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RESEARCH ARTICLE

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Immunomodulatory effects of mesenchymal stem cell–derived exosomes on experimental type-1 autoimmune diabetes

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Abstract

Exosomes derived from adipose tissue-derived mesenchymal stem cells (AD-MSCs) have immunomodulatory effects of T-cell inflammatory response and reduction of clinical symptoms on streptozotocin-induced of the type-1 diabetes mellitus (T1DM). Beside control group and untreated T1DM mice, a group of T1DM mice was treated with intraperitoneal injections of characterized exosomes derived from autologous AD-MSCs. Body weight and blood glucose levels were measured during the procedure. Histopathology and immunohistochemistry were used for evaluation of pancreatic islets using hemotoxylin and eosin (H&E) staining and anti-insulin antibody. Isolated splenic mononuclear cells (MNCs) were subjected to splenocytes proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, immunophenotyping of regulatory T cells and cytokines. A significant increase in the levels of interleukin-4 (IL-4), IL-10, and transforming growth factor-β, and a decrease in the levels of IL-17 and interferon-γ in concordance with the significant increase in the Treg cell ratio in splenic MNCs ($P < 0.05$) was shown in T1DM mice treated with AD-MSC's exosomes as compared to T1DM untreated mice. This amelioration of autoimmune reaction after treatment of T1DM mice with the AD-MSC exosomes was confirmed with a significant increase in islets using H&E staining and Immunohistochemistry analyses. As expected, body weight, blood glucose levels in a survival of T1DM mice treated with AD-MSC's exosomes were maintained stable in comparison to untreated T1DM mice. It can be concluded that AD-MSC's exosomes exert ameliorative effects on autoimmune T1DM through increasing regulatory T-cell population and their products without a change in the proliferation index of lymphocytes, which makes them more effective and practical candidates.

KEYWORDS

adipose tissue-derived mesenchymal stem cells, exosomes, type-1 diabetes mellitus mice

Abbreviations: AD-MSCs, adipose tissue-derived mesenchymal stem cells; IFN-γ, interferon-γ; IL, interleukin; MNCs, mononuclear cells; STZ, streptozotocin; T1DM, type-1 diabetes mellitus; TGF, transforming growth factor.

1 | INTRODUCTION

Diabetes is a metabolic disorder caused by an impairment in the metabolism of glucose, which has increased dramatically in recent decades, with an estimated 422 million cases suffering from the disease in 2016.[1] This impairment of metabolic cycle causes short-term and chronic cardiovascular, renal, neurological, ocular, and other complications in two known major types of the disease. Type-1 diabetes mellitus (T1DM) has resulted from the absence of insulin and type-2 diabetes (T2DM) mellitus results from a heterogeneous group of impairments with varying degrees of resistance to secreted insulin.[2,3]

T1DM is primarily an autoimmune disorder caused by the irreversible immunological degeneration of insulin-secreting cells (β) of the pancreatic islet of Langerhans.[4-6] The main treatment using insulin for the control of the disease is only a symptomatic treatment which cannot control the serious symptoms of the disease.[1] Considering the role of both humoral and cellular arms of the immune system, including autoantibodies, autoreactive T lymphocytes, and their products in the pathophysiology of the disease, efforts in improving the treatment continues.[1] In this regard, the use of immunotherapy, pancreatic, or Langerhans transplantation, as well as cell therapy, especially the use of stem cells, is being studied.[3,7-9] Among the stem cells used in regenerative medicine, we can refer to mesenchymal cells (MSCs) with proven immunomodulatory properties.[10-17] These immunomodulatory effects of MSCs have been studied in various autoimmune diseases, including T1DM.[18-20]

The main concern is the consequences of using a complete cell with all of its components and subsequent unwanted side effects despite the evidence of the effectiveness of these cells in treatment.[21-23] For this reason, the use of effective components such as different products of these cells is increasing.[24,25] A group of these cellular secretions is extracellular microvesicles which are called exosomes, that contain significant amounts of RNA and protein.[26-30] The content of exosomes is proven to be directly related to the type and activity of the producer cell, and its role in intercellular signaling and the transfer and management of secreted has been proven.[31]

In several studies, the effects of MSC-derived exosomes on autoimmune diseases, such as T1DM, have been studied.[32-35] In 2016, Nakano et al[36] indicated the improvement of cognitive impairment in T1DM mice model after treatment with an intravenous injection of MSC-derived exosomes through the repair of damaged neurons and astrocytes. Moreover, another study by Wen et al[37] showed the improvement of β-islet transplantation with the administration of MSC-derived exosomes as a consequence of a decrease in the proliferation of PBMCs and improvement of the suppressor T-cell function in T1DM mice model.

MSCs can be obtained from various tissues, in which, due to the possibility of isolating high amounts of MSCs from abdominal fat tissue, adipose tissue-derived MSCs (AD-MSCs) were indicated to be more popular.[38] In this regard, the effect of AD-MSCs derived exosomes on the clinical, pathological, and immunological findings of streptozotocin (STZ)-induced T1DM mice model have been investigated in the current study.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

C57BL/6 male mice; 6- to 8-week old, were obtained from the Pasteur Institute, Tehran, Iran. Also in each group, we have seven mice for the experiment (n = 7 animals/group). Animals were housed in standard conditions in accordance with the Guidelines for Care & Use of Laboratory Animals provided by Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.2 | Isolation, culture, and characterization of MSCs

In sterile condition, the abdominal adipose tissue of C57BL/6 mice was removed and washed three times with phosphate-buffered saline (PBS) and minced. Tissue's extracellular matrix digested using 0.1% of collagenase type-I in Dulbecco modified Eagle medium (DMEM) (Gibco, UK) for 20 minutes at 37°C. After centrifugation (15 minutes, 500g), pellet was transferred to DMEM containing 10% fetal bovine serum (Gibco, UK) and 2 mM L-glutamine, penicillin, and streptomycin (all from Invitrogen, Waltham, MA) as MSCs culture media and incubated (humidified air, 37°C in 5% CO_2).

At adherent cells optimum confluency (>80%), these cells harvested and at second passage, assessment of AD-MSCs was done with immunophenotyping of CD_{90}, CD_{105}, CD_{29}, CD_{45}, and CD_{11b} (all from Abcam, Cambridge, UK). For this purpose, harvested AD-MSCs were detached with 0.25% trypsin or EDTA and resuspended to 10^5 cells/mL in PBS. After the incubation of cells with the specific or isotype control antibodies (mouse IgG1-fluorescein-5-isothiocyanate (FITC) and mouse IgG1-PE; eBioscience, San Diego, CA) in 3% bovine serum albumin (Sigma, St. Louis, MO) in PBS for 45 minutes at 4°C, cells were fixed with 1% paraformaldehyde (Sigma). Eventually, analyses were done using Attune Acoustic Focusing Cytometer (RIC Facility, Boston, MA) and FlowJo software (San Jose, CA).

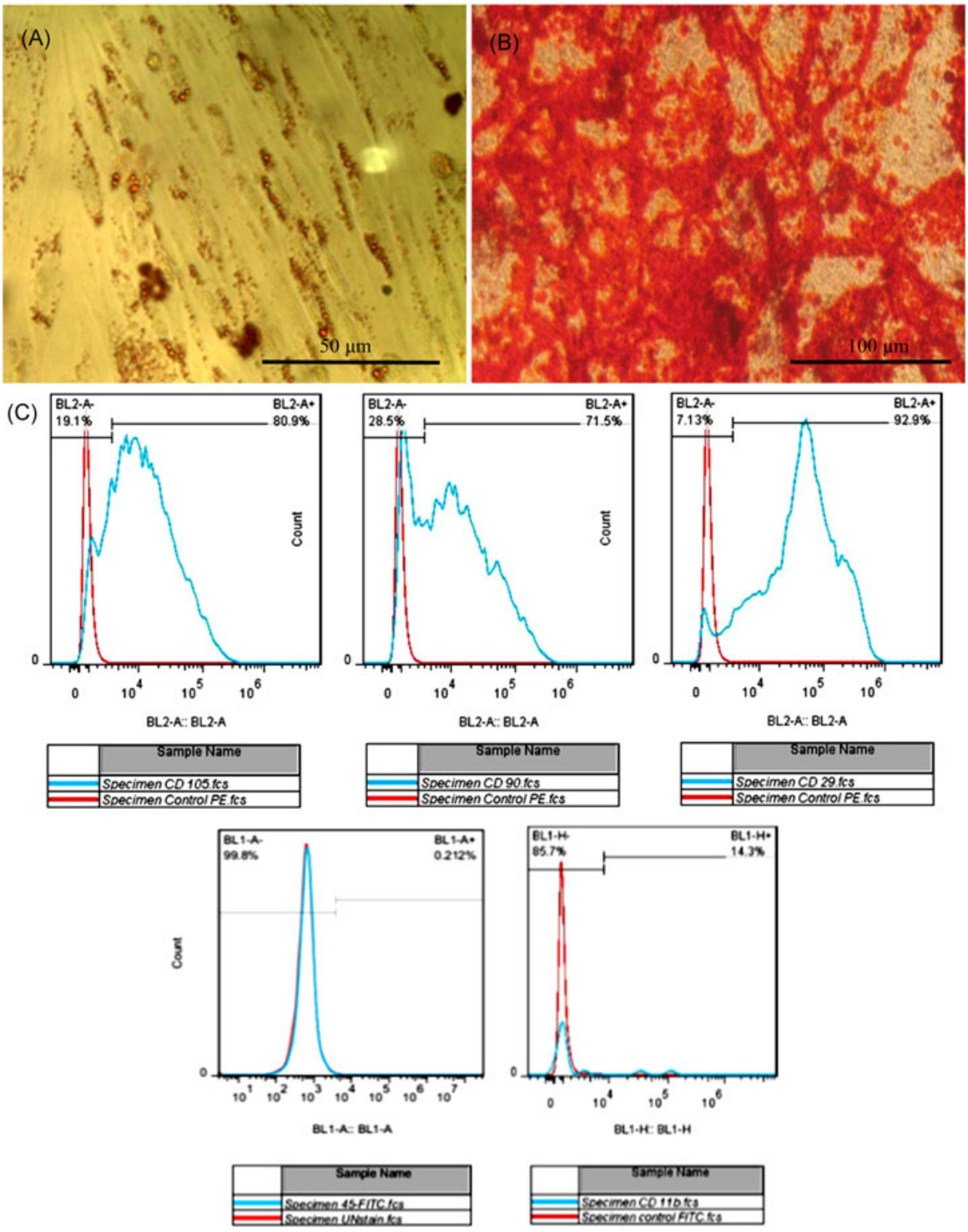

FIGURE 1 Visualization of AD-MSCs differentiation into adipocytes and osteocytes by ORO (A) and AR (B) staining using phase contrast microscopy. Flowcytometric immunophenotyping for surface markers of AD-MSCs (C). AD-MSCs, adipose tissue-derived mesenchymal stem cells; AR, alizarin-red; ORO, oil-red O

AD-MSCs potency was proved through the evaluation of their ability to differentiate into osteoblasts and adipocytes. Mineralization in cells as a sign of osteoblast differentiation was assessed using alizarin-red staining, after 3 weeks of culturing AD-MSCs in medium containing 10 mM β-glycerophosphate (Merck), 50 mg/mL ascorbic acid biphosphate (Sigma), and 100 nM dexamethasone (Sigma). Accumulation of oil droplets in the cytoplasm as a sign of adipogenic differentiation was assessed using oil-red O staining, after 3 weeks of culturing AD-MSCs in medium containing 250 nM dexamethasone (Sigma), 0.5 mM

3-isobutyl-1-methylxanthine (Sigma), 5 mM insulin (Sigma), and 100 mM indomethacin (Sigma).

2.3 | Isolation and characterization of MSC-derived exosomes

After the characterization of AD-MSCs, isolation of exosomes was performed on the cell culture supernatant at second passage 48 hours after culturing in serum-free DMEM. Obtained culture supernatant became the subject of several ultracentrifugation steps at 300*g* for 10 minutes, 2000*g* for 10 minutes, 10 000*g* for 30 minutes, 100 000*g* for 70 minutes which was resulted in the elimination of the cells, large dead cells, and debris, leaving purified exosomes as a final pellet.[39] For the preparation of protein solution of exosomes, the pellet was washed with PBS and centrifuged for 70 minutes at 100 000*g* and protein content determined using Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). Briefly, 10 μL of protein solution and serial dilutions of bicinchoninic acid were added to 200 μL of BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic, acid and sodium tartrate in 0.1M sodium hydroxide) and BCA reagent B (4% cupric sulfate) in 96-well plate. Absorbances of the plate in 570 nm were reported and content of protein solution was calculated using standard curve obtained from serial dilutions of BCA.

For characterization of particles size, samples diluted five times in PBS (refractive index 33.1 and viscosity 1.08) were subjected to dynamic light scattering and ζ potential measurements which were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

Obtained fresh exosomes were placed on the carbon coated grid after fixation with 1% glutaraldehyde (Sigma). The sample was examined using LEO 906 transmission electron microscope (TEM, Zeiss, Germany), operating at 80 kV after washing grids twice using PBS for 5 minutes and staining them using 1% uranyl acetate for 10 minutes. Images of Transmission electron microscopy (TEM) were recorded with Orius 200 camera (Gatan Inc, Washington, DC) using DigitalMicrograph Software (Gatan Inc).

Investigations for characterization of particles ended with Scanning Electron Microscopy: EM 3200 (SEM; KYKY, Bejing, China) using 1 to 5 μL of the dried sample on silicon chip after fixation with 2% paraformaldehyde. Images were captured with SEM at 30 kV following gold–palladium sputtering.

2.4 | Treatment of T1DM-induced mice model using low-dose streptozotocin with MSC-derived exosomes

C57BL/6 mice were rendered diabetic using standard protocols.[40] Briefly, multiple low doses (50 mg/kg) of STZ

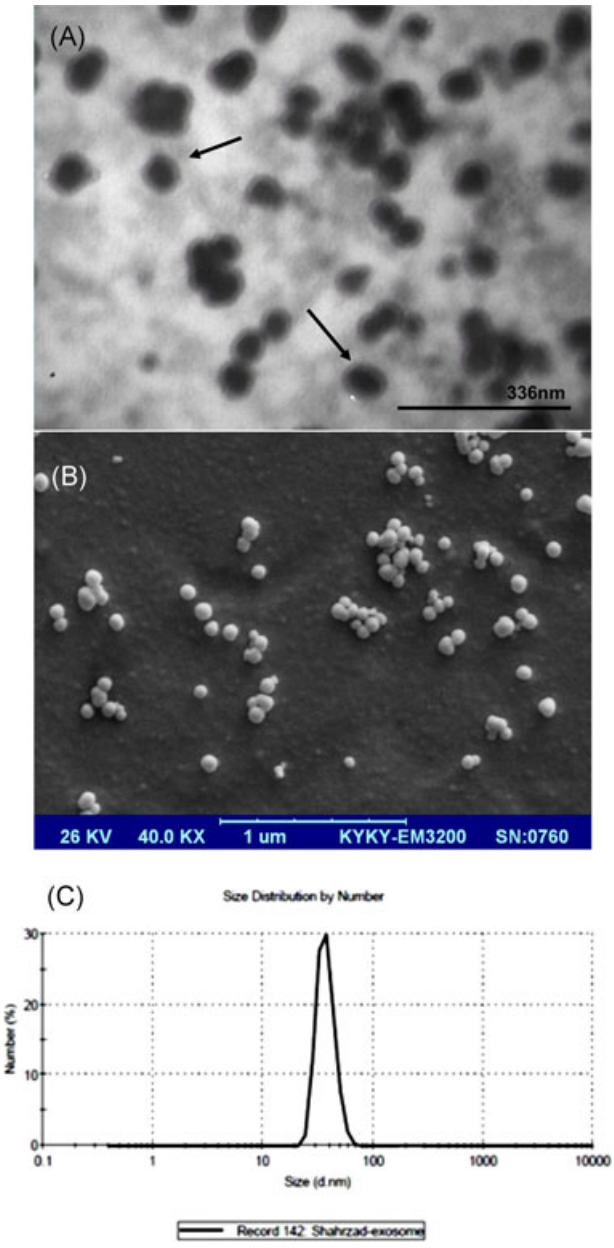

FIGURE 2 Micrographs of scanning electron microscopy showed spheroid shaped vesicles at the diameter of about 40 to 100 nm (A) transmission electron microscopy confirmed the average diameters of exosomes to be ≤100 nm with preserved intact spherical morphology (B). also results of dynamic light scattering showed that almost 68% of the solution ingredients had an average diameter of 41.1 nm (C)

(Sigma) solubilized in the sodium citrate buffer (pH 4.5), were injected intraperitoneally for five consecutive days. Four weeks after the last injection, mice blood glucose levels were measured using Glucometer (Accu-Chek; Performa, Roche, Basel, Switzerland) and mice with blood glucose >250 mg/dL considered to be diabetic. T1DM-induced mice were divided into T1DM untreated control group (T1DM) and T1DM under treatment (treated T1DM) with an intraperitoneal injection of

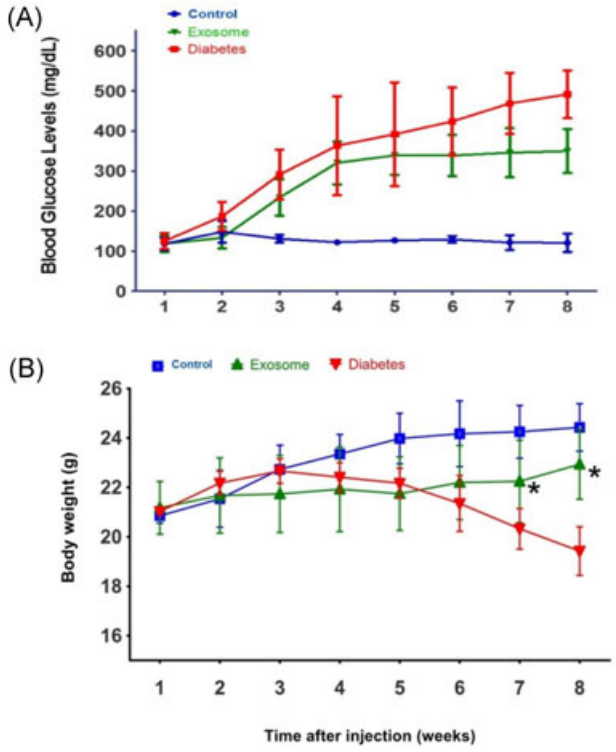

FIGURE 3 Exosomes derived from AD-MSCs ameliorates body weight and blood glucose levels of T1DM mice. Blood glucose levels of the T1DM mice were over 400 mg/dl during the 28 days after the last injection, but T1DM mice treated with exosomes showed a stable glycemic state (A) which have been shown to be in concordance with lower weight loss in T1DM mice treated with exosomes derived from AD-MSCs as compared to T1DM control mice (B). Data are presented as mean values of n = 7 animals/group ± SD. Statistical significant differences were tested using ANOVA test; *$P < 0.05$. SD, standard deviation. AD-MSCs, adipose tissue-derived mesenchymal stem cells; ANOVA, analysis of variance; T1DM, type-1 diabetes mellitus

50 μg of characterized exosomes solubilized in 1 mL of PBS twice a week. A group of healthy mice without any treatment were also regarded as a healthy control group (control). During 2-month of the experimental procedure, all groups of mice weights and blood glucose levels were monitored regularly with 7-day intervals.

2.5 | Histopathology and immunohistochemistry

At the end of the experimental procedure, mice were dissected by cervical dislocation and mice pancreas and spleen quickly removed. Mice pancreas after fixation with 10% formaldehyde, were processed for histopathological examinations using hemotoxylin and eosin staining due to instructions of a standard protocol.[41] After deparaffinization and retrieval of prepared sections, staining with Mouse monoclonal anti-insulin (R&D System, Minneapolis, MN) as primary and FITC-conjugated rat anti-mouse IgG (Bioscience) as secondary antibodies were performed using standard protocols of the manual.

2.6 | Isolation, purification, and proliferation assay of splenocytes

Cellular content of removed spleens was isolated through perfusion of 10 mL DMEM and pretreated with ammonium chloride for lysis and removal of erythrocytes. The remaining cellular content was washed twice with DMEM and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test for evaluation of the splenocyte number.

2.7 | Cytokine assays and immunophenotyping of regulatory T cells in splenic mononuclear cells

Mononuclear cells isolated from the spleen of three distinct groups were adjusted to 1.5×10^6 cells per well and stimulated with a cell activation cocktail containing brefeldin A due to the manufacturer's instructions (eBioscience). After 6 hours of incubation, activated cells were collected and washed with cell staining buffer (eBioscience). Activated cells with 0.3×10 cells per 100 μL distribution were stained for cell surface and intracellular markers, including eBioscience Mouse Regulatory T-Cell Staining Kit #3 (Anti-Mouse CD_4 FITC, Anti-Mouse CD_{25} PE, Anti-Mouse/Rat Foxp3 PE-Cy5 from eBioscience). Analyses of stained cells were performed using Attune Acoustic Focusing Cytometer (RIC Facility) and FlowJo software.

MNCs from three distinct groups of mice were adjusted to 3×10^6 cells per well containing DMEM supplemented with 15% fetal bovine serum as duplicates. It should be noted that phytohemagglutinin (Invitrogen) were added to the wells containing splenocytes of the control group. Mitogen phytohemagglutinin is a complex glycoprotein that stimulates T-cell lymphocytes in cell cultures. After 72 hours of incubation, the fresh supernatant of wells was collected for assessment of Mouse TGF-β1 (DuoSet ELISA DY1679-05; R&D Systems) and interleukin-17A (IL-17A) (homodimer ELISA Ready-SET-Go!; eBioscience) and IL-10, IL-4, and IFN-γ (Bender MedSystems, Wien, Austria) levels using ELISA due to the manufacturer's instructions.

3 | RESULTS

3.1 | Characterization of AD-MSCs

Adipogenic and osteogenic differentiation of AD-MSCs at second passage were confirmed using ORO and AR

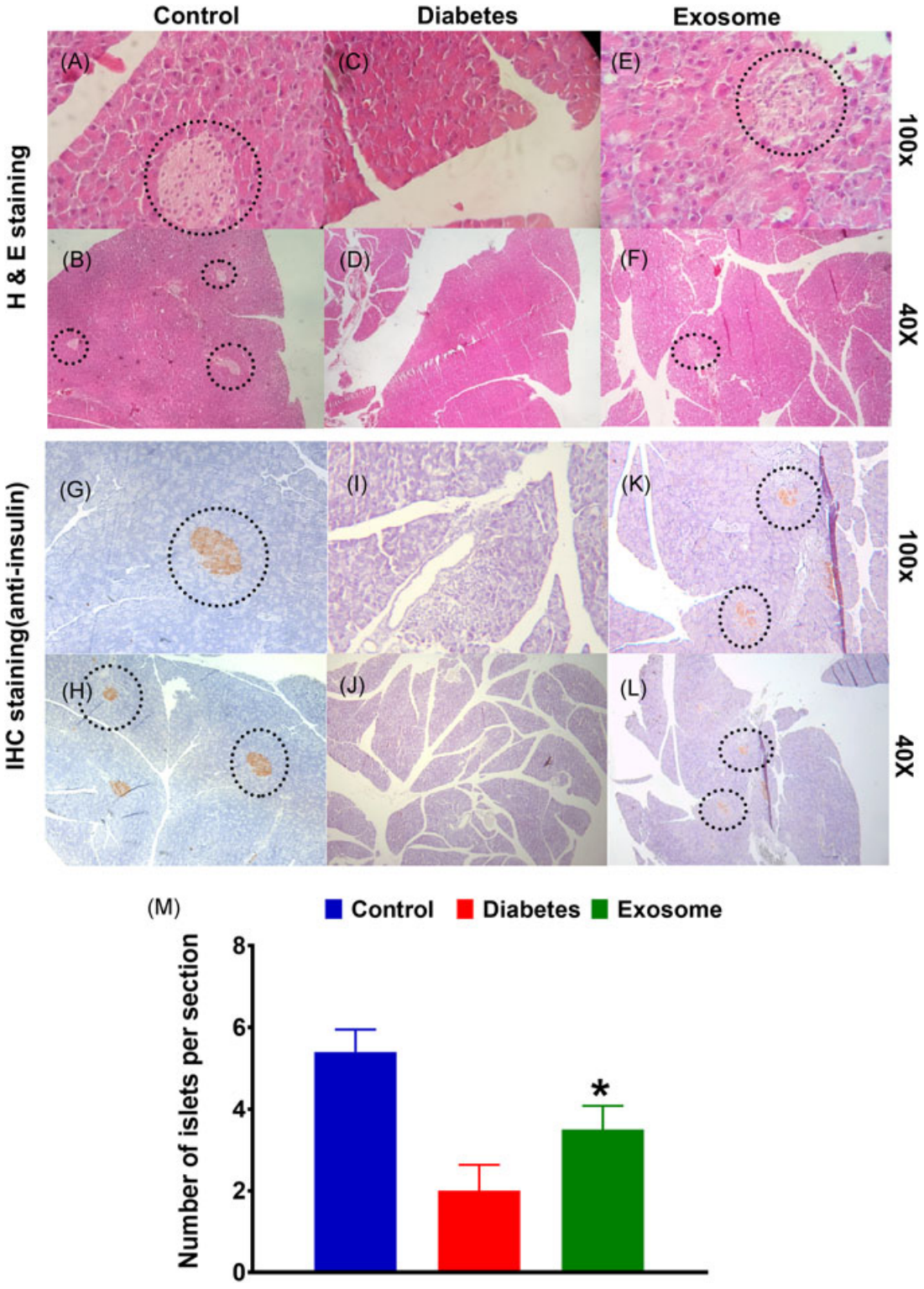

FIGURE 4 Histopathological and immunohistochemistry assessment of pancreas. (A,B) Control mouse pancreas in between normal pancreatic acini and normal pancreatic ducts with insulin producing β cells stained with anti-insulin using IHC (G,H). (C,D) Diabetic pancreas showing ruptured and destructed islet of Langerhans with damaged β cells which were not shown to produce insulin using IHC (I,J). (E,F) Diabetic pancreas of mice treated with exosomes derived from AD-MSCs showing more islets as compared to untreated T1DM mice which were stained with anti-insulin using IHC (K,L). Scale bars represent 40 and 100 nm. (M) Investigating the number of islets in the pancreas with β cells (insulin positive). Statistically significant differences were investigated using one-way ANOVA; $^*P < 0.05$. AD-MSCs, adipose tissue-derived mesenchymal stem cells; ANOVA, analysis of variance; IHC, immunohistochemistry; SD, standard deviation

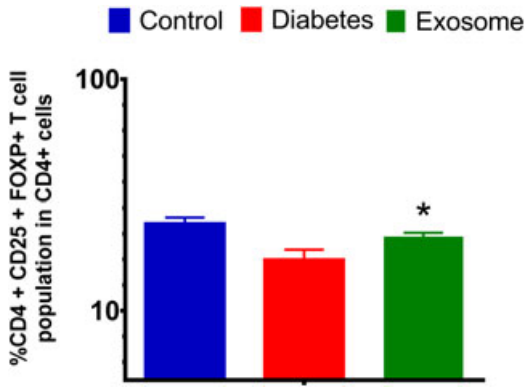

FIGURE 5 (A) Flowcytometric analyses showing significant changes in T-regulatory cells (%Treg) as a subpopulation in mononuclear cells derived from the spleen. Data are presented as mean ± SD values of n = 7 animals/group. Statistically significant differences were tested with one-way ANOVA and the Tukey's multiple comparison test; *$P < 0.05$. ANOVA, analysis of variance; SD, standard deviation

staining of lipid droplets and calcium droplets, respectively (Figure 1A,B). The ideal immunophenotype of isolated AD-MSCs also was confirmed using flow cytometry which was indicative of lower levels of surface expression for CD_{11b} and CD_{45} along with the high levels of CD_{29}, CD_{90}, and CD_{105} surface expression (Figure 1C).

3.2 | Exosome characterization

Prepared solution of isolated exosomes from the supernatant of AD-MSCs confirmed to contain 630 mg/mL protein using BCA assay. Size distribution in the exosome solution using dynamic light scattering (Figure 2C) were confirmed the method of isolating the exosomes which were shown to be an inconsistency with the results of SEM and transmission electron microscopy (Figure 2A,B).

3.3 | Treatment with exosomes derived from AD-MSCs is helpful in maintaining blood glusoce on stable levels

Increasing blood glucose levels, four weeks after the last injection of STZ, were seen in diabetic mice (Figure 3A) which is coordinated with the decrease in body weight in corresponding groups (Figure 3B).

3.4 | Pancreatic islets were regenerated in T1DM mice treated with exosomes derived from AD-MSCs

Histopathological analyses indicated that STZ induced massive destruction of pancreatic islets in T1DM mice (Figure 4C,D) have been ameliorated in T1DM mice treated with exosomes derived from AD-MSCs (Figure 4E,F) as compared to healthy control group (Figure 4A,B). Additionally, these restored islets of T1DM mice treated with exosomes derived from AD-MSCs (Figure 4K,L) have been supporetd to be functional since staining with anti-insulin antibody in this group were shown to be positive in comparison to T1DM and healthy control groups (Figure 4G-J). We Investigate the number of islets in the pancreas with beta cells (insulin positive) at each section of the tissue in Anti-insulin IHC (Figure 4M).

3.5 | The effect of treatment with exosomes derived from AD-MSCs on the splenic MNCs cytokines and regulatory T-cell population

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was not indicative of any significant differences in the stimulation index of splenic MNCs of different groups (Figure 6A). Yet, a significant increase in the population of Treg cells in T1DM mice treated with exosomes derived from AD-MSCs in comparison to the untreated T1DM group have been reported ($P < 0.05$) (Figure 5). A significant increase also was reported in the levels of IL-4, IL-10, and TGF-β along with the significant decrease in the levels of IFN-γ and IL-17 in T1DM mice treated with exosomes derived from AD-MSCs as compared to untreated T1DM mice ($P < 0.05$) (Figure 6B-F).

4 | DISCUSSION

MSCs have been supported to attribute therapies in engraftment of different tissues since these cells benefit from the potency of differentiation into multiple cell types and modulation of immune responses. MSCs derived from different sources produce these therapeutic factors constantly but do not survive for long in vivo and also as for the risk of genetic instability and hypersensitivity reactions related to using whole cells as therapeutic agents, the application of produced biofactors secretome derivatives may present considerable advantages.[42] As it was assumed in vitro role of MSC-secreted exosomes in inducing paracrine signaling resulting in the protective immunomodulatory role of MSCs on T1DM mice model on the other hand[20] provided supporting evidence for the probable therapeutic application of exosomes derived from AD-MSCs on autoimmune diabetes. In the current study, for the first time, we have shown that intraperitoneal application of exosomes derived from autologous

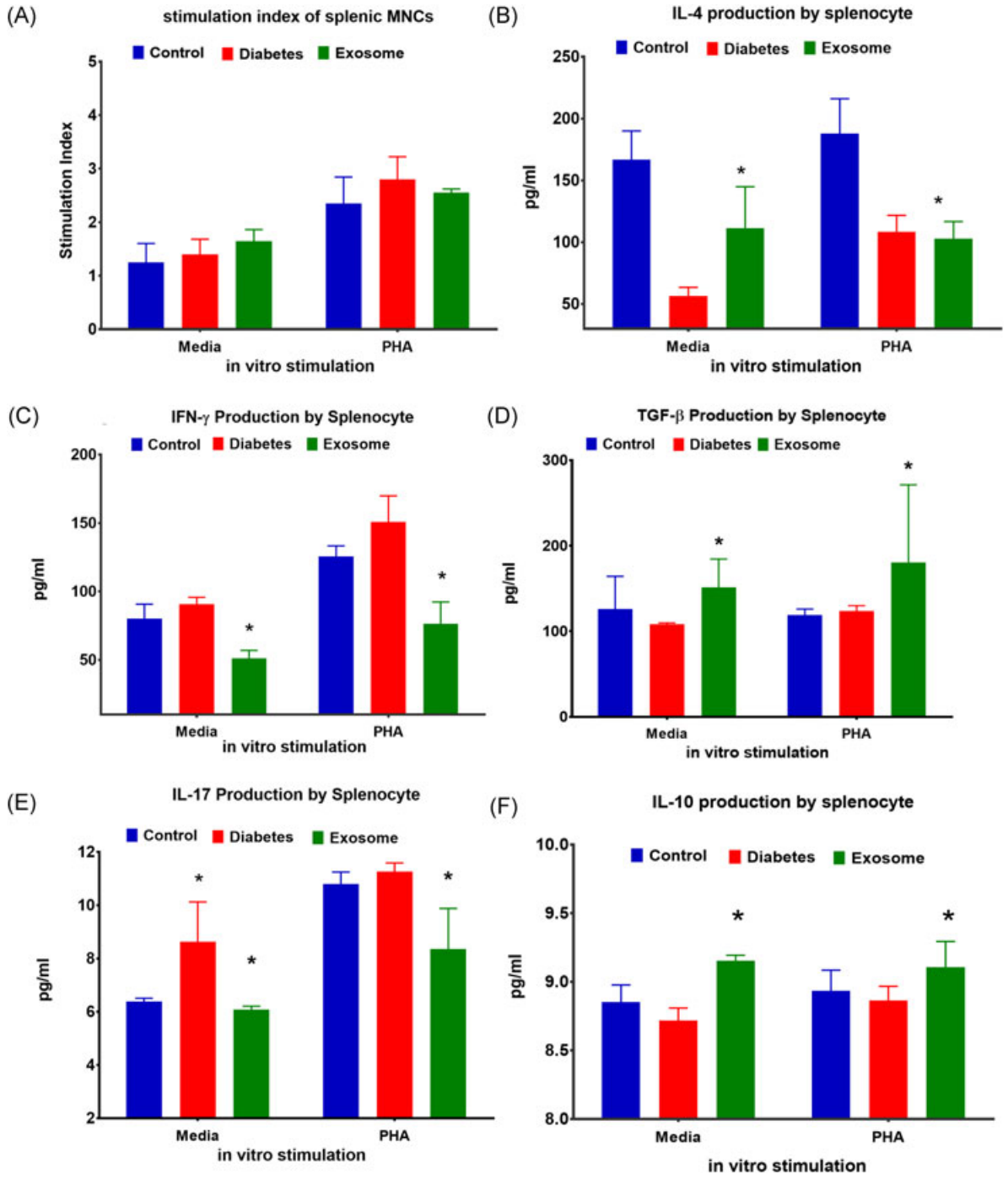

FIGURE 6 Assessment of splenic MNCs proliferation and secreted cytokines. The stimulation index of splenic MNCs was not indicative of any significant differences between groups even in the presence of PHA (A). Immunomodulatory effects of exosomes derived from AD-MSCs resulted in a significant increase in measured anti-inflammatory cytokines including IL-4, IL-10, and TGF-β (B, D, F) which were in concordance with a significant decrease in inflammatory cytokines including IFNγ and IL-17 (C, E) in the supernatant of splenic MNCs using ELISA. Data are presented as mean ± SD values of n = 7 animals/group. Statistically significant differences were investigated using one-way ANOVA; $^*P < 0.05$. AD-MSCs, adipose tissue-derived mesenchymal stem cells; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; IFNγ, interferon γ; IL, interleukin; MNC, mononuclear cells; PHA, polyhydroxyalkanoate; SD, standard deviation; TGF-β, transforming growth factor β

AD-MSCs can ameliorate the in vivo autoimmune response of STZ induced T1DM mice model.

Results of the current study demonstrated a significant increase in the potential of splenic MNCs for production of TGF-β, IL-4, and IL-10 as prominent anti-inflammatory cytokines with a significant decrease in the production of IL-17 and IFN-γ as leading inflammatory cytokines without any significant changes in the stimulation index of these MNCs (Figure 5). These changes in the dominant pattern of cytokines without any change in the stimulation index have been concluded to result from the changes in the polarization of splenic MNCs into $CD_{25}^{+}Foxp3^{+}$ Treg population. In this regard, Bai et al[43] showed similar results in their study on a rat model of experimental autoimmune uveitis in which they showed the ameliorative effect of local administration of MSCs exosomes as a result of significant reduction in IL-17 and IFN-γ producing T-cell subsets along with the significant increase in the $CD_{25}^{+}Foxp3^{+}$ T cells of lymph nodes with no inhibitory effects on the proliferation of T cells. The underlying cause of the changes in the polarization of T cells have been proposed to be a shift in the balance of M1/M2 macrophages in favor of M2 macrophages as a result of MYD88-dependent toll-like receptor 4 signaling induced after the failure in suppressing splenic MNCs proliferation by exosomes derived from MSCs.[44]

In a previous study on drug-induced liver injury models, promotion of anti-apoptotic activity as a consequence of treatment with MSC-derived exosomes have been shown to be associated with higher survival rates.[45] In this study, the pancreas of T1DM mice treated with exosomes derived from AD-MSCs has been shown to contain more islets in comparison with T1DM mice in which these islets insulin production have been confirmed through the staining with the anti-insulin antibody used in IHC (Figure 4). This improvement of the pancreas function in producing insulin has been shown to result in an maintained stable glycemic state of treated T1DM mice thus ameliorating the complications of the disease and improving survival of T1DM mice (Figure 3).

Possible immunomodulatory roles of exosomes have been investigated before in different experimental models which were indicative of conflicting results. Exosomes derived from insulinoma were demonstrated to contain strong innate stimuli and expresses candidate diabetes autoantigens thus exacerbating diabetes-related autoimmune responses to nonobese diabetic mice.[46] Similarly, immune-stimulatory effects of exosomes derived from islet MSC-like cells isolated from nonobese diabetic mice empowered the hypothesis indicative of autoantigen content of potent adjuvant activities for this exosome.[47] Yet, microvesicles but not exosomes derived from pathfinder cells, another cell type used in cell-based therapies isolated from the pancreas, were shown to stimulate functional recovery of the pancreas in STZ induced T1DM mice.[48] On the other hand, ameliorative effect of treatment with exosomes derived from MSCs in different autoimmune experimental models including osteoarthritis, systemic lupus erythematosus, rheumatoid arthritis in concordance with the results of the current study may be indicative of the importance of different sources of exosomes and even the source of the MSCs, and the importance of dominant microenvironment.[49,50]

5 | CONCLUSION

Since transplantation of multipotent stem cells renders potential risks, secretome derivatives of these cells may be considered as promising practical therapeutics in regenerative medicine for treatment of T1DM. The findings of the current study demonstrated that investigation into involved mechanisms can pave the way for cell-free therapeutic strategies in regenerative medicine and optimization of various aspects in further experimental settings seems to be essential.

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