ectosomes or microparticles, are released into the extracellular environment after directly budding or shedding from the plasma membrane (27). Exosomes originate from the endosomal pathway. Extracellular material fuses with early endosomes (ESEs) through membrane invagination and endocytosis, and gradually matures and develops into late endosomes (LSEs). The invagination of LSEs leads to the formation of intraluminal vesicles (ILVs), and multiple vesicles assemble to form MVBs, which fuse with the cell membrane and are subsequently released (**Figure 1**) (20). With regards to the delivery of exosome contents, exosomes can be bound by target cells through multiple pathways, of which the main mechanisms include endocytosis, ligand-receptor binding, or direct binding (**Figure 1**) (28–31). Exosomes are formed by budding through the endosome pathway and are wrapped in a lipid bilayer, contents of which can be protected by the external environment to maintain exosome integrity.

Exosomes contain a large number of proteins, lipids, transcription factors, as well as DNA, mRNA, and miRNA (32, 33). Exosome membranes contain lipid raft structures composed of cholesterol, spingomyosin, and ceramide and the tetraspanin protein family (CD63, CD81, and CD9) as an exosome marker (34). Exosomes also contain other common proteins, including MVB biogenesis proteins (Alix, TSG101, and ESCRT Complex), membrane transporter and fusion proteins (RAB protein, GTPases, and annexins), heat shock proteins (HSP60, 70, and 90), lipid related proteins, and phospholipases (35). Notably, MSC-EXOs express not only common surface markers such as CD81 and CD9, but also mesenchymal stem cell surface markers (CD44, CD73, and CD90) by flow cytometry (34). Proteomic analysis of exosomes isolated from human bone marrow-derived mesenchymal stem cells (hBM-MSC)provided evidence of 730 functional proteins associated with MSC proliferation, adhesion, migration, and morphogenesis (36). Surprisingly, protein packaging in exosomes is not random, because human primed MSCs secrete exosomes (pMEX), compared to human primed MSCs (pMSC), has a high concentration of specific subcategories of proteins, including secretory proteins and extracellular matrix (ECM) associated proteins, which may provide the molecular basis for its unique functional properties (37). In addition to proteins, MSC-EXOs also contain numerous RNA. Interestingly,

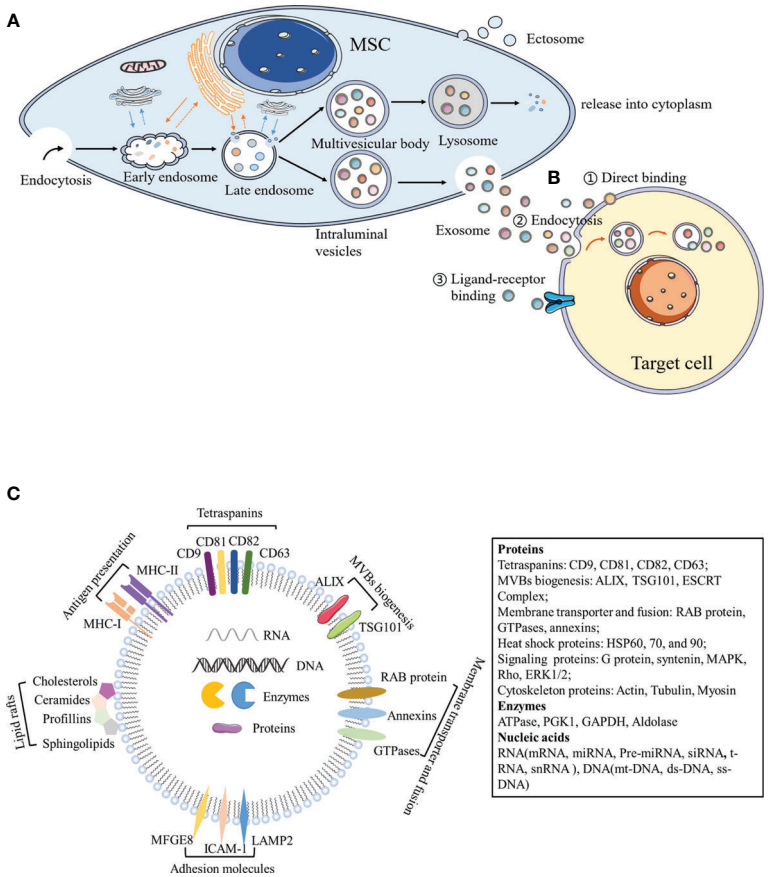

FIGURE 1 | Biogenesis and components of exosomes. **(A)** Exosomes originate from the endosomal pathway. Extracellular material enters the cytoplasm through plasma membrane depression and endocytosis, and fuses with early endosomes, endoplasmic reticulum and preformed Golgi bodies, to develop into late endosomes, which are interlinked with the cell membrane network structure to form ILVs containing a vesicle structure. Different concentrations and sizes of ILVs constitute MVBs. On the one hand, MVBs fuse with lysosomes, degrade the contents, and release them into the cytoplasm. On the other hand, MVBs are transferred to the cytoplasmic membrane through the membrane system and vesicles are released outside the cell, which are termed exosomes. **(B)** Exosomes can act by binding to receptors present on the surface of target cells, by binding to endocytosis, or by the direct binding to recipient cells. **(C)** Exosome components. MFGE8, milk fat globule-EGF factor 8 protein; ICAM-1, intercellular adhesion molecule 1; MHC I and II, major histocompatibility complex I and II; LAMP2, lysosomal-associated membrane protein 2; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PGK1, phosphoglycerate kinase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RNA is specifically incorporated into exosomes, where it accumulates and then enters the recipient cell to do its job (38). Interestingly, enrichment of MSC-EXOs with network-informed miRNA further enhanced the intrinsic ability of MSC-EXOs to prevent apoptosis, promote angiogenesis and induce myocardial cell proliferation (39). MSC-EXOs change the activity and function of target cells primarily through the horizontal transfer of these substances (**Figure 1**). Due to the lack of specific markers, MSC-EXOs are currently prepared according to size or density. Most laboratories separate exosomes from conditioned media *via* hypercentrifugation, which cannot differentiate exosomes from other EVs or biological macromolecules (40). The MISEV2018recommends using the generic term "small/medium/large EVs" based on its size or density, rather than the classic terms "exosome", "vesicle", and "apoptotic body" (19). However, most of the current articles continue to use classic terms. The sizes of small/medium/large EVs are partially overlapped and cannot be strictly distinguished. Therefore, this review takes a cautious attitude towards the absolute definition of different types of vesicles, and focuses on the effects of nanoscale EVs (e.g., exosomes on immune cells) and various autoimmune diseases.

Numerous studies have shown that MSC-EXOs exhibit similar functions to that of MSCs (e.g., repairing damaged tissues, regulating immune responses, and playing anti-inflammatory effects) (10, 41). Although MSC therapy is widely regarded as an effective therapy for several immunological diseases, the direct therapeutic effect of MSCs remains limited. Due to the relatively large size of MSCs, intravascular administration may lead to vascular obstruction, which can result in pulmonary embolism and death in severe cases (42). In addition, allogenic immune rejection or abnormal chromosomal differentiation may occur

during *in vivo* transplantation, and even malignant tumors may form (43). Also, MSCs cells age rapidly and are expensive to have a large-scale production (44). Using MSC-EXOs in humans has several potential advantages over MSCs. First, nano-scale exosomes are free to pass through various biological barriers without blocking microvascular circulation. Second, their application effectively prevents the metastasis of DNA-mutated cells and hinders the tumor development. Third, the number of MSCs decreases rapidly following transplantation, whereas the delivery of MSC-EXOs can continue to function in the body (10). In addition, MSC-EXOs are considered to be non-immunogenic and can be produced in a large-scale production for clinic application (44). However, toxicological studies of nanoparticles *in vivo* still need to be fully evaluated, particularly following long-term exposure (45). Moreover, the discovery of a broad therapeutic effect of exosome-mediated MSCs eliminates many of the challenges associated with the use of living replicative cells, as it fundamentally shifts living-cell-based MSCs therapy to a "cell-free" treatment, reducing the risk of living cell therapy. Therefore, MSC-EXOs, as cell-secreted natural EVs, has the advantage of being an ideal nanoscale drug carrier.

3 IMMUNOMODULATORY FUNCTION OF MESENCHYMAL STEM CELL-DERIVED EXOSOMES

3.1 Innate Immunity

3.1.1 Macrophages

As an important aspect of the innate immune system, macrophages originate from either the yolk sac during embryonic development or bone marrow-derived monocytes (46). Under microenvironmental activation, macrophages may evolve into an M1 phenotype of pro-inflammatory macrophages or an M2 phenotype of anti-inflammatory macrophages. In general, M1 macrophages secrete pro-inflammatory molecules, including TNF-α and IL-1β, whereas M2 macrophages secrete immune regulatory factors (e.g., IL-10) (47). Recent data support the findings that the anti-inflammatory effect of MSC-EVs is inseparably related to macrophage polarization. MSC-EVs inhibit pro-inflammatory M1 macrophage activation and promote their polarization to M2 macrophages, which are consistent with a decrease in the levels of VEGF-A, IFN-γ, IL-12, and TNF-α, and an upregulation of IL-10 (48, 49). Previous studies have reported that macrophages are the main target cells for MSC-EVs to alleviate colon inflammation. In dextran sodium sulfate (DSS)-induced colitis, MSC-EVs effectively alleviate colitis by inducing an immunosuppressive M2 phenotype exhibited by colonic macrophage polarization. Compared with the control group, MSC-EV-treated mice produced a greater number of IL-10-producing M2 macrophages (49). Similarly, in a study of sepsis conducted by Song et al., the authors demonstrated that MSC-EVs promoted M2 macrophage polarization. Human umbilical cord-derived MSCs(hUC-MSCs) pretreated with IL-1β effectively induce macrophage polarization into an anti-inflammatory M2 phenotype *via* exosomal miR-146a, which ultimately resulted in prolonging the survival of sepsis mice (50). Moreover, the results showed that MSC-EVs down-regulated the production of IL-23 and IL-22, enhanced the anti-inflammatory phenotype of mature human regulatory macrophages (Mregs), which led to a weakened Th17 response (51). Zhao et al. reported that adipose-derived MSC-EXOs(AD-MSC-EXOs) promoted an M2 macrophage polarization by activating STAT3 transcription, up-regulating the expression of IL-10 and Arg-1 in macrophages, thereby inhibiting the inflammatory response of macrophages (52). In addition, in the rat model of experimental autoimmune uveitis (EAU), Bai et al. demonstrated that MSC-EXOs treatment downregulated the proportion of $CD68^+$ macrophages in the retina and demonstrated the down-regulation of MSC-EXOs on macrophage migration to the retina (**Figure 2**) (53). Interestingly, MSC-EXOs also enhanced the immunosuppressive function of macrophage precursor, which is called myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature Myeloid derived cells. IL-6 secreted by olfactory ecto-mesenchymal stem cell-derived exosomes (OE-MSC-EXOs) activates the JAK2/STAT3 pathway in MDSCs, and enhances the inhibitory function of MDSCs by upregulating the levels of arginase, ROS and NO (54). In addition, abundant S100A4 in OE-MSC-EXOs mediated endogenous IL-6 production of MDSCs through TLR4 signaling, and along with exosomal-derived IL-6 promotes the immunosuppressive function of MDSC (54).

3.1.2 Natural Killer Cells

NK cells are important effector cells in the innate immune response and play an important role in the host pathogen defense. When pathogens invade a host, they kill target cells by releasing cytotoxic particles containing perforin and granzyme (55). Although autoimmune diseases are primarily caused by T and B lymphocytes, NK cells possess excessive activation and inhibitory receptors, which can play a role in regulating autologous cell reactivity (56). In addition, NK cells may regulate the activity of other immune cells by secreting cytokines and influence the development of the adaptive immune response through these pathways (57). MSC-EVs primarily induce an immunosuppressive effect on NK cells, including the proliferation, activation, and cytotoxicity of NK cells. In a rat model of EAU, an injection of MSC-EXOs around the eye restored EAU damage by downregulating the transport of $CD161^+$ NK cells in the lesion (53). Recent studies have shown that human fetal liver (FL) MSC-derived exosomes(hFL-MSC-EXOs) mediated downstream TGF-β/Smad signal transduction through the surface expression of TGF-β to inhibit the proliferation and activation of NK cells (58). In human graft-versus host disease (GVHD) experiments, researchers noted that MSC-EXOs suppressed the release of IFN-γ and TNF-α by activated NK cells, which reduced the cytotoxic effect of NK cells, and lessened the inflammatory response (**Figure 2**) (59).

3.1.3 Dendritic Cells

DCs play an immunological role as antigen presenting cells (APCs), which can ingest, process, and present antigens to T and B lymphocytes (60). Most DCs in the body are immature DCs (iDCs) under normal circumstances and express low levels

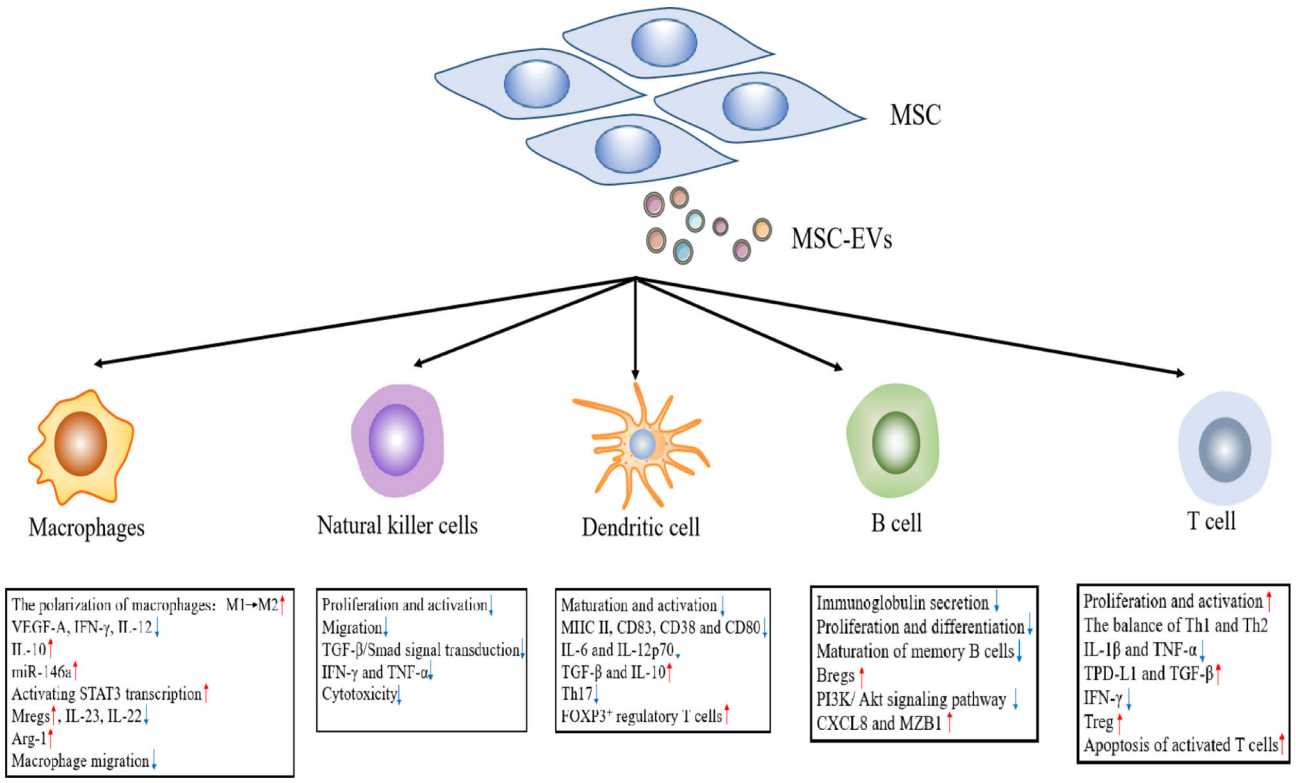

FIGURE 2 | The effects of MSC-EVs on immune effector cells.

of class II major histocompatibility complex (MHC II) and T cell costimulatory molecules (CD80, CD86, and CD40). In addition, iDCs up-regulate the surface expression of MHC II and costimulatory molecules after ingesting antigens or cytokine stimulation, and are converted into mature DC (mDCs) (61). Previous studies indicate that the immunosuppressive effect of MSC-EVs on DCs is primarily realized by inhibiting DC maturation and activation. In the presence of MSC-EVs, iDCs are impaired in their ability to receive antigens and differentiate mDCs, which results in the decreased expression of mature and activation markers (e.g., CD83, CD38 and CD80), and correspondingly decreased the secretion of pro-inflammatory cytokines (i.e., IL-6 and IL-12p70) (62). At the same time, MSC-EVs can also enhance the release of TGF-β and IL-10 in $CD11c^+$ DCs, thereby inhibiting lymphocyte proliferation (63). Researchers suggest that MSC-EVs treatment inhibits cell surface expression of MHC II and costimulatory molecules on $CD11c^+$ cells in a dose-dependent manner. The results showed that DCs exhibited a hypoactive phenotype and thereby suppressed the subsequent T cell activation and proliferation (64). Moreover, the co-culture of MSC-EV-treated DCs with T cells reduced Th17 cell counts and IL-17 levels, and increased $Foxp3^+$ regulatory T cells. This finding is consistent with the previous conclusion (65). In brief, there was a weakened ability of iDCs co-cultured with MSC-EXOs to differentiate into mDCs. This led to an inability to perform antigen presentation and effectively stimulate T lymphocyte proliferation. In contrast, it promoted an increase in Treg cells, which indirectly reflected that MSC-EXOs can enhance host immune tolerance (**Figure 2**).

3.2 Adaptive Immunity

3.2.1 B Cells

B cells are important adaptive immune cells, whose main functions are to mediate humoral immunity and secrete antibodies. The regulation of MSC-EVs on B lymphocytes was investigated *in vitro*. In the CpG-stimulated PBMC co-culture system, MSC-EVs can completely replicate the inhibitory effect of MSCs on immunoglobulin secretion, B cell proliferation and differentiation in a dose-dependent manner (66, 67). Budoni et al. also claimed that MSC-EVs were internalized by activated $CD19^+/CD86^+$ B cells to inhibit B cell proliferation, differentiation, and antibody production, as well as inhibit memory B cell maturation (68). There are functional B cell subsets, known as regulatory B cells (Bregs). The key function of Bregs is to release IL-10, which inhibits the production of proinflammatory cytokines and supports regulatory T cell differentiation (69). MSC-EVs exert dose-dependent anti-inflammatory effects by inhibiting B cell maturation and inducing Bregs in lymph nodes in a murine model of collagen-induced arthritis (CIA) and delayed-type hypersensitivity (DTH). Moreover, MSC-EVs also regulate cellular function

through the differential expression of mRNA in related genes. Researchers found that MSC-EVs induced B cells to down-regulate the PI3K/Akt signaling pathway through miR-155-5p, inhibited B cell proliferation and reduced the activation capacity of B lymphocytes (70). Real-time PCR analysis confirmed that following exosome treatment from MSC sources, the expression of genes that played an important role in B cell immune regulation (e.g., CXCL8 and MZB1) were upregulated (67). These studies confirmed that MSC-EXOs can play an immunomodulatory role by acting on B cells (**Figure 2**).

3.2.2 T Cell

T cell proliferation and activation contribute to the occurrence and development of many autoimmune diseases. MSC-EVs play a negative role in T cell proliferation and activation. MSC-EVs have been shown to carry a variety of active molecules, including TGF-β (68, 71), IDO protein (72), and miR-125a-3p (73). These molecules give MSC-EVs the ability to inhibit T cell proliferation and activation. Studies have revealed that adenosine is associated with a robust immunosuppressive effect, and MSC-EVs inhibits T cell proliferation *in vitro* through adenosine signal transduction (74). MSC-EVs can also adjust the balance of T helper type 1 (Th1) and T helper type 2 (Th2) cells and reconstruct the stable state of Th1 and Th2. Chen et al. co-cultured bone marrow mesenchymal stem cell (BM-MSC)-derived exosomes (BM-MSC-EXOs) and peripheral blood mononuclear cells. The authors subsequently found that it could promote the Th1 to Th2 conversion of helper T cells, significantly reduced the levels of pro-inflammatory factors, IL-1β and TNF-a, and improved the levels of anti-inflammatory TGF-β (66). In a similar study, human adipose tissue MSC-EXOs inhibited the differentiation and activation of T cells and the release of pro-inflammatory factors (e.g., IFN-γ) (75). In addition, MSC-EXOs can facilitate the differentiation of Tregs. Treg cells are immune cells with negative feedback regulation. Similarly, inflammatory IL-1β-primed MSC-EVs upregulated PD-L1 and TGF-β expression, which induced the apoptosis of activated T cells, and increases the proportion of Treg cells in a mouse model of autoimmune encephalomyelitis (76). Previous studies in our laboratory have also found that OE-MSC-EXOs had a potent inhibitory effect on the proliferation of $CD4^+$ T cells in experimental colitis mice. At the same time, the secretion of IL-17 and IFN-γ by T cells was reduced, whereas the secretion of TGF-β and IL-10 was enhanced, which could significantly slow disease progression (77). Following treatment with OE-MSC-EXOs, the Th1/Th17 subsets were significantly reduced, while Treg cells were increased (77). It was found that transgene-free human induced pluripotent stem cells (iPSCs) derived EVs could prevent the progression of Sjögren's syndrome (SS) by inhibiting the differentiation of follicular helper T (Tfh) and Th17 cells (78). In addition, other studies have reported that MSC-EXOs can promote the differentiation of monocytes into an M2 macrophage phenotype, thereby activating the differentiation of $CD4^+$ T cells into Treg cells and delaying the occurrence of immune rejection (**Figure 2**) (79).

4 APPLICATIONS OF MESENCHYMAL STEM CELL-DERIVED EXOSOMES IN AUTOIMMUNE DISEASES

To develop EVs as a cell-free therapy and elucidate its potential role in stem cell effects *in vivo*, the following contents can assess the role of exosomes released by MSCs in the treatment of autoimmune diseases, including Multiple sclerosis (MS), Systemic lupus erythematosus (SLE), Type-1 diabetes (T1DM), uveitis, Rheumatoid arthritis (RA), and Inflammatory bowel disease (IBD) (**Table 1**).

4.1 Multiple Sclerosis

MS is the most common inflammatory disease of the central nervous system (CNS), which is characterized by demyelination, neuronal damage and loss, and ultimately neurological dysfunction (80). Experimental autoimmune encephalomyelitis (EAE) is the commonly used animal model of MS (81). As the primary immune cells of the central nervous system, microglia can maintain tissue homeostasis and contribute to central nervous system development under normal physiological conditions (82). Following pathogen invasion, activated microglia with an M1 phenotype as the first line of defense secrete pro-inflammatory cytokines to eliminate invading pathogens. However, when tissue homeostasis is restored, microglia exhibiting an M2 phenotype need to be activated, otherwise the excessive release of pro-inflammatory cytokines can lead to neuronal damage (83). Microglia have both neurodestructive and neuroprotective functions. An imbalance of the M1/M2 phenotype inhibits the nerve protection function and promotes the occurrence of MS (84). Therefore, M1 to M2 polarization of microglia may be beneficial to the neuroprotective function of microglia, thereby preventing disease progression. The study by Zijian et al. demonstrated that BM-MSC-EXOs therapy inhibits microglia from developing into an M1 phenotype, promotes M2 phenotype polarization, and secretes anti-inflammatory cytokines (e.g., TNF-α, IL-10, and TGF-β). Moreover, BM-MSC-EXOs treatment significantly improved the neurobehavioral symptoms of EAE rats and relieved the inflammation and demyelination of CNS (85). Human periodontal ligament stem cells (hPDLSCs)-EVs from MS patients has been shown to inhibit NALP3 inflammasome activation in an EAE model (86). Although substantial progress has been made in the treatment of MS, the appropriate treatment for MS remains controversial. The currently available drugs may have potentially harmful side effects and cannot meet future needs (87). MSC-EXOs is a natural non-toxic vesicle that can deliver mRNA, miRNA, and proteins, as well as alleviate the condition of EAE by regulating microglia polarization (88). As the carrier of MSC-specific tolerance molecules (e.g., PD-L1, Galecin-1 [GAL-1], and TGF-β), MSC-EVs can effectively inhibit the activation and proliferation of self-responding lymphocytes and promote the secretion of anti-inflammatory cytokines derived from lymphocytes, thereby alleviating EAE disease progression (76). In another study, it was also shown that

TABLE 1 | The role of MSC-EVs in the treatment of autoimmune diseases, as discussed in the text.

Disease types	Animal model	Exosomes sources	Exosomes reactive molecules	Target cells/ tissue	Mechanism of action	Effect	Ref
Multiple sclerosis (MS)	Experimental autoimmune encephalomyelitis (EAE)	BM-MSCs	Immune response/inflammatory response/myellination-related proteins (unspecified)	Microglia	The polarization of microglia from M1 to M2	Relieves the neurobehavioral symptoms and attenuates the inflammation and demyelination of CNS	(85)
		BM-MSCs	PD-L1, GAL-1 and TGF-β	Lymphocyte	Inhibits the activation and proliferation Promotes the secretion of anti-inflammatory cytokines	Alleviates the disease progression	(76)
		hPDLSCs	IL-10 and TGF-β	Spinal cords	NALP3 inflammasome inactivation, and NF-kB reduction	Attenuates the inflammation Infiltration	(86)
		hBM-MSCs	Anti-inflammatory RNAs, anti-inflammatory and neuroprotective proteins(unspecified)	Microglia	Infiltration of microglia reduces The formation of Treg	Alleviates the development of EAE	(89)
Type-1 diabetes (T1DM)	Nonobese diabetic (NOD)	BM-MSCs	unspecified	Neurons and astrocytes	Repairs damaged neurons and astrocytes	Improves cognitive impairment	(100)
		AD-MSCs	unspecified	T/B lymphocytes	Increases the expression of anti-inflammatory factors and the population of Treg cells	Prevents overactivation and autoimmune damage	(101)
		hBM-MSCs	unspecified	Islet cells	Inhibits islet inflammation	Increases the plasma insulin level	(64)
Uveitis	Experimental autoimmune uveitis (EAU)	hBM-MSCs	unspecified	Th1 and Th17 cells	Decreases the number of Th1 and Th17 cells	Inhibits the development of EAU	(64)
		hUC- MSCs	unspecified	$CD3^+$Tcells Macrophages	Decreases $CD3^+$T cells infiltration and macrophages migrating to the retina	Alleviates the development of EAU	(53)
		hBM-MSCs	unspecified	$CD4^+$T cells	Induces the transformation of $CD4^+$T to Treg cells	Plays immunomodulation function on EAU	(64)
Rheumatoid arthritis (RA)	Collagen-induced arthritis (CIA)	BM-MSCs	miRNA-150-5p	Fibroblast-like synoviocytes (FLS)	Targets MMP14 and VEGF.2	Decreases migration and invasion in RA FLS and downregulates tube formation in HUVECs	(112)
		BM-MSCs	miRNA-320a	FLS	Suppresses CXCL9 expression	Attenuates arthritis and bone damage	(113)
		BM-MSCs	miRNA-192-5p	FLS	Inhibits the levels of pro-inflammatory factors and suppresses synovial hyperplasia by RAC2	Delays the event of the inflammatory response	(114)
		BM-MSCs	unspecified	B cell	Expands Breg cells and decreases plasmablast differentiation	Lowers disease incidence and deceases clinical score Reduces levels of serum auto-antibodies	(48, 115)
Sjögren's syndrome (SS)	Experimental Sjögren's syndrome (ESS)	OE-MSCs	IL-6	MDSC cells	Activates the Jak2/Stat3 pathway in MDSCs	Upregulates arginase expression and increases ROS and NO levels attenuates disease progression	(54)
		BM-MSCs and iPSC-MSCs	unspecified	SG epithelial cells (SGECs) and immune cells	Inhibits the differentiation of Tfh and Th17 cells and the activation of APCs	Decreases the lymphocyte infiltration in salivary glands and serum autoantibody levels	(78)
Inflammatory bowel disease (IBD)	Experimental Colitis	hUC -MSCs	unspecified	Macrophages	Inhibits the expression of iNOS and IL-7	Relieves inflammatory responses, and attenuates DSS induced colitis	(121)
		hUC -MSCs	miR-326	Human colorectal mucosa cells (FHC)		Inhibits the neddylation process and achieves the effect of relieving IBD	(122)

(Continued)

TABLE 1 | Continued

Disease types	Animal model	Exosomes sources	Exosomes reactive molecules	Target cells/ tissue	Mechanism of action	Effect	Ref
					Targets the expression of NEDD8 (neural precursor cell-expressed, developmentally downregulated gene 8)		
		BM-MSCs	miR-146a	Colonic epithelial cells	Inhibits TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) expression	Ameliorates the disease severity	(123)
		hUC -MSCs	TSG-6	Th2 cells Th17 cells	Enhances the immune response of Th2 cells in MLN and reduces the immune response of Th17 cells	Ameliorates IBD symptoms and reduces mortality rate	(119)
		OE-MSCs	unspecified	Th1/Th17 cells Treg cells	Regulates Th-cell responses	Alleviates the severity of disease	(77)
		AD-MSCs	unspecified	Treg cells	Regulate the Treg population	Improves inflammation in DSS-induced acute colitis	(124)
		hBM -MSCs	Metallothionein-2	Macrophages	Polarizes M2b macrophages	Attenuates mucosal inflammation	(125)

the infiltration of microglia in spinal cord sections treated with MSC-EXOs was significantly reduced, and MSC-EXOs induced the formation of Tregs and alleviated the development of EAE (89). Therefore, MSC-EXOs may hold great potential for the treatment of MS.

4.2 Systemic Lupus Erythematosus

SLE is a chronic autoimmune disease characterized by immune inflammation and multiple organ damage. In addition, SLE pathogenesis is extremely complex, primarily due to the comprehensive influence of genetic, environmental, hormonal, epigenetic, and other factors (90). With the help of Tfh cells, anti-nuclear antibodies (ANA) are produced, leading to the deposition of immune complexes in vital organs. The immune complex triggers an influx of large inflammatory cells by activating the complement cascade, causing tissue inflammatory damage (e.g., nephritis) (91). Nephritis represents the leading cause of morbidity and mortality in SLE and occurs in approximately half of all patients (92). In previous studies, researchers found that the infusion of hBM-MSCs into mouse models of lupus nephritis reduced the level of autoantibodies, improved the survival rate in mice, and alleviated the clinical symptoms of glomerulonephritis by inhibiting the development of Tfh (91). Moreover, the loss of BM-MSC/osteoblast function in an SLE mouse model results in an impairment of the osteoblastic niche and an imbalance of immune homeostasis. Allogeneic bone marrow mesenchymal stem cell transplantation (MSCT) plays a positive role in reconstructing the osteoblast niche and restoring immune homeostasis; thus, effectively reversing multiple organ dysfunction (93). The above findings confirm a positive therapeutic effect of MSCs on SLE; however, cellular therapy continues to face technical, cost, and regulatory challenges in clinical trials. Recent studies have demonstrated that the immunoregulatory activity of MSCs was mainly mediated by paracrine factors (e.g., MSC-EVs) (66). In a novel porcine model of coexisting metabolic syndrome (MetS) and renal artery stenosis (RAS), MSC-EVs isolated from adipose tissue were capable of improving renal structuring and function, and reducing renal injury and dysfunction by up-regulating the level of IL-10 expression (94). Although MSC-EVs have been observed to inhibit autoimmune diseases *in vitro*, there have been no studies on the use of MSC-EVs for the treatment of SLE in mice or humans. In the future, MSCs are expected to provide a novel and safe treatment for SLE patients.

4.3 Type-1 Diabetes

T1DM is considered a chronic autoimmune disease that is influenced by genetic, immune, and environmental factors (95). T1DM is primarily caused by the autoimmune destruction of beta-cells in the islets of Langerhans, leading to insufficient insulin secretion (96). Non-obese diabetic (NOD) mice are the preferred spontaneous disease model of T1DM. NOD mice exhibited the same clinical symptoms of diabetes as human beings-hyperglycemia, polyuria, and polydipsia (95). Although insulin therapy remains the main treatment method in the short term, MSCs have recently attracted wide attention as a promising method for the treatment of diabetes (96). MSCs display immunomodulatory properties in inflammatory diseases,

and their immunomodulatory effects have been studied in T1DM (97, 98). Human MSCs have the ability to delay the onset of autoimmune diabetes by inhibiting the development of Th1 cells, which may improve the efficacy of islet transplantation in patients with T1DM (99). Currently, it is generally believed that a paracrine mechanism of action is more direct in MSCs *in vivo*, particularly MSC-EXOs (10). In previous studies, the effect of MSC-EXOs on diabetes has been investigated. Previous studies have shown that exosomes released by BM-MSCs had similar functions to those of BM-MSCs, and they had the ability to improve cognitive impairment in diabetic mice by repairing damaged neurons and astrocytes (100). In another study, AD-MSC-EXOs were found to have a significant mitigatory effect on T1MD by increasing the expression of anti-inflammatory factors (e.g., IL-10), and the population of Tregs that are equipped to suppress the immune response, preventing immune overactivation and autoimmune damage (101). In addition, the results of the study by Shigemoto-kuroda et al. also confirmed that MSC-EVs could inhibit islet inflammation, significantly increasing the plasma insulin levels, and effectively delaying the occurrence of T1DM in mice (64). These results suggested that MSC-EVs have great potential as a cellular therapy for the prevention of T1DM.

4.4 Uveitis

Uveitis is the leading cause of visual disability worldwide. Uveitis can be divided into three categories according to etiology: 1) infectious uveitis; 2) non-infectious uveitis; and 3) masquerade uveitis, of which non-infectious uveitis is believed to be caused by autoimmunity, and EAU is used as a mouse model (102). Traditional immunosuppressive drugs (e.g., corticosteroids) and novel biological agents have been shown to be effective for the treatment of uveitis; however, side effects and unknown long-term safety often limit the use of these drugs (103). A large number of experimental results have suggested that MSC-EXOs have a positive effect on inflammatory eye disease. EAU mice that do not MSC-EXOs therapy have severely damaged retinal photoreceptors and the infiltration of inflammatory cells, whereas EAU mice with exosomes injected through tail vein displayed eyes similar to that of normal mouse retinas, with almost no structural damage and inflammatory infiltration (64). Compared with EAU mice treated with PBS, mice treated with MSC-EXOs exhibited a significant decrease in $CD3^+$ T cells infiltrating the retina and a decrease in the number of macrophages migrating to the retina (53). In addition, Th1 and Th17 cells represent important pathogenic factors in the development of EAU disease. Flow cytometry results of the cervical draining lymph nodes (CLNs) showed that the number of IFN-γ^+CD4^+ cells (Th1) and IL-17^+CD4^+ cells (Th17) in EAU mice treated with MSC-EXOs was significantly lower than that in the PBS-treated mice (64). The above results indicate that MSC-EXOs can inhibit the development of EAU by inhibiting Th1 and Th17 cells. Part of the immunomodulatory function of MSCs on EAU is to induce the transformation of $CD4^+$ T cells into antigen-specific $CD4^+CD25^+Foxp3^+$ Tregs by secreting TGF-β in a paracrine manner (104). In another study, MSC-EVs alleviated EAU by directly inhibiting the development of Th1 and Th17 cells rather than inducing Tregs to inhibit T cell proliferation (64). Although there may be some differences between MSC-EVs and MSCs in various immunomodulatory pathways, the treatment of EAU using MSC-EVs also represents a potential new method.

4.5 Rheumatoid Arthritis

RA is a chronic inflammatory disease characterized by synovial hyperplasia and immune cell infiltration, leading to joint destruction (105, 106). The microvesicles derived from BM-MSCs carry the regulatory molecules that exist in the mother cell, including PD-L1, GAL-1, and TGF-β1 (76). As a PD-1 receptor, PD-L1 plays a key role in regulating the development of inducible T regulatory cells (iTregs) (107). In addition, GAL-1 is an endogenous lectin that has been shown to induce growth arrest and the apoptosis of activated T cells, as well as the immunoregulation mediated by regulatory T cells (108, 109). Researchers found that the injection of co-gene DBA/1 fibroblasts secreting GAL-1 inhibited the progression of arthritis through T cell apoptosis in collagen-induced arthritis (110).

Moreover, exosomal miRNA also plays an important role in alleviating the development of RA (111). For example, MSC-derived miRNA-150-5p-expressing exosomes decreased the migration and invasion in RA fibroblast-like synoviocytes (FLS) and downregulated tube formation in HUVECs by targeting MMP14 and VEGF (112). In 2020, researchers found that BM-MSCs-secreted exosomal miRNA-320a and miRNA-192-5p also acted on FLS, reducing inflammatory response and alleviating the progression of RA (113, 114). Despite the pathogenic role of B cells in RA, recent studies have demonstrated the therapeutic effect of MSC-EXOs *via* expanding Bregs (48, 115). BM-MSC-EXOs-treated CIA mice exhibited a lower disease incidence and deceased clinical score, accompanied by reduced levels of serum auto-antibodies (115). Furthermore, such phenomena was associated with decreased plasmablast differentiation and the generation of Bregs (48). Interestingly, similar therapeutic effects have been revealed in osteoarthritis (OA). The lncRNA KLF3-AS1 was significantly enriched in MSC-EXOs, which promoted cartilage repair and chondrocyte proliferation in OA rat models (116). In addition, in a model of porcine synovitis induced by bovine serum albumin, the intraarticular injection of BM-MSC-EVs into pigs had an anti-inflammatory effect, with a reduced number of synovial lymphocytes and down-regulated level of TNF-α transcription (117). These results provide evidence for a role of MSC-EVs for the treatment of inflammatory diseases (e.g., arthritis). MSC-EVs provide novel insight into the treatment of RA, which may lead to new therapeutic opportunities and strategies for RA.

4.6 Inflammatory Bowel Disease

IBD is a chronic, nonspecific, relapsing inflammatory gastrointestinal disease associated with mucosal immune system disorders and gastrointestinal injury. IBD, which primarily includes ulcerative colitis (UC) and Crohn's disease (CD), has become a global disease with an increasing incidence (118). Studies of this disease have mainly used DSS and 2,4,6-trinitrobenzenesulfonic acid (TNBS) to induce IBD in mouse models (119). Existing drugs to treat IBD are still very limited,

Therefore, there is an urgent need to develop safe and effective treatments for IBD (120). At present, several studies have shown that MSC-EXOs/exosome components have potential functions in the development of IBD and can serve as potential targets for the diagnosis and treatment of IBD. In a model of IBD induced by DSS, hUC-MSC-EXOs treatment decreased the infiltration of macrophages in colon tissue and inhibited the expression of IL-7 (121). hUC-MSC-EXOs also inhibited neddylation and alleviated IBD by miR-326 (122). Another study also showed that exosomal miRNA of MSCs, such us miR-146a, downregulated TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) expression, inhibited pro-inflammatory cytokine and enhanced the expression of IL-10 (123).Moreover, tumor necrosis factor-α stimulated gene 6 (TSG-6), as detected by hUC-MSC-EXOs, regulated the immune response of Th2 and Th17 cells in mesenteric lymph nodes (MLN), down-regulated the levels of pro-inflammatory cytokines in colon tissue, and up-regulated the levels of anti-inflammatory cytokines to protect the intestinal barrier (119). Other tissue-derived MSC-EXOs showed similar efficacy to that of hUC-MSC-EXOs in the treatment of DSS induced IBD. OE-MSC-EXOs significantly improved the severity of experimental colitis in mice primarily by modulating the immune response of Th-cells, including a significant reduction in Th1/Th17 subsets and an increase in Treg cells (77). Neda Heidari et al. reported that AD-MSC-EXOs therapy could restore the percentage of Treg in the spleen to a baseline level similar to that in normal mice and improved inflammation in DSS induced acute colitis (124). Furthermore, metallothionein-2 in hBM-MSC-EXOs inhibited inflammatory responses, polarized M2b macrophages, and maintained intestinal barrier integrity (125). With the further research on exosomes, exosomal-structure design of novel drugs may provide new insights for IBD.

5 CONCLUSION AND PROSPECTS

The treatment of autoimmune diseases is challenging and there is currently no effective cure. In this review, we discussed the regulatory role of MSC-EXOs on immune cells and the new progress of MSC-EXOs in the treatment of autoimmune diseases, suggesting that MSC-EXOs may be a new cell-free drug for the treatment of autoimmune diseases.

In view of the therapeutic potential of MSC-EXOs in preclinical studies, there are currently 19 clinical trials looking at its application in a variety of diseases (Available online: http://www.clinicaltrials.gov/). Although encouraging results have been achieved with MSC-EXOs, several uncharted territories remain to be explored before MSC-EXOs can be used as drug vectors for clinical use. First, although it has been reported that clinical-grade exosomes can be produced using good manufacturing techniques and standard operating procedures, large-scale production of exosomes for clinical use remains to be explored (126). Second, support materials can be used to maximize the therapeutic power of MSC-EXOs. In recent years, hydrogel has attracted much attention among biocompatible auxiliary materials. In the experimental model of ischemia, the retention time of MSC-EXOs with an injectable hydrogel was prolonged, which enhanced the therapeutic effect of exosomes (127). In the experiment of preventing hyperplastic scar in rabbit ear model, adipose-derived stem cell conditioned Medium (ADSC-CM) was combined with polysaccharide hydrogel to prolong the therapeutic effect of cytokines (128). Third, in order to ensure the safety of patients treated with MSC-EXOs, the route and dose of exosomes must be explored. At present, the main route of administration in clinical studies is intravenous infusion. However, aerosol inhalation of MSC-EXOs was used in clinical trials to explore the efficacy of MSC-EXOs in severe pulmonary diseases (Clinical Trials. Gov Identifier: NCT04313647). In addition, the clinical trial assessing the safety and efficacy of MSC-EXOs in Patients with Alzheimer's disease was conducted with nasal drip (ClinicalTrials.gov Identifier: NCT04388982). Therefore, the route of exosome administration needs to be determined according to the actual situation of the disease. In addition, clinical trials need to monitor patients treated with MSC-EXOs in real time to ensure that the smallest dose is most effective. Therefore, future work should focus on the combination of basic research on MSC-EXOs with emerging technologies to bring new breakthroughs for the treatment of autoimmune diseases.

AUTHOR CONTRIBUTIONS

ZS, WH, and JL drafted and revised the manuscript. JT and SW discussed and revised the manuscript. KR conceived the topic and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Therapeutic Potential of Mesenchymal Stromal Cell-Derived Extracellular Vesicles in the Prevention of Organ Injuries Induced by Traumatic Hemorrhagic Shock

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Severe trauma is the principal cause of death among young people worldwide. Hemorrhagic shock is the leading cause of death after severe trauma. Traumatic hemorrhagic shock (THS) is a complex phenomenon associating an absolute hypovolemia secondary to a sudden and significant extravascular blood loss, tissue injury, and, eventually, hypoxemia. These phenomena are responsible of secondary injuries such as coagulopathy, endotheliopathy, microcirculation failure, inflammation, and immune activation. Collectively, these dysfunctions lead to secondary organ failures and multi-organ failure (MOF). The development of MOF after severe trauma is one of the leading causes of morbidity and mortality, where immunological dysfunction plays a central role. Damage-associated molecular patterns induce an early and exaggerated activation of innate immunity and a suppression of adaptive immunity. Severe complications are associated with a prolonged and dysregulated immune–inflammatory state. The current challenge in the management of THS patients is preventing organ injury, which currently has no etiological treatment available. Modulating the immune response is a potential therapeutic strategy for preventing the complications of THS. Mesenchymal stromal cells (MSCs) are multipotent cells found in a large number of adult tissues and used in clinical practice as therapeutic agents for immunomodulation and tissue repair. There is growing evidence that their efficiency is mainly attributed to the secretion of a wide range of bioactive molecules and extracellular vesicles (EVs). Indeed, different experimental studies revealed that MSC-derived EVs (MSC-EVs) could modulate local and systemic deleterious immune response. Therefore, these new cell-free therapeutic products, easily stored and available immediately, represent a tremendous opportunity in the emergency context of shock. In this review, the pathophysiological environment of THS and, in particular, the crosstalk between the immune system and organ function are described. The potential therapeutic benefits of MSCs or their EVs in treating THS are

discussed based on the current knowledge. Understanding the key mechanisms of immune deregulation leading to organ damage is a crucial element in order to optimize the preparation of EVs and potentiate their therapeutic effect.

Keywords: mesenchymal stromal cell, extracellular vesicles, inflammation, traumatic hemorrhagic shock, multi-organ failure, acute injury

1 INTRODUCTION

Severe trauma is the main cause of death among young people worldwide (1, 2), one-third being attributed to hemorrhage (3). In the military population, during modern conflicts, 90% of preventable deaths are of hemorrhagic origin (4).

Hemorrhage secondary to trauma is an emergency that can evolve into traumatic hemorrhagic shock (THS). Hemorrhagic shock in such condition is a complex association of tissue injuries and a severe hypovolemia due to blood loss. This leads to circulatory failure and inadequate tissue perfusion that induces a switch from aerobic to anaerobic metabolism (5). This phenomenon is responsible for secondary insults with tissue damage and inflammation, which can progress in the worst cases to organ dysfunction and multi-organ failure (MOF). The incidence of MOF is high in cases of severe trauma and remains a major cause of morbidity and mortality (≈33%) (6, 7).

Severe trauma is most often accompanied by significant tissue damage. Tissue attrition will rapidly lead to significant inflammation. The current challenge in the management of THS patients is preventing organ injuries, which currently have no etiological treatment available. Indeed, whereas post-hemorrhage resuscitation improves tissue perfusion, it does not treat the complex mechanisms that occur with reperfusion (ischemia/reperfusion, I/R) and activation of inflammatory and immune responses. Inflammatory and immune burst after trauma are major contributors of MOF (8, 9). The immune cells then become adherent to the vascular wall and decrease distal blood flow. These phenomena then induce tissue hypoperfusion, responsible for dysfunction of the microcirculation, hypoxia, and cellular acidosis, rapidly leading to organ failure and MOF. To improve the prognosis of patients, there is a critical need for new therapies to prevent and treat organ dysfunction and MOF after trauma.

Modulation of the immune and inflammatory response is a promising therapeutic strategy to treat complications of THS.

Mesenchymal stromal cells (MSCs) were discovered in the 1970s. Alexander Friedenstein, demonstrated the ability of culture-isolated fibroblast cells (now designated as MSCs) to recreate a hematopoietic environment *in vivo* after heterotopic grafting (10). These pioneering experiments provided the first clues to the existence of a cellular memory of the function they exerted in their original tissue. MSCs in the medullary microenvironment participate in the regulation of self-renewal and differentiation of hematopoietic stem cells (HSCs). More recently, clinical trials have shown that the co-graft of MSCs and HSCs allowed for better engraftment of HSCs while decreasing the risk of graft *vs.* host disease (GvHD) (11–14). Since then, many studies have shown the immunomodulatory capacities of MSCs in different contexts *in vitro* and *in vivo* and notably after trauma (15–17). MSCs exert their immunomodulation capacities by cell-to-cell contact or paracrine pathway *via* the secretion of various types of anti-inflammatory molecules and extracellular vesicles (EVs) (18).

In this review, we discuss the therapeutic potential and rationale for the application of EV-enriched MSC secretome for the prevention of organ injuries in an emergency context of THS.

2 TRAUMATIC HEMORRHAGIC SHOCK

2.1 Epidemiology

Hemorrhagic shock is responsible for 1.9 million deaths per year worldwide, 79% of which are caused by physical trauma (1). According to the World Health Organization, 5.8 million deaths per year are due to trauma, which represents 10% of the causes of death (19). The majority of deaths occur at the site of the trauma or in the first hours of medical management, mainly as a result of brain injury or circulatory collapse following hemorrhage. Hospital deaths are the result of sepsis or MOF (20, 21). In modern conflicts, blast injuries have become predominant and account for nearly 75% of combat casualties in Iraq and Afghanistan (22). These injuries mainly concern poorly protected areas (limbs and the head and neck axis) in 34% of cases (23). Among soldiers killed in action, 87% died before reaching a medical facility, 24% of these deaths being considered to be potentially preventable. More than 90% of these potentially preventable deaths are associated with hemorrhage (4). During the last decade, the strategy to decrease the mortality rate was to prevent pre-hospital exsanguination. This has been partially achieved by the large diffusion of massive bleeding control strategies based on compressive devices such as tourniquets (24). However, the time of the pre-hospital phase has been considerably increased in recent conflicts (Sahel), promoting the duration of the shock and the onset of complications (25, 26).

2.2 Pathophysiology

The pathophysiology of THS is complex. We describe this phenomenon from the clinical to the cellular aspect, then discuss the 2021 guidelines for the management of critically ill patients without comorbidity factors.

THS associates tissue trauma and hemorrhagic shock, a form of hypovolemic shock in which sudden and severe blood loss leads to inadequate oxygen delivery at the cellular level (5). Hypovolemia causes a drop in venous return, blood pressure,

and stroke volume. The clinical manifestations of shock include tachycardia, tachypnea, sweat, pallor, oliguria, and confusion. The clinical definition of shock associates one or several of these signs to a systolic blood pressure <90 mmHg. Metabolic cell activity is strongly dependent on the oxygen supply (DO_2). The dioxygen artery concentration (CaO_2) depends first on O_2 binding to hemoglobin (Hb) and dioxygen saturation (SaO_2) and, second, on dissolved (PaO_2) (27). During hemorrhage, DO_2 decreases because of a drop in Hb, cardiac output, or SaO_2. Because of this drop, aerobic cell metabolism switches from aerobic to anaerobic metabolism, allowing the cell to maintain a minimal energy production (cf. *Section 2.2.1*). To maintain a sufficient DO_2, the number of perfused capillaries increases (i.e., capillary recruitment) in proportion to the degree of tissue hypoxia, the oxygen extraction ratio increases, and regional vascular resistance is lowered to induce blood flow redistribution (28).

The adaptive mechanisms allowing the adaptation of the organism are neurological, renal, and hormonal. These can lead to the three phases of THS: compensated, decompensated and exceeded (29).

2.2.1 Compensated THS

In the compensated shock phase, tissue hypoperfusion is counterbalanced by adaptive mechanisms.

The decrease in blood pressure is quickly detected by cardiopulmonary and arterial baroreceptors that induce an increase in sympathetic activity, resulting in arteriolar and venous vasoconstriction and an increase in heart rate to preserve vital organs such as the heart, lungs, and brain (30, 31). The renin–angiotensin–aldosterone system is also activated. Angiotensin promotes a ubiquitous vasoconstriction and stimulates aldosterone and anti-diuretic hormone production, sympathetic heart stimulation, thirst sensation, and decreased glomerular filtration rate (GFR) (32). Altogether, these compensatory mechanisms maintain the cardiac output, perfusion pressure, and circulating volume. All cellular functions are maintained as long as the combined yields of the aerobic and anaerobic sources of energy provide sufficient ATP (28). Nevertheless, these compensations can be overwhelmed.

2.2.2 Decompensated THS

When blood loss reaches a critical level (30%–40%) (29, 30), the compensatory mechanisms are overwhelmed: there is a massive decrease of reflex-activated sympathetic drive and an increase in cardiac vagal drive, resulting in reductions in heart rate and arterial blood pressure and loss of peripheral resistances (30). Uncompensated THS resulting in irreversible tissue damage occurs when the combined aerobic and anaerobic ATP supplies are not sufficient to maintain cellular function (28).

2.2.3 Exceeded THS

This last phase is associated with a "no reflow," even if volemia is restored. Neutrophils adhere to the damaged endothelium, block capillaries, and aggravate local ischemic injuries. This worsens lesions such as coagulopathy, endotheliopathy, microcirculation failure, inflammation, and immune activation. All of these lead to secondary organ failure, MOF, and death (29).

2.3 From Cellular Insults to MOF

2.3.1 Cellular Insult Due to Ischemia/Reperfusion

The shift from aerobic to anaerobic metabolism results in the formation of lactate and protons and a decrease in ATP production. pH is maintained *via* H^+/Na^+ and Na^+/Ca^{2+} pumps, causing an elevation of cytosolic Ca^{2+} (33). Moreover, ATP production is insufficient to maintain the function of these pumps. A disruption of the mitochondrial architecture also occurs, which destabilizes the mitochondrial membrane potential. This membrane potential is further affected by the opening of the mitochondrial permeability transition pore and inner membrane anion channels, finally impairing ATP production (34). The damaged mitochondria are no longer able to efficiently reduce O_2 in H_2O in the electron transport chain, leading to reactive oxygen species (ROS) formation (35). Oxidative stress is usually defined as an imbalance between the production of ROS and antioxidants. The ensuing pathophysiological consequences and oxidative damages correspond to protein nitrosylation, lipid peroxidation, or DNA damage and can lead to cell death. Necrotic cells and damage to the extracellular matrix release various intracellular and extracellular molecules, which act as "alarmins" triggering inflammatory cascades (36).

2.3.2 Activation of Inflammation During THS

2.3.2.1 Alarm Signals

"Alarmins," among which damage-associated molecular patterns (DAMPs) are released with tissue injuries, trigger both an intense pro-inflammatory systemic immune response syndrome (SIRS) and a counterbalancing anti-inflammatory response syndrome (CARS) within 30 min post-injury (37). Every DAMP proven to induce efferent pro-inflammatory pathways can be involved in the development of SIRS (38). This highlights the critical role of DAMPs in SIRS-associated MOF following THS. Moreover, it has been recently described that suppressing inducible DAMPs (SAMPs) (39), mainly produced by activated leukocytes and macrophages upon stress and injury (e.g., lipid mediators such as prostaglandin E2 or annexin A1) (40, 41), could trigger the pro-resolving pathways in CARS. An excessive CARS could lead to posttraumatic immunosuppression. In this review, we mainly focus on the mechanisms of THS-induced SIRS. DAMPs are passively released by necrotic cells, but also actively secreted by stressed or activated cells (e.g., high mobility group box protein 1, HMGB1). Elevated levels of HMGB1 (42–46), mtDNA (47–52), heat shock proteins (53–57), Ca^{2+}-binding protein S100 (58), histones (59–63), ATP (64), interleukin 33 (IL-33) (65), or IL-1 (66) have been described after trauma in preclinical and clinical studies. DAMPs activate immune cells *via* their binding to pattern recognition receptors (PRRs), a group of receptors involved in the innate immune response, and induce the transcription of inflammatory factors (67, 68). Toll-like receptors (TLRs) form the most prominent group (69), and Nod-like receptors (NLRs) such as NLRP3 (70), receptor for

advanced glycation end products (RAGE) (71), and purinergic (72) or complement receptors (73) also contribute to inflammation. The activation of these receptors triggers multiple pathways, notably the tumor necrosis factor alpha (TNF-α)/nuclear factor kappa B (NF-κB)/c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling cascade (42, 66, 74–77), and the activation of NLRP3 inflammasome with production of IL-1β or IL-18 (78). In the case of major THS, the massive release of DAMPs may induce an excessive innate immune response, leading to coagulopathy, endothelial dysfunction, and an increase in vascular permeability, promoting the circulation of new DAMPs. This amplifies a vicious cycle of cell and tissue injuries that heightens the immunological response (73, 78). Resident inflammatory cells have the role of sentinels. They detect an increase in circulating DAMPs, and then they trigger the recruitment of circulating immune cells by releasing TNF-α, IL-6, IL-1β, etc. (34, 79, 80). DAMPs could also be secreted by activated immune cells such as neutrophils or monocytes and are also potent activators of the complement, leading to the generation of C3a, iC3b, and C5a (81, 82). Elevated plasma C3a, C5a, and C5b-9 levels correlate with trauma severity (83–85), and complement activation also contributes to neutrophil and monocyte recruitment (34).

2.3.2.2 Granulocytes: In the First Line

Knowledge of the immune changes during the early phase is limited. A study on severe trauma patients revealed a massive leukocytosis, elevated serum pro- and anti-inflammatory cytokines, and evidence of innate cell activation within minutes of trauma (86).

The SIRS-primed circulating neutrophils home to the tissues and become activated by local inflammatory stimuli (87). Notably, data obtained in a cohort of trauma patients suggest that circulating platelet-activating factor (PAF) and IL-8 are potential mechanisms of circulating neutrophil priming. Indeed, the use of a PAF antagonist inhibits neutrophils priming 3 h after injury, and plasmatic levels of IL-8 increase between 6 and 12 h after injury. Moreover, at 12 h, IL-8 may also be an early predictive marker of the onset of MOF (88). Circulating neutrophil activation is associated with reduced surface expressions of CXCR2 (CD182) and C5aR (CD88) 3–4 h after injury, followed by gradual restoration (86, 89). Then, the expressions of CD62L (L-selectin) and CXCR1 (CD181) start decreasing at about 4–12 h (86), and CD62L remains low at 24 h (90). These phenotypic changes are directly related with inflammation (87) and phagocytosis (91). C5aR promotes phagocytosis, and its expression is downregulated by the binding of C5a (92). Conversely, the expression of CD11b is increased (93). Traumatic injury also leads to marked alterations in the phenotype, function, and life span of circulating neutrophils (94–96).

Circulating neutrophil counts increased sharply 3 h after injury and then decreased within 12 h, suggesting end organ sequestration. The drop in circulating neutrophils was significantly greater in MOF than that in non-MOF patients (93). Neutrophils reach the damaged tissues by diapedesis in the post-capillary venules. Neutrophil binding to the endothelium is first controlled by selectins (CD62L that binds to CD62E and CD62P), which promote the initial rolling or tethering. Then, integrins (the β2 integrins CD11a and CD11b) induce firm adhesion. Examination of autopsy specimens from patients with MOF revealed the presence of neutrophils that varies from renal blood vessels to large-scale tissue infiltration of the lung (97). Neutrophil apoptosis was profoundly delayed in severely injured patients, as well as their tissue clearance, correlating with a high risk of MOF (98, 99). When neutrophils are exposed to pro-inflammatory signals, they release not only ROS and proteases but also neutrophil extracellular traps (NETs), which induce injuries in healthy tissues. During NETosis, neutrophils release decondensed chromatin and proteins including neutrophil elastase, cathepsin G, and myeloperoxidase (MPO), as well as histones in NETs (100–102), which participate in the pathophysiology of trauma (103). The level of circulating cell-free DNA (used as a marker of NET formation) is higher in SIRS trauma patients than that in healthy subjects (104, 105).

2.3.2.3 Antigen-Presenting Cells: Pivot of the Inflammatory Reaction

Antigen-presenting cells (APCs), such as dendritic cells (DCs) and monocytes/macrophages, are important effector cells whose functional capacities are deeply influenced during tissue-induced injury (**Figure 1**). After THS, resident inflammatory cells serve as sentinels, then circulating neutrophil recruitment is rapidly followed by monocytes and macrophages. DAMPs bind to macrophage PRRs, leading to their pro-inflammatory activation, and can also trigger inflammasome formation, which does not support any direct transcriptional activity but allows the caspase-1-dependent cleavage of pro-IL-1β and pro-IL-18 into mature forms (37). It was recently demonstrated that inflammasomes, like TLRs, could trigger innate immune responses to aggression.

Functional phenotypical changes of macrophages from pro-inflammatory (M1) to anti-inflammatory (M2) occur to support tissue repair at the damaged sites. The clearance of neutrophils in tissues by efferocytosis represents a central element in the induction of the M1-to-M2 switch (106). These M2 macrophages secrete growth factors and anti-inflammatory cytokines such as IL-10, transforming growth factor beta (TGF-β), and IGF-1, which enhance tissue remodeling (79, 107) mediators of resolution (e.g., lipoxins and resolvins) (108) and increase their expression of the receptors programmed cell death ligands 1 (PD-L1) and 2 (PD-L2) (109, 110). Within 2–4 h after injury, the activation of the p38 MAPK, ERK1/2, and JNK pathways triggers macrophage activation in the liver, which releases TNF-α, IL-6, and remarkably high levels of monocyte chemoattractant protein-1 (MCP-1) and keratinocyte-derived chemokine. Macrophages are the major producers of MCP-1 and IL-6 after trauma–hemorrhage and contribute, at least in part, to the trauma/hemorrhage-associated neutrophil infiltration (111, 112).

As observed in sepsis, suppression of the function of monocytes/macrophages is directly associated with the severity of trauma (113). SIRS and CARS occur concomitantly, but when the CARS is

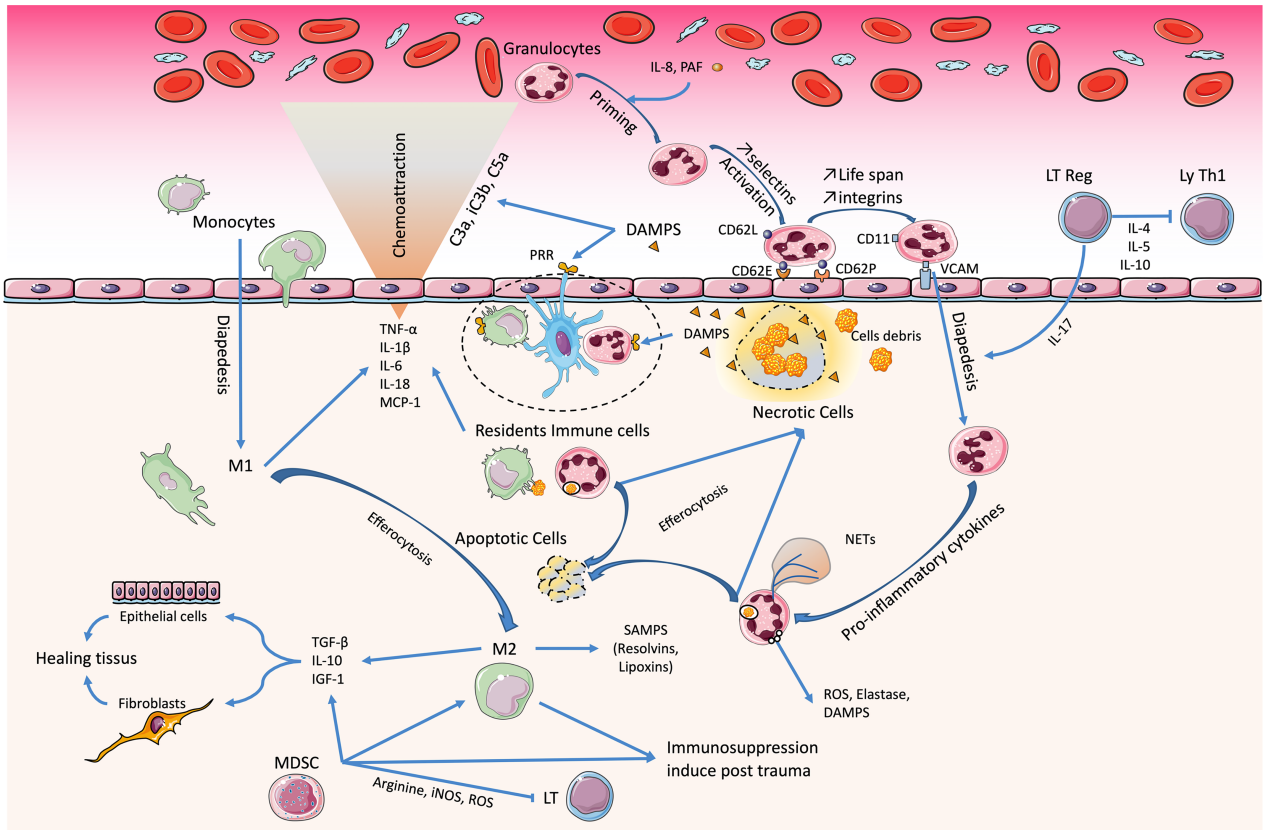

FIGURE 1 | Immunological imbalance during traumatic hemorrhagic shock (THS). Damage-associated molecular patterns (DAMPs) play a key role in pathophysiology. The resident immune cells detect them and carry out the first reactions of phagocytosis and amplification of the inflammatory response. The circulating granulocytes infiltrate the tissue and maintain this reaction. Later-onset macrophages are pivotal in resolving this inflammatory phase (M1→M2) and initiate the healing phase. However, during THS, the abundance of DAMPs promotes the acquisition and maintenance of a pro-inflammatory phenotype. The trio of Treg, platelets, and endothelial cells co-stimulates and causes immunomodulation, with inhibition of Th1 lymphocytes. Bone marrow dysfunction induces an immunosuppression that favors the occurrence of sepsis.

excessive or persistent, it promotes immunosuppression, secondary infections, and late or persisting organ dysfunctions (114). Macrophage dysfunction is a significant contributor to both innate and adaptive immune suppression (115). This suppressive function is related to a decrease in human leukocyte antigen DR (HLA-DR) and CD86 expression (116, 117). This impairment in the antigen presentation of macrophages appears early after injury and is maintained for several days (118–121). In addition, DCs, which represent the most potent APCs for the induction of primary T-cell responses, show a reduced responsiveness to bacterial components within a few hours after trauma–hemorrhage, secrete reduced levels of TNF-α and IL-6, as well as INF-γ, IL12, and IL-12p40, and are less potent to induce T-cell proliferation (122).

2.3.2.4 Bone Marrow Dysfunction

Maintaining the immune response following trauma requires the mobilization of bone marrow progenitors.

However, the formation of bone marrow granulocyte–macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and erythrocyte colony-forming units (CFU-E) was significantly reduced, while peripheral blood CFU-GM, BFU-E, and CFU-E were increased in trauma patients. Bone marrow stroma failed to grow to confluence by day 14 in >90% of trauma patients. These data indicate that trauma induces a bone marrow dysfunction that releases immature white blood cells into circulation and may also contribute to a failure to clear infection and an increased propensity to organ failure (123, 124). Moreover, in pathophysiological conditions such as trauma, a partial blockade in the differentiation of immature myeloid cells into mature myeloid cells results in an expansion of this population called myeloid-derived suppressor cells (MDSCs), which have remarkable ability to suppress T-cell responses and to modulate macrophage cytokines (125). Moreover, MDSCs, like all APCs, interact and modulate the behavior of the adaptive immune system, notably T helper (Th) lymphocytes *via* major histocompatibility complex class II (MHCII), CD40, CD80, or CD86. MDSCs express low concentrations of MHCII and CD80/CD86 (126). The expansion of MDSC populations is proportional to the severity of the inflammatory insult (126, 127). Therefore, MDSCs could contribute to the post-trauma immunosuppression leading to the development of late sepsis and MOF (128).

2.3.2.5 Adaptive Immune Response

The persistence of high levels of pro- and anti-inflammatory cytokines promotes T-cell exhaustion. There is a progressive decrease in the ability of T cells to produce cytokines (IFN-γ and TNF-α), higher expressions of CD28 and PD1 on $CD4^+$ and a lower expression of CD127 on T cells, a loss of proliferative capacity, and a decreased cytotoxicity, which can lead to apoptotic cell death (129). Lymphocyte apoptosis occurs early after severe trauma and usually peaks at day 3 after the injury. There is a correlation between the injury severity score (ISS) and lymphopenia, aggravating the risk of subsequent major infection and sepsis (130). Apoptosis affects more the $CD4^+$ and natural killer (NK) T lymphocytes than the $CD8^+$. In contrast, the $CD4^+/CD25^+$ lymphocyte populations, regulatory T cells (Tregs), are more resistant to sepsis or burn-induced apoptosis (129, 131). Tregs are important mediators of the suppression of T-cell activation and the reduction in Th1 cytokine production after injury (132). Tregs also play a role in regulating neutrophils during I/R by modulating, for example, their sequestration diapedesis (133).

2.3.2.6 Imbalance of Immunological Response

A leukocyte "genomic storm" occurs in critically injured patients, in which up to 80% of the leukocyte transcripts were altered in the first 12 h. It activates a large number of inflammatory mediators or pattern recognition receptors, but also suppresses genes involved in antigen presentation, T-cell proliferation and apoptosis, T-cell receptor function, or NK cell function (**Figure 2**). The unfavorable clinical course of the patients correlates with a higher and longer duration of expression of these genes (28 days, against 7–14 days for a favorable course), but not with the expression pattern (134). These results are consistent with another study describing an increase in blood Th17 $CD4^+$ T cells and peripheral monocytes, as well as changes in the NK profile, and the plasma increase in IL-17F and IL-22, TNF-α, IFN-γ, and MCP-1 at 5 days of trauma (135). This

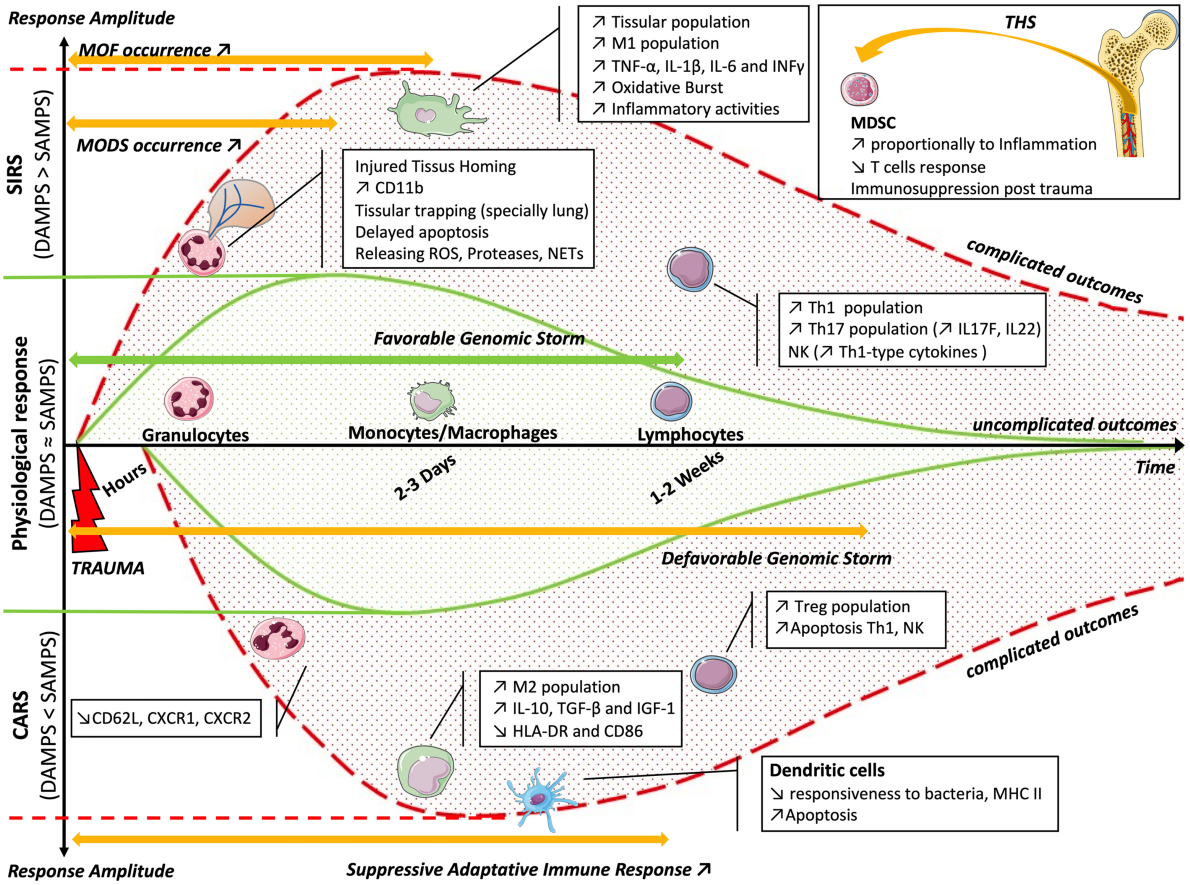

FIGURE 2 | Balance of the inflammatory reaction (CARS or SIRS) as a function of time. The solid green curves represent the physiological response, following the favorable genomic storm and the balance between the effects of DAMPs and SAMPs. In the case of imbalance, the genomic storm becomes unfavorable. The *upper dotted red curve* represents the imbalance toward SIRS, with an increased effect of DAMPs, appearance of MODS and MOF, and cellular modifications. The *lower dotted red curve* represents the imbalance toward CARS, with an increased effect of SAMPs, appearance of suppressive adaptive immune response, and cellular changes. The *box* summarizes bone marrow dysfunction during THS. *CARS*, counterbalancing anti-inflammatory response syndrome; *SIRS*, systemic immune response syndrome; *DAMPs*, damage-associated molecular patterns; *SAMPs*, suppressing inducible DAMPs; *MODS*, multi-organ dysfunction syndrome; *MOF*, multi-organ failure; *THS*, traumatic hemorrhagic shock.

suggests that it is illusory to imagine finding a specific marker or a single therapeutic agent that allows avoiding complicated outcomes of patients.

There is a concomitant and synchronous evolution of SIRS and CARS. To restore homeostasis, their evolution must be mirrored. If not, there is an imbalance on the SIRS side and, therefore, the appearance of deregulated inflammation, and even an MOF, or there is an imbalance on the CARS side and the occurrence of infection or delayed healing.

This balance could be the target of therapeutic strategies and help improve the prognosis of patients in the medium and long term after THS (**Figure 2**). Cell therapy or therapy by EVs could therefore be an interesting future strategy in this field.

2.3.3 Microvascular Dysfunction, Endotheliopathy, and Coagulopathy

2.3.3.1 Microvascular Dysfunction

Microcirculation is made up of three levels: arterioles, capillaries, and venules. All three are affected during THS. The vasoconstriction induced by epinephrine maintains local hypoxia and limits tissue exchange and, therefore, the clearance of lactic acid, for example. This association—coagulopathy, inflammation, anaerobiosis, and oxidation—promotes endotheliopathy (3). In this case, the arteriolar endothelium exhibits a dysfunction in relaxation linked to the local overproduction of ROS by CD11/CD18$^+$ cells. In the capillaries, there is an adhesion of activated leukocytes to damaged endothelial cells. There is also a local exudate (**Figure 3**).

The endothelium of post-capillary venules plays a key role in the onset of complications secondary to THS. Firstly, ROS cause complement (C5) activation and the production of several factors (PAF and leukotriene B4), which are able to induce the adhesion and activation of leukocytes on the endothelium. ROS also induce the release of Weibel–Palade bodies, which are large endothelial vesicles that stock von Willebrand factor (vWF) and P-selectin. Then, ROS lead to the production, *via* the NFκB and AP-1 pathways, of E-selectin, intercellular adhesion molecules (ICAM), or even vascular cell adhesion molecules (VCAM). These elements allow the adhesion and diapedesis of CD11/CD18$^+$ activated cells such as neutrophils. The inflammatory response is amplified by mast cells and macrophages, which release inflammatory mediators like TNF-α, nitric oxide (NO), histamine, or ROS. All these elements limit downstream blood flow, called microcirculation failure (136, 137).

2.3.3.2 Endotheliopathy

Ischemia and inflammation often result in the disruption of endothelial tight junctions, adherent junctions and glycocalyx

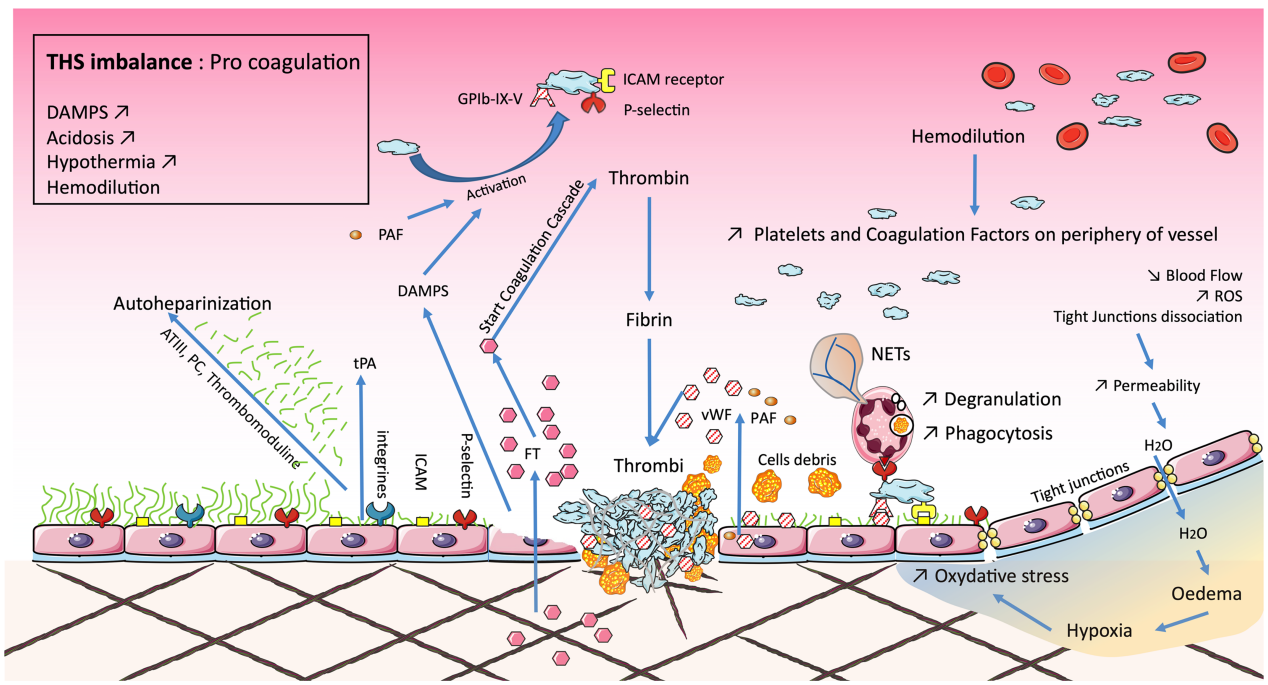

FIGURE 3 | Microvascular dysfunction occurring during traumatic hemorrhagic shock (THS) induces the permeability of tight junctions, responsible for edema, increased oxidative stress, and, ultimately, local inflammation. Endotheliopathy is either direct from tissue damage or secondary to microvascular dysfunction. Endothelial damage degrades the glycocalyx resulting in local autoheparinization. The shedding of the glycocalyx exposes integrins and selectins, promoting the adhesion of platelets and polynuclear neutrophils. Their association stimulates endothelial cells, which release factors such as sCD40L, von Willebrand factor (vWF), and platelet-activating factor (PAF). Endothelial damage is also associated with the release of damage-associated molecular patterns (DAMPs) and tissue factor (TF). This activates the coagulation cascade reducing the downstream blood flow, forming the bed of coagulopathy in 15% of cases, the latter leading to disseminated intravascular coagulopathy (DIC). These phenomena are associated with the lethal triad: coagulopathy, acidosis, and hypothermia.

components (138–140). The decrease in blood flow is a mechanical stimulus inducing the activation of the adhesion molecule PECAM, vascular endothelial growth factor (VEGF) receptors, and VE-cadherin, which results in the depolarization of the endothelial cell membrane and subsequent ROS generation. These events finally disrupt the integrity of the endothelial cell–cell junction and compromise the endothelial barrier, leading to hyperpermeability (141).

The glycocalyx is an intravascular coat composed of glycosaminoglycans (e.g., heparan sulfate) and proteoglycans (e.g., syndecan) (142). The thickness of the glycocalyx decreases during hemorrhagic shock, in proportion to the reduction in blood flow (143). During I/R, glycocalyx shedding increases the circulating blood concentrations of syndecan-1 (which is highly associated with mortality) (144) and heparan sulfate (145). This results in the exposure of the injured endothelium to pro-inflammatory leukocytes, leading to the alteration of its structural integrity and hyperpermeability (146). Activated neutrophils cause glycocalyx disruption during trauma because they release proteolytic enzymes such as neutrophil elastase and degranulation, which promotes local inducible nitric oxide synthase (iNOS) or ROS synthesis (143).

2.3.3.3 Coagulopathy

Coagulopathy occurs in up to 15% of THS patients. It worsens the bleeding and is associated with excess mortality (139, 147). Tissue factor (TF) is the key element in initiating the coagulation cascade. Tissue damage exposes both TF and collagen, capable of binding factor VII and vWF (148), respectively, and initiating coagulation. At the damaged vascular site, the platelets come into contact with the thrombin formed during the initiation phase of the coagulation cascade and are then massively activated. Activated endothelial cells become procoagulant by secretion of plasminogen activator inhibitor-1 (PAI-1). Moreover, the damaged glycocalyx exposes P-selectin or ICAM-1, favoring platelet and neutrophil adhesion, respectively. In turn, neutrophils promote local fibrin activation and platelet adhesion (143). Observational data suggest a correlation between high levels of circulating syndecan-1 and higher catecholamines, IL-6, IL-10, histone-complexed DNA fragments, HMGB1, thrombomodulin, D-dimer, tissue plasminogen activator (tPA), and urokinase plasminogen activator and a threefold increased mortality (139). In addition, hypotension and hypovolemia during THS cause the release of tPA by endothelial cells (3). This could limit the procoagulant effects of the activated endothelium proteins (e.g., protein C and protein S), which inhibit the coagulation pathways and prevent an inappropriate extension of coagulation beyond the damaged vascular site. Nevertheless, this equilibrium may be broken and trigger trauma-associated coagulopathy. As previously described in the literature (139), there is a continuum between local and initial coagulopathy and disseminated intravascular coagulopathy (DIC), which appears later (hours/day). This DIC is the consequence of extensive trauma, overwhelmed anticoagulant capacity, and major inflammation. Coagulopathy is aggravated as part of the lethal triad *via* acidosis and hypothermia, which are traumatic or iatrogenic, but also by hypovolemia (149–151).

2.4 Multiple Organ Failure

MOF is defined as alterations in the function of at least two organ systems, ranging from mild dysfunction to irreversible failure. Risk factors are related to the severity, type, and distribution of injuries (thoracic trauma), as well as the duration of hemorrhagic shock (152). As described previously, the pathogenesis of MOF is complex, with interrelated mechanisms involving neurohumoral and cellular cascades leading to generalized inflammatory reaction, capillary damage and permeability, interstitial edema, and, finally, organ dysfunction/failure (153). MOF should be distinguished from multi-organ dysfunction syndrome (MODS), which occurs frequently during resuscitation. Much of these early organ dysfunctions return to normal within 48 h of injury. The peak of MOF occurs within the first 3 days after injury. Disparate patterns were described: early MOF occurring within the first 3 days post-injury depending on shock severity, carrying high mortality, or late MOF whose incidence increases with age (154, 155). A retrospective study showed that lung failure was the most common organ failure, whereas cardiac and pulmonary system dysfunction decreased and renal and liver failures persisted at similar levels (155). Liver, kidney, or gastrointestinal tract injuries are directly linked to blood flow redistribution to vital organs such as the brain and heart after THS (155, 156).

A large number of scoring systems have been proposed to define MOF, without gold standard. All scoring systems [Denver, Marshall, and Sequential Organ Failure Assessment (SOFA)] include at least the monitoring of cardiac (e.g., mean arterial pressure), respiratory (e.g., PaO_2/FiO_2), hepatic (e.g., bilirubin), and renal (e.g., creatinine) functions (157, 158). Serum cytokine expression evaluated each 4 h during 24 h on 48 trauma patients revealed six candidate predictors of MOF occurrence: CXCL10, macrophage inflammatory protein-1 (MIP-1), IL-10, IL-6, IL-1Ra, and eotaxin (159), and the IL-4, IL-6, IL-8, and TNF-α levels are predictors of unfavorable outcomes (160).

However, depending on the type of scoring used and the classification of patients, retrospective studies can have very different conclusions. For example, over the years from around 2000 to 2010, some indicate a decrease in the incidence of MOFs with a MOF-related death rate that did not change. In contrast, others observed a significant increase of MOF prevalence and a decrease of mortality after multiple trauma and notably in the subgroup with MOF (84, 155).

2.4.1 THS-Induced Intestinal Injury

The gastrointestinal tract and the tissues vascularized by the superior mesenteric artery are particularly sensitive to reduced blood perfusion (156). The loss of gut barrier integrity is hypothesized to be the "motor" of MOF by allowing the translocation of organisms from the external environment (including not only bacteria but also proteolytic enzymes) and by limiting systemic access for necessary nutrients (161, 162). Therefore, prevention of gut injury associated with intestinal ischemia could be a key therapeutic strategy. The decrease of

mesenteric perfusion after THS leads to hypoxia of the villi (73). DAMPs govern the activation of resident leukocytes, the recruitment of circulating leukocytes, and also the activation of local and systemic complement (45, 82, 163). Inflammatory response, ROS production, and intraluminal pancreatic proteases also lead to mucus layer injury (164–166). Loss of the mucus layer was associated with increased gut permeability (164, 165, 167). Critical illness has a profound effect on the number of cells in the mucosal immune system (161). The lamina propria contains enteric glial cells (EGCs), and DCs; they can both recognize DAMPs and pathogen-associated molecular pattern molecules (PAMPs). EGCs are central in the homeostasis of the intestinal epithelium (168). Moreover, tissue damage can drive the dysregulation of pro-inflammatory group 3 innate lymphoid cell (ILC3s) response, which can contribute to immunopathology (169). In contrast, successful integration of environmental cues by ILC3s allows homeostasis of the gut–blood barrier by the production of IL-22. This route allows the restoration of local intestinal homeostasis after trauma (73, 169). It was demonstrated that severe THS caused an increase in bacterial translocation from the gut to the blood and organs, such as the liver and spleen. Moreover, it induces a modification toward a naive Th2 phenotype of $CD4^+$ and a tolerogenic phenotype of DC in mesenteric lymphatic nodes, which is consistent with the clinical forms of immunosuppression observed in severe patients (170). Interestingly, a recent study of gut I/R showed that mice displayed a significant inflammatory response with neutrophil infiltration into mucosal areas, but also in the lung. Mesenteric lymph duct ligation, which had no effect on gut injury, attenuated lung injury following gut I/R. This study highlights the central role of the gut in the development of systemic inflammation and MOF, including acute lung injury (ALI) (171). Thus, the digestive tract can be both an instigator and a victim of MOF (172).

2.4.2 THS-Induced ALI

ALI and acute respiratory distress syndrome (ARDS) are serious complications of traumatic injury. ALI/ARDS constitute a pathophysiological continuum that is defined by a lung disease with acute onset, non-cardiac, diffuse bilateral pulmonary infiltrates and a PaO_2/FiO_2 ≤300 for ALI or ≤200 for ARDS (173).

In a recent study, 30% of patients developed ARDS as a result of trauma, with a death rate three times higher. Lung damage can be caused by pulmonary contusion, shock, administration of blood products including platelets, and an element of volume overload that can occur in the presence of increased pulmonary vascular permeability (174). Following hemorrhagic shock, neutrophils (activated *via* NF-kB and NLRP3 signaling) and macrophages (*via* HMGB1/TLR4 pathway) induce pulmonary inflammation (175). Moreover, in a model of THS lymph-induced ALI, the lung injury was totally abrogated in neutrophil-depleted animals (176). This inflammation locally damaged tight junctions and endothelial cells and ultimately led to the production of edema and the deterioration of capillary alveolar exchanges (107). Furthermore, pulmonary edema is aggravated by the decreased production of surfactant by injured endothelial cells (177).

2.4.3 THS-Induced Acute Liver Injury

During hemorrhage, the spongy hepatic structure and vascular response, modulated by hepatic sympathetic nerves, could temporarily compensate for the volume of blood lost. Moreover, hepatic glycogenosis could also compensate for hypovolemia by an osmotic effect toward the vessels (178). Nevertheless, THS inducing liver ischemia rapidly leads to endothelial and hepatocyte cell death (179). The diagnostic evaluation includes a combination of biochemical tests with, for example, the determination of serum hepatobiliary enzymatic activities [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and gamma-glutamyl transpeptidase (GGT). Histopathologic changes include cellular swelling, vacuolization, endothelial cell disruption, neutrophil infiltration, and hepatocellular necrosis (180, 181). Following THS, the number of activated Ito cells (perisinusoidal fat-storing cells, stellate cells, and lipocytes) and Kupffer cells (KCs, resident hepatic macrophages) are increased. Activated KCs migrate from hepatic sinusoids into the injury areas, increase phagocytosis, and release ROS and various cytokines such as TNF-α, IL-1, IL-6, or IFN-γ (34, 181). This results notably in neutrophil activation and their sequestration in different vascular beds of the liver (156, 180). Neutrophil release NETs, proteases, and ROS, inducing hepatocyte injury and their release of DAMPs (133, 182). DAMPs (the most described in the liver is HMGB1) and also the complement pathway can activate KCs. The pro-inflammatory cytokines and ROS released by activated KCs also exert cytotoxic effects by inducing changes in the cell membrane receptors of hepatocytes and endothelial cells. They also activate other KCs and produce chemotactic factors for neutrophils and $CD4^+$ lymphocytes (181, 183–187), which aggravate microvascular/hepatocellular injury by the formation of cellular thrombi (133).

2.4.4 THS-Induced AKI

The incidence of acute kidney injury (AKI) is indicated at 13% in trauma and increases to 42.5% in THS; 96% of AKI appear within the first 5 days (188). AKI is the clinical endpoint of multiple processes and results in a decrease in the GFR, which is a measure of global renal function. The injury mechanisms identified are I/R, inflammation, and rhabdomyolysis. In the nephron, the glomerulus is exposed to vasoconstriction of the afferent glomerulus artery, resulting in a decrease in the GFR by injury of the glomerular–blood barrier. Cellular debris can precipitate in the tubule, further decreasing renal filtration and reabsorption. I/R injury is among the most common causes of AKI, and the underlying pathogenesis involves injury to the nephron by both ischemia and oxidative stress survival/death mechanisms. Proximal renal tubular cells along the nephron segments are particularly sensitive to hypoxia. One of the early events in renal I/R is the activation of the endothelium (increased expressions of E-selectin, ICAM-1, and CX3CL1), increasing vascular permeability and promoting leukocyte extravasation. Moreover, tubular epithelial cells increase complement binding and upregulate TLRs, leading to cytokine/chemokine production. A study in patients suffering from AKI post-blunt trauma showed a rapid increase in concentrations on D0 (time of

measurements after injury within the first 12 h = D0, 24–96 h = D1–D4, and ≥96 h ≥ D4) in inflammatory factors [e.g., IL-8, MCP-1 (alias CCL2), and IL-6] and anti-inflammatory factor (e.g., IL-1ra), followed by a drop on D4, in IL-1ra, IL-4, and IL-6 (189). In the tubules, neutrophils are observed between 3 and 24 h, followed by an ascending plateau up to 72 h after I/R injury (190). Macrophages are recruited at D1, with a peak at D5. The M1 is the dominant population from D1 to D3, then the M2 from D5 to D7. The authors demonstrated the role of M1 in the onset of tissue lesions and more of M2 in tubular repair (191). The I/R model also induces a maturation of the DC phenotypes and their production of TNF-α, IL-6, or MCP-1 in the first 24 h (192). Moreover, the injured epithelium releases fraktaline, which recruits more DCs (193). Finally, the kidney disposes type 2 innate lymphoid cells (ILC-2 cells), which appear to be involved in the anti-inflammatory phase. ILC-2 releases IL-4 and IL-13, allowing the polarization of macrophages and lymphocytes to M2 and Th2/Treg phenotypes, respectively (194). Finally, rhabdomyolysis is a classic complication of severe trauma ranging from the elevation of serum myoglobin and creatinine kinase (CK) activity to AKI and disseminated intravascular coagulation. It induces disturbances in intracellular ionic gradients, leading to increased concentrations of intracellular Ca^{2+}. The pathogenesis of AKI by rhabdomyolysis involves myoglobin-induced intrarenal vasoconstriction, direct ischemic injury, and tubular obstruction (195). Moreover, in a model of rhabdomyolysis-induced AKI, the heme-activated platelets enhanced the production of macrophage extracellular traps (METs) by increasing intracellular ROS generation and histone citrullination (196). There is a need today to find new therapies to prevent/treat kidney damage in order to avoid the clinical consequences associated with AKI and progress to chronic renal failure (197).

3 CURRENT MANAGEMENT OF THS

3.1 Current Support

The current management of hemorrhagic shock is based on two main pillars: stopping the bleeding and damage control resuscitation. This is applied during the pre-hospital and intrahospital phases (198). Bleeding control is first achieved by local compression, placing tourniquets or hemostatic dressings. The definitive management of these wounds requires surgical hemostasis (150, 198). The aim of damage control resuscitation is to maintain permissive hypotension (80–90 mmHg) as long as surgical hemostasis is not achieved; it is a compromise between tissue perfusion and aggressive resuscitation with high doses of fluids (199). Moreover, this limits hemodilution by overfilling, which helps maintain DO_2 above the critical limit (<8/10 ml min^{-1} kg^{-1}) (28, 198).

Preserving blood pressure begins with vascular filling. It is recommended to use a plasma first (200). Treatment with plasma during massive bleeding allows restoration of the glycocalyx (145). The use of vasopressors or sympathomimetics is only recommended as a second-line treatment (198, 201–203). Hemoglobinemia is not the only criterion for optimal transfusion. It is recommended to start the plasma transfusion at the same time, with a plasma/blood cell ratio between 1:2 and 1:1. Platelet infusion should be administered to maintain a minimum count, depending on the clinical situation (150, 198). To finish, tranexamic acid must be used before the third hour after THS for anti-fibrinolytic action. Other treatments such as coagulation factor concentrate, fibrinogen supplementation, or calcium supplementation could be used against coagulopathy (150, 198).

3.2 Frontiers in Current Management

The complexity and heterogeneity of the multiple factors involved in the pathophysiology of THS can give rise to MOF despite constant improvement in patient care. Deregulations of the immune system are at the heart of systemic deregulations after injury; therefore, modulating the immune response is a promising therapeutic strategy for preventing the complications of THS.

Preclinical and clinical proof-of-concept studies have analyzed the efficacy of new and emerging therapeutic candidates in the context of individual organ failure. Although informative, these studies do not address the full complexity of THS. Hence, hypothesis-driven research studies targeting the multi-organ dysfunction of THS are urgently needed. The therapeutic potential of MSC therapy has been well characterized and demonstrated to improve tissue function and regeneration. The established immunomodulation capacity and ability to restore tissue damage may also be applied in the treatment of THS-induced MOF. THS is a life-threatening emergency requiring immediate medical intervention. While cell-based therapy carries multiple advantages, the drawback is the delay of the supply of MSCs that require *in vitro* expansion and the complex storage and transport before administration. EVs, on the other hand, are secretory products of MCS. The major advantage of using cell derivatives rather than cells is the immediate availability of the product, which may be prepared, amplified, characterized, and easily stored for future use in the emergency context of THS patients. Existing evidence indicates that MSC-derived EVs are able to prevent immunological disturbances that lead to organ failure.

4 MSC-DERIVED EXTRACELLULAR VESICLES: TOWARD CELL-FREE THERAPEUTIC APPLICATIONS

4.1 Mesenchymal Stromal Cells

MSCs have been described since 1970 (204). These cells of mesenchymal origin have been found in both perinatal tissues and numerous adult tissues (205). Although isolated from various tissues, they share common properties described in 2006 by the International Society for Cellular Therapy (ISCT), which proposed minimal criteria to define MSCs. These plastic-adherent fibroblastic-like cells express a panel of antigenic surface markers (positive for CD73, CD90, and CD105 and negative for hematopoietic markers) and have an *in vitro* multipotency

capacity in the three canonical pathways: osteoblastic, chondroblastic, and adipocytic (206). They have many capacities: trophic support and immunomodulation, as described above, but also anti-apoptosis, pro-angiogenesis, or even antioxidation (207). MSCs were first described as key regulators of the HSC niche homeostasis. Later, in the 2000s, it has been described that allogeneic MSC transplantation given intravenously is well tolerated (11), can promote hematopoietic engraftment (208), accelerate lymphocyte recovery (209), reduce the risk of graft failure, and reduce the incidence of GvHD (12, 210). MSCs can modulate innate immunity by promoting the repolarization of monocytes and macrophages from a type 1 (pro-inflammatory) to a type 2 (anti-inflammatory) (211), by suppressing the proliferation, cytokine secretion, and NK cell cytotoxicity (212), and by inhibiting the maturation and migration of DCs (213), as well as modulate polymorphonuclear cell apoptosis and activity (214). MSCs can also modulate both adaptive immune effector activity by inhibition of T-cell (215) and B-cell (216) functions. These data open the way to their utilization as cell therapy products in degenerative and/or inflammatory diseases lacking appropriate treatments (217). Presently, hundreds of clinical trials are using MSCs to evaluate their therapeutic effects in numerous severe diseases (217). The first clinical trial in this context, using the systemic administration of allogeneic MSCs, did not exacerbate the elevated cytokine levels in the plasma of septic shock patients, consistent with a safe response. This cohort also revealed patient-specific and dose-dependent perturbations in cytokines, including an early but transient dampening of pro-inflammatory cytokines (218).

This immunomodulation potential has been extensively documented. Caplan and Dennis (219), in 2006, postulated that MSCs could mediate their therapeutic activity *via* the secretion of soluble factors such as prostaglandin E2 (40), IL-1 receptor antagonist (IL-1RA) (220), TGF-β (221), hepatocyte growth factor (HGF) (222), indoleamine 2,3-dioxygenase (IDO) (223), or tumor necrosis factor-stimulated gene 6 (TSG-6) (224–226) rather than by direct cellular interactions. In 2007, it was then demonstrated that MSC-conditioned media rich in small EVs could exert cardioprotective effects in a myocardial infarction model (227). Another team described the beneficial effects of MSC-conditioned media enriched with larger EVs in a mouse model of AKI (228). Consequently, today, there is a growing interest in MSC-derived EVs (MSC-EVs). More recently, it was also demonstrated that MSC-EVs can be a promising therapy for preventing chronic GvHD by exhibiting potent immunomodulatory effects (229, 230). Moreover, in several preclinical studies, it was shown that MSC-EV therapy reduced inflammation in kidney injury animal models (231) and decreased the inflammatory cell influx, altering alveolar macrophages toward an anti-inflammatory phenotype in lung injury models (232, 233) or the pro-inflammatory cytokine messenger RNA (mRNA) levels in liver injury (234). Finally, it is important to understand that MSCs are sensitive to their environment. Their properties and those of their by-products may vary depending on growing conditions, known as the concept of "priming" (235, 236). Stimulating MSCs with pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1α, or IL-1β induces the secretion of soluble or EV-encapsulated anti-inflammatory factors (237–240). Interestingly, the secretome of MSCs primed with IL-1β and the sera of polytrauma patients share important characteristics. IL-1β priming enhances the secretion of pro-inflammatory and pro-angiogenic factors (IL-6 and VEGFA) and chemokines (CXCL1 and CCL2). Moreover, MSC-IL-1β priming may improve their therapeutic effects by inducting cell adhesion molecules and anti-inflammatory and anti-fibrotic molecules (241).

The few studies using MSCs in THS showed that their administration early after hemorrhagic +/− traumatic shock limited vascular permeability by preserving the barrier junction proteins (VE-cadherin, claudin-1, and occludin-1), inhibiting the expressions of leukocyte adhesion molecules (VCAM-1 and ICAM-1) on endothelial cells, and decreasing both serum concentrations of inflammatory molecules and CD68- and MPO-positive cell tissue infiltration (17). We recently showed that IL-1β-primed MSCs attenuated hemorrhagic shock-induced early hepatic and kidney injury and dysfunction and reduced the SIRS/CARS syndrome, as shown by the decreases in the plasma cytokine concentrations and the phenotypic activation of circulating $CD11bc^{+}$ cells (242). MSCs would also prevent the decrease in hematopoietic progenitors induced by THS in the bone marrow (15, 17, 243). Whether the use of MSCs could alleviate or potentially exacerbate THS-induced coagulopathy is unclear. MSCs express TF (244, 245) and phosphatidylserine (246), which are thrombogenic and tend to increase the clotting rates. MSCs can therefore behave as beneficial hemostatic agents, but can also be excessively procoagulant, depending on the dose, the time of administration, and the method of preparation, which, in this case, may require the concomitant administration of anticoagulants to prevent venous thromboembolism or disseminated coagulopathy (247). Moreover, prothrombotic factors on their surface could trigger the instant blood-mediated inflammatory reaction (IBMIR) after blood exposure. IBMIR is characterized by the activation of complement/coagulation cascades, the binding of activated platelets to the MSCs, and clot infiltration by neutrophils and monocytes, which could lead to cell destruction. It is important to note that the induction of IBMIR depends on the MSC source and the dose administered and increases when their *in vitro* expansion has been high (passages 5 to 8) (245). This means that the choice of the modes of preparation and administration of MSCs can modulate their thrombotic activity. In contrast, cultured fibrin-embedded human MSCs can dissolve the surrounding fibrin mesh. This fibrinolytic capacity may be related to the transcriptional expression of the urokinase plasminogen activator (uPA) and its receptor (uPAR), the tPA, and the PAI (248). In conclusion, MSCs are being employed as an experimental therapy in a variety of human diseases and represent an important hope in the context of lesions induced by THS. They act on several biological processes including inflammation and reprogramming of immune cells, but also by the activation of endogenous repair pathways. Current dogma indicates that they improve disease through the secretion of paracrine-acting factors and, more recently, *via* the production of EVs.

In the emergency context of THS, requiring very quick availability of the therapeutic product, a ready-to-use EV solution, appears to be a particularly interesting innovative strategy.

4.2 MSC-Derived EVs

4.2.1 EV Definition

Cells use multiple and sophisticated modes of communication. Besides direct cellular communication through the expression of cell surface markers, they communicate not only by the secretion of soluble molecules but also *via* the production of EVs. The term "extracellular vesicle" corresponds to a generic term that refers to particles naturally released from the cells, delineated by a lipid bilayer, and are devoid of replicative activity (i.e., without functional nucleus). The three main EV subtypes found in the literature include microvesicles (MVs; also known as microparticles or ectosomes), exosomes (exo), and apoptotic bodies. They are characterized by their size (small vesicles, <100–200 nm; medium/large, >200 nm), density, cellular origin, and their biochemical composition (tetraspanin, annexin V, etc.) or according to their biogenesis process (249). The biogenesis of small vesicles (exosomes) occurs in early endosomes; then, during the process of maturation, the early endosomes become endosomes or multivesicular bodies and accumulate intraluminal vesicles, which can either be degraded by lysosomes or released as exosomes in the extracellular space. The biogenesis of medium/large vesicles (MVs) occurs *via* the direct budding of the cell membrane and are released into the extracellular space (250). Apoptotic bodies are large-sized vesicles that specifically originate from apoptotic cells (251). These EVs contain bioactive soluble molecules (mRNA, miRNA, proteins, lipids, etc.) and membrane-bound molecules (CDs and enzymes). The most currently available EV isolation methods [ultracentrifugation, tangential filtration, immunocapture, or precipitation (252), including those used for clinical grade isolation] do not allow the precise isolation or purification of a specific EV subpopulation (exo or MVs) (253–255). Therefore, the International Society for Extracellular Vesicles (ISEV) has suggested minimal information for studies of extracellular vesicles (MISEV). These guidelines indicate that "EV" remains a collective term describing a complex continuum of vesicles of different sizes and composition and resulting from various mechanisms of formation and release (249). Moreover, in most cases, EV preparations are composed of different vesicles and a greater or a lesser amount of soluble proteins that may participate in the biological activity of the final product. We must therefore also take into consideration the heterogeneity of the final preparations used in the different studies, which mostly include soluble factors. The most suitable term would ultimately be "EV-enriched secretome" rather than "EVs."

Intercellular communication *via* extracellular cargo is highly conserved across species (from bacteria to human); therefore, EVs are likely to be a highly efficient, robust, and economic manner of exchanging information between cells (256).

The specific combinations of molecules in EVs generally reflect the unique characteristics of their original cells and influence their functional properties; therefore, these EVs could recapitulate most effects of the cells from which they originate from and be used as substitutes of those cells in therapeutic objectives (18). EVs can be harvested from all body fluids and take part in many physiological and pathophysiological processes (18). Indeed, EVs are frequently produced in greater abundance in stressed than in unstressed cells; therefore, they can promote the activation of immune cells such as macrophages, which can, in turn, also release EVs and soluble factors and promote stress cell and tissue inflammation and injury (257).

The most extensive studies on EV-mediated communication have been performed between tumor and immune cells and between different types of immune cells. Currently, dendritic cells and mesenchymal stromal cells are the sources for which the prospects for clinical use in humans are most advanced. Since the first descriptions of the therapeutic potential of MSC-EVs in AKI and MI models (227, 228), many studies have addressed the therapeutic functions of MSC-EVs. They could provide new therapeutics and have to be better described and understood (249, 258).

4.2.2 Immunomodulatory Capacity of MSC-EVs

4.2.2.1 Interaction Between MSC-EVs and Innate Immune Cells

As described above, MSCs release a unique signature of proteins (259), lipids (260), and membrane receptors or various types of nucleic acids through EVs (258), which participate in the protection and the regeneration process of damaged cells notably by mitigating the immune response (261, 262). Proteome analysis of MSC-EVs provided by the ExoCarta database showed that the MSC-EV proteome is rich in IL-10, HGF, and leukemia inhibitory factor (LIF) anti-inflammatory cytokines. Moreover, some cytokines, chemokines, and chemokine receptors involved in immune cell recruitment, cell migration, immunosuppression, or neutrophil degranulation, such as CCL2, VEGFC, CCL20, as well as chemokine ligand 2 (CXCL2), CXCL8, CXCL16, defensin α1, HERC5, and IFITM2, are also expressed (261). Similarly, they carry microRNAs (miRNAs) involved in immune function, like miR-146b, identified as an IL-10 effector on macrophages by targeting the TLR4 pathway (263), or miR-181c, which also decreases the expression of TLR4 and the activation of the NF-κB pathway (264). In addition, the pro-inflammatory priming of MSCs, for example by TNF-α and IFN-γ, generates modifications of the protein content and the transcripts of MSC-EVs, notably a greater expression of COX2 leading to an increased release of PGE2, which could promote their anti-inflammatory activity (260). Hypoxia also modulates the MSC-EV miRNA expression profile with notably significant overexpressions of miR-223 and miR-146b, which are implicated in the inflammatory phase of the healing process (265).

Concerning the anti-inflammatory effects on DCs, the authors described 49 miRNAs enriched in MSC-EVs, including miR-21-5p, miR-142-3p, miR-223-3p, and miR-126-3p, known for their role in DC maturation and function (266).

Macrophages have an important role in the inflammatory phase firstly by their pro-inflammatory phenotype and then by their switch to a pro-resolving, anti-inflammatory phenotype. A study in which unfractionated PBMCs were co-cultured with $PKH26^{+}$-MSC-derived EVs showed that EVs were mostly

internalized by monocytes and scarcely by lymphocytes after 24 h or 4 days, but inflammatory priming of MSCs increases EV internalization by lymphocytes (267). It was already described that PBMC or macrophage co-culture with adipose-derived MSC-Exo (MSC-derived exosomes) could induce M2 macrophage polarization (265, 268). MSC-EVs also inhibited TNF-α and IL-6 production by inflammatory glial cells and limited their activation (loss of CD45 and CD11b expressions) and induction of CCL2, one of the membrane markers of M2 polarization (269). Finally, bone marrow MSC-EVs could also downregulate the production of IL-23 and IL-22 by macrophages and pro-inflammatory cytokines, inducing Th17 effector T cells. Therefore, MSC-EV-educated macrophages could promote resolution *via* the decrease of Th17 pathogenicity (270).

4.2.2.2 Interaction Between MSC-EVs and Adaptive Immune Cells

MSC-EVs limit the proliferation and differentiation of activated $CD4^+$ and $CD8^+$ lymphocytes (271). They induce $CD3^+$ and $CD4^+$ lymphocyte apoptosis and increase the Treg/T effector balance (272) by promoting the passage from Th1/Th17 to Th2 (273–275). Otherwise, in co-culture with activated PBMCs, MSC-EVs inhibit the secretion of TNF-α and IL-1β, but increase the concentrations of TGF-β (276) and IL-10 in the co-culture medium (272). As described above, monocytes and, to a lesser extent, lymphocytes were able to internalize $PKH26^+$-MSC-derived EVs. Interestingly, the uptake of MSC-derived EVs occurred in resting but mostly in activated immune effector cells, allowing presumption of a possible role of EVs in immunosuppression, and the inhibition of EV secretion impairs the immunosuppressive capacities of MSCs. Moreover, EV uptake by stimulated B lymphocytes and NK cells is more important than that by T lymphocytes and correlates with the immunosuppressive activity of EV, observed only for B lymphocytes and NK cells, but not for T lymphocytes. Finally, pro-inflammatory priming of MSCs induced an increase in the levels of the anti-inflammatory miRNA-155 and miRNA-146 in both MSCs and their EVs (267, 277, 278). Another study also reported a concentration-dependent immunosuppressive effect of MSC-derived exosomes on B lymphocytes (263).

All these elements show MSC-EVs representing a promising therapy for inflammatory diseases.

5 IMMUNO-PROPERTIES OF MSC-EVS AND MOF

There is significant expanded literature concerning the use of MSC-EVs in multiple preclinical models, in particular on isolated organ damage (**Table 1**). However, no data are currently available on their use in the context of THS. In the following paragraphs, the beneficial effects of MSC-EVs on immunological deregulations and the endothelial dysfunctions of several critical organs injured in MOF are exposed. Otherwise, although inflammation and coagulation are interdependent processes that can initiate a vicious cycle in which each process intensifies the other, the potential benefit of MSC-EVs on coagulopathy has been poorly explored. However, as discussed previously in the section on MSCs, EVs express phosphatidylserine and TF on their surfaces, which were functionally thrombogenic and tended to increase the clotting rates (246) or IBMIR.

5.1 MSC-EVs and Intestinal Injury

To our knowledge, no study has investigated the role of EV administration in THS-induced intestinal ischemia. Most studies have investigated the role of EVs in inflammatory bowel diseases (IBDs), mainly ulcerative colitis and Crohn's disease. A number of rodent models of colitis have been developed; among them, chemical models, notably dextran sulfate sodium (DSS), are widely used (308). The data listed below will relate to the results obtained in this context.

The intravenous injection of MSC-EVs from different sources [bone marrow, umbilical cord, and adipose-derived stromal cells (ADSCs)] attenuated the severity of colitis. Indeed, they exert antioxidative and anti-apoptotic effects, they also reduce the mRNA and protein levels of NF-kB, numerous cytokines, chemokines (TNF-α, IFN-γ, IL-12, IL-1β, IL-6, IL-7, CCL-24, and CCL-17) and enzymes (iNOS and COX2) and they increase IL-10 and TGF-β in the injured colon (279, 280, 282). However, it was observed that TSG-6 depletion in EVs reduced their immunomodulatory efficacy. TSG-6 in EVs plays a key role in increasing the population of Tregs and for macrophage polarization from M1 to M2 in the colon (269). Moreover, intraperitoneal injection of MSC-Exo in a mouse model of inflammatory bowel disease indicated a protective role in the intestinal barrier not only by preventing the destruction of tight junctions, therefore decreasing permeability, but also by modulating the responses of Th2 and Th17 cells in the mesenteric lymph nodes. Again, the knockdown of TSG-6 abrogated the therapeutic effects of MSC-Exo; conversely, administration of a recombinant of TSG-6 showed beneficial effects similar to those of MSC-Exo (286). Therefore, TSG-6 appears to play a major role in the anti-inflammatory effects of MSC-EVs in inflammatory bowel pathologies. Moreover, another study revealed that bone marrow MSC-EVs could inhibit the differentiation of Th17 cells in ulcerative colitis by regulating histone H3 lysine-27 trimethylation (H3K27me3) that is closely associated with the differentiation of Th17 cells. Therefore, MSC-EVs, which regulate H3K27me3, could be promising agents for inflammatory immune diseases associated with abnormal Th17 cell differentiation (283). Administration of MSC-EVs also increases the percentages of $CD4^+$ $CD25^+Foxp3^+$ Tregs in lymph nodes and the spleen (281).

Moreover, as is described in other pathologies, TNF-α and IFN-γ MSC priming increased the immunosuppression of MSC-EVs (270). Finally, Yu et al. evaluated the effect of EphB2-overexpressing bone marrow MSC-EVs. EphB2 is a signaling receptor involved in the regulation of inflammatory response, immune homeostasis, and cell migration. They showed that the overexpression of EphB2 improved the colonic targeting ability of EVs and demonstrated a robust immunomodulatory effect by the modulation of the Th17/Treg balance (278).

TABLE 1 | Overview of the applications of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in preclinical experimental studies.

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
GUT	TNBS induced colitis	Human bone marrow derived MSC	EV	ultracentrifugation	/	male Sprague-Dawley	3 days after colon lesions	systemic	50, 100 , or 200µg EV diluted in 1mL	EV ↘ histological lesions, inflammation (expression of TNFα,IL-1β, NF-κBp65, iNOS or COX2↘, and expression of IL-10 ↗), and apoptosis (Cleavage od caspase 3, 8 and 9), ↗antioxydant effect. Dose response effects	Yang et al. (279). doi: 10.1371/journal.pone.0140551
	DSS induced colitis	Dog Adypocytes tissu derived MSC	EV	ultracentrifugation	transfection (TSG-6 siRNA)	C57BL/6 male mice	during the intoxication week at day 1, 3 and 5	intraperitoneal	100 µg of EV diluted en 100µL/mouse	EV ↘ histological lesions, inflammation (expression of TNF-α, IFN-γ, IL-1β or IL-6↘, and expression of IL-10 ↗). Polarization M2 via TSG-6 pathway	An et al. (269). https://doi.org/10.1371/journal.pone.0220756
						vitro : dogs PBMC, LPS stimulated	coculture with EV	/	100 µg/well		
	DSS induced colitis	Dog Adypocytes tissu derived MSC	EV	ultracentrifugation	24 h with TNF-α and IFN-γ	C57BL/6 J male mice	during the intoxication week at day 1, 3 and 5	intraperitoneal	100 µg of EV diluted in 100µL/mouse	Pro inflammatory primed EV, over express immunosuppressive protein (HGF, TSG-6, PGE2or TGF-β). EVs ↘ histological lesions, inflammation, ↗ Tregs, and M2 polarization	An et al. (270) https://doi.org/10.1038/s41598–020–58909–4
						vitro 1: RAW 264.7 - vitro 2: DH82, LPS stimulated - Vitro 3: canine PBMC concavalin A stimutated	coculture with EV	/	50 µg/well		
	DSS induced colitis	Mouse bone marrow derived MSC	EV	ultracentrifugation	/	BALB/c male mice	1 per day, during seven intoxications days	intraperitoneal	50 µg of EVs/mouse	Ev ↘ symptoms, histological lesions, and VEGF-A, IFN-γ, IL-12, TNF-α, CCL-24, or CCL-17 levels. EV ↗ IL-10 and TGF-β levels. EV allow polarization M2.	Cao et al. (280) https://doi.org/10.1016/j.intimp.2019.04.020
						Vitro: macrophage stimulated LPS	coculture with EV	/	100 µg/mL EVs for 24 h		
	DSS induced colitis	Mouse Adypocytes tissu derived MSC	Exosome	exosome isolation kit		C57BL/6 female mice	during the intoxication week at day 2, 4 and 6	intraperitoneal	100 µg exosome diluted in 200 µl	Ev ↘ symptoms, histological lesions, inflammatory cells penetration. In spleen end lymph nodes, Treg, TGF-β, IL-4, and IL-10 ↗and IFN-γ, TNFα, IL-12, or IL-17↘	Heidari et al. (281), https://doi.org/10.1002/jcp.30275
	DSS induced colitis	hUC-MSCs	Exosome	ultracentrifugation	/	male KM mice	during the intoxication the 11 days	intraperitoneal	400 µg exosomes/mouse	hUC-MSCs-Ev ↘ symptoms, histological lesions, inflammatory	Mao et al. (282) https://doi.org/10.1155/2017/5356760

(Continued)

TABLE 1 | Continued

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
							at 3, 6, and 9			cytokines levels (TNF-α, IL-1β, iNOS, IL-6 or IL-7), ↘ macrophages infiltration, and ↗ IL-10 levels, in colon and spleen.	
						vitro : Mice macrophage	coculture with EV	/	exosomes 160 µg/ml		
	TNBS induced colitis	Rat bone marrow derived MSC	EV	ultracentrifugation	/	male Sprague-Dawley	3 days after colon lesions	systemic	50µg /100µg / 200µg EV	Ev ↘ symptoms, histological lesions. Ev limit Th17 polarization via increase H3K27me3 levels. Dose response effects	Chen et al. (283) https://doi.org/10.1016/j.molimm.2019.12.019
	Small bowell transplantation rejection	Rat bone marrow derived MSC	Exosome	Exosome separation kits	transfection (Heme Oxygen-1)	Allograft (Lewis rat (donor) Brown Norway rat (Recipient))	/	/	/	HO1-MSC derived exosomes ↘ inflammatory injury , via miR-200b which ↘ Hmgb3 gene expression in intestinal epithelial cells.	Sun et al. (284), https://doi.org/10.1038/s41419-020-2685-8
						vitro : Rat intestinal epithelial cells, inflammation injured wirh TNF-α (100 ng/mL) and lymphocytes	coculture with CSM	/	100 µg/mL Exosome		
	TNBS induced colitis	Rat bone marrow derived MSC	EV	ultracentrifugation	transfection (miR-146a)	male Sprague-Dawley	3 days after colon lesions	systemic	100 µg EV diluted in 1ml	Ev ↘ histological lesions. MiR-146a negatively regulates TRAF6 and IRAK1 and decrease inflammatory (↘TNF-α, IL-6 or IL-1β) via suppressing NF-κB activation pathway.	Wu et al. (285) https://doi.org/10.1016/j.intimp.2018.12.043
	DSS induced colitis	hUC-MSCs	Exosome	ultrafiltration	/	C57BL/6 male mice	during the intoxication at days 3, 6, and 9	systemic	1 mg Exosome	Exosomes ↘ histological lesions, pro-inflammatory factors (IL-1β, IL-6), and ↗ IL-10. miR-326 overexpressed in hucMSC-Ex inhibit neddylation process and NF-κB pathway.	Wang et al. (277) https://doi.org/10.1002/ctm2.113
						vitro : Human colorectal cells LPS stimulated	coculture with exosomes	/	200 µg Exosome		

(Continued)

TABLE 1 | Continued

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
	DSS induced colitis	Rat bone marrow derived MSC	EV	ultracentrifugation	transfection (EphB2)	male Sprague-Dawley	after the intoxication week at day 8 and 11	systemic	100 µg of EV diluted in 100µL	EphB2-EV ↘ symptoms, histological lesions, inflammation (NF-κB level, TNF-α, IFN-γ, IL-1β, and IL-2 ↘), STAT3 expression, and oxydative stress. EphB2-EV ↗ Treg polarization.	Yu et al. (278) https://doi.org/10.1186/s13287–021–02232–w
	DSS / TNBS induced colitis	hUC-MSCs	Exosome	ExoQuick-TC	transfection (siTSG-6)	C57BL/6 male mice	TNBS: 24h after colon lesions	intraperitoneal	200µg Exosome/ mouse	↘ mortality, symptoms, histological lesions, pro-inflammatory cytokines, ↗ anti-inflammatory cytokines, switch toward Th2. Effects via TSG-6	Yang et al. (286), https://doi.org/10.1186/s13287-021-02404-8
							DSS: 5 days after intoxication	intraperitoneal	200µg Exosome/ mouse		
LUNG	Traumatic ALI	Rat bone marrow derived MSC	Exosome	exosome EVtant/ centrifugation	overexpressed plasmid vectors (miR-124-3p)	male Sprague-Dawley	30 min before procedure	sytemic	25 µg of exosomes	↘ oxidative stress injury, inflammatory response. Mediated by miR-124-3p	Li et al. (287), doi:10.1152/ajplung.00391.2018
	I/R induced ALI	Rat bone marrow derived MSC	Exosome	ultracentrifugation	/	male Sprague-Dawley	end of procedure	sytemic	5 - 10 µg of exosomes	↘ TNF-α, IL-6 and IL-1β . ↘ TLR4 and NF-κB levels in rat lung tissue	Liu et al. (288), doi: 10.7150/ijms.35369
	Histone induced ALI	Mice Adypocytes tissu derived MSC	Exosome	exosome precipitation kit	GW4869 (N-Smase inhibitor)	male C57BL/ 6 N mice	ADCS Injection 30 min prior to injury	sytemic	3 × 10'5 cells/mice	Exosomes ↘endothelial damage via the PI3K/Akt pathway, modulate by miR-126.	Mizuta et al. (289) https://doi.org/10.1186/s13287–020–02015–9
					/	human umbilical vein endothelial cells exposed to histones	coculture with exosomes	/	/		
	HS induced lung vascular permeability	Human bone marrow derived MSC	EV	ultracentrifugation	/	C57BL/6 male mice	end of HS	systemic	30 µg of EVs	In vivo: ↘ vascular permeability, via cytoskelatal proteins phosphorylation. In vitro, MSC CM but not MSC-EVs prevented thrombin-induced endothelial cell permeability.	Potter et al. (290), doi:10.1097/TA.0000000000001744
						vitro: Human lung	coculture with EV	/	/		

(Continued)

TABLE 1 | Continued

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
	I/R and ex vivo lung perfusion induced lung injury	hUC-MSCs	EV	ultracentrifugation	/	microvascular EC cells C57BL/6 wild-type mice	1 h before ischemia	intratracheal	MSCs or EVs (1 × 10'6)	↘ edema,neutrophil diapedesis and proinflammatory cytokine (IL-17, TNF-α, HMGB1, CXCL1, MCP-1, IL-6, MIP-1α,). Immunomodulatory effect.	Stone et al. (291), doi 10.1186/s12931-017-0704-9
						vitro 1: murine iNKT cells and macrophages - vitro 2: mice primary lung microvascular endothelial cells	coculture with EV	/	/		
LIVER	I/R induced liver injury	Mouse bone marrow derived MSC	EV	ultracentrifugation	/	C57BL/6 mice	30 min before ischemia	systemic	2 × 10'10 EV diluted en 200µL	Ev ↘ histological lesions, apoptosis, hepatic enzymes releasing (AST, ALT, BUN), NFκB and ROS activity. Immunomodulatory effect (TNF-α, IL-1α, IL-1β, IL-6, IL-12, or IFNγ↘, and CXCL1 or MCP-1 ↗).	Haga et al. (292), doi: 10.1002/lt.24770
						vitro: AML12 and hypoxia culture	coculture with EV	/	1,8 ×10'8 EV		
	I/R induced liver injury	hUC-MSCs	EV	ultracentrifugation	/	male Sprague-Dawley	/	systemic	10 mg/kg EV	hUC-MSC-EVs ↘ histologic lesions, inflammation, neutrophil infiltration, oxydative stress, apoptosis, ALT, AST, and ALP level. hUC-MSC-Evs carry antioxidant enzyme.	Yao et al. (293), doi: 10.1096/fj.201800131RR
						vitro 1: human LO2 cells - vitro 2: neutrophils LPS activated	/	/	20 µg EV		
	I/R induced liver injury	Human-induced pluripotent stem cell	Exosomes	ultrafiltration/ ultracentrifugation.	/	male Sprague-Dawley	end of procedure	inferior veina cave	600 µg suspended in 400 µL	Evs, ↘ histological lesions, hepatic enzymes levels, oxydative stress, inflammation (infiltration	Nong et al. (294) https://doi.org/10.1016/j.jcyt.2016.08.002

(Continued)

TABLE 1 | Continued

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
		derived MSC								cells,HMGB1, TNF-α and IL-6↘). Protect hepatocyte (apoptosis↘ proliferation↗).	
	I/R induced liver injury	hUC-MSCs	Exosomes	ultracentrifugation	Transfection with : miR-1246 inhibitor	C57BL/6 mice	0h after reperfusion	/	2.5 × 10'12 exosome diluted in 500 µL	hUCB-MSCs-derived exosomes ↘ apoptosis in vitro, histological lesions, enzymatic release (AST, ALT) and cytokines (TNF-α, IL-6 and IL-1β↘). via miR-1246 and GSK3β-Wnt/β-catenin pathway activation.	Xie et al. (295), https://doi.org/10.1080/15384101.2019.1689480
						vitro: LO2 cells exposed to hypoxia/ reoxygenation (H/R)	coculture	/	/		
	I/R induced liver injury	hUC-MSCs	Exosomes	exosome isolation kit	/	C57BL/6 male mice	0h after reperfusion	systemic	10 µg/100 µL exosomes	hUCB-MSCs-derived exosomes ↘ histological lesions, enzymatic release and Th17/Treg ratio in CD4+ T cells in vitro, via the IL-6-gp130-STAT3 pathway	Xie et al. (296), 2019 doi: 10.1002/iub.2147
						vitro: naïve human CD4+ T cocultured with LO2 and tranfected with IL-6 signal transductor	coculture	/	/		
	I/R induced liver injury	hUC-MSCs	EV	ultracentrifugation	/	C57BL/6 male mice	0h after reperfusion	systemic	100 µg/100 µL EV	Evs ↘ inflammatory response by decrease CD154 expression on T CD4+, via CCT2 and NFAT1 signaling pathway.	Zheng et al. (297), doi: 10.1002/advs.201903746
						vitro 1: intrahepatic mononuclear cells - vitro 2: CD4+ T	/	/	/		
	I/R induced liver injury	Human bone marrow	EV	ultracentrifugation	/	C57BL/6 female mice	5 min before procedure	systemic	1 x 10'9 EV/ 200µL	MSC-derived EV ↘ serum transaminase levels, hepatic necrosis,	Anger et al (298), doi: 10.1089/scd.2019.0085

(Continued)

TABLE 1 | Continued

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
		derived MSC								transcription of inflammation-associated genes, and ↗ number of Ki67-positive hepatocytes	
KIDNEY	Glycerol induced AKI	Human bone marrow derived MSC	EV	ultracentrifugation	/	male CD1 nude mice	3 days after injury	systemic	200 µg	EVs accumulated specifically in the kidneys of the mice with AKI compared with the healthy controls	Grange et al. (299), DOI: 10.3892/ijmm.2014.1663
						vitro: Human renal proximal tubular epitheial cells	coculture with EV	/	50 µg/mL EV		
	I/R induced AKI	Human bone marrow derived MSC	MV	ultracentrifugation	/	male Sprague-Dawley rat	end of procedure	systemic	30 µg of MV	MV ↘ apoptosis, functionnal lesions, ↗ stimulating tubular epithelial cell proliferation.	Gatti et al. (300), doi: 10.1093/ndt/gfr015
	I/R induced AKI	Human amnion epithelial cell derived exosomes	Exosomes	ultracentrifugation	/	Male C57BL/6j mice	end of procedure	systemic	3 × 10'8 exosomes	Exosomes ↘histological, functionnal lesions, apotosis, ↗ cells proliferation, density of peritubular capillars, M2 polarization, and anti-inflammatory effects (IL-4, IL-13↗, TNF-α,IFN-γ ↘)	Ren et al. (301), https://doi.org/10.1186/s13287-020-01917-y
						vitro: HK-2 cells exposed to hypoxia during 48h	coculture with EV	/	1 × 10'8/ml exosomes		
	I/R induced AKI	Rat Adypocytes tissu derived MSC	Exosomes	ultracentrifugation	/	male Sprague-Dawley rat	3h after injury	systemic	exosome (100 µg)], and/or ADMSC (1.2 × 10'6 cells)	Exosomes ↘ histological, functionnal lesions, apoptosis , oxydative stress, inflammation (TNF-α, NF-κB, IL-1β, MIF, PAI-1 and COX-2 ↘at 72h)	Lin et al. (302) https://doi.org/10.1016/j.ijcard.2016.04.061
	Rhabdomyolisis via glycerol induced AKI	Human bone marrow derived MSC	MV	ultracentrifugation	Transfection with shRNAmiR targeting Drosha	SCID Mice	3 days after injury	systemic	2.2×10'8MV diluted in 150µL	miARN allow MV therapeutics effects	Collino et al. (303). doi: 10.1681/ASN.2014070710
						vitro: tubular epithelials cells, for C57BL/6 female mice	coculture with MV	/	/		

(Continued)

TABLE 1 | Continued

Organ	Model	Microparticles			Priming CSM	Model	Administration			Results	References
		Origins	Type	Purification			Timing	Route	Dose		
	I/R induced AKI	hUC-MSC	EV	ultracentrifugation	/	male Sprague-Dawley rat	end of procedure	systemic	100 µg of MV diluted in 0,5 mL	EV ↘ NK (and CX3CL1 - TLR2) up-regulation. EV action is allowed by carriying ARN	Zou et al. (304), doi: 10.1089/hum.2016.057
						vitro: human umbilical vein endothelial cells	coculture with EV	/	/		
	I/R induced AKI	hUC-MSC	MV	ultracentrifugation	IFN-γ during 24 or 48h	male Sprague-Dawley rat	end of procedure	systemic	/	MV without priming are better to protect kidney (Histological and functionnal lesions ↘). MV promote Treg proliferation. Priming with INFγ modulate MV material carrying and origin.	Kilpinen et al. (305), http://dx.doi.org/10.3402/jev.v2i0.21927
						vitro: PBMC	coculture with MV	/	/		
	Rhabdomyolisis via glycerol induced AKI	Human bone marrow derived MSC	EV	ultracentrifugation	/	SCID Mice	3 days after injury	systemic	165 × 10'6 particules diluted in 120µL	EV population enriched in exosomes ↘ histlogical and functionals lesions comparable with total EV population. Enriched in specific mRNAs (CCNB1, CDK8, CDC6) in comparaison with EV population enriched in MV	Bruno et al. (306), doi: 10.1089/ten.tea.2017.0069
						vitro: murine epithelials cells	coculture with particules	/	/		
	I/R induced AKI	hUC-MSC	EV	ultracentrifugation	Rnase pre treatement of EV	male Sprague-Dawley rat	end of procedure	systemic	100 µg of MV diluted in 1mL	EV ↗ renal VEGF, ↘ fibrosis and HIF-1α. Rnase treatement abrogate benefits	Zou et al. (307), doi:8(10): 4289–4299.
						vitro1: rat tubular epithelial cells	coculture with EV	/	/		

↗ mean: increase, and ↘ mean: decrease

TABLE 2 | Overview of the applications of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in clinical studies.

NCT Number	Title	Status	Conditions	Interventions	Outcome Measures	Sponsor / Collaborators	Phases	Enrollment	Study Type	Study Designs	Other IDs or DOI	Start Date	Locations	Results, if published
NCT04384445	A Phase I/II Randomized, Double Blinded, Placebo Trial to Evaluate the Safety and Potential Efficacy of Intravenous Infusion of Zofin for the Treatment of Moderate to SARS Related to COVID-19 Infection vs Placebo	Recruiting	Covid19 Corona Virus Infection SARS (Severe Acute Respiratory Syndrome) Acute Respiratory Distress Syndrome	Drug: ZofinTM Versus Placebo	Incidence of any infusion associated adverse events | Incidence of Severe Adverse Events Safety | Survival Rate|Cytokine Levels | D-dimer Levels | C-reactive protein Levels |Quantification of the COVID-19 | Improved Organ Failure | Chest Imaging Changes	Organicell Regenerative Medicine	Phase I / II	20	Interventional	Allocation: Randomized | Intervention Model: Parallel Assignment | Intervention Model Description: parallel | Masking: Double| Double blind |Primary Purpose: Treatment	19881	September 8, 2020	Larkin Community Hospital Miami,and Hospital South Miami, Florida, United States	Not Published
NCT04602104	A Multiple, Randomized, Double-blinded, Controlled Clinical Study of Allogeneic Human Mesenchymal Stem Cell Exosomes (hMSC-Exos) Nebulized Inhalation in the Treatment of Acute Respiratory Distress Syndrome	Not yet recruiting	Acute Respiratory Distress Syndrome	Biological: Exosome of MSC (High, medium or low dose)	Incidence of adverse reaction | Murray lung injury score | PaO2/FiO2 | SOFA score | ApachII score | Number of deaths | The number of days that survivors were offline for mechanical ventilation | The number of days the survivor was out of ICU | Incidence of treatment emergent adverse event	Ruijin Hospital / Cellular Biomedicine Group Ltd.	Phase I / II	169	Interventional	Allocation: Randomized |Intervention Model: Parallel Assignment | Masking: Double (Participant, Investigator) | Primary Purpose: Treatment |	MEXARDS	October 2020	Ruijin Hospital, Medical School of Shanghai Jiaotong University and Shanghai, Shanghai, China	Not Published
NCT04798716	Mesenchymal Stem Cell Exosomes for the Treatment of COVID-19 Positive Patients With Acute Respiratory Distress Syndrome and/or Novel Coronavirus Pneumonia	Not yet recruiting	Covid19 Novel Coronavirus Pneumonia Acute Respiratory Distress Syndrome	Drug: MSC-exosomes delivered intravenously every other day on an escalating dose: (2:4:8) or (8:4:8) or (8:8:8)	Measure and report treatment-related-adverse events | Quantify safety of ARDOXSO™ | Tabulate and report the number of IMV days | Analyze and report organ failure, associated with ICU mortality | Correlate and analyze the SOFA score| Record and analyze respiratory measures (Berlin Score/PEEP) | Quantify efficacy of interventional exosome therapy in COVID-19	AVEM HealthCare	Phase I / II	55	Interventional	Allocation: Randomized |Intervention Model: Sequential Assignment | Masking: Double (Participant, Care Provider) | Primary Purpose: Treatment	20582	September 2021	Mission Community Hospital Panorama City, California, United States	Not Published
NCT04493242	Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment for COVID-19 Associated Acute Respiratory Distress Syndrome (ARDS): A Phase II Clinical Trial	completed	Covid19 ARDS Pneumonia, Viral	Biological: DB-001 Versus Placebo	PaO2/FiO2 ratio | Time to Recovery | Incidence of Serious Adverse Events | All-cause Mortality | (SARS-CoV-2) Ribonucleic Acid (RNA) Level | Viremia | CRP, D-dimer, Ferritin, IL-6, TNF-α | Immune Cell Counts |SOFA scoring |Standardized Quality of Life Metric	Direct Biologics, LLC	Phase II	120	Interventional	Allocation: Randomized | Intervention Model: Parallel Assignment | Masking: Triple (Participant, Care Provider, Investigator) | Double-blinded |	DB-001 // doi: 10.1089/scd.2020.0080. Epub 2020 May 12.	September 24, 2020	Helen Keller Hospital Sheffield, Alabama, United States, 35660| St. Joseph Hospital Heritage Fullerton, California, United States, 92835 | Donald Guthrie Foundation/ Robert Packer Hospital Sayre, Pennsylvania, United States, 18810 | Covenant Health Lubbock, Texas,	Safty profile | Restore oxygenation | Downregulate cytokine storm | Reconstitute immunity

(Continued)

TABLE 2 | Continued

NCT Number	Title	Status	Conditions	Interventions	Outcome Measures	Sponsor / Collaborators	Phases	Enrollment	Study Type	Study Designs	Other IDs or DOI	Start Date	Locations	Results, if published
										Primary Purpose: Treatment			United States, 79410 | PRX Research Mesquite, Texas, United States, 75149	
NCT04276987	A Pilot Clinical Study on Aerosol Inhalation of the Exosomes Derived From Allogenic Adipose Mesenchymal Stem Cells in the Treatment of Severe Patients With Novel Coronavirus Pneumonia	Completed	Coronavirus	Biological: MSCs-derived exosomes	Adverse reaction and severe adverse reaction |Time to clinical improvement | Number of patients weaning from mechanical ventilation | Duration (days) of ICU monitoring | Duration (days) of vasoactive agents usage | Duration (days) of mechanical ventilation supply | Number of patients with improved organ failure | Rate of mortality | (SOFA) score | Biologicals measure	Ruijin Hospital / Shanghai Public Health Clinical Center Wuhan Jinyintan Hospital, Wuhan, China Cellular Biomedicine Group Ltd.	Phase I	24	Interventional	Allocation: N/A | Intervention Model: Single Group Assignment | Masking: None (Open Label) | Primary Purpose: Treatment	MEXCOVID	February 15, 2020	Ruijin Hospital Shanghai Jiao Tong University School of Medicine Shanghai, Shanghai, China	Not Published
NCT04602442	The Extended Protocol of Evaluation of Safety and Efficiency of Method of Exosome Inhalation in COVID-19 Associated Two-Sided Pneumonia	Enrolling by invitation	Covid19 SARS-CoV-2 PNEUMONIA COVID-19	Drug: EXO 1 inhalation Drug: EXO 2 inhalation Drug: Placebo inhalation	Number of participants with non-serious and serious adverse events during trial |Time to clinical recovery |SpO2 concentration changes | Chest Imaging Changes | CRP | Procalcitonin concentration | Ferritin concentration | Creatinine concentration |Urea concentration	Clinics of the Federal State Budgetary Educational Institution SSMU Samara Regional Clinical Hospital V.D. Seredavin	Phase II	90	Interventional	Allocation: Randomized | Intervention Model: Parallel Assignment| Masking: Double (Participant, Care Provider) | Primary Purpose: Other	COVID-19 EXO Extended	October 1, 2020	Medical Centre Dinasty Samara, Russian Federation	Not Published
NCT04356300	Exosome of Mesenchymal Stem Cells for Multiple Organ Dysfuntion Syndrome After Surgical Repaire of Acute Type A Aortic Dissection	Not yet recruiting	Multiple Organ Failure	Biological: Exosome of MSC	survival after intervention|sequential organ failure assessment score| interleukin-6|The number of allergic reactions|The number of people who get cancer|the effects on kidney function|the effects on liver function| the effects on lung function|the effects on coagulation function| the effects on central nervous system	Fujian Medical University	Not Applicable	60	Interventional	Allocation: Randomized | Intervention Model: Parallel Assignment| Masking: Single (Outcomes Assessor)| Primary Purpose: Treatment	2020005	September 1, 2020	Fujian Medical University, Fujian Province, China	Not Published

Reaserch terms in Clinical Trials (https://clinicaltrials.gov/ct2/home) on August 25th 2021

Statues	Conditions or disease	Other Terms
All studies	lung disease lung dysfunction lung injury acute lung injury acute respiratory distress syndrom kidney disease kidney injury kidney dysfunction acute kidney injury liver disease	microvesicules exosomes microparticules extracellular vesicules exosomes of mesemchymal cells MSC derived

(Continued)

TABLE 2 | Continued

Reasearch terms in Clinical Trials (https://clinicaltrials.gov/ct2/home) on August 25th 2021		
Statues	**Conditions or disease**	**Other Terms**
All studies	lung disease	microvesicules
	lung dysfunction	exosomes
	lung injury	microparticules
	acute lung injury	extracellular vesicules
	acute respiratory distress syndrom	exosomes of mesenchymal cells
	kidney disease	MSC derived
	kidney injury	
	kidney dysfunction	
	acute kidney injury	
	liver disease	
	liver injury	
	liver dysfunction	
	acute liver injury	
	gut disease	
	gut injury	
	gut dysfunction	
	bowell disease	
	bowell injury	
	bowell dysfunction	
	inflammation	
	ischemia reperfusion injury	
	multiple organ failure	
	multiple organ dysfunction	
	multiple trauma	
	blunt trauma	
	blunt injury	
	haemorrhagic shock	
	traumatic hemorrhage	
	traumatic hemorrhage shock	
	war injury	
	war related trauma	
	war related injuries	

Regarding the potential beneficial effect of the miRNA content in EVs in this pathology, a crucial role of exosomal miR200b has been described by using heme oxygenase-1 (HO-1)-modified bone marrow MSC-Exo, which overexpresses miR200b. This miRNA targets the *HMGB3* gene involved in intestinal inflammation (284). Moreover, EVs overexpressing miR-146a seem to exert better anti-inflammatory effects in an experimental rat model of colitis (285). Furthermore, the study of Wang et al. demonstrated a stronger therapeutic effect of human umbilical cord (hUC)-MSC-derived exosomes that highly expressed miR-326. Indeed, this miRNA played an important role in the inhibition of the neddylation process that indirectly activated NF-κB pro-inflammatory transcription factor (277).

5.2 MSC-EVs and ALI or ARDS

MSC-EVs have been extensively studied in septic ALI, including in clinical trials (309) and, more recently, in COVID-19 patients (**Table 2**). In the few models of ALI induced by THS, it was demonstrated that bone marrow MSC-EVs can modulate cytoskeletal signaling and attenuate lung vascular permeability (290). Moreover, ADSC-MSC-EVs could decrease endothelial damage *via* the PI3K/Akt signaling pathway (289). In a mouse model of lung I/R injury, EV treatment significantly attenuated lung dysfunction and injury by decreasing edema, neutrophil infiltration, and myeloperoxidase levels. Moreover, significant decreases in pro-inflammatory cytokines (IL-17, TNF-α, and CXCL1) and HMGB1 were observed. An upregulation of KGF, PGE2, and IL-10 in the bronchoalveolar fluid was also shown. Finally, MSC-EVs significantly downregulated the iNKT-produced IL-17 and the macrophage-produced HMGB1 and TNF-α in an *in vitro* model of hypoxia/reoxygenation (291). Moreover, as described previously, intestinal I/R is a common clinical occurrence caused by a number of pathophysiological contexts, including THS. ALI is a primary component of MOF triggered by intestinal I/R. In a rat model of ALI induced by occlusion/reperfusion of the superior mesenteric artery, intravenous treatment by rat bone marrow MSC-derived exosomes attenuated lung damage by decreasing apoptosis and the pulmonary levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, accompanied by a downregulation of the expressions of TLR4 and NF-κB (288).

EV miRNAs also play a role. miR-124-3p, abundantly expressed in rat MSC-derived exosomes, inhibits the expression of the purinergic receptor P2X ligand gated ion channel 7 (P2X7). Inhibition of P2X7, which is overexpressed in traumatic ALI rats, improves oxidative stress and decreases the levels of inflammatory factors, including TNF-α, IL-6, and IL-8, and increases IL-10 (287). Furthermore, the transfer of MSC-EV miR-451 to macrophages *in vitro* not only inhibits TNF-α and macrophage migration inhibitory factor secretion but also represses their TLR signaling. This repression allowed the mitochondrial transfer of MSC-EVs to macrophages. All these immunomodulatory effects on macrophages were exerted by different MSC-EV populations (310). Altogether, these data indicate that MSC-EVs, by limiting oxidative stress and vascular permeability and by downregulating the activity of immune cells in the lungs, represent a novel therapeutic option in the treatment of traumatic ALI.

5.3 MSC-EVs and Acute Liver Injury

As for ALI, many studies have evaluated MSC-EVs in models of hepatic injury induced by the administration of D-galactosamine/TNF-α, various toxic drugs, or LPS. Systemic administration of human MSC-EVs on hepatic I/R injury suppressed not only hepatocyte necrosis and sinusoidal congestion but also AST and ALT injury markers (294, 298, 311). Moreover, in a model of I/R-induced hepatic apoptosis, hUC-MSC-EVs reduced neutrophil infiltration and, therefore, their respiratory burst. This alleviated oxidative stress in hepatic tissue (293). This suggests that MSC-EVs could reduce hepatic injury by suppressing inflammatory responses (of TNF-α, IL-6, and HMGB1) and attenuating the oxidative stress response [by increasing glutathione, glutathione peroxidase, and superoxide dismutase (SOD)] and apoptosis (by decreasing caspase-3 and Bax) (292, 294). hUC-MSC-EVs could induce anti-apoptotic and pro-survival effects in a human liver cell line and ameliorated the I/R injury-induced hepatic dysfunction in mice. This study highlighted the crucial role of miR-1246 *via* the regulation of the GSK3β-Wnt/β-catenin pathway to mediate these effects (295). Subsequently, exosomes expressing miR-1246 had protective effects against hepatic I/R by regulating Th17/Treg imbalance *via* the interaction of miR-1246 and IL-6-gp130-STAT3 (296). Another team described in an I/R mouse model that hUC-MSC-EVs significantly modulated the membranous expression of CD154 of intra-hepatic $CD4^+$ T cells, which initiated the inflammatory response in the liver and can aggravate liver I/R (297). As shown in the few studies exploring the effects of treatment with MSC-EVs after I/R, their capacity to inhibit immune cell activation (mainly neutrophils) and pro-inflammatory cytokine release, as well as their capacity to attenuate oxidative stress and to inhibit hepatic cell apoptosis, makes MSC-EVs a promising therapy to treat liver injury following THS.

5.4 MSC-EVs and AKI

Many studies have shown the beneficial effects of the administration of MSC-EVs in AKI (312). As in the previous sections, only studies using models of I/R or rhabdomyolysis were discussed since toxicity studies (cisplatin) are not relevant to THS. The therapeutic effects of EVs are mediated by different biological processes, including anti-apoptosis, anti-inflammation, angiogenesis, and anti-fibrosis (303, 306, 307). After systemic injection, labeled MSC-EVs accumulated specifically in the kidneys of mice with AKI, but not in healthy controls (299). This suggests a homing capacity of EV-derived MSCs on the site of injury.

In an I/R-induced AKI mouse model, exosomes from human amnion epithelial cells (hAEC-Exo) could improve animal survival and renal function and induce M2 macrophage polarization. This M1/M2 shift was associated with increases in the IL-4 and IL-13 levels and decreases in the TNF-α and IFN-γ levels, which helped reduce the inflammatory response (301). Similarly, EVs from ADSCs decreased the protein levels of NF-κB, TNF-α, IL-1β, and MIF, as well as PAI-1 and COX-2 in the kidney parenchyma, 72 h after I/R (302). Moreover, administration of human Wharton jelly MSC-EVs also alleviated inflammation (decreased TNF-α and increased IL-10 expressions in the kidney) in the first 48 h, but also suppressed the expression of CX3CL1 (a potent chemo-attractant factor for macrophages) and decreased the number of $CD68^+$ macrophages in the kidney (231). Several studies suggest that the therapeutic effects of EVs can be mediated by functional mRNAs and miRNAs (228, 300, 303). MSC-EVs express high levels of miR-15a, miR-15b, and miR-16 that may modulate CX3CL1 expression (231). The same team also described that the number of NK cells increased in the kidney after I/R injury. EVs also decreased the percentage of NK cells in ischemic kidney, suggesting that MSC-EVs could alleviate kidney injury by regulating NK cells (304). Several proteins expressed by both naive and IFN-γ-primed EV-MSCs, such as galectin-1 and galectin-3 described as mediators of MSC T-cell immunosuppression, or the membrane markers CD90 and CD73 are also associated with MSC-immunosuppressive capacity (305). Finally, EV-MSCs contain anti-inflammatory and anti-oxidative apolipoprotein A1 (ApoA1). ApoA1 is described to have therapeutic effects in kidney injury, leading to the reduction of serum creatinine levels, serum TNF-α and IL-1β levels, and tissue MPO activity. Moreover, ApoA1 can suppress the expressions of ICAM-1 and P-selectin in the endothelium, thus diminishing neutrophil adherence (313). This literature, reduced here to I/R and rhabdomyolysis injuries, indicates the benefit of treatment with MSC-EVs of AKI by limiting the leukocyte chemoattraction and activation through inducing a shift from M1 to M2 macrophages or by decreasing pro-inflammatory and increasing anti-inflammatory cytokine production. All these encouraging arguments suggest that there is a potential interest in the use of MSC-EVs in the context of THS.

5.5 MSC-EVs: A New Hope for the Prevention of MOF?

The pathophysiology of THS-induced MOF is complex and still not fully understood. The aim of most treatments currently used in the clinic is to compensate for the function of the affected organ with, for example, dialysis, parenteral nutrition, or controlled ventilation and oxygenation. Limited options are available to prevent the occurrence or limit the extent of organ failure in THS. The imbalance between SIRS and CARS is a key mechanism in MOF, but because it is at the crossroad of multiple system dysfunctions, no unique physiological or molecular therapeutic target can be identified. As shown in previous sections, MSCs and their EVs have an important potential to treat isolated organ failure through multiple intricate molecular mechanisms that target notably inflammation and oxidative stress. This is the reason why we believe that taking advantage of the pleiotropic effects of MSC-EVs could be a precious new approach in a pathophysiological situation as complex and multifactorial as that of THS leading to MOF.

6 MSC-EVS: TOWARD A CLINICAL GRADE PRODUCTION

MSC-EVs represent a great hope for the treatment of THS. Their use can have important advantages, but unknowns

persist. Although a number of preclinical studies have explored the biology of MSC-EVs, only a few clinical trials have been listed concerning acute injuries of isolated organs, systemic immune dysfunctions, I/R injuries, or trauma and MOF (**Table 2**). A significant increase has occurred with the SARS-CoV-2 pandemic, and complete studies indicated that the administration of MSC-EVs decreases systemic inflammation and allows restoration of pulmonary oxygenation; most other studies are in progress.

Regarding the systemic administration of EVs, which seems the most relevant in the context of THS-induced MOF, although the biodistribution/homing of MSCs has been explored, it is still poorly understood for EVs. However, it was demonstrated that 70% of near-infrared lipophilic dye-labeled human MSC-EVs accumulated in the liver after systemic administration in healthy mice. Interestingly, dendritic cell-derived EVs showed an increased accumulation in the spleen, suggesting that the homing pattern of EVs reflects those of their original cells (314). On the other hand, the unpredictable nature of THS, as well as the need for emergency administration of the therapeutic product, requires the use of EVs from allogeneic MSCs. It is known that the survival of allogeneic MSCs is limited after administration, but during this time, they continuously secrete soluble factors/EVs, adapted to the pathophysiological context that they encounter. This is not the case with EVs, but iterative administrations can be more easily considered. Indeed, it will be a product already prepared/qualified, immediately available, and easily stored and transportable, allowing patients to be treated anywhere without the need for nearby production facilities. In addition, we hope that the lack of adaptability of EVs to the pathophysiological context could be compensated by the use of optimized priming upstream.

In addition, as has been the case with MSCs, there may be a mismatch between the hope raised by exciting preclinical publications and the ability to enter daily clinical practice. These difficulties could result not only from differences between human and animal species but also from the heterogeneity of the products used. Variability in MSC-EVs is associated with the variability of the cells from which they are produced. The variability of MSCs arises from several key factors such as the tissue origin (bone marrow, adipose tissue, perinatal tissues, etc.), donor, culture condition media/support (platelet lysate, fetal bovine serum, bioreactor, priming by hypoxia, or cytokines), age (age of donor and culture passage), or cryopreservation. Moreover, depending on the therapeutic target, a strategic choice between primary MSCs or cell line, native or modified, must be carefully considered. Therefore, EVs could be selected based on the advantages of MSC sourcing/efficacy. A study comparing the protein profiles of MSC-EVs with the proteome profiles of EVs from other cells showed a specific protein signature of MSC-EVs, despite the huge diversity in the sources of MSCs or the preparation methods of MSC-EVs. However, 22 proteins were exclusively found in the bone marrow-derived MSC-EV profiles. Identification of the functional markers of potency and the development of easily deployable and standardized methods of evaluation would benefit the field of EVs, as it did for cell therapy, and in this study, it was also suggested that several membrane and extracellular proteins (i.e., COL6A2 or COL6A3 and THY1) could be used as a standard for the quality control of production either in research or in clinical settings (259). EVs can also be transformed/loaded (without prior transformation of the producing MSCs) to improve their targeting or their therapeutic properties. All these provide a large field of possibilities for the clinical use of EVs (315). Moreover, in most EV manufacturing processes, the therapeutic product is composed of a continuum of different types of vesicles (size and origin) and certain amounts of soluble proteins that may participate in the biological and therapeutic activity of the final product (**Figure 4**). In fact, most of the studies described in the literature are based on products that do not consist solely of EVs (due very often to isolation by ultracentrifugation), but which contain a greater or a lesser proportion of soluble proteins. The most effective product could therefore be an "EV-enriched secretome." In preclinical studies, EVs are isolated using different techniques: ultracentrifugation, tangential filtration, immunocapture, or precipitation (252). However, not all of them are easy to consider when moving to clinical grade production. Indeed, although ultracentrifugation is the most widely used, it is time-consuming and additional stages of purification (washing and microfiltration) are generally necessary to increase the purity of the EV products. Tangential flow filtration, for example, already validated for industrial-scale productions, seems more suitable. On the other hand, specifically concerning MSCs, the culture media used for the expansion phases are enriched with fetal calf serum or platelet lysate, which contain large amounts of EVs that cannot be distinguished or separated from MSC-derived EVs. To overcome this problem, in many studies, the culture medium is removed after the expansion of MSCs, rinsed, and replaced by a medium without these additives during the entire period of MSC secretion. The cellular stress generated by starving must, however, be taken into consideration since it generates modifications in the state of the cells and, therefore, in the quality/functionality of the EVs produced. On the other hand, the secretion times of the EVs, and therefore the potential quantity of EVs recovered, are limited. The alternatives for the clinic consist in the use of serum-free or platelet lysate-free media (containing specific cocktails of growth factors and additives), but these media are very specific of a cell type and very expensive, which is problematic for the large-scale production of conditioned medium. Commercial "exosome-free" media also exist. Depletions are performed by ultracentrifugation; however, the levels of depletion are not optimal, with variations depending on the centrifugation conditions and durations. Likewise, tangential flow filtration appears to be a possible

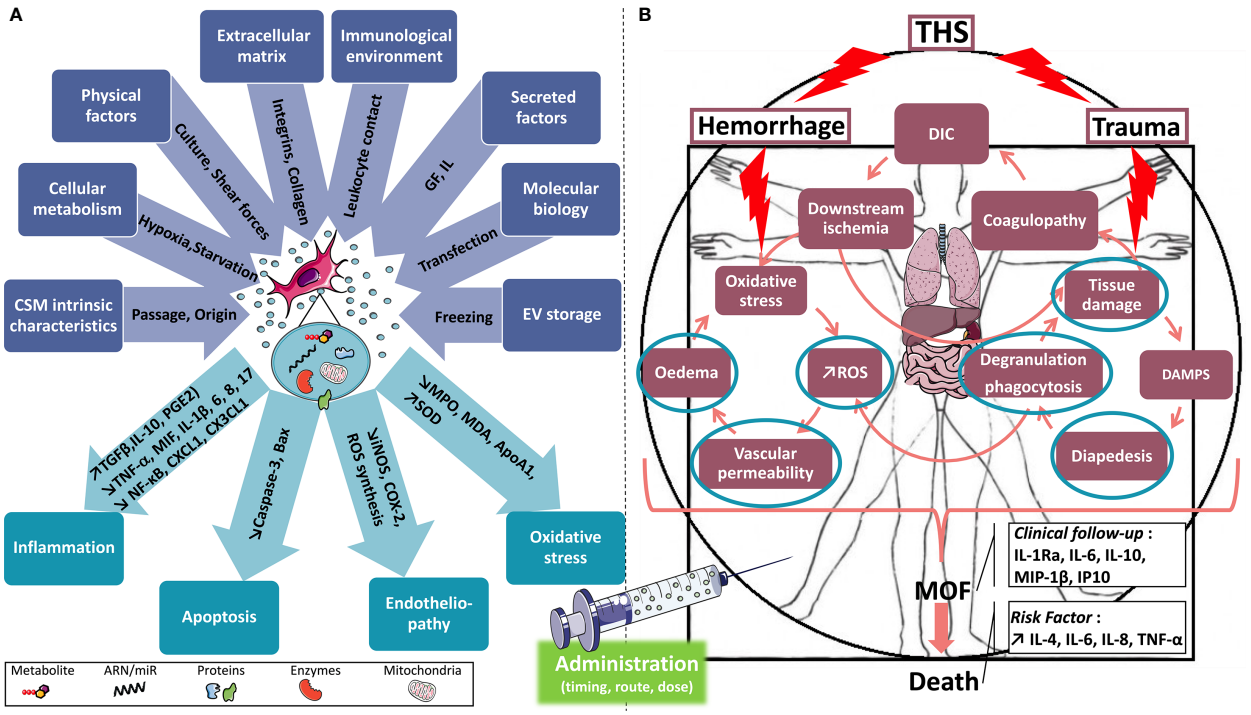

FIGURE 4 | MSC-EVs in THS, or how to apply recent knowledge at the service of those seriously injured. **(A)** In *blue* are the main factors modulating the production of EVs. EVs in the *center* contain, depending on the priming, organelles, proteins, enzymes, RNA, and miR in variable quantities. In *aquamarine* are the possible main pathways of the potential beneficial effects of EVs in THS models. The administration methods vary by model and must be explored. **(B)** Simplified consequences of THS. Three loops (coagulopathy, inflammation, and endotheliopathy) are involved in the vicious circle leading to MOF. Cytokines of clinical interest are predictors of the onset of MOF. IL-4, IL-6, IL-8, and TNF-α are significantly increased in trauma patients with MOF and not surviving it. Items circled in aquamarine are potential targets for EV action. EVs, extracellular vesicles; MSC-EVs, mesenchymal stromal cell-derived extracellular vesicles; THS, traumatic hemorrhagic shock; MOF, multi-organ failure; MPO, myeloperoxidase; MDA, malondialdehyde; SOD, superoxide dismutase; GF, growth factor; IL, interleukin.

solution concerning the purification of culture media for large-scale clinical productions.

Finally, from a regulatory point of view, EV-derived products are classified as medicinal products. Within the framework of medicinal products, EV-derived products are categorized as "biological medicinal products" (Directive 2003/63/EC of June 25, 2003, amending Directive 2001/83/EC). However, MSC-EVs could be subcategorized. When they originate from unmodified primary cells or from genetically modified cells that do not contain a transgene product (immortalized cells), they belong to the biological medicinal product category, without any further subcategory. In contrast, MSC-EVs containing a transgene considered as a gene therapy product (e.g., recombinant mRNA and miRNA) are classified as gene therapy products, a subclass of advanced therapy medicinal products (ATMP). This means that the active substance and mode of action of MSC-EVs are decisive for their regulatory classification and can have significant repercussions on the manufacturing process. The use of primary MSCs may have some limitations for large clinical-scale manufacturing due to their limited life span and the donor-to-donor or batch-to-batch heterogeneity. Therefore, EVs produced from immortalized MSCs could be the most promising strategy to prevent MOF.

7 CONCLUSION/DISCUSSION

In recent years, a considerable number of studies have contributed to a better understanding of the biology of EVs and paved the way for their therapeutic use. In cases of isolated organ injuries, MSC-EVs can help restore local homeostasis by decreasing inflammation and oxidative stress, by having an anti-apoptotic effect, or even inhibiting endotheliopathy. Locally protecting the onset of organ damage is a means to prevent the onset of SIRS and the depression of CARS at the systemic level, which promote the development MOF.

This new therapeutic tool could revolutionize the field of cell therapy because it opens the way to treatments that can be administered as early as possible for the care of patients, not only in civilian life but also in hostile contexts such as those encountered in theaters of military operations.

AUTHOR CONTRIBUTIONS

GV and JP wrote the manuscript with input from all authors. All authors (NL, CM, EV, and SB) provided critical feedback and helped shape the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of microRNA Shuttled in Small Extracellular Vesicles Derived From Mesenchymal Stem/Stromal Cells for Osteoarticular Disease Treatment

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Osteoarticular diseases (OD), such as rheumatoid arthritis (RA) and osteoarthritis (OA) are chronic autoimmune/inflammatory and age-related diseases that affect the joints and other organs for which the current therapies are not effective. Cell therapy using mesenchymal stem/stromal cells (MSCs) is an alternative treatment due to their immunomodulatory and tissue differentiation capacity. Several experimental studies in numerous diseases have demonstrated the MSCs' therapeutic effects. However, MSCs have shown heterogeneity, instability of stemness and differentiation capacities, limited homing ability, and various adverse responses such as abnormal differentiation and tumor formation. Recently, acellular therapy based on MSC secreted factors has raised the attention of several studies. It has been shown that molecules embedded in extracellular vesicles (EVs) derived from MSCs, particularly those from the small fraction enriched in exosomes (sEVs), effectively mimic their impact in target cells. The biological effects of sEVs critically depend on their cargo, where sEVs-embedded microRNAs (miRNAs) are particularly relevant due to their crucial role in gene expression regulation. Therefore, in this review, we will focus on the effect of sEVs derived from MSCs and their miRNA cargo on target cells associated with the pathology of RA and OA and their potential therapeutic impact.

Keywords: microRNA, small extracellular vesicles, mesenchymal stem cells, osteoarthritis, rheumatoid arthritis

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INTRODUCTION

An excessively prolonged imbalance of the immune system response can lead to a vast array of inflammatory and autoimmune disorders. Moreover, genetic predisposition and epigenetic regulations, including environmental factors and age, promote autoimmune, inflammatory, and degenerative diseases development (1). These illnesses imply a high economic burden for the healthcare system and those who suffer from them (2, 3). Osteoarticular diseases (OD), such as osteoarthritis (OA), and rheumatoid arthritis (RA), have raised particular concern in the last decades due to the increase of medical consults. They affect roughly 23% of the population over 40 worldwide for knee OA (the most common articulation affected by OA) (4, 5), and around 0.5% of the worldwide population for RA (6). Moreover, both OA and RA cause a great deal of pain and discomfort to the patients, impacting their quality of life (7). Without a cure for OD, patients rely mainly on non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, and glucocorticoids as the primary options to manage the symptoms (8, 9). Unfortunately, these treatments lack disease- and structural-modifying capabilities and even worse, their prolonged use is associated with severe side effects (9, 10).

Thus, alternative therapies are still needed to treat autoimmune/inflammatory and degenerative diseases like OA and RA. Both diseases are mainly defined by the loss of articular cartilage and are known to affect people of all races, genders, and ages (11, 12). Numerous therapeutic efforts have been made to restore the affected joints, including tissue engineering to promote tissue regeneration. Recently, cell-based therapies have had a considerable rise, such as the regulatory T cell therapy. However, their high cost and the technical difficulties in producing off-the-counter cell therapies remain significant hurdles for their clinical application (13). Three types of cell treatment are used in clinical trials for OA or degenerative environments; articular chondrocytes, meniscal fibrochondrocytes, and mesenchymal stem/stromal cells (MSCs), where the latter has shown encouraging results (11, 14–17). MSCs are multipotent stem cells of mesodermal origin that can be defined as a cell population with the hallmark self-renewal properties and differentiation into chondrogenic, osteogenic, and adipogenic lineages (18). Although therapy using MSCs has achieved significant progress, stem cell-based therapies have not fulfilled the initial promise. Some remaining drawbacks include the inconveniences associated with high costs and potential side effects, leading to inconsistency among preclinical and clinical trials (19).

In recent years, the therapeutic benefit of MSCs has been attributed to their functions through cell–to–cell contact and, more prominently, paracrine communication. The main mediators of paracrine communication are small extracellular vesicles (sEVs), which play an essential role as an alternative mechanism by which MSCs regulate different biological processes (20, 21). sEVs are heterogeneous particles that are delimited by a lipid bilayer membrane, whose primary function is to act as vehicles of cellular communication, transporting and transferring several bioactive molecules, such as proteins, peptides, lipids, messenger RNA (mRNA), and microRNA (miRNA) (22). miRNAs are small 20–22-nucleotide-long non-coding RNAs, which mediate post-transcriptional gene silencing by binding to the 3'-untranslated region (UTR) or open reading frame (ORF) region of target mRNAs (23) unpairing protein translation and causing a rapid tuning of cell fate decisions in response to environmental cues (24). Although sEVs can carry different types of cargo, increasing evidence points at miRNAs as significant mediators for the effects of these vesicles over the target cells (25, 26). Noteworthy, miRNAs regulate the immune system and signaling pathways related to extracellular matrix synthesis, chondrocyte survival, and proliferation (27–29). In addition, the auspicious use of sEVs as "cell-free cellular therapies" provides substantial advantages in contrast to whole-cell therapy, such as their easy handling and minimizing the risks of rejection (30). This review summarizes the current knowledge of MSC derived sEVs (MSC-sEVs) and their miRNA cargo as a potential and attractive substitute for treating autoimmune/inflammatory and degenerative disorders.

MSC-BASED THERAPY FOR OD TREATMENT

MSCs are multipotent fibroblast-like cells of mesodermal origin that have been described in several mammals, including humans and mice (31). According to the International Society of Cell Therapy (ISCT), three major criteria define MSCs: their capacity to adhere to plastic surfaces under culture conditions (32), their ability to self-renew and differentiate toward mesodermal lineages, such as adipogenic, chondrogenic and osteogenic (33) lineages, as well as the expression of surface markers CD105, CD73, and CD90 in the absence of hematopoietic markers including CD45, CD34, CD14 or CD11b, CD19, and HLA-DR (18, 34). These cells are found in various tissues, including bone marrow, adipose tissue, dental pulp, endometrium, amniotic fluid, placenta, and umbilical cord, among others (35). However, bone marrow and adipose tissues represent the most common sources for MSCs isolation because of their availability (36–47).

MSCs display a wide variety of biological functions, such as secretory (48), immunomodulatory (49) and homing (50) properties, representing a stem cell population with demonstrable progenitor cell functionality (33, 51) and a promising candidate for cell-based therapies. Illustrating this, ClinicalTrial.gov (https://clinicaltrials.gov/) lists 10406 phase I or II trials using MSCs in skin, bone, cartilage, heart, kidney, lung, liver, diabetes, immune/autoimmune diseases and even for COVID-19. Among these trials, 222 registered studies are using MSCs for OA and 55 for RA. OD are well–documented candidates for MSC treatment. Recent studies have shown that OA patients treated with an intra-articular injection of MSCs display a substantial enhancement in cartilage coverage and quality, relieving pain, ameliorating disability, and significantly

improving their quality of life (11, 12, 52, 53). Similarly, a phase Ia clinical trial in RA demonstrated the reduction of pro-inflammatory cytokines in patients injected with MSCs and revealed no short-term safety concerns (54). This data supports the potential of MSCs as an effective treatment for OA and RA patients.

Several studies have shown that MSCs can replace several damaged tissues *in vivo*. Mirza and collaborators showed that undifferentiated MSCs seeded on a graft were able to grow and restore a thick multicellular layer mimicking mature vascular tissue (55), whereas Sheng and collaborators were able to successfully transplant MSCs and regenerating sweat glands in patients *in vivo* (55, 56). Previous studies have demonstrated that MSCs can regulate the inflammatory response by suppressing mononuclear cells and promoting anti-inflammatory subsets from innate and adaptive immunity, including T-cells (57, 58). It has been well described that MSCs regulate T-cells activation and proliferation without the need for the cell to cell contact, suggesting the involvement of secreted soluble factors as the mechanism of action (59, 60). Additionally, MSCs negatively regulate natural killer cells (NK) activity, dendritic cells (DC) maturation, and B-cells proliferation while promoting Treg induction [Reviewed in (61, 62)]. It has also been shown that one of the hallmarks of MSC therapeutic potential is the regulation of cytokine production, including IFN-γ, TNF-α, and IL-10 (62). By modulating different immune cells involved in autoimmune diseases' pathogenesis, MSCs have a promising therapeutic potential. Although some mechanisms require the cell to cell contact, MSCs secretome seems to mediate most of their therapeutic effects in several pathologies, including OD (63, 64).

In the last few years, several studies suggest that MSC therapies in clinical applications do not show severe adverse effects showing promising therapeutic benefits (65). Nonetheless, the clinical application of MSCs and the fast development of commercial products show contradicting outcomes in clinical application and unsatisfactory therapeutic effects, primarily due to their low survival and homing capacity *in vivo* (19). Site-specific injection seems to be better to obtain more efficiency results [Reviewed in (66, 67)]. Therefore, to use MSCs as a successful treatment, these difficulties must be overcome. The most critical challenges are donor heterogeneity, stemness stability and differentiation capacities, limited expansion capacities, homing capacity, and rejection risks (68). In this regard, their derivatives including extracellular vesicles come as a promising solution as a cell-free based therapy due to their role as molecule delivery vehicles that mimic the effects of the parent on the target cell (66).

MSC-DERIVED SMALL EXTRACELLULAR VESICLES AS THERAPEUTIC TOOLS TO TREAT OSTEOARTICULAR DISEASES

Extracellular vesicles (EVs) are membrane-bound nanostructures released that act as essential mediators of cell-to-cell communication under physiological and pathological conditions (69). According to their size, EVs can be classified as apoptotic bodies (more than 1000nm), microvesicles (between 40-1000nm), and exosomes (50-200nm) (70). EVs can be generated directly by budding from the plasma membrane (microvesicles) or after fusion of multivesicular bodies (related to the endocytic pathway) with the plasma membrane to release intraluminal vesicles (exosomes). EVs are normally obtained by differential centrifugation protocols and the exosome enriched fraction also contains small microvesicles (smVs) commonly referred to as small extracellular vesicles (sEVs) (71, 72). sEVs can be further characterized by the expression of exosome-associated markers such as TSG101, ALIX, and tetraspanin proteins such as CD9, CD63 or CD81 (70). Released sEVs can either be readily taken up by neighboring or by distant cells due to their ability to travel through body fluids and mimic the parent cell's effect on the target cell (70). Due to the natural role of sEVs in cell-to-cell communication, they are readily taken up through phagocytosis, micropinocytosis, and endocytosis mediated by lipid raft, caveolin or clathrin (73, 74). Although sEVs can be delivered to any cell type, they are internalized in a highly cell type-specific manner that depends on recognizing typical sEV surface molecules by the cell or tissue, making them ideal therapeutic delivery systems [Reviewed in (74)].

A substantial advantage of using sEVs as therapeutic carriers is that they are nearly non-immunogenic and are capable of homing to distant tissues where the inflammation is located (75, 76) Indeed, mice injected with both wild-type and engineered sEVs showed no toxicity nor a significant immune response, further adding to the safety of sEV based therapies (77). However, the delivery and the frequency of sEVs injection on patients still needs to be addressed, in order to determine the most efficient strategy to obtain positive clinical outcomes.

In preclinical models, it has been described that MSCs-sEVs inhibit TNF-α induced collagenase activity and promote cartilage regeneration in chondrocytes derived from OA patients *in vitro* (75, 76). Moreover, MSCs-sEVs significantly improve OA progression by inhibiting cartilage degeneration in the collagenase-induced OA murine model (78). MSCs-sEVs were also shown to enhance the production of immature DCs that secrete IL-10, which are involved in suppressing inflammatory T-cell responses (76, 79, 80). On the other hand, Zhu and colleagues demonstrated that sEVs could reduce arthritis index, leukocyte infiltration, and, most importantly, destruction of the joint in a CIA mice model. These sEVs lowered Th1 and Th17 cells' frequencies through miRNA targeting of STAT3 and T-bet, having a potential role in treating arthritis (81). Munir and colleagues also proved that treating CIA in mice with MSCs decreased the severity of the disease by dampening the pathogenic immune response. Mice that received this treatment had reduced IL-6 and TNF-α, increased IL-10 in their joints and increased the frequency of Tregs in their spleen and lymph nodes, and a lower Th1:Th17 ratio (66). Other studies have demonstrated that sEVs can decrease the clinical signs of inflammation present in the CIA model by polarizing B lymphocytes into Breg-like cells (82).

Therefore, evidence supports the repairing properties of MSCs-sEVs in joint tissue, especially after intra-articular administration (83). These and other preclinical studies of MSCs-sEVs show that these potential treatments are safe and scalable for clinical application (20).

Since phase III clinical trials have shown inconsistent results in RA and OA without cartilage regeneration despite the promising preclinical studies (52, 84), their derived sEVs could also display conflicting results for RA and OA treatment. Several techniques to improve MSCs therapy have been recommended to overcome these issues [Reviewed in (85)]. For example, hypoxia preconditioning and 3D culture can increase the production of pro-chondrogenic factors (86). Additionally, sEVs action can be strengthened by modifying their specific cargo (87, 88), or by treatment with immunosuppressive cytokines, such as IL-10 (89), enhancing their anti-inflammatory and chondroprotective properties. Moreover, it has been shown that the genetic engineering of MSCs affects their derived sEVs, improving their immunosuppressive and chondroprotective abilities (87), where sEVs demonstrated to enhance chondrogenesis and suppress cartilage degradation (88).

The therapeutic effect of sEVs in the target cell is directly dependent on their cargo, which can be composed of a wide variety of molecules, including proteins, peptides, lipids, and several nucleic acids such as DNA, messenger RNA and microRNAs [Reviewed in (21)]. Although the effects of other sEV cargos cannot be excluded, proteins and miRNAs are considered the main mediators of the effect of sEVs in target cells. Proteomic analysis in sEVs has identified thousand proteins implicated in key biological processes such as sEV biogenesis, cellular structure, tissue repair and regeneration, and inflammatory response [Reviewed in (90)] Indeed, Chaubey and collaborators, validated TSG-6 as one of the protein mediators of MSC-sEV for immunomodulation by inducing a decrease in neutrophil infiltration in a murine model of hyperoxia-induced lung injury (91). However, to determine the role of proteins and miRNA in mediating the therapeutic efficacy of sEVs, a relation between the concentration of miRNA and proteins in their cargo is needed (92). Moreover, it is not well defined whether proteins and miRNAs work independently or synergistically in target cells, indicating that further studies are needed in this field. On the other hand, miRNAs encompass an important fraction of the exosome content and arise as the main regulators of MSC-sEVs function (26, 93). miRNAs are small non-coding RNA highly conserved among species, which control gene expression through its binding capacity to the three prime untranslated region (3'-UTR) of the targeted mRNAs, for repressing the expression of the corresponding gene at a post-transcriptional level (94). Compared with transcriptional and epigenetic regulation, post-transcriptional processes are fast and therefore can instantly tune cell fate decisions in response to environmental cues (94). Moreover, miRNAs contained in sEV are protected from RNAse degradation and through their integrins and opsonins the delivery of their internal content is efficient (24). Indeed, Neviani and collaborators demonstrated that sEVs derived from inactivated natural killer (NK) cells showed an equal cytotoxic activity when compared to sEVs derived from activated NK cells. Indeed, inactivated NK derived sEVs showed low levels of killer proteins in their cargo (perforin 1, granzyme A, granzyme B) while still retaining their cytotoxic activity, showing that the protein cargo is not the main bioactive mediator (95). In line with these results, RNA-depleted sEVs lose their immunosuppressive activity on T-cells, demonstrating their pivotal role on MSC-sEVs immunoregulation.

RELEVANCE OF miRNA IN THE PATHOGENESIS OF OD

miRNAs are critical regulators in maintaining a healthy joint as they participate in chondrocyte homeostasis and in the regulation of inflammatory mediators (96, 97). Proof of this is the phenotype observed in Dicer (a key enzyme in the miRNA biosynthesis pathways) knock-out mice, whose growth plates exhibited a reduction in proliferating chondrocytes and accelerated differentiation into a hypertrophic type, resulting in severe skeletal growth defects and premature death (98). Accordingly, an imbalance of some miRNAs has been associated with OD in both human and murine models. Illustrating this, a study using the serum transfer mouse model of RA in C57BL/6 mice identified a total of 536 upregulated genes and 417 downregulated genes that are predicted targets of miRNAs with reciprocal expression in arthritic mice (99). Twenty-two miRNAs whose expression was most significantly changed between nonarthritic and arthritic mice regulated the expression of proteins involved in bone formation, specifically Wnt and BMP signaling pathway components.

While activation of canonical Wnt signaling promotes bone formation (100), Wnt signaling antagonists such as Dkk inhibit this pathway and have been shown to regulate the erosive process in RA (101, 102). Among the most upregulated miRNAs found by Maeda and colleagues was miR-221-3p, which is induced in the TNF-driven model of arthritis and fibroblast-like synoviocytes (FLS) from RA patients (103). In bone, synovium-derived miRNAs, including miR-221-3p, may control skeletal pathways that inhibit osteoblast differentiation from augmenting bone erosion in RA by regulating Dkk2. Similar studies in OA patients have revealed significant miRNA imbalance in cartilage, synovial fluid, and plasma (104). Several studies have shown that there is differential expression of several miRNAs in OA *versus* a healthy joint. By evaluating the expression of 365 miRNA in OA patients versus healthy donors, Iliopoulos and colleagues found 16 altered miRNA, providing one of the earliest insights on the osteoarthritic chondrocytes miRNA signature (105). A subsequent study showed that a set of 17 miRNA that contribute to cartilage remodeling presented an altered expression and suggested that these changes were due to epigenetic regulation (106). Murata and colleagues investigated whether, in plasma and synovial fluid, miRNA could be used as possible biomarkers for RA and OA, finding that some miRNAs can effectively differentiate between both diseases (107).

Interestingly, 12 miRNA were overexpressed under the OA condition, all targeting important genes in chondrocyte maintenance and differentiation such as SMAD1, IL-1B, COL3A, VEGFA, and FGFR1 (104). Other reports point out imbalances in miRNAs associated with the regulation of ECM degradation enzymes. For example, the increase of miR-146a/miR-145/miR-22 and the decrease in miR-149/miR-125b/miR-558 causes ECM degradation. Some miRNAs such as miR-27b, miR-140, and miR-320 have been reported to target MMP13, a regulator of tissue repair and remodeling (108–110), while miR-92a-3p and miR-27b regulate ADAMTS expression, an enzyme that plays an important function in the degeneration of cartilage in RA and OA (111). Furthermore, it has been shown that the down-regulation of miR-140 inhibits IL-1β by inducing ADAMTS expression and that miR-27b regulates MMP-13 expression in human chondrocytes. Importantly, miR-27b, miR-140, and miR-146a are dysregulated in OA patients, suggesting a role for them in OA pathogenesis (108, 112, 113).

It has been widely reported that TGF-βs and BMPs regulate postnatal joint cartilage homeostasis and that dysregulated TGF-β and BMP signaling are often associated with OD [Reviewed in (114)]. These TGF-β superfamily members bind to the heteromeric receptor complex, comprised type I and II receptors at the cell surface, that transduce intracellular signals by activating Smad complex or mitogen-activated protein kinase (MAPK) cascade. BMPs have a chondroprotective role in different animal models of RA (115); specifically, it has been suggested that endogenous expression of BMPs is required to maintain chondrocytes phenotype *in vitro* (116, 117). However, its dynamic regulation has been observed in the CIA murine model, supporting a role for this pathway in RA (118). During CIA, BMP-2 and BMP-7 are upregulated in a TNF-dependent manner, a phenomenon accompanied by an increase in Smad-5 phosphorylation: thus, there is an increase in BMP signaling activity. Similarly, in an OA rat model, it was shown that IL1b upregulated BMP-2 through the MEK/ERK/Sp1 signaling pathways and that the administration of the BMP antagonist Noggin prevented cartilage degeneration and OA development (119). An observational study in OA patients showed that the levels miR-22, which targets BMP2, are increased in the progression of the disease (120). Furthermore, the inhibition of miR-22 has been shown to prevent inflammatory activity (105, 121). On the contrary to miR-22, miR-140 also targets BMP2 but in a different position of the 3′-UTR region and is associated with increased BMP2 expression (120). Notably, the levels of synovial miR-140 were significantly reduced in the patients with OA and were negatively correlated with OA severity compared to controls (120, 122). Furthermore, after arthroscopic debridement, the levels of these miRNAs and BMP2 were restored (120), suggesting miR-22 and miR-140 play a role in the development of OA by regulating BMP-2. It has also been shown that BMP targeting miRNAs' dysregulation is associated with the pathogenesis of RA. It has been demonstrated that sEVs derived from fibroblast-like synoviocytes with elevated levels of miR-486-5p promoted osteoblast differentiation and proliferation by repressing Tob1, thus activating the BMP/Smad signaling pathway, alleviating the severity of RA in the CIA model (123).

On the other hand, TGF-β has been implicated in cartilage ECM production and maintenance, specifically by increasing COL2A1, perlecan, fibronectin, and hyaluronan (124, 125). Furthermore, TGF-β also has anti-inflammatory functions, counteracting IL1b and IL-6 mediated inflammation in the joint (124, 125). Importantly, several miRNAs target different proteins of these pathways, which has been reviewed elsewhere (126). It has been shown that miR-455-3p promotes TGF-β/Smad signaling in chondrocytes and inhibits cartilage degeneration by directly suppressing PAK2, a kinase that inhibits TGF-β signaling. Accordingly, the miR-455-3p levels were decreased, and both PAK2 and phospho-PAK2 were increased in OA cartilage compared with control cartilage. Moreover, miR-455-3p KO mice displayed significant degeneration of the knee cartilage (127). In OA cartilage, miR-150-5p is overexpressed. It has been shown that miR-140-5p directly targets TGF-b3 signaling by altering the expression of TGF-b3 and Smad-3 in mandibular condylar chondrocytes, thus having a role in the regulation of mandibular cartilage homeostasis and development (128). Furthermore, this miRNA is increased in the cartilage of OA patients compared to control cartilage from femoral neck fracture patients, where it suppresses the Smad2/3 pathway, a process that promotes cartilage destruction and the progression of the disease (129). Using miR-140-null mice, which showed different changes related to OA such as fibrillation of articular cartilage, Miyaki and collaborators demonstrated that miR-140 regulates cartilage development and homeostasis (113). Interestingly, miR-140 knockout mice presented proteoglycan loss and fibrillation of articular cartilage emulating age-related OA. On the contrary, transgenic mice overexpressing miR-140 in cartilage were resistant to antigen-induced arthritis. Another miRNA involved in TGF-β signaling modulation is miR-125-5p, which downregulates the Smad2 expression and leads to the dysfunction of TGF-β signaling. Noteworthy, the circular ribonucleic acids (circRNAs), CircCDK14, which is down-regulated in the joint wearing position, regulates metabolism, inhibits apoptosis, and promotes chondrocyte proliferation by miR-125a-5p sponging (130). Taking together, studying miRNA dysregulation in OD and the underlying mechanisms could provide new insights towards more effective treatments. At the same time, TGF-β exerts an anabolic repairing response on articular cartilage. On the other hand, proinflammatory cytokines such as IL-1β and TNF-α which exert a strong catabolic effect (131). As follows, the balance between TGF-β and the IL-1β or TNF-α signaling pathways is a critical regulator of articular cartilage homeostasis (131), thereby its disruption contributes to the pathogenesis of OA.

In OA, NF-κB signaling orchestrates chondrocyte catabolism, survival, and synovial inflammation. Growing evidence suggests that miRNAs targeting either matrix-degrading enzymes or components of the NF-κB pathway can suppress chondrocyte catalytic activity. While some miRNAs such as miR-138 and miR-9 directly suppress the NF-κB subunits p65 or p105/50 (132, 133), others like miR-210, miR-26a/b, miR-93, miR149,

and miR-146a act indirectly by targeting upstream regulators of NF-κB (134) such as death receptor 6 (DR6), KPNA3, Toll-like receptor 4 (TLR4), TAK1, and TNF-receptor associated factor 6 (TRAF6)/interleukin-1 receptor-associated kinase 1 (IRAK1). Additionally, synovial inflammation in the context of OA or osteoblastogenesis is associated with miR-146/miR-155/miR-218/miR-135, among others (135–137).

In RA, miRNA dysregulation is implicated in the activation of multiple cytokine-signaling pathways that leads to synovial tissue lesions and dysregulation of immune cells, thereby contributing to pathogenesis (139). Many studies have demonstrated that miR-16, miR-146a, miR-155, and miR-223 present an increased expression level in synovial fluid of RA patients. Moreover, inflamed joints of RA patients show an increased expression of miR-133a, miR-142-3p, miR-142-5p, miR-146a, miR-155, miR-203, miR-221, miR-222, miR223 (103, 107, 140, 141). On the other hand, the expression of miR-124a and miR-34a is decreased in the context of RA (142, 143). Furthermore, miR-181a, miR-17–92 overexpression enhances the inflammation, while upregulation of miR-146a and miR-573 suppresses the autoimmunity (144). Although several miRNAs related to inflammation are dysregulated in RA, miR-146a appears to be essential in controlling the inflammation. miR-146a targets TNF-α/TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), elevating TNF-α production through TRAF6/IRAK1 mediated pathway [Reviewed in (126, 145)]. miR-146a is also able to regulate genes such as FAF1, IRAK2, FADD, IRF-5, Stat-1, and PTC-1 (146), making it a possible therapeutic target for the treatment of RA. Besides miR-146, miR-155 can also stimulate the proinflammatory mediators TNF-α, TLRs, LPS, and IL-1 [Reviewed in (145)]. Upregulation of miR-155 has been observed in synovial tissue, FLS, peripheral and blood mononuclear cells. Supporting a role for targeting miR-155 in RA, miR-155 knockout mice do not develop collagen-induced arthritis (146). Therefore, miR-155 may be a promising therapeutic target for RA.

miRNAs and their levels in plasma and synovial fluids are associated with the occurrence of OD. Therefore they could serve as predictive biomarkers and even as therapeutics targets. Owing to the fact that miRNAs play a crucial role in the maintenance of healthy joints, restoring their balance could be an effective way to treat OA and RA. To accomplish an effective therapeutic strategy, the delivery system is the main barrier that has to be overcome (147). Given that miRNAs are naturally carried by sEVs, they are protected from RNAse degradation and the delivery to target cells is efficient thanks to the integrins and opsonins (147–150).

miRNA SHUTTLED BY sEVs DERIVED FROM MSCs AND THEIR THERAPEUTIC FUNCTION ON OSTEOARTICULAR DISEASES

Since MSC-sEVs are natural carriers of therapeutic miRNA, they have arisen as an attractive therapeutic tool to treat several diseases including OD. There are copious amounts of studies reporting the different effects of miRNA transfer *via* sEVs, and their relevance in cell to cell communication. Indeed, miRNAs have gained more attention than proteins or other variety molecules contained in sEVs, due to their regulatory roles in gene expression. Goldie and collaborators demonstrated that the proportion of miRNA is higher in sEVs than in their parent cells (151). Moreover, a profiling study of miRNAs has demonstrated that miRNAs are not randomly packaged into sEVs. Guduric-Fuchs and collaborators have shown that a subset of miRNAs (miR-150, miR-142-3p, and miR-451) are preferentially incorporated in sEVs (152). Although the effects of other sEV cargos cannot be excluded, miRNAs are considered the key functional elements on recipient cells. Several thousand miRNAs have been identified in humans, and their studies have increased in the last decade, moreover miRNAs are frequently deregulated in multiple human diseases which offers many opportunities for diagnosis and treatment for various pathological conditions.

The use of sEVs as a therapeutic treatment for different immune diseases is still challenging, since safety evaluations are still pending. Multiple experiments must be done in large and proper animal models in order to prove their therapeutic efficacy and safety in this area before applying this approach in the clinic. Given that it primarily affects the joints, we suggest that the optimal form of delivery should be intra-articular injection.

Chen and collaborators, have shown that, both *in vitro* and *in vivo*, BM-MSC-sEV enriched in miR-150-5p suppress the expression of MMP14 and VEGF, and decrease the expression levels of IL-β, TNF-α, and TGF-β, resulting in the inhibition of the proliferation and migration of fibroblast-like synoviocytes (FLS) and alleviation of inflammation (153). Similarly, BM-MSC sEV derived miR-320a targets CXCL9 and thereby suppresses FLS activation, migration and invasion in RA (154). Additionally, the overexpression of miR-124a in MSC-sEV significantly increased the expression of apoptosis-related proteins inducing an inhibition on the proliferation, invasion and migration of RA-FLS cells (155).

It has been well documented that miRNAs in MSC-sEVs have a chondroprotective role in OA (156). Illustrating this, MSC-sEVs shuttled miR-92a-3p increases chondrocyte proliferation and the levels of COL9a2 and aggrecan, and effect mediated by targeting noggin3 and Wnt5a while activating the PI3K/AKT/mTOR pathway, thus increasing the levels of [Reviewed in (21)], (88). On the other hand, MSC-sEVs-derived miR-135b stimulates cartilage regeneration by binding to the transcription factor Sp1 (SP1), which regulates apoptosis and proliferation (157). Moreover, miR-140-5p upregulates Sox9 and promotes MSCs chondrogenesis (**Figure 1**). Additionally, recent studies show that sEV-mediated transfer of miR-140 from dendritic cells improves OA *in vitro* by inhibiting proteases associated with cartilage degradative processes in the joint and alleviates the progression of OA in a rat model *in vivo* (158). In contrast, another study reported that miR-155 levels are significantly upregulated in human OA cartilage biopsies and

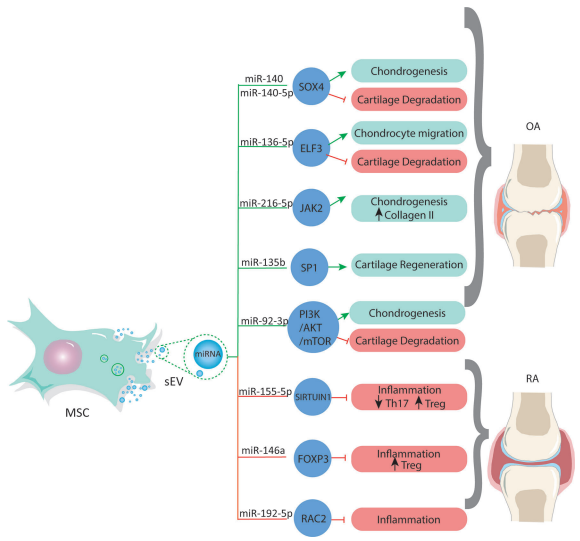

FIGURE 1 | MSCs release sEVs with a miRNAs cargo that regulate gene expression by targeting transcription factors associated to different processes in osteoarticular diseases. These miRNA can be used to develop new and effective therapies for OA and RA.

primary chondrocytes stimulated by IL-1ß. Moreover, miR-155 overexpression promotes IL-1ß-induced apoptosis and catabolic activity in chondrocytes *in vitro* (159). Chen et al. reported that MSC-sEV-shuttled miR-136-5p promotes chondrocyte migration *in vitro* and inhibits cartilage degeneration *in vivo* (**Figure 1**) both in human chondrocytes *in vitro* and in mice *in vivo* (160).

On the other hand, the involvement of MSC-sEVs-derived miRNAs in the context of immune modulation has been reported (149). MSC-sEVs are immunologically active, meaning that they can attenuate the immune system through increasing anti-inflammatory cytokines, such as IL-10 and TGF-β and the induction of Tregs, modulating immune activity. Indeed, RNA-depleted sEVs lose their immunosuppressive activity on T-cells (161), demonstrating their pivotal role on MSC-sEVs immunoregulation and therefore their potential use on autoimmune diseases such as RA (75, 80, 83). Indeed, the downregulation of miR-192-5p has been reported in RA patients, and its transfer *via* sEVs derived from BM-MSCs reduced the inflammatory response by downregulating the Ras-related C3 botulinum toxin substrate 2 protein (RAC2) (**Figure 1**), attenuating the severity of the disease in rats (162). It has been reported that sEVs derived from TNFα and IFNγ pretreated-MSCs improve their suppressive activity over T cells (75). This pretreatment was associated with a higher expression of miR-155 and miR-146, two miRNAs involved in activating and inhibiting T cells inflammatory reactions (163). Similarly, miR-155-5p loaded in sEVs derived from LPS-stimulated periodontal ligament stem cells (PDLSCs) inhibited pro-inflammatory Th17 cells favoring their conversion into Treg through inhibition of Sirtuin-1 (Sirt1) (164). Moreover, the therapeutic role of miR-146a-5p contained in MSC-sEVs has been shown *in vivo* in a model of allergic airway inflammation (161). In this study, the authors demonstrated that the miRNA signature of MSC-sEVs was enriched in miR-146a-5p compared to sEVs derived from less immunosuppressive cells such as fibroblasts (161). In addition, miR-146a-5p mimic improves the immunosuppressive capacities of fibroblast sEVs, while miR-146a-5p inhibition impairs the immunosuppressive activity of MSC-sEVs on T-cell proliferation (161). In RA, miR-146a is downregulated, but its upregulation associated with the administration of MSC-sEVs increased the frequency of Treg cell population by increasing the expression of some key autoimmune response genes and their protein products, such as TGFβ, IL-10 and FOXP3 (**Figure 1**), resulting in a beneficial anti-inflammatory response (165, 166). Rong and collaborators showed that the hypoxic pre-treatment of rat BM-MSC (a known method for the improvement of the therapeutic properties of MSCs [Reviewed in (167)]) promotes the release of miR-216a-5p enriched sEVs that target JAK2 in chondrocytes, resulting in an increase in chondrocyte proliferation and migration, while inhibiting their apoptosis. The miR-216a-5p enriched sEVs also reduced ECM degradation through the inhibition of MMP expression and increasing COL-II expression levels (168).

In summary, several miRNAs are known to be associated with different processes relevant to OD (169), such as inflammation (miR-22, miR-320) (105, 110), extracellular matrix synthesis (miR-148a, miR-27, miR-218) (170, 171) and chondrocyte proliferation. Additionally, several miRNAs have been shown to be involved in processes associated with MSCs differentiation into chondrocytes (miR-19a, miR-410) (172, 173), and processes such as chondrocyte hypertrophy (miR-381, miR-140) (174, 175), apoptosis and autophagy (miR-30b) (176) (**Table 1**). The therapeutic potential of miRNAs both in degenerative diseases such as OA and autoimmune diseases such as RA is very promising, and their delivery through sEVs greatly facilitates escalation to later-stage clinical trials. Still, more work needs to be done concerning the full effect of miRNAs both in target cells and other types of cells to assess the safety of the therapeutic application of miRNAs.

CONCLUDING REMARKS

As mentioned in the previous sections, MSC-sEVs arise as a potential cell-free based therapy that can reduce the risks associated with MSC. Strikingly, several reports show that MSC-sEVs mimic the biological effects of MSCs. Therefore, MSC-sEVs represent a hopeful alternative to MSC therapy.

The main functional components of MSC-sEVs are miRNAs, which can regulate the expression of multiple target genes and participate in various cell signaling processes. The miRNA profile of MSC-sEVs is associated with their effect. Although there are tools to identify miRNAs in sEVs, the principal target genes of sEVs derived miRNAs remain unspecified. However, this work summarizes some of the miRNAs involved in OD pathogenesis and some of the miRNAs that mediate the therapeutic effects of sEVs in OD. These miRNA could be

TABLE 1 | Summary of the literature reporting the role of miRNAs in OD.

miRNA	Context	Target cell	Effect on the target cell	Mechanism of action	Reference
miR-92a-3p	sEVs from miR-92-3p-overexpressing MSCs; OA	Chondrocytes	Enhancement of chondrogenesis and suppression of cartilage degradation	Targeting the PI3K/AKT/mTOR pathway	88
miR-135b	sEVs from TGF-β1-stimulated MSCs	Chondrocytes	Cartilage regeneration	Binding to transcription factor (SP1)	157
miR-22	OA; inflammation	Chondrocytes	Decrease inflammation and ECM degradation	Targeting the PPARα and BMP-7 signaling pathway	105
miR-140	OA; MSC-sEVs	Chondrocytes; MSCs;	Inhibition of cartilage degradation; suppression of chondrocytes hypertrophy; Promotion of chondrogenesis	Suppression of the expression of cartilage degrading enzymes; controlling the BMPs signaling pathway; Upregulation of Sox9	158; 175; 177
miR-320	Cartilage homeostasis	Chondrocytes	Regulation of chondrogenesis	Targeting the expression of MMP-13	110
miR-27	OA	Chondrocytes	Decreasement of inflammation	Inhibition of the NF-κB pathway	170
miR-149	OA inflammation	Chondrocytes	Suppression of chondrocyte inflammatory response	Downregulation of the TAK1/NF-κB pathway	135
miR-19a	OA	Chondrocytes	Promotion of cell viability and migration	Upregulation of Sox9 *via* the/NF-κB pathway	173
miR-410	OA	MSCs	Chondrogenic differentiation	Targeting the Wnt signaling pathway	172
miR-381	OA pathogenesis	Chondrocytes	Chondrocyte hypertrophy	Targeting histone deacetylase 4 (HDAC4)	174
miR-125b	OA	Chondrocytes	ECM degradation	Targeting of ECM-degrading enzyme ADAMTS-4	178
miR-558	OA	Chondrocytes	Cartilage homeostasis	Inhibiting COX-2 and IL-1β-induced catabolic effects	178
miR-9	OA	Chondrocytes	Suppression of apoptosis and promotion of cell proliferation	Binding to NF-kB1	132
miR-138	OA	Chondrocytes	Decrease in the chondrocyte inflammatory response	Suppressing the protein levels of p65, COX-2 and IL6	133
miR-136-5p	OA; MSC-sEVs	Chondrocytes	Increase in chondrocyte migration and decrease in cartilage degradation	Inhibiting the expression of ELF3	160
miR-153	OD	MSCs	Decrease in osteogenic differentiation	Interacting with bone morphogenetic protein receptor type II (BMPR2)	134
miR-194	Bone homeostasis	MSCs	Increase in osteogenic differentiation	Suppressing STAT1	179
miR-216a	OD; MSC-sEVs	MSCs; chondrocytes	Increase in osteogenic differentiation; increase in chondrocyte proliferation and migration	Downregulation of c-Cbl; inhibiting JAK2	180; 168
miR-126a-5p	OA	Chondrocytes	Reduction in ECM degradation	Increasing expression of collagen II and decreasing expression of MMP	168
miR-146a	RA; MSC-sEVs	Tregs	Increase in anti-inflammatory response	Increasing the expression of FOXP3	83

considered as promising candidates to use for effective treatment of these diseases. Further studies in this field are required to develop MSC-sEVs therapeutics based on miRNA delivery for autoimmune/inflammatory and degenerative diseases. Furthermore, delving into the role of miRNAs in the pathogenesis of disease, would also improve therapeutic strategies that can restore their normal levels, because not all miRNAs have beneficial effects. In this context it is also important to study the regulation of miRNAs and their biological functions, and also increase the knowledge of other non-coding RNAs that can be involved in OD. On the other hand, studies on the enrichment of sEVs in beneficial miRNAs and/or other non-coding RNAs that regulate disease-promoting miRNAs and evaluating strategies for the targeted delivery of sEVs to particular cell types to increase efficiency remain one of the following challenges.

AUTHOR CONTRIBUTIONS

EL-B and MA wrote the main part of the manuscript with inputs from CH, FB-B, AO, CG, FG, CP, NL-C, GM, RE-V, and FD. PL-C and AV-L design the original idea of the review and critical review the manuscript. All authors contributed to the article and approved the submitted version.

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Serum Alpha-1-Acid Glycoprotein-1 and Urinary Extracellular Vesicle miR-21-5p as Potential Biomarkers of Primary Aldosteronism

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Primary aldosteronism (PA) is the most common cause of secondary hypertension and reaches a prevalence of 6-10%. PA is an endocrine disorder, currently identified as a broad-spectrum phenotype, spanning from normotension to hypertension. In this regard, several studies have made advances in the identification of mediators and novel biomarkers of PA as specific proteins, miRNAs, and lately, extracellular vesicles (EVs) and their cargo.

Aim: To evaluate lipocalins LCN2 and AGP1, and specific urinary EV miR-21-5p and Let-7i-5p as novel biomarkers for PA.

Subjects and Methods: A cross-sectional study was performed in 41 adult subjects classified as normotensive controls (CTL), essential hypertensives (EH), and primary aldosteronism (PA) subjects, who were similar in gender, age, and BMI. Systolic (SBP) and diastolic (DBP) blood pressure, aldosterone, plasma renin activity (PRA), and aldosterone to renin ratio (ARR) were determined. Inflammatory parameters were defined as hs-C-reactive protein (hs-CRP), PAI-1, MMP9, IL6, LCN2, LCN2-MMP9, and AGP1. We isolated urinary EVs (uEVs) and measured two miRNA cargo miR-21-5p and Let-7i-5p by Taqman-qPCR. Statistical analyses as group comparisons were performed by Kruskall-Wallis, and discriminatory analyses by ROC curves were performed with SPSS v21 and Graphpad-Prism v9.

Results: PA and EH subjects have significantly higher SBP and DBP ($p < 0.05$) than the control group. PA subjects have similar hs-CRP, PAI-1, IL-6, MMP9, LCN2, and LCN2-MMP9 but have higher levels of AGP1 ($p < 0.05$) than the CTL&EH group. The concentration and size of uEVs and miRNA Let-7i-5p did not show any difference between groups. In PA, we found significantly lower levels of miR-21-5p than controls ($p < 0.05$). AGP1 was associated with aldosterone, PRA, and ARR. ROC curves detected AUC for AGP1 of 0.90 (IC 95 [0.79 – 1.00], $p < 0.001$), and combination of AGP1 and

EV-miR-21-5p showed an AUC of 0.94 (IC 95 [0.85 – 1.00], p<0.001) to discriminate the PA condition from EH and controls.

Conclusion: Serum AGP1 protein was found to be increased, and miR-21-5p in uEVs was decreased in subjects classified as PA. Association of AGP1 with aldosterone, renin activity, and ARR, besides the high discriminatory capacity of AGP1 and uEV-miR-21-5p to identify the PA condition, place both as potential biomarkers of PA.

Keywords: primary aldosteronism (PA), biomarker, lipocalin, miR-21-5p, extracellular vesicles, AGP1, Alpha-1-acid glycoprotein-1

INTRODUCTION

The etiology of arterial hypertension (AHT) is unknown in more than 80-90% of cases, which is named essential hypertension (EH). One third of EH has been suggested to be associated with endocrine disorders (1). Primary aldosteronism (PA) is an endocrine disorder, currently identified as a broad-spectrum phenotype, spanning from normotension (4% prevalence) to hypertension (10% prevalence) (2–6). PA is characterized by an inappropriately high circulating aldosterone independent of known physiological regulators such as renin, angiotensin II, potassium, and sodium status (e.g., high saline intake) (7). The diagnosis of PA is relevant, not only for its association to high blood pressure but also for the harmful effects in extra-renal tissues, generally associated with the mineralocorticoid receptor (MR) activation by aldosterone which induces inflammation (8, 9), tissue remodeling, and fibrosis (8, 10–14), affecting the renal, heart, the vascular system (endothelial cells and smooth muscles cells), the immune system (15) and the adipose tissue (16).

Several studies have tied to advance in the identification of novel biomarkers for PA that support its early detection and also other reported effects as inflammation, endothelial dysfunction, renal damage, vascular remodeling and (17, 18), and oxidative stress (19, 20). Early "surrogate biomarkers" have been previously evaluated, such as high sensitive C-reactive protein (hs-CRP), Plasminogen inhibitor activator-1 (PAI-1), matrix metallopeptidase 9 (MMP-9) and malondialdehyde (MDA) (8, 9), free Cystatin-C (CysC), and neutrophil gelatinase associated lipocalin (NGAL or LCN2) (21–23). However, none of these biomarkers are currently available in clinical diagnoses for arterial hypertension or PA. Recent proteomic studies have shown that urinary and serum alpha-1-acid glycoprotein-1 (AGP1), also known as ORM1, have been proposed as prognostic biomarkers for inflammatory diseases such as chronic heart failure (24), some types of cancer (25), and lately for PA (26).

Experimental and clinical studies demonstrate that small extracellular vesicles (sEVs) or exosomes are potential biomarkers of disease (27), including in cancer, metabolic disorders, and cardiovascular diseases (28, 29). Urinary EVs originated mainly from cells lining the renal tubules carrying proteins, lipids, RNA, and miRNA, and have been recognized recently as a source of diagnostic biomarkers for different renal and endocrine pathologies (30–36), including primary aldosteronism (26).

MicroRNAs (miRNAs) are short non-coding RNA molecules genome-encoded, that are approximately 22 nucleotides in length and modulate downstream gene expression by post-transcriptional mechanisms, specifically by binding to the 3′ untranslated regions (UTR) of a target messenger RNA (mRNA), leading to mRNA degradation or repression of translation (37–39). Recent literature (30, 37, 40–46) proposes that microRNAs in exosomes are involved in physiological and pathophysiological processes correlated with hypertension (47) response to sodium intake (48) and PA (26, 30). miRNAs are packaged into EVs for transport into different biofluids (e.g., blood, urine) and change according to the metabolic microenvironment (e.g., inflammation) of the parent cell. In endocrine hypertension phenotypes, such as nonclassic-AME (31) and PA, have been identified in the differential expression of EV-associated miRNAs, such as miR-192, miR-204 (31), miRNA-21, and Let-7i. miR-21 has been found in EVs isolated from urine (43), plasma (44), and endothelial cells (45). Romero et al. reported on the protective role of miR-21 in the cardiac pathology triggered by excess aldosterone in the heart of mice and rats (49, 50). Let-7i negatively regulates cardiac inflammation and fibrosis in presence of angiotensin II or aldosterone (49–51). Similarly, Deccman et al. identified circulating miR-30e-5p, miR-30d-5p, miR-223-3p, and miR-7-5p in PA patients with bilateral adrenal hyperplasia (BAH) and aldosterone-producing adenoma (APA). Altogether, these reports highlight the potential role of both miRNA and EV-associated miRNAs as biomarkers or mediators of PA (46).

The current study aimed to evaluate lipocalins LCN2 and AGP1, and specific urinary extracellular vesicles miR-21-5p and Let-7i-5p as novel biomarkers of primary aldosteronism.

METHODS

This study used the serum, plasma, and urine samples from a biobank obtained from a cohort of 206 adult Chilean subjects of both genders, between 18 and 65 years old. The subjects were recruited from outpatient centers associated with the UC-Christus Health Network in Santiago, Chile, following the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Faculty of Medicine of the Pontificia Universidad Católica de Chile (Certification CEC-MEDUC 12-

207 and 14-268, and updated by CEC-MEDUC 190823001 and 200619004).

All subjects had a sodium diet ad libitum and declared that they did not ingest any herbal products or extreme diets during the month prior to the analysis. Subjects with a BMI >30 kg/m^2, kidney disease, diabetes mellitus, liver, and heart failure were excluded. Subjects using glucocorticoids, contraceptives, or some interfering drugs, such Ag-II-receptor blockers (ARB), ACE-Inhibitors (ACEI), and spironolactone (MR antagonist), were also excluded.

After exclusion criteria were applied, 132 subjects were included in the study. The subjects were classified as normotensive controls (CTL), have clinical and biochemical parameters in the normal range, essential hypertensives (EH) according to the 2017 ACC/AHA Guidelines for High Blood Pressure (52), and subjects having a positive screening for PA (Aldosterone >9ng/dl, PRA <1 ng/ml*h), according to The Endocrine Society 2016 guidelines (53) and Vaidya et al. (5, 54–56). All studied subjects (PA, EH, and CTL) have a clinical record including medical history and physical examination, as well a biochemical profile, creatinine, electrolytes, aldosterone, plasma renin activity (PRA), serum, and 24-hour and morning urine samples. Aldosterone and PRA were measured by immunoassay using a commercial kit (DiaSorin, Stillwater, MN). Urine samples for uEV isolation were stored at -80C with a 1X protease inhibitor cocktail (Roche, USA).

Evaluation of the Parameters Associated With Inflammation, Endothelial, and Renal Damage in PA Subjects

The inflammatory status of all subjects was evaluated by measuring hs-CRP with a nephelometric assay (BN ProSpec Systems; Siemens Healthcare Diagnostics Products, Marburg, Germany) and IL-6 by an ELISA with commercial reagents and standards (D6050, R&D Systems, Minneapolis, MN), according to the manufacturer's protocols. Endothelial damage was evaluated by surrogate markers such as PAI-1, MMP9, and MMP2 activities. PAI-1 was determined by ELISA (HYPHEN BioMed, Neuville sur Oise, France), and MMP9 and MMP2 activities by zymography, as previously described (57). Early renal damage was evaluated with 24-hour urine albuminuria to creatinine ratio (UACR). Albumin is measured by a turbidimetric immunoassay (Roche, Germany), and urine creatinine was measured with a colorimetric assay (Roche, Indianapolis, IN) in a Hitachi Automatic Analyzer 7600 (Roche/Hitachi, Kobe, Japan). Plasma and urinary electrolytes (sodium and potassium) were evaluated with methods previously described (58).

Determination of Serum Lipocalins AGP1, LCN2, and LCN2-MMP9 in PA Subjects

We measured the serum levels of lipocalins AGP1, LCN2, and LCN2-MMP9 proteins (26) by commercial ELISA immunoassay for AGP1 (Human α1-Acid Glycoprotein Immunoassay, DAGP00, USA R&D Systems, Inc.) according to the manufacturer's protocol, LCN2 (DLCN20, R&D Systems, Minneapolis, MN), LCN2-MMP9 (DM9L20, R&D Systems, Minneapolis, MN).

Isolation and Characterization of Urinary Extracellular Vesicles From PA Subjects

Urinary EVs (uEVs) were isolated by a sequential ultracentrifugation protocol previously described by Barros et al. (26). Urinary creatinine was used to normalize samples of uEVs (59, 60). Isolated uEVs were characterized as previously described (26, 31) and according to the International Society for Extracellular Vesicles guidelines (27) using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and western blot with characteristic EV proteins (61).

TEM was performed with 15 µl of uEVs suspension were absorbed onto a 200 mesh carbon-coated copper grid for 1 min. Samples were negatively stained with 2% uranyl acetate solution for 1 min. Grids were visualized in a Phillips Tecnai transmission electron microscope at 80 kV and images were acquired using a SIS-CCD Camera Megaview G2 (62). The concentration and size of uEVs were determined by nanotracking analyses (NTA) performed in a low-volume flow cell (LVFC) of a NanoSight NS300 and NTA 3.2 software (Malvern Instruments Ltd, Malvern, UK). Camera level and detection threshold was optimized to identify individual particles and minimum background noise during recordings (camera level = 12-14; detection threshold = 3-5; flow speed = 50). Particles were tracked by passing a laser beam through the liquid sample and the scattered light was detected and captured in short videos by a sCMOS camera (3 videos of 20 seconds each). The Brownian motion of particles was determined, and the distance moved by the detected particles will be used to calculate the diameter (mean and mode size) and concentration of vesicles using the Stokes-Einstein equation (63).

Western Blot of Exosome Markers TSG101 and CD9 Proteins

Similar quantities of EVs were resuspended in Laemmli buffer and then separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, CA, USA), blocked with 5% skim milk in PBS-Tween20 (PBST) 0.1% (vol/vol) for 1 hour and probed with primary rabbit monoclonal anti-TSG101 (1:10.000 Ab125011, Abcam, MS, USA), rabbit monoclonal anti-CD9 (1:500 (D801A) cat#13174; Cell Signaling Technology, MA, USA). After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG-HRP (1:10.000; ab6939; Abcam, USA) for 1 hour at RT. Proteins were detected using chemiluminescence (ECL Western Blotting substrate reagent, Pierce, USA) in a Chemi-Doc MP imaging system (Bio-Rad, CA, USA).

Urinary EV RNA Isolation

RNA from the extracellular vesicle was isolated by organic extraction using the Trizol® reagent according to the manufacturer's instructions. Two microliters of each RNA sample were pipetted on the NanoQuant Plate™ of the Infinite® M200 PRO spectrophotometer (TECAN; Männedorfl;

Switzerland) to quantify the RNA concentration (A260 nm) and purity (A260/A280 nm ratio) using Tecan i-control™ software.

Expression of miR-21-5p and Let-7i-5p in Urinary Extracellular Vesicles

Reverse transcription of miRNA samples was performed using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (A28007), according to the manufacturer's instructions. The expression of miRNAs (Hsa-miR-21-5p and Hsa-let-7i-5p) were evaluated with TaqMan™ Advanced miRNA Assay (A25576) and the TaqMan™ Fast Advanced Master Mix (4444557, Applied Biosystems) in the RotorGene 6000 thermocycler (Corbett Research, Sydney, Australia). The amplification reactions were performed as follows: Enzyme activation at 95°C for 20 seconds and 40 cycles of 95°C for 3 seconds, anneal/extend at 60°C for 30 seconds. RNU6 snRNA was used as an internal normalization control (TaqMan™ MicroRNA Assay, ID001973). The fold changes of miRNA expression were calculated using the relative cycle threshold (2−ΔΔCt) method and further normalized by the spot urinary creatinine. Unpaired Kruskal-Wallis test was performed to identify differences in PA patients versus EH and healthy controls.

Statical Analyses

Clinical, biochemical, and expression data are expressed as median [Q1-Q3]. Data normality was determined by Kolmogorov-Smirnov test. For parametric and non-parametric comparisons between two sets of data, an unpaired Student *t*-test or a Mann-Whitney test were performed. To assess differences between groups of data and an independent variable, a one-way Analysis of Variance (ANOVA) or Kruskal Wallis was performed using a Tukey or Dunn *post hoc* test, respectively. Associations were performed by linear regression by Pearson or Spearman regression according to data normality.

Receiver operating characteristic (ROC) analysis was used to test the ability of lipocalins (LCN2, AGP1) and uEV-associated miRNAs (miR-21-5p and Let-7i-5p) to discriminate PA patients from EH and control subjects. A p value < 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism v9.1 (GraphPad, CA, USA) or SPSS v21 (IBM, USA) software.

RESULTS

Clinical and Biochemical Characteristics of Subjects With PA

We identified 11 PA subjects (8.3%) in our cohort of study according to the PA criteria described in the Methods section. Clinical and biochemical baseline characteristics are shown in **Table 1**. PA, EH, and CTL groups were similar in age, gender, and body mass index. Systolic (140 [125-153] vs. 134 [123-139] vs 116 [110-121] mmHg, p<0.05) and diastolic blood pressure (89 [76-98] vs. 87 [81-93] vs. 75 [71-78] mmHg, p <0.05) were higher in the PA and EH group compared to healthy controls, respectively (**Figure 1**).

Serum aldosterone was higher in PA in respect to EH, but similar to the control group (12.7 [10.4-13.7] vs. 7.8 [6.1-8.4] vs. 9.8 [6.9-12.5] ng/dL, p <0.0001). PRA was significantly lower in PA in respect to EH and controls (0.8 [0.5-0.9] vs. 1.9 [1.4-2.8] vs. 1.8 [1.3-2.3] ng/mL*h, p <0.0001). The ARR was higher in PA than EH and controls (17.9 [13.7-20.8] vs. 4.1 [2.6-5.4] vs. 5.4 [3.2-7.3], p<0.0001) (**Table 1**, **Figure 2**). No differences were found in plasma and urinary sodium and potassium electrolytes, nor in the fractional excretion of potassium (FEK) or the fractional excretion of sodium (FENa) in PA, EH, and controls (**Table 1**).

Evaluation of Parameters Associated With Inflammation, Endothelial, and Renal Dysfunction in PA Subjects

We found similar plasma levels of hs-CRP (1.4 [1.1-2.0] vs 2.1 [0.5-4.0] vs 1.1 [0.9-2.9] mg/L, p NS) and Interleukin 6 (IL-6)

TABLE 1 | Clinical and biochemical parameters of subjects identified as control, EH, and primary aldosteronism.

	CONTROL	EH	PA
N	13	17	11
Age (years old)	37 [28-47]	39 [29-47]	48 [37-53]
Man (%)	46	58	55
BMI (kg/m^2)	26.1 [24.7-27.7]	27.7 [24.4-29.7]	28.5 [27.5-29.1]
SBP (mmHg)	116 [110-121]	134 [123-139] b	140 [125-153][a]
DBP (mmHg)	75 [71-78]	87 [81-93]	89 [76-98][a]
Serum aldosterone (ng/dl)	9.8 [6.9-12.5]	7.8 [6.1-8.4]	12.7 [10.4-13.7][a]
Plasma renin activity (ng/mL*h)	1.8 [1.3-2.3]	1.9 [1.4-2.8]	0.8 [0.5-0.9][a,b]
ARR	5.4 [3.2-7.3]	4.1 [2.6-5.4]	17.9 [13.7-20.8][a,b]
Plasma sodium (mEq/l)	140 [139-141]	141 [140-142]	140 [139-142]
Plasma potassium (mEq/l)	4.1 [3.9-4.4]	4.2 [3.8-4.5]	4.2 [3.9-4.4]
Urinary sodium (mEq/24 h)	136 [73-202]	162 [114-216]	125 [99-176]
Urinary potassium (mEq/24 h)	47 [31-62]	54 [39-66]	53 [41-67]
Sodium excreted fraction (%)	0.63 [0.53-0.83]	0.64 [0.34-0.78]	0.57 [0.51-0.88]
Potassium excreted fraction (%)	7.2 [5.6-8.2]	7.9 [5.1-9.3]	8.0 [7.4-8.7]

Data are presented as a median and interquartile range [Q1-Q3]. BMI, Body Mass Index; PAS, Systolic Pressure; PAD, Diastolic Pressure; ARR, Aldosterone/Plasmatic Renin Activity Ratio.
[a]Different from the HE group and [b]the control group. Analysis was performed using Kruskal-Wallis, p < 0.05, and χ^2 test, p < 0.05.

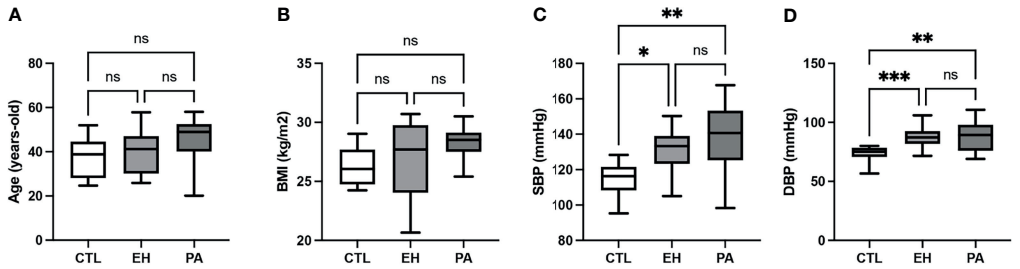

FIGURE 1 | Clinical characteristics of subjects with PA. **(A)** Age (years old). **(B)** Body mass index (BMI; kg/m^2) **(C)** Systolic blood pressure (SBP; mmHg). SBP was higher in PA and EH subjects in the CTL group. **(D)** Diastolic blood pressure (DBP; mmHg). DBP was higher in PA and EH subjects in the CTL group. Comparison between groups was performed by unpaired one-way ANOVA or Kruskal-Wallis test. Data are presented as median and interquartile range [Q1-Q3], N.S: No significative difference, *p < 0.05, **p < 0.01, ***p < 0.001.

(3.0 [1.5-3.1] vs. 3.2 [2.7-3.9] vs. 3.0 [1.7-3.2] pg/ml, p NS) in PA from those found in EH and controls, respectively. Endothelial markers PAI-1, MMP9 and MMP2 were also evaluated, showing no differences in PA respect to EH or control subjects (**Table 2**, **Figure 3**). Renal dysfunction was evaluated with the urinary albumin to creatinine ratio (UACR) (3.2 [2.0-4.6] vs. 3.6 [1.5-5.4] vs. 4.3 [1.9-6.6] mg/gr Crea) which was also similar in PA and EH and Controls (**Table 2**).

Determination of Serum AGP1, LCN2, and LCN2-MMP9 in PA Subjects

We detected higher levels of AGP1 in PA (934.1 [736.5-1255] vs 62.50 [47.1-365.9] and 60.7 [18,0-609,0] ug/ml, p<0.01) compared to EH and controls subjects. LCN2 and LCN2-MMP9 were similar between the groups (p NS) (**Table 3**). Total LCN2 was found to be higher in EH with respect to the control group, meanwhile, PA was similar to EH but did not reach a significant difference when compared to the control (**Figure 4**). We observed significant associations of AGP1 with Aldosterone (rho = 0.34, p <0.05), with PRA (rho = -0.44, p <0.01) and with ARR (rho = 0.38; p <0.05) (**Figure 5**).

Characterization and Quantification of Urinary Extracellular Vesicles

We isolated uEVs from all subjects in this study. **Figure 6** shows a representative image of isolated uEVs with a donut-shape morphology by TEM (**Figure 6A**), a characteristic plot size/concentration from NTA with the main peak near to 150 nm (**Figure 6B**), and the western-blot of EV markers CD9 and TSG101 (**Figure 6C**). No differences were observed in concentration, mean and mode size of uEVs measured by NTA in PA, EH, and controls (**Table 4** and **Figure 7**).

Expression of miR-21-5p and Let-7i-5p in Urinary Extracellular Vesicles

We identified a low expression of miR-21-5p in uEVs in PA and EH in the control group. No difference was detected of miR-21-5p between the PA and EH group (**Table 5**). Concerning Let-7i-

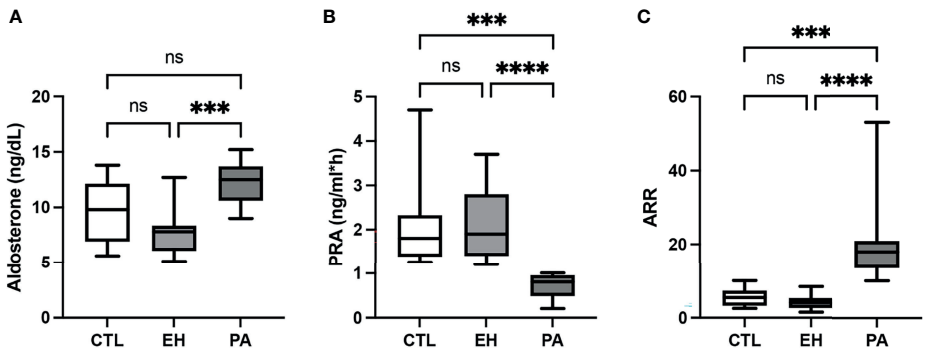

FIGURE 2 | Biochemical characteristics of subjects with PA. **(A)** Serum aldosterone concentration (ng/dL). Serum aldosterone levels were higher in PA subjects in the EH group. **(B)** Plasmatic renin activity (PRA; ng/mL*h). Plasmatic renin activity was lower in PA subjects in both the EH and CTL groups. **(C)** Aldosterone to renin ratio (ARR). ARR was higher in PA subjects in both the EH and CTL groups. Comparison between groups was performed by unpaired one-way ANOVA or Kruskal-Wallis test. Data are presented as median and interquartile range [Q1-Q3], N.S, No significative difference; ***p < 0.001, ****p < 0.0001.

TABLE 2 | Evaluation of parameters associated with inflammation, endothelial and renal damage in PA subjects, EH, and controls.

	CONTROL	EH	PA
Hs-CRP (mg/l)	1.1 [0.9-2.9]	2.1 [0.5-4.0]	1.4 [1.1-2.0]
IL-6 (pg/ml)	3.0 [1.7-3.2]	3.2 [2.7-3.9]	3.0 [1.5-3.1]
PAI-1 (ng/ml)	14.0 [11.5-19.5]	15.8 [11.4-21.2]	21.1 [7.3-24.4]
MMP9 (activity FC)	1.2 [0.8-2.2]	1.4 [1.2-2.4]	1.4 [1.0-1.5]
MMP2 (activity FC)	1.2 [1.0-1.5]	1.2 [1.0-1.9]	1.1 [1.0-1.3]
Urinary albumin (mg/g creatinine)	4.3 [1.9-6.6]	3.6 [1.5-5.4]	3.2 [2.0-4.6]

hs-PCR, High sensitivity C reactive protein; IL-6, Interleukin-6; PAI-1, Plasminogen activator inhibitor-1; MMP9, Matrix metalloproteinase-9 activity (fold change); MMP2, Matrix metalloproteinase-2 activity (fold change); LCN2, Serum LCN2 concentration; LCN2-MMP9, Serum LCN2-MMP9 concentration; LCN2+MMP9, Serum LCN2+LCN2-MMP9 concentration; AGP1, Serum AGP1 concentration. Data are presented as a median and interquartile range [Q1-Q3]. Statistical analyses were performed using Kruskal-Wallis (Dunn's) with significance $p < 0.05$.

TABLE 3 | Determination of serum AGP1A, LCN2, and LCN2-MMP9 in PA subjects.

	CONTROL	EH	PA
AGP1 (mg/ml)	60.7 [18-609]	62.5 [47.1-365.9][a,c]	934.1 [736.5-1255][a,b]
LCN2 (ng/ml)	96 [61-117]	104 [88-133]	123 [80-131]
LCN2-MMP9 (ng/ml)	28 [16-43]	45 [29-65]	52 [29-75]
Total LCN2 (ng/ml)	107 [81-162]	179 [156-202][c]	190 [172-214][b]

AGP1, Serum AGP1 concentration. LCN2, Serum LCN2 concentration; LCN2-MMP9, Serum LCN2-MMP9 concentration; Total LCN2, sum of free LCN2 and LCN2-MMP9 complex. Data are presented as a median and interquartile range [Q1-Q3]. Statistical analyses were performed using Kruskal-Wallis (Dunn's) with significance $p < 0.05$. [a]PA different from the EH group, [b]PA different from the control group, and [c]EH different from the control group.

5p, we did not detect any differences between all groups (**Table 5** and **Figure 8**).

Receiver Operating Characteristic Curve Analyses for AGP1 and miR-21-5p

Receiver operating characteristic (ROC) analysis showed that a serum AGP1 concentration of 647.9 mg/ml had the best sensitivity (90%) and specificity (83%) to discriminate PA from EH and control subjects. In this analysis, the area under the curve (AUC) for AGP1 was 0.90 (IC 95 [0.79 – 1.00], p <0.001) (**Figure 9**) and for miR-21-5p (AUC 0.63 [0.40-0.86], p NS]. The ROC curve for both AGP1 + EV-miR-21-5p showed a sensitivity of 90% and specificity of 85% with an AUC of 0.94 (IC 95 [0.85 – 1.00], p<0.001) (**Figure 9**).

DISCUSSION

In the present study, we evaluated the concentration of lipocalins AGP1 and LCN2, and the expression of miR-21-5p and Let-7i-5p in uEVs as potential biomarkers of PA. We observed a higher concentration of AGP1 in PA subjects, which is associated with the critical variables used to screen PA, as plasma aldosterone, renin, and ARR. Further to these novel findings, we noted a low expression of miR-21-5p in PA subjects, which is interesting since it supports a combinate model for the identification of PA conditions. We suggest that both AGP1 and miR-21-5p are associated with the pathogenic course of the primary aldosteronism and can be useful in the design of a novel diagnostic algorithm for PA. There is also a widely accepted

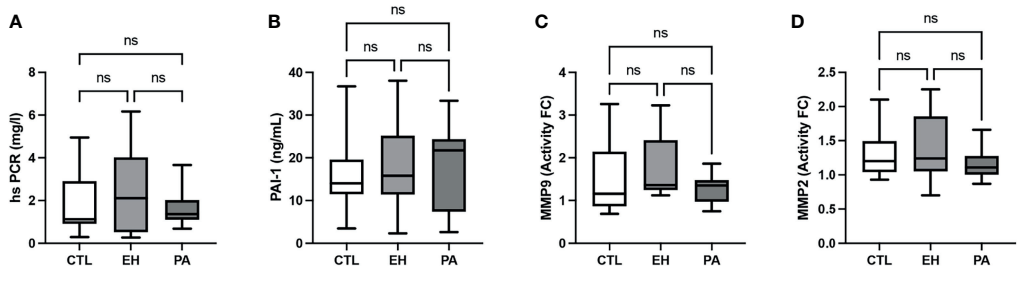

FIGURE 3 | Evaluation of parameters associated with inflammation, endothelial and renal dysfunction in PA subjects. **(A)** High sensitivity C reactive protein (hsPCR; mg/L). **(B)** Plasminogen activator inhibitor – 1 (PAI-1; ng/mL). **(C)** Metalloproteinase 9 (fold change activity). **(D)** Metalloproteinase 2 (fold change activity). No differences of parameters associated with inflammation, endothelial and renal dysfunction were found between groups. Comparison between groups was performed by unpaired one-way ANOVA or Kruskal-Wallis test. Data are presented as a median and interquartile range [Q1-Q3], N.S, No significative difference.

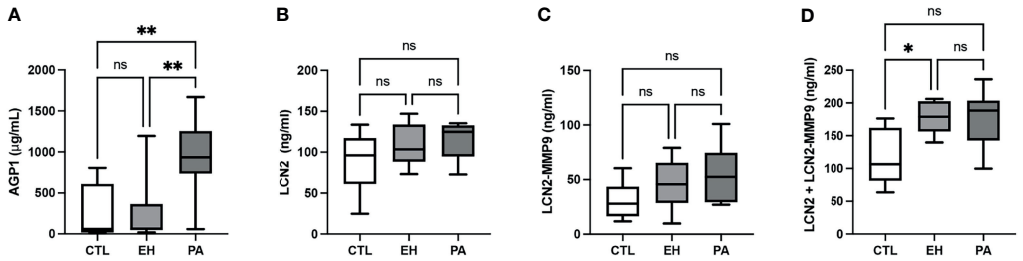

FIGURE 4 | Determination of serum AGP1, LCN2, LCN2-MMP9, and in PA subjects. **(A)** Serum AGP1 concentration (µg/mL). We detected higher levels of AGP1 in PA subjects in both EH and CTL groups. **(B)** Serum LCN2 concentration (ng/mL). LCN2 concentration was similar between groups **(C)** Serum LCN2-MMP9 concentration. LCN2-MMP9 concentration was similar between groups (ng/mL). **(D)** Serum LCN2 + LCN2-MMP9 concentration (ng/mL). Serum levels of LCN2 + LCN2-MMP9 were higher in EH subjects in the CTL group. LCN2 + LCN2-MMP9 concentration was similar between PA and EH subjects and PA and CTL subjects. Comparison between groups was performed by unpaired one-way ANOVA or Kruskal-Wallis test. Data are presented as a median and interquartile range [Q1-Q3]. N.S, No significative difference, *p < 0.05, **p < 0.01.

consensus regarding a positive screening for PA is an ARR >30 ng/dL per ng/mL/h, with suppressed renin (PRA<1.0 ng/mL*h or DRC <10 uUI/ml) and an aldosterone concentration >15 ng/dL. Some studies have been identified that can improve the detection of milder forms of primary aldosteronism when using less conservative ARR thresholds with suppressed renin activity and plasma aldosterone levels >9 ng/dL (5, 53, 54), which is in agreement with the outcome of this study.

We found similar levels of hs-CRP and IL-6 as markers of inflammation in PA subjects, which were similar to EH and control groups, according to previous studies (12, 64). Similarly, endothelial damage markers (PAI-1, MMP9, and MMP2) and renal function markers (Urinary albumin (UACR)) do not show any significant changes in PA compared with EH and controls. It suggests these subjects, currently classified as subclinical PA (54, 65) do not have chronic inflammation, vascular compromise, or renal function impairment as is seen in overt or classic PA. Hence, is highly necessary novel and sensitive biomarkers aimed to detect subclinical PA and avoid complications associated with the renal and extra-renal effects reported in classic PA.

This perspective is the first to report findings that show a higher serum AGP1 concentration in PA than EH and controls subjects (**Figure 4**). We also observed a significant association of AGP1 with classic screening parameters for PA (e.g., aldosterone,

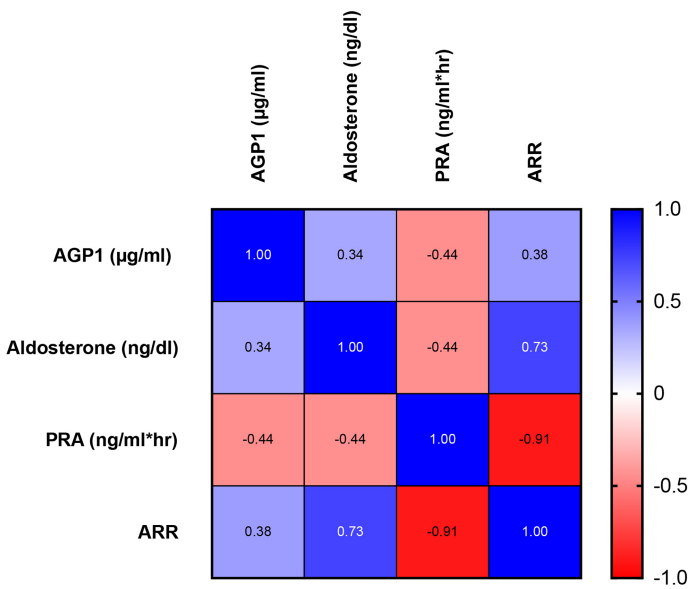

FIGURE 5 | Heat map of AGP1 associations with serum Aldosterone, PRA, and ARR in PA, EH, and CTL subjects. Positive associations are presented in blue gradient with the respective ρ (rho) value. Similarly, negative associations are presented in the red gradient. We observed a significant association between AGP1 concentration and the 3 relevant biochemical parameters in primary aldosteronism screening. Association studies were performed by Spearman test, p < 0.05.

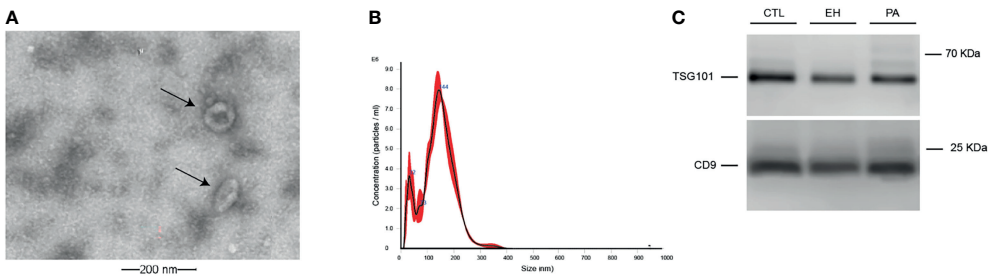

FIGURE 6 | Characterization and quantification of urinary EVs. **(A)**. Identification of uEVs by Transmission Electron Microscopy (TEM) (indicated by black arrows). **(B)** Representative size distribution plot from uEVs using a NanoSight NS300 instrument. **(C)**. Western blot of classic extracellular vesicles markers TSG101 and CD9.

TABLE 4 | Characterization by NTA of urinary extracellular vesicles.

	CONTROL	EH	PA
uEV (particle/g crea)	1.63x10^{11} [1.14 x10^{11}-1.95 x10^{11}]	2.21 x10^{11} [1.55 x10^{11}-2.63 x10^{11}]	2.0 x10^{11} [1.18 x10^{11}- 3.89 x10^{11}]
uEV mean size (nm)	142 [129-149]	141 [138-161]	145 [139-152]
uEV mode size (nm)	121 [109-129]	130 [117-169]	135 [122-155]

Statistical analyses were performed using Kruskal-Wallis (Dunn's) with significance p <0.05.

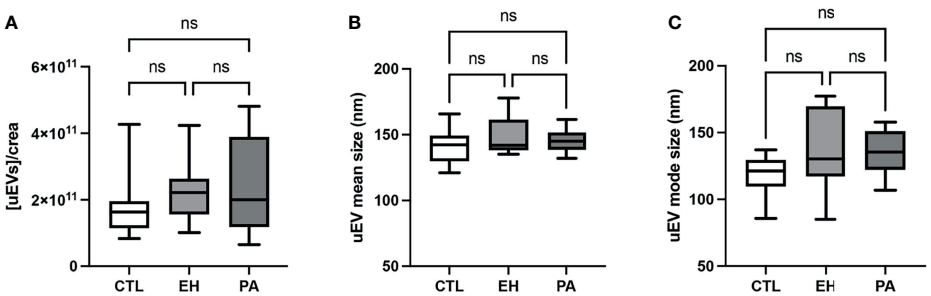

FIGURE 7 | Quantification of uEVs by NTA. **(A)** Urinary creatinine normalized uEVs concentration (uEVs particles/mg creatinine). **(B)** Mean diameter of uEVs particle size distribution (nm). **(C)** Mode diameter of uEVs particle size distribution (nm). uEVs concentration and diameter were similar between groups. Comparison between groups was performed by unpaired one-way ANOVA or Kruskal-Wallis test. Data are presented as a median and interquartile range [Q1-Q3]. N.S: No significant difference.

TABLE 5 | Expression of miR-21-5p and Let-7i-5p in urinary extracellular vesicles.

	CONTROL	EH	PA
miR-21-5p (RU/Crea)	2194 [143.5-12311]	34.1 [5.1-101.7][c]	7.3 [0.6-667.5][b]
Let7i-5p (RU/Crea)	157.2 [16.7- 374.5]	70.1 [14.9 -515.4]	26.7 [0.2-684.9]

RU/Crea, Relative units/mg creatinine. Statistical analyses were performed using Kruskal-Wallis (Dunn's) with significance p <0.05. [b]PA different from the control group, and [c]EH different from the control group.

PRA, and ARR) (**Figure 5**). Moreover, we found by discriminative analyses by ROC curves that AGP1 can identify PA from HE&CTL subjects with high sensitivity and specificity. All these results suggest that circulating AGP1 protein is a novel and potential biomarker of PA, which was also suggested for AGP1 protein in urinary exosomes (26). Since AGP1 is a protein from the family lipocalin associated with the acute phase response with immunomodulatory properties (66, 67), affected by glucocorticoids (68–70) and mineralocorticoids (71), we suggest that AGP1 expression is modified by high aldosterone

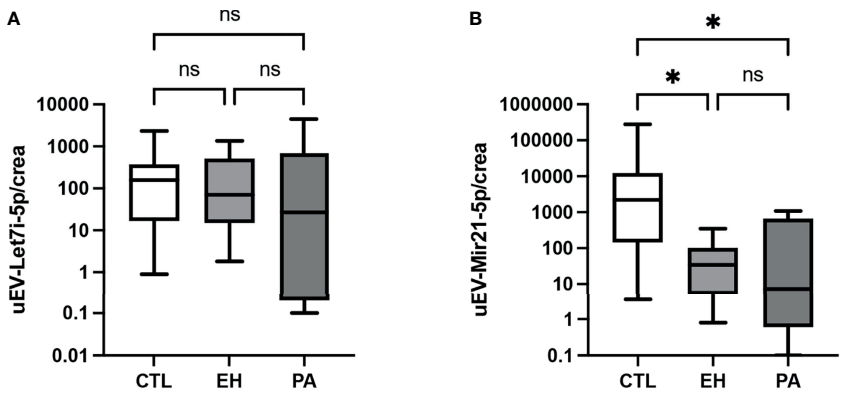

FIGURE 8 | Expression of miR-21-5p and Let-7i-5p in uEVs. **(A)** miR-Let7i-5p expression in uEVs normalized by urinary creatinine (RU/mg creatinine). No differences in miR-Let7i-5p levels were found between groups. **(B)** miR-21-5p expression in uEVs normalized by urinary creatinine (RU/mg creatinine). uEVs miR-21-5p expression was higher in PA and EH subjects respect CTL group. Comparison between groups was performed by unpaired one-way ANOVA or Kruskal-Wallis test. Data are presented as a median and interquartile range [Q1-Q3]. N.S: No significative difference, *p < 0.05.

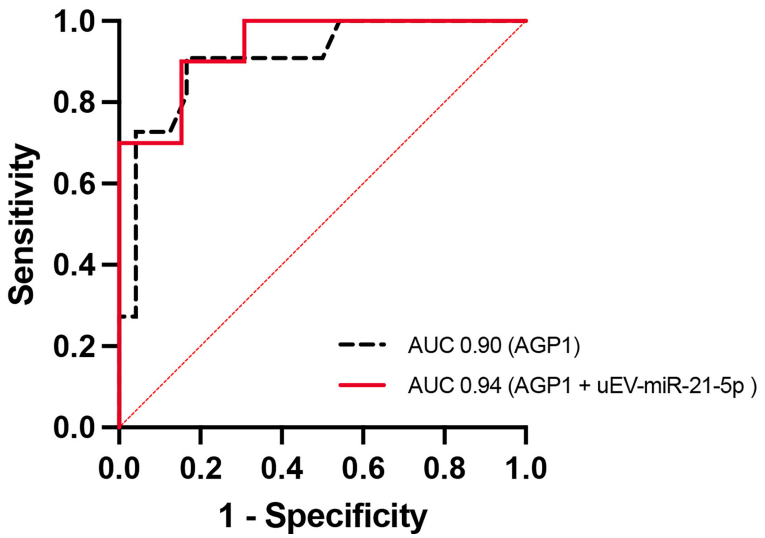

FIGURE 9 | Regression model and Receiver operating characteristic (ROC) curve. ROC curve for serum AGP1 levels (black) and serum AGP1 levels + uEVs associated miR-21-5p (red) can discriminate the PA condition from EH and CTL groups.

levels through MR activation, having a dual role as a potential biomarker of PA, and possible mediator of the tissue response to high aldosterone. Further clinical and animal model or *in-vitro* studies using MR antagonists should be performed to support this hypothesis.

Similarly, we measured free LCN2 and LCN2 conjugated with matrix metalloproteinase 9 protein as a potential biomarker of PA (72). We found an increase only in total LCN2 (the sum of free and complexed LCN2) in EH, but it did not reach a significant difference in PA when compared with the control or EH. LCN2 is a proinflammatory molecule upregulated in obese individuals or patients with cardiometabolic syndrome, as also has been described in classic PA (73, 74) and is suggested as an MR sensitive protein (75, 76). LCN2 expression is influenced by several factors including obesity, salt intake, aging, infection, and inflammatory status (72, 74, 77–79). Since these subjects have a middle or subclinical PA, with no clear evidence of inflammation, renal/vascular damage (**Figure 3**), or concomitant cardiometabolic disease, we hypothesize the LCN2 fails to increase in these PA subjects since they require a concomitant hit as inflammation (78, 79), obesity (high adipose tissue) (74), or high salt intake (72) to increase the circulating LCN2 levels.

We studied the urinary extracellular vesicles as a source of potential biomarkers for PA (26, 30). In the present study, uEVs

TABLE 6 | Target genes of miR-21-5p and Let-7i-5p, biological process associate and its predicted renal and global effect.

miRNA	Gene target	Biological process	Predicted effect	Global effect
Hsa-miR-21-5p	IL1B IL12A IL10	- regulation of lymphocyte mediated immunity - regulation of adaptive immune response	promote an inflammatory state characterized by vascular infiltration of immune cells	Increase inflammation
	COL10A1 COL12A1 COL13A1 COL1A1 COL4A1	- collagen catabolic process - extracellular matrix disassembly	degradation and reorganization of extracellular matrix scaffold	Hypertrophy or hyperplasia of cardiac myocytes and vascular smooth muscle cells (VSMCs)
	NEDD4	protein polyubiquitination	Regulates ENaC function by controlling the number of channels at the cell surface	Increase plasma volume
	SLC12A2	- Mediates sodium and chloride reabsorption. - Plays a vital role in the regulation of ionic balance and cell volume	Increased renal Na+ reabsorption	Increase plasma volume
	TIAM1	GEFs mediate the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP).	Regulator involved in the activation of Rac1 induced by salt loading and aldosterone.	Salt sensitive hypertension
	YWHAZ	positive regulation of signal transduction by binding to phosphoserine-containing proteins	14-3-3 proteins modulate the expression of epithelial Na+ channels	Increase plasma volume
Hsa-let-7i-5p	TGFBR1	Is a multifunctional cytokine affecting many cell types and tissue remodeling processes, including angiogenesis and organ fibrosis. TGF-β mediates tissue fibrosis associated with inflammation and tissue injury.	TGF-β increased fibroblast activation, proliferation, and excessive extracellular matrix (ECM) production	increased fibroblast activation, proliferation, and excessive ECM production. Increase fibrosis
	AQP2	renal water homeostasis	increasing the retention of water and sodium	Increase plasma volume
	COL1A1 COL1A2 COL24A1 COL3A1	extracellular matrix organization	degradation and reorganization of extracellular matrix scaffold	hypertrophy or hyperplasia of cardiac myocytes and vascular smooth muscle cells (VSMCs)
	DNMT3A DNMT3B	- DNA methylation on cytosine within a CG sequence - S-adenosylmethioninamine metabolic process - methylation-dependent chromatin silencing - regulation of gene expression by genetic imprinting	Increased promoter methylation of HSD11B2 gene	Decreased cortisol to cortisone metabolism; High F/E ratio
	IL10 IL12A IL13 IL15 IL17RA IL6 IL6R IL8	- positive regulation of cytokine production - inflammatory response	promote an inflammatory state characterized by vascular infiltration of immune cells	Increase inflammation
	NEDD4	protein polyubiquitination	Regulates ENaC function by controlling the number of channels at the cell surface	Increase plasma volume
	ORM1 ORM2	- acute-phase response - response to stress	Functions as transport protein in the blood stream.	Increase due to acute inflammation
	SCNN1A	- sodium ion homeostasis	Increased renal Na+ reabsorption	Increase plasma volume
	SLC12A1	- It plays a key role in concentrating urine and accounts for most of the NaCl resorption	Increased renal Na+ reabsorption	Increase plasma volume
	YWHAZ YWHAE	- mediate signal transduction by binding to phosphoserine-containing proteins.	14-3-3 proteins modulate the expression of epithelial Na+ channels	Increase plasma volume

Gene target identification for identified miRNAs was performed using 5 miRNA gene target databases: miRmap, miRWalk, TargetScan, miRanda, and RNA22.

showed similar particle concentration and size in PA subjects with respect to EH and controls (**Table 4**). Previous studies in PA show similar findings in uEVs concentration (26) but differ from studies in circulating EVs in PA (75, 76), where they reported an increased concentration of circulating EVs in the serum of PA patients when compared with essential hypertensives and attributed it to an enhanced biological response of the endothelium to aldosterone *in vivo* (75), which has also been observed *in vitro* (80–82). These differences could be related to the PA classification, overt PA versus subclinical PA, and also the different biofluids used to quantify the impact of high aldosterone in EV concentration, serum versus urine, which is

associated with distinct mechanisms and the rates of EV shedding that have different tissues (e.g., vascular endothelium vs renal epithelia).

Based on previous reports, we measured the expression of two miRNA in urinary EVs, miR-21-5p, and Let-7i-5p, as potential biomarkers of PA. We observed that uEV-associated miR-21-5p expression in uEVs from PA were lower than controls (**Figure 8**) and similar to EH, however a trend to lower levels was observed in PA. This result suggests that uEV-miR-21-5p is downregulated and associated with pathophysiological mechanisms depending on both high BP and PA conditions. miR-21-5p expression is regulated by cytokines, inflammatory modulators (e.g., NF1, AP1), and steroids. Downregulation of miR-21-5p would affect the downstream target genes related with inflammation (83) as IL-1B gene, aldosterone effect as NEDD4, YWHAZ, SCL12A2 genes, and fibrotic processes (42, 84) as COL1A and COL4A1 genes (**Table 6**). Prospective animal models and *in vitro* studies with miR-21-5p are necessary to gain depth of understanding about the role of this miRNA in high aldosterone conditions in renal epithelia, as occurs in PA.

With respect to uEV-associated Let-7i-5p, we did not observe any differences in Let-7i-5p expression in all groups. Let-7i has been found in either urine (31) and plasma exosomes (44) and is associated with RAAS, mediating inflammation and fibrosis, in both *in vitro* models and experimental models of kidney disease (51, 85). Let-7i regulates downstream target genes TGFBR1, IL6, IL10, COL1A1, COL3A1, DNMT3A, NEDD4, ORM1, VIM, FN1, ACTIN, SCL12A1, and YWHAZ, among others (85–87) (**Table 6**). In the current study, we did not find differences in inflammation parameters, and were unable to measure other important parameters related to fibrosis in these PA subjects, such as the procollagen type 1 protein (PINP, COL1A1).

The ROC curves analyses with AGP1 and miR-21-5p as significant variables associated with PA subjects, support a simple (AGP1) or combinate model (AGP1 + miR-21-5p) to discriminate PA with significant AUC of 90% or 94%, respectively. This AUC is similar to previous reports on AGP1 in uEVs (92%), which support free or uEV-associated AGP1 as potential biomarkers of PA (26).

In summary, we found higher levels of serum AGP1 and lower uEV-miR-21-5p expression in subjects classified as PA with respect to EH and controls. Besides the high discriminatory capacity identified by ROC curves, the association of AGP1 with aldosterone, PRA, and ARR, place both as potential biomarkers of PA. Further studies examining the possible role of AGP1 and miR-21-5p as a mediator of the pathogenic course of PA are encouraged.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Unidad de Ética y Seguridad de la Investigación, CEC-SaludUC, Pontificia Universidad Católica de Chile. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CC, AT-C, and CF contributed to conception and design of the study. CC and CF provide with the contribution of patients or study material. CC, AT-C, JP performed the collection and/or obtaining of results, organized the database, and performed the statistical analysis. CC and AT-C achieve the analysis and interpretation of the results. CC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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