ORIGINAL SCIENTIFIC ARTICLE

EXO-D-MAPPS ATTENUATES PRODUCTION OF INFLAMMATORY CYTOKINES AND PROMOTED GENERATION OF IMMUNOSUPPRESSIVE PHENOTYPE IN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Mesenchymal stem cells (MSCs) produce immunomodulatory factors that regulate production of cytokines and chemokines in immune cells affecting their functional properties. Administration of MSCs-sourced secretome, including MSC-derived conditioned medium (MSC-CM) and MSC-derived exosomes (MSC-Exos), showed beneficial effects similar to those observed after transplantation of MSCs. Due to their nano-size dimension, MSC-Exos easily penetrate through the tissue and in paracrine and endocrine manner, may deliver MSC-sourced factors to the target immune cells modulating their function. MSCs derived from amniotic fluid (AF-MSCs) had superior cell biological properties than MSCs derived from bone marrow. We recently developed "Exosomes Derived Multiple Allogeneic Proteins Paracrine Signaling (Exo-d-MAPPS)", a biological product in which the activity is based on AF-MSC-derived Exos capable to deliver immunomodulatory molecules and growth factors to the target cells. Herewith, we analyzed immunosuppressive capacity of Exo-d-MAPPS against human peripheral blood mononuclear cells (pbMNCs) and demonstrated that Exo-d-MAPPS efficiently suppressed generation of inflammatory phenotype in activated pbMNCs. Exo-d-MAPPS attenuated production of inflammatory cytokines and promoted generation of immunosuppressive phenotype in Lipopolysaccharide-primed pbMNCs. Exo-d-MAPPS treatment reduced expansion of inflammatory Th1 and Th17 cells and promoted generation of immunosuppressive T regulatory cells in the population of Concanavalin A-primed pbMNCs. Similarly, Exo-d-MAPPS treatment suppressed pro-inflammatory and promoted anti-inflammatory properties of α-GalCer-primed pbMNCs. In summing up, due to its capacity for suppression of activated pbMNCs, Exo-d-MAPPS should be further explored in animal models of acute and chronic inflammatory diseases as a potentially new remedy for the attenuation of detrimental immune response.

Keywords: *mesenchymal stem cells, amniotic fluid, secretome, immunosuppression, mononuclear cells.*

INTRODUCTION

Mesenchymal stem cells (MSCs) are the plastic adherent, fibroblast-like multipotent cells capable to self-renew and under appropriate culture conditions, differentiate into cells of the mesodermal, endodermal and ectodermal lineage (1-4). MSCs are present in virtually all postnatal tissues and organs and after isolation (from the bone marrow, umbilical cord blood, placenta, adipose tissue, amniotic fluid, Wharton's jelly) may be easily propagated to reach appropriate cell number for autologous or allogeneic transplantation (2, 3). Therefore, large number of experimental and clinical studies indicated that MSCs could be considered as new remedy in cell-based therapy of degenerative diseases (5).

Additionally, MSCs are able to modulate phenotype of immune cells and may suppress detrimental, local and systemic immune response (6). In juxtacrine, the cell to cell contact-dependent manner and paracrine manner (through the production of soluble mediators), MSCs alter the function of all immune cells (macrophages, dendritic cells (DCs), natural killer (NK), natural killer T cells (NKT), T and B lymphocytes) that have essential role in the pathogenesis of autoimmune, acute and chronic inflammatory diseases (7-9).

Production of immunoregulatory factors in MSCs and their capacity for immunosuppression was identified by Haynesworth and co-workers (10). They reported that MSCs produce and release a broad repertoire of growth factors, chemokines, and cytokines that modulate production of inflammatory cytokines in immune cells affecting their functional properties (10). Additionally, further studies revealed that MSC-sourced factors promote neo-angiogenesis, reduce apoptosis and enhance survival of parenchymal cells, regulate remodeling of extracellular matrix and prevent fibrosis in injured tissues, enabling the enhanced tissue repair and regeneration (11, 12).

Among immunomodulatory factors, MSCs produce transforming growth factor-β (TGF-β), hepatic growth factor (HGF), nitric oxide (NO), indolamine 2,3-dioxygenase (IDO), IL-10, IL-6, leukocyte inhibitory factor (LIF), IL-1 receptor antagonist (IL-1Ra), tumor necrosis factor a-stimulated gene 6 (TSG-6), human leukocyte antigen-G (HLA-G), hemeoxygenase-1 (HO-1), and prostaglandin E2 (PGE2) (6, 13, 14). Therefore, local as well as systemic administration of MSCs-sourced secretome, including MSC-derived conditioned medium (MSC-CM) and MSC-derived exosomes (MSC-Exos), showed beneficial effects similar to those observed after transplantation of MSCs. Due to their nano-size dimension, MSC-Exos easily penetrate through the tissue and in paracrine and endocrine manner, deliver MSC-sourced factors to the target immune cells modulating their function (15).

Several lines of evidence suggested that MSCs derived from amniotic fluid (AF-MSCs) had superior cell biological properties than MSCs derived from the bone marrow (BM-MSCs) (16-19). Roubelakis and colleagues revealed that AF-MSCs have 78 unique proteins which are responsible for their increased proliferation rate and plasticity (20). Furthermore, AF-MSCs more efficiently suppressed detrimental T cell-driven immune response than BM-MSCs (21). In line with these findings, we recently developed: "Exosomes Derived Multiple Allogeneic Proteins Paracrine Signaling (Exo-d-MAPPS)", a biological product in which activity was based on AF-MSC-derived Exos capable of delivering immunomodulatory molecules and growth factors to the target cells (22). Herewith, we analyzed immunosuppressive capacity of Exo-d-MAPPS against activated human peripheral blood mononuclear cells (pbMNCs) and demonstrated that Exo-d-MAPPS efficiently down-regulated production of inflammatory cytokines and enhanced production of immunosuppressive cytokines in pbMNCs, suggesting its potential therapeutic use in the treatment of acute and chronic inflammatory diseases.

MATERIALS AND METHODS

Preparation of AF and Exo-d-MAPPS samples

Amniotic fluid and tissues were collected from healthy, full-term, scheduled cesarean sections. Samples of collected material were tested by laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) and were found negative using United States (U.S) Food and Drug Administration (FDA) licensed tests for detection of at minimum: Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus Types 1/2, Treponema Pallidum. All samples were obtained with patient consent as well as institutional ethical approval, as previously described (23). Exo-d-MAPPS samples were engineered as AF-derived sterile product containing AF-MSC-Exos, manufactured under current Good Manufacturing Practices (cGMP), regulated and reviewed by the FDA (22). Sterile Exo-d-MAPPS incorporate Regenerative Processing Plant's (RPP) proprietary patented sterilization process to provide for a safe, sterile product. Exo-d-MAPPS samples, used in this study, were manufactured under specific conditions in order to be applicable for bioavailability testing and for different therapeutic use.

Isolation of pbMNCs

Serum samples (2 ml) were obtained from healthy volunteers at the Center for Molecular Medicine and Stem Cell Research, the Faculty of Medical Sciences of the University of Kragujevac, and pbMNCs were isolated by the use of Histopaque (Sigma-Aldrich, Munich, Germany) density gradient centrifugation. Briefly, the serum was diluted by equal volume of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/L of L- glutamine, 1 mmol/L penicillin-streptomycin, and 1 mmol/L of mixed nonessential amino acids, (Sigma Aldrich, Munich, Germany). Heparinized peripheral blood (10 ml) was centrifuged at 400 g for 10 min to separate plasma and cells. Lymphocyte separation liquid (3 ml) was filled into a 10-ml centrifuge tube. After 10 min, the mononuclear cell layer was transferred to a sterile tube by a fresh sterile pipette (capillary tube), gently mixed with five volumes of DMEM and

centrifuged at 2700 r/min for 20 min, then washed with DMEM twice. After the supernatant was discarded, cells were resuspended in DMEM containing the 10% fetal bovine serum (Gibco, United States) for lymphocyte count. Then the cell suspensions were diluted to 1^x10^6 cells/ml for further *in vitro* experiments.

Activation of pbMNCs

After isolation, pbMNCs were plated in a 24-well plate (1^x10^6 cells per well) and subsequently primed with 10 ng/ml Lipopolysaccharides (LPS) (24), or 5 μg/ml Concanavalin A (Con A)-potent activator of T cells (25), or 100 ng/ml of alpha galactosyl ceramide (α-GalCer)-selective stimulator of NKT cells (8, 25, 26). Isolated pbMNCs were cultured in complete DMEM for 48h in the presence or in the absence of Exo-d-MAPPS and AF. After 48 hours of culture, activated pbMNCs were harvested for the ELISA assay or flow cytometry.

Measurement of cytokines in supernatants

The ELISA assay was conducted according to the handbook provided by the ELISA kit (R&D Systems Minneapolis, MN for IL-12, IL-17 and IL-10; BD Biosciences San Diego, CA for IFN-γ). The optical density of each sample at 450 nm was detected by an ELISA microplate reader (Zenyth, 3100) and the concentrations of IL-12, IL-17, IFN-γ and IL-10 levels in supernatants were determined.

Flow cytometry analysis of pbMNCs

To detect the cell surface expression of a variety of molecules, isolated pbMNCs were analyzed by the flow cytometry (FACS) using standard staining methods (27). Briefly, the prepared cell suspension fluid (1 ml) was centrifuged at 250 g for 5 min and rinsed twice with suspension fluid. The supernatant was discarded and cells were suspended with PBS to 10 μl, adding human CD14, CD56, HLA-DR and CD4 antibody conjugated with fluorescein isothiocyanate (FITC; BD Biosciences, Franklin Lakes, NJ), phycoerythrin (PE; BD Biosciences) or allophycocyanin (APC; BD Biosciences or isotype-matched controls (BD Pharmingen/BioLegend) (about 1.25 μg, suggested by the manual) respectively, and incubated at 4°C in the dark for 30 min. Then, the cell suspension was supplemented with 2 ml PBS, centrifuged at 250 g 5 minutes, and washed with suspension fluid followed by staining with flow cytometry staining buffer. For the intracellular staining, cells were previously stimulated with phorbol myristate acetate (PMA) and ionomycin for 4 h at 37 °C with the addition of 1 μg/mL Golgi plug. Intracellular staining for forkhead box P3 (Foxp3), IL-10, tumor necrosis factor alpha (TNF-α), IL-17, interferon gamma (IFN-γ) was performed using the BD Bioscience fixation/permeabilization buffer kit. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analyzed by the application of the flowing software analysis program.

Statistical analysis

Results were analyzed using the Student's t test. All data in this study were expressed as the mean ± standard error of the mean (SEM). Values of $p<0.05$ were considered as statistically significant.

RESULTS

Exo-d-MAPPS attenuated production of inflammatory cytokines and promoted generation of immunosuppressive phenotype in LPS-primed pbMNCs

LPS significantly enhanced production of inflammatory IL-12 in pbMNCs (Fig.1A). Exo-d-MAPPS significantly attenuated concentration of IL-12 in supernatants of LPS-primed pbMNCs (Fig.1A). Importantly, both room temperature (RT) and fridge (4°C) stored Exo-d-MAPPS suppressed production of IL-12 more efficiently than RT and 4°C stored AF (Fig.1A). Since LPS mainly activates CD14-expressing macrophages, we analyzed whether Exo-d-MAPPS affected percentage of this cell population. As it is shown in Fig.1B, the percentage of LPS-primed pbMNCs that expresses CD14 was significantly lower after Exo-d-MAPPS treatment. Additionally, Exo-d-MAPPS down-regulated expression of HLA-DR molecule and production of inflammatory TNF-α in CD14-expressing pbMNCs (Fig.1C-D). In similar manner as it was observed in the attenuation of IL-12 production, Exo-d-MAPPS-treated LPS-primed CD14+pbMNCs produced lower amount of TNF-α than AF-treated LPS-primed CD14+pbMNCs (Fig. 1C-D). In line with these findings, Exo-d-MAPPS treatment induced generation of immunosuppressive phenotype in LPS-primed CD14+pbMNCs (Fig.1E). Significantly higher percentage of IL-10-producing CD14+ cells and significantly higher concentration of IL-10 was observed in supernatants of Exo-d-MAPPS treated LPS-primed pbMNCs than in supernatants of AF-treated LPS-primed pbMNCs (Fig.1E)

Exo-d-MAPPS treatment reduced expansion of inflammatory Th1 and Th17 cells and promoted generation of immunosuppressive Tregs in the population of Con A-primed pbMNCs

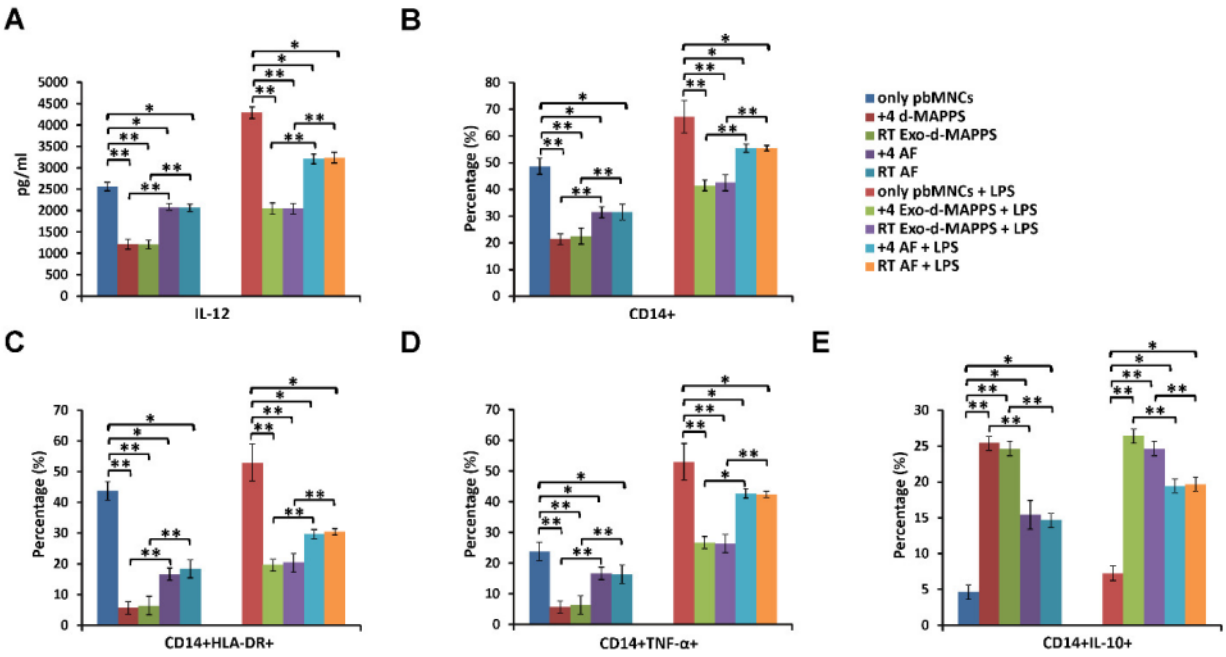
Con A treatment induced expansion of CD4+ cells and, particularly, inflammatory, IFN-γ-producing Th1 and IL-17-producing Th17 CD4+ T cells within the population of pbMNCs (Fig.2A-C). Exo-d-MAPPS significantly attenuated expansion of CD4+ cells and alleviated production of IFN-γ and IL-17 in Con A-primed CD4+ T cells (Fig.2A-C). Importantly, treatment with either RT or 4°C stored Exo-d-MAPPS more efficiently reduced expansion of inflammatory Th1 and Th17 cells than AF (Fig.2A-C), indicating superior immunosuppressive properties of Exo-d-MAPPS over AF. Additionally, Exo-d-MAPPS promoted generation of immunosuppressive phenotype in CD4-expressing pbMNCs, as evidenced by higher percentage of FoxP3-expressing and IL-10-producing CD4+ cells in the population of Exo-d-MAPPS-treated Con A-primed pbMNCs compared to Con A-only and AF+Con A-treated pbMNCs (Fig.2D-E). In line

with these findings, significantly lower concentration of IL-17 and higher concentration of IL-10 were noticed in supernatants of Exo-d-MAPPS+Con A-treated pbMNCs compared to Con A-only and AF+Con A-treated pbMNCs (Fig.2F-G).

Exo-d-MAPPS treatment suppressed pro-inflammatory and promoted anti-inflammatory properties of α-GalCer-primed pbMNCs

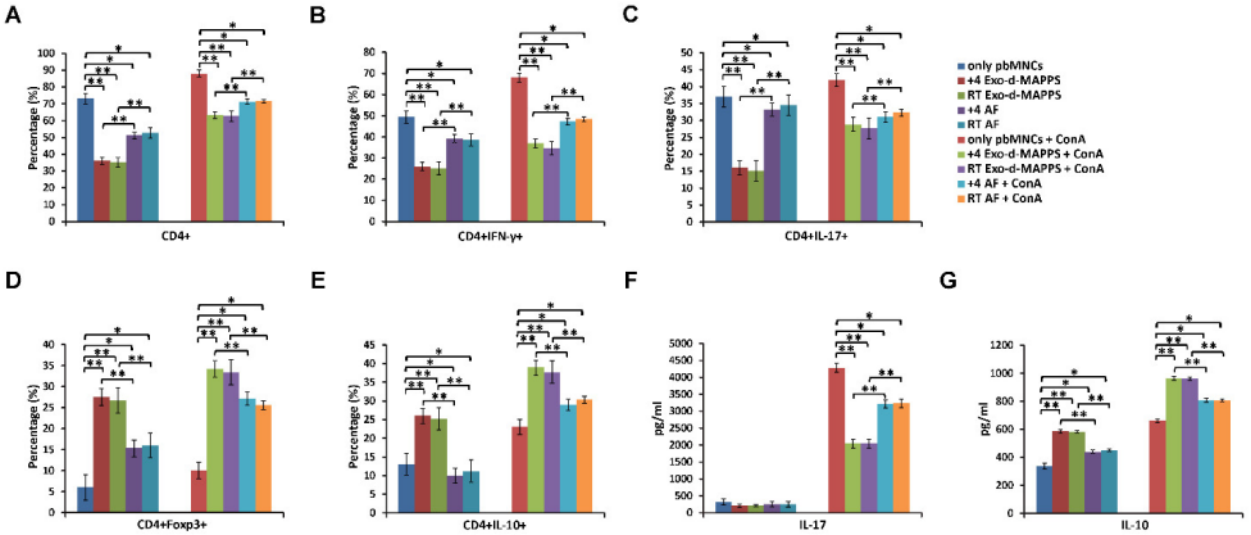
As it is shown in Fig.3A, α-GalCer treatment stimulated expansion of inflammatory, IFN-γ-producing and IL-17-producing CD56-expressing cells within population of pbMNCs. Both RT and 4°C stored Exo-d-MAPPS more efficiently reduced proliferation of IFN-γ-producing and IL-17-producing CD56+ cells than RT or 4°C stored AF (Fig.3B-C). In similar manner as it was observed with Con A-primed pbMNCs, Exo-d-MAPPS treatment promoted generation of immunosuppressive phenotype in α-GalCer-activated pbMNCs (Fig.3D-E). Significantly higher percentage of FoxP3-expressing and IL-10-producing CD56+cells were observed in the population of α-GalCer+Exo-d-MAPPS-treated pbMNCs than in α-GalCer-only and α-GalCer+AF treated pbMNCs (Fig.3D-E). In line with these findings, significantly lower concentration of immunosuppressive IL-10 was measured in supernatants of α-GalCer+Exo-d-MAPPS-treated pbMNCs than in supernatants of α-GalCer-only and α-GalCer+AF-treated pbMNCs (Fig.3F).

Figure 1. Exo-d-MAPPS decreased production of proinflammatory cytokines and promoted generation of immunosuppressive phenotype in LPS-primed pbMNCs



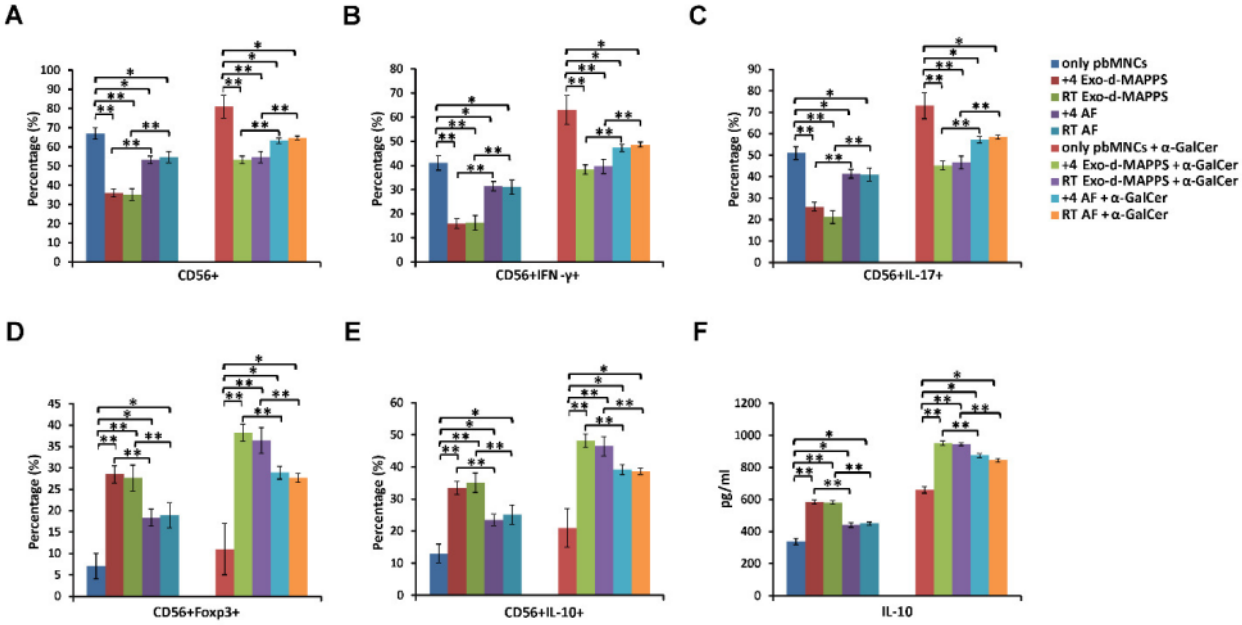
(A) Level of pro-inflammatory IL-12 in supernatants of pbMNCs
(B-E) Percentage of CD14+, CD14+HLA-DR+, CD14+TNF-α+ and CD14+IL-10+ pbMNCs primed with LPS after treatment with Exo-d-MAPPS and AF.
Values are mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Treatment with Exo-d-MAPPS diminished expansion of inflammatory Th1 and Th17 cells and promoted generation of immunosuppressive Tregs in the population of Con A-primed pbMNCs.



(A-C) Flow cytometry data showing decrease in percentage of CD4+ T cells as well as IFN-γ and IL-17-producing CD4+ T cells after Exo-d-MAPPS treatment. **(D, E)** Significant increase in percentage of regulatory CD4+ cells (Foxp3+ and IL-10+) after Exo-d-MAPPS treatment. **(F, G)** Level of the pro-inflammatory IL-17 and anti-inflammatory IL-10 in supernatants of ConA-primed pbMNCs.
Data presented as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Exo-d-MAPPS treatment suppressed pro-inflammatory and promoted anti-inflammatory properties of α-GalCer-primed pbMNCs



(A-C) The percentage of CD56+ cells as well as IFN-γ and IL-17-producing CD56+ cells after Exo-d-MAPPS and AF treatment. **(D, E)** Significant increase in the percentage of regulatory CD56+ cells (Foxp3+ and IL-10+) after Exo-d-MAPPS treatment. **(F)** Level of anti-inflammatory IL-10 in supernatants of α-GalCer-primed pbMNCs. Values are mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

A large number of experimental and clinical evidences suggested that MSCs, due to their immunomodulatory properties, should be considered as new therapeutic agents for the treatment of autoimmune and incurable inflammatory diseases (28-31). Despite promising results observed after autologous and allogeneic transplantation of MSCs, safety issues regarding MSCs-based therapy are still a matter of debate (32). Due to their multipotency, MSCs may spontaneously differentiate into the undesired cell type, particularly osteocytes and chondrocytes (32). Although malignant transformation of transplanted MSCs has not been validated, the high proliferation rate and the capacity for self-renewal indicate possible risk of mutations which may result in tumor development and therefore the long-term follow up of patients that received MSCs is required (32). Several studies revealed that despite of low engraftment rate therapeutic effects of MSCs remained long after transplantation, suggesting that beneficial effects of MSC-based therapy were relied on the activity of MSC-sourced factors rather than on the differentiation of engrafted MSCs (33-35). Recently published studies indicated that MSC-derived immunosuppressive factors might be delivered to the target immune cells within MSC-Exos which, due to their nano-sized dimension and lipid envelope, easily avoid biological barriers in the body (28). In line with these findings, herewith we demonstrated that Exo-d-MAPPS, soluble product which contains a broad number of MSC-derived immunomodulatory factors (22), efficiently suppress inflammatory properties of pbMNCs and could be considered as a potentially new agent for the treatment of acute and chronic inflammatory diseases.

Capacity of LPS-primed CD14-expressing monocytes for the production of inflammatory cytokines (TNF-α and IL-12) was significantly attenuated by Exo-d-MAPPS treatment (Fig.1). CD14 is a LPS-binding protein, expressed on the membrane of macrophages and DCs, playing crucial role in the immune recognition of the microbial cell wall components from Gram-negative bacteria (36). Cross-talk between LPS-activated, CD14-expressing monocytes and IFN-γ-producing CD4+ Th1 cells has crucially important role in the pathogenesis of many autoimmune and chronic inflammatory diseases (diabetes mellitus, multiple sclerosis, Crohn's disease, etc.) (37-38). CD14-expressing macrophages and DCs, through the production of "pro-Th1 cytokines" (TNF-α and IL-12), induce generation of IFN-γ-producing CD4+ Th1 effector cells, which in turn, through the secretion of IFN-γ promote the phagocytic activity and capacity for antigen presentation of CD14-expressing monocytes (37, 38). Exo-d-MAPPS treatment resulted in attenuated production of TNF-α and IL-12 in activated CD14-expressing monocytes (Fig.1A, D) and alleviated production of IFN-γ in activated CD4+ cells (Fig.2B), indicating its capacity for suppression of CD4+Th1: the macrophage crosstalk and therapeutic potential for the treatment of chronic inflammatory diseases. Additionally, Exo-d-MAPPS significantly attenuated production of IL-17 in activated CD4+T cells (Fig.2F) and inhibited expansion of Th17 cells (Fig.2C). IL-17 and Th17 cells have important pathogenic role in the chronic organ-specific and systemic inflammatory disorders and, therefore, alleviation of IL-17-driven immune response has been responsible for beneficial effects of MSCs and MSC-derived secretome in the therapy of liver fibrosis, multiple sclerosis, systemic lupus erythematosus and rheumatoid arthritis (39-42). Several lines of evidence indicated that MSCs in IDO/Kynurenine, TGF-β and PGE2-dependent manner increased Tregs/Th17 ratio by promoting generation of immunosuppressive Tregs during the differentiation process of Th17 cells (42-44). In line with these findings, Exo-d-MAPPS, containing MSC-derived Treg-promoting factors (22), concomitantly suppressed proliferation of Th17 cells and induced enhanced expansion of IL-10-producing Tregs (Fig.2C-D).

Similarly, Exo-d-MAPPS induced expansion of FoxP3-expressing and IL-10-producing CD56+ cells and suppressed proliferation of inflammatory IFN-γ and IL-17 α-GalCer-primed pbMNCs (Fig.3). Having in mind that most of α-GalCer-primed CD56-expressing pbMNCs are NKT cells that play crucially an important role in the development of fulminant hepatitis, Exo-d-MAPPS might be considered as a potentially new remedy for the attenuation of NKT cell-dependent acute liver failure.

Since there was not a significant difference in immunomodulatory potential of RT and 4ºC Exo-d-MAPPS samples (Fig.1-3), Exo-d-MAPPS may be used either as RT or 4ºC storage soluble product. Importantly, although Exo-d-MAPPS is an AF-MSC-derived product, Exo-d-MAPPS-suppressed generation of inflammatory phenotype in pbMNCs is significantly better than AF (Fig.1-3). Exo-d-MAPPS contains AF-MSC-derived Exos, extracellular vesicles which diameter is smaller than 100 nm and do not contain apoptotic bodies (22). During the production of Exo-d-MAPPS, due to the centrifugation and filtration, large extracellular vesicles (with diameter bigger than 100 nm), including apoptotic bodies and microvesicles, were removed from the secretome (22). Since apoptotic bodies may induce an activation of inflammatory cascade in immune cells (45), we believe that their deficiency in Exo-d-MAPPS samples resulted in their better immunosuppressive potential compared to the AF samples (Fig.1-3).

CONCLUSION

Due to its capacity for suppression of activated pbMNCs, Exo-d-MAPPS should be further explored in animal models of acute and chronic inflammatory diseases as the potentially new remedy for the attenuation of detrimental immune response.

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