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In vitro analysis of immunomodulatory effects of mesenchymal stem cell- and tumor cell -derived exosomes on recall antigen-specific responses

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ABSTRACT

Background: The aim of the present study was to evaluate in vitro effects of exosomes derived from mesenchymal stem cells (MSCs) or tumor cells on recall-antigen-specific immune responses.
Methods: The exosomes were isolated from the supernatant of the cultures of the adipose-derived MSCs, and 4T1 cell line. The splenocytes isolated from experimental autoimmune encephalomyelitis (EAE) mice were utilized to evaluate the effects of exosomes on recall-antigen-specific responses. The expression of master regulators for T cell sub-types and the levels of their corresponding cytokines were evaluated.
Results: Treatment by disease-inducing peptide ($MOG_{35–55}$) combined with MSC-EXO or by MOG + TEX enhanced the expression of Foxp3 as the master regulator for Treg cells; by comparing with splenocytes which were treated by MOG. Nonetheless, the production of IL-10 and TGF-β were increased only in splenocytes treated by MOG + TEX. Additionally, treatments of splenocytes by MOG + TEX and MOG + MSC-EXO decreased the expression of Tbx21 and Gata3, as the master regulator for T helper (T_H)1 and T_H2 responses. However, the IFN-γ level did not decrease. The expression of Rorc and Elf4, which are the activator and inhibitor for differentiation of T_H17 respectively were increased after splenocytes was treated by MOG + TEX. However, a reduction in Rorc and Elf4 levels was observed when splenocytes were treated by MOG + MSC-EXO. Indeed, the concentration of IL-17 did not alter significantly following the treatment by MOG + exosomes.
Conclusion: It was ultimately attained that TEX and MSC-EXO utilized various mechanisms to modulate the recall immune responses. TEX was more potent than MSC-EXO to induce regulatory responses by upregulating the production of Foxp3, IL-10, and TGF-β.

1. Introduction

Mesenchymal stem cells (MSCs) are a group of multipotent progenitor cells that play indispensable roles in maintaining cellular homeostasis via differentiation into a variety of cell types. Moreover, they produce a broad spectrum of bioactive molecules, which modulate the immune responses and inflammation; as well, support the tissue regeneration following the injury. MSCs home into the inflammatory sites to dampen the inflammation and lead to the regeneration of injured tissues [1,2]. In recent studies, it has been shown that MSCs also home to tumor sites in response to inflammatory mediators released by the tumor and stromal cells [3–6]. At the first glance, it seems that MSCs and tumor cells have entirely different properties and activities; nevertheless, they are in some extent similar; for example, stemness is a common feature for MSCs and tumor cells [7,8]. As well, both of them suppress the immune responses [9,10] by utilizing similar tools including exosomes.

Cell secreted exosomes are a sub-population of nanometer-sized extracellular vesicles, which they actively secreted by a diverse range of living cells. Exosomes deliver multiple types of molecules, such as proteins, lipids, and genetic materials (mRNA and miRNAs); participate in multiple biological processes relevant to the functions of the secreting cells [11].

Exosomes derived from MSCs (MSC-Exo) and Tumor-derived exosomes (TEX) suppress the immune response in a similar way to MSCs and tumor cells. These exosomes deliver the negative messages to all types of immune cells distinctly to T lymphocytes by using multiple mechanisms. For example, it has been shown that exosomes from both the MSC and tumor sources could suppress the proliferation of lymphocytes. TEX which gets released by breast cancer was shown previously that directly suppresses both $CD4^+$ and $CD8^+$ T lymphocytes [12]. Similarly, MSCs from human adipose tissue demonstrated the

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potential to reduce T cells proliferation [13]. Another mechanism which is also implemented by exosomes is through inducing apoptosis in the immune effector cell. Microvesicles or exosomes have shown to express FASL and TRAIL on their surface to target cells including lymphocytes [14–16]. Suppressing the production of inflammatory cytokines, such as IFN-γ and IL-17 is another mechanism which is used by the MSC-derived microvesicles to dampen the immune responses [17]. The other vital mechanism that is employed by exosomes is the ability to induce Treg cell differentiation through reprogramming. For example, tumor microvesicles induce Foxp3 expression by transferring miR-214 to the target cells [18]. However, MSC-EXO or TEX ability for reprogramming the committed T_H1, T_H2, and T_H17 lymphocytes have not been studied thoroughly yet.

The aim of the current study was to determine whether MSC-EXO and TEX could modulate the recall, antigen-specific immune responses through altering the expression levels of the master regulators which are corresponding to the subtypes of T helper cells. In the present study, we employed Experimental Autoimmune Encephalomyelitis (EAE) mice as the animal model to evaluate the aforementioned hypothesis. EAE is a mice model for human multiple sclerosis (MS) induced by autoreactive T_H1 and T_H17 cells that promote inflammation and demyelination in the central nervous system (CNS) [19]. Additionally, Treg and T_H2 impairment are important hallmarks of EAE [20,21].

In the current in vitro-study, the effects of MSC-EXO and the TEX on the recall-antigen-specific immune responses in splenocytes of EAE mice were appraised by measuring the messenger RNA (mRNA) levels of transcription factors including Tbx21, Gata3, Rorc, Elf4, and Foxp3. Additionally, we analyzed the production of pro- and anti-inflammatory cytokines that are commonly controlled by these transcription factors.

2. Materials and methods

2.1. Animals, induction and assessment of the EAE model

The study design is schematically represented in Fig. 1. Female C57BL/6 mice (6–8 week old) were purchased from the Pasteur Institute of Tehran (Iran). The animals were housed, handled, and treated according to the ethical protocols of animal treating and handling of Shahid Beheshti University of Medical Sciences. EAE was induced in C57BL/6 mice according to the standard protocols [22]. Briefly, the mice immunized by using an intradermal injection of 150 μg of oligodendrocyte glycoprotein peptide $MOG_{35–55}$ ($MOG_{35–55}$; Amino acid sequence-Purity ≥95% (HPLC): Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys; KJ Ross-Petersen ApS, Copenhagen, Denmark) emulsified in 500 μg complete Freund's adjuvant (CFA) (Sigma, USA) in a final volume of 200 μl divided and injected in both flanks. Pertussis toxin (List Biological Lab, USA) (300 ng) solved in 100 μl PBS and was administered intraperitoneally, 2 h after immunization. The pertussis toxin injection was repeated the next day. The assessment of the diseased animals was performed according to the following clinical scoring system: score 0: no sign, score 0.5: tip of the tail paralysis, score 1: complete tail paralysis score, 2: hind limbs' paresis, score 2.5: one hind limbs' paralysis, score 3: both hind limbs' paralysis, scores 3.5: both hind limbs' paralysis and front limbs' weakness, score 4: front limbs' paralysis, score 5: moribund [23]. The clinical assessments were performed by two independent examiners.

2.2. Mesenchymal stem cell isolation and culture

A total of 5 female C57BL/6 mice (8–10 weeks old) were sacrificed and the abdominal adipose tissue was harvested. In order to isolate MSCs, the tissue was primarily chopped by a scalpel in a petri-dish and other tissues were removed from the margins if any was present. The chopped tissue of each mouse was transferred into a 15 ml centrifuge tube. After that, the tissue was digested by 0.075% type 1 collagenase enzyme (Gibco; Thermo Fisher Scientific, Inc.) in a volume of 1 ml of incomplete medium, and was incubated for 30 min at 37 °C. During the incubation time, the tubes were shaken by finger flicking vigorously every 7 min. After 30 min, the tissue was completely digested, with a two-phase appearance. The enzyme was neutralized by adding at least 3 ml complete DMEM-F12 medium (Biosera, France) with 20% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), and centrifuged for 10 min at 300 ×*g*. Then, the pellet was re-suspended in the complete DMEM-F12 medium that contained FBS 20%; then was transferred to the T25 treated tissue culture plates. After 24 h, in order to remove non-adherent cells and tissue debris, the supernatant was replaced with fresh media.

2.3. Tumor cell

4T1 cells were provided from the Pasteur Institute of Iran. 4T1 is a murine breast cancer cell line which is an adherent epithelial mammary carcinoma with highly tumorigenic and invasiveness [24]. The cells were cultured in complete DMEM-F12 media with 10% FBS and at the confluence of 80% were passaged.

2.4. Characterization of mesenchymal stem cell

The MSCs were characterized at passage 2 (P2) by functional differentiation assay by culturing in osteogenic and adipogenic differentiation media. To investigate the differentiation potential, MSCs were

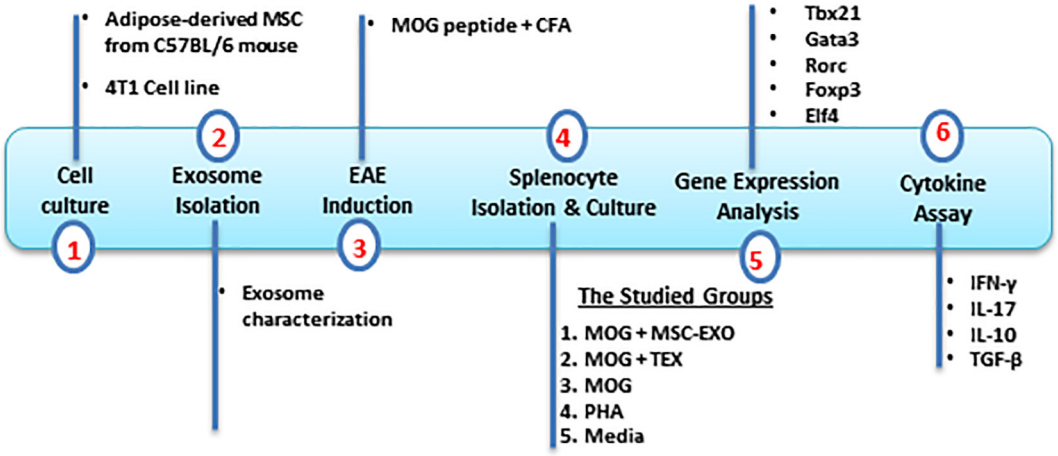

Fig. 1. The schematic representation of the study design.

Table 1
The sequence of primers used for gene expression assays.

Gene	Forward	Reverse
β-Actin	5′-AAGCCATGCCAATGTTGTCTCT-3′	5′-CCTAGCACCATGAAGATCAAGATCA-3′
Tbx21	5′-CCACAAGCCATTACAGGATGTT-3′	5′-GGAGTCTGGGTGGACATATAAGC-3′
Gata3	5′-CTCGGCCATTCGTACATGGAA-3′	5′-GGATACCTCTGCACCGTAGC-3′
Foxp3	5′-ACTCGCATGTTCGCCTACTT-3′	5′-GTCCACACTGCTCCCTTCTC-3′
Rorc	5′-ACAGCCACTGCATTCCCAGTTT-3′	5′-TCTCGGAAGGACTTGCAGACAT-3′
Elf4	5′-CGGAAGTGCTTTCAGACTCC-3′	5′-GGTCAGTGACAGGTGAGGTA-3′

cultured in 4-well plates at 6×10^3 cells/well in the DMEM-F12 medium supplemented with 15% FBS. The process of differentiation was induced 48 h later. The adipocyte differentiation was induced by adding of 250 mM dexamethasone (Merck, Germany), 100 mM indomethacin (Merck, Germany), 5 mM insulin (Merck, Germany), and 0.5 mM 3-isobutylmethylxanthine (Merck, Germany) for 3 weeks. Oil Red O dye was used to identify adipocytes (Merck, Germany). To induce osteogenic differentiation, the cells were treated by 10 mM beta-glycerolphosphate (Merck, Germany), 50 mg/ml ascorbic acid biphosphate (Merck, Germany), and 100 mM dexamethasone (Merck, Germany) for 3 weeks. Alizarin Red staining was used for osteogenic differentiation process.

In addition, the identity of MSCs was confirmed by flow cytometry. The cells at P2 detached using a trypsin solution (0.125%). Following washing with PBS the cells were re-suspended in PBS containing 5% FBS. The count and viability of cells were evaluated by using Trypan blue in a Neubauer hemocytometer. The following monoclonal antibodies (BD, USA) were used: the cluster of differentiation (CD) CD34-PE, CD11b-PE, CD90-PE, CD44-FITC, CD45-FITC, and D105- PerCP-Cy5. Isotype controls were included in the analysis for determining nonspecific binding and defining cut-off values. Totally 2×10^4 MSCs harvested and were incubated at 4 °C for 15 min with the appropriate amount of antibodies, subsequently, washed twice with PBS. For each sample, at least 10,000 events were acquired using a BD FACSCalibur® flow cytometer and the results were analyzed using FlowJo™ 7.6 software.

2.5. Exosome separation and characterization

To collect the exosome from serum free-media, when cultured cells reached the 80% confluence (for MSCs at P2), the concentration of FBS was reduced gradually over a period of 12 to 14 days, and was replaced by Insulin-Transferrin-Selenium (ITS) (Gibco; Thermo Fisher Scientific, Inc.) as a basal medium supplement. The final concentration of FBS and ITS in the exosome isolation medium was 0% and 1%, respectively. The supernatants were collected 2 times with three days' intervals from MSCs or 4T1 cells which were cultured in T75 flasks (SPL, Korea), and stored at −70 °C for further analysis. The exosome was isolated by Exospin exosome purification kit (Cell Guidance Systems, UK) according to the manufacturer's protocol. The protein content for the purified exosomes was measured by bicinchoninic acid (BCA) assay kit (Aryatus, Mashhad, Iran) as an indicator of the amount of exosome. The morphology of isolated exosomes was evaluated by using scanning electron microscopy (SEM), and particle size distribution in a Dynamic light scattering (DLS) instrument (Malvern Instruments, UK). Scanning electron microscopy (SEM) was performed on KYKY-EM 3200 (KYKY, China) to confirm the spherical shape and size of the purified exosomes. In addition, to characterize the exosomes further, the presence of CD63 as an exosome marker was demonstrated by using dot blot technique. Concisely, 30 μg of exosomes in 10 μl of buffer was blotted on the nitrocellulose membrane. Following blocking by 5% skimmed milk, 10 μl of both the primary and secondary antibodies were added, respectively. The detection of mouse CD63 antigen on the exosomes was performed by 10 μl of 100 μg/ml (1:5 diluted) purified rat anti-mouse CD63 antibody (BioLegend, USA) and 10 μl of 1:30 diluted HRP-conjugated anti-rat secondary antibody (R&D Biosystems). The substrate was added and the ECL signal was recorded on Photographic films.

2.6. Co-incubation of splenocytes with exosomes

The EAE mice were sacrificed 28 days after disease induction. The splenocytes were cultured in 24-well plates; in a cell concentration of 10^6/ml/well, by using RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. According to stimulants that were used, the wells of the culture plate were divided into 5 groups. The cells in test groups (TEX and MSC-EXO) were treated by 10 μg/ml $MOG_{35–55}$ to re-stimulate the antigen-specific clones of lymphocytes; at the same time, 15 μg/ml of either TEX or MSC-EXO, were added to each corresponding group. The control group re-stimulated only with MOG, but TEX or MSC-EXO exosomes were not added. The fourth and fifth groups including negative control (media) and positive controls (PHA) received neither MOG nor exosomes. The cells were incubated at 37 °C with 5% CO_2 and then were collected after 48 h for gene expression analysis by using Real-Time PCR; as well as the supernatant of another plate was collected after 5 days for cytokines measurement by using ELISA assay.

2.7. Real-time PCR

Following incubation with TEX or MSC-EXO, total RNA was extracted by using RNeasy Mini Kit (Qiagen, Germany). Afterward, cDNA was synthesized by cDNA Synthesis Kit (TaKaRa Bio, Japan). The levels of mRNA of Tbx21, Gata3, Foxp3, Rorc, and Elf4 were measured by SYBR® Green Real-Time PCR method (BioFact, Korea). The expression level of β-actin mRNA was used as normalizer. The sequences of the forward and reverse primers that were used are presented in Table 1.

2.8. Cytokine assay

The level of IL-10, IL-17, and IFN-γ were measured by ELISA kits (Mabtech, Sweden) base on the manufacturer's instructions. The concentration of TGF-β in the supernatant of cultured cells was assayed by using DuoSet ELISA Development System kit (R&D Systems, UK). Immediately before measurement, the TGF-β was activated by 1 N HCl and neutralized by 1.2 N NaOH/0.5 M HEPES.

2.9. Nitric oxide assay

The nitric oxide concentration on the conditioned media was measured as aforementioned [25]; briefly, each sample was mixed with equal amounts of Sulfanilamide 1% and 0.1% NED (N-(1-naphthyl) ethylenediamine) sequentially and following the addition of each one, the plate was incubated for 10 min in dark at room temperature. The absorbance was measured at 520–550 nm and the concentrations were calculated in accordance with the standards.

2.10. Statistics

The cell culture and ELISA and real-time PCR tests were performed duplicated. Statistical analysis were performed using SPSS version 22

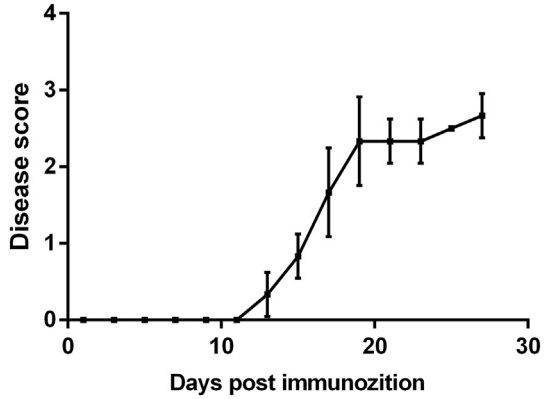

Fig. 2. The EAE disease score of the mice.

(SPSS, Chicago, IL, USA). To compare means of nonparametric data, Mann-Whitney *U* test was used. Differences with p values < 0.05 were considered significant. The entire data are presented as the mean ± S.E.M (standard error of the mean). The graphs were generated by GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, California, USA).

3. Results

3.1. EAE induction confirmation

The mice developed the primary signs of EAE two weeks after induction (Fig. 2). Our findings of disease progression were consistent with other reports that have employed a similar protocol to induce EAE [26]. The mice sacrifice was done two weeks later (day 28), when the mice demonstrated strong complications, scored 2 to 2.5 by both the examiners.

3.2. Mesenchymal stem cells show typical cellular and functional phenotype

Instantly, after 24-h culture, the isolated primary cells from abdominal adipose tissues adhered to the flask. When the medium was changed, the majority of non-adherent cells were eliminated and adherent cells gradually proliferated. MSCs were irregular spindle-shaped. After 1–5 days, they were confluent (Fig. 3C). The primary cells became round when treated with trypsin. Thereafter, 3 to 4 h after subculture, the majority of cells adhered. The MSC functional differentiation to osteocytes and adipocytes was verified following staining the cells (Fig. 3D and E). In addition, the presence of CD105, CD44 and CD90 molecules, and absence of CD34, CD45 and CD11b markers on the surface of cells confirmed the identity of MSC cells that were used in this study (Fig. 3A and B). It should be notified that adipose-derived MSC is similar to bone marrow-derived MSC; both of them are multipotent and competent to differentiate into mesenchymal lineages. Nevertheless, adipose-derived MSCs are more stable in long-term cultures [27], in which indicate a minor senescence, and major viability; furthermore, they have a considerable proliferation ability and maintain their high rate of differentiation capacity in long-term culture in comparison to BM-MSCs. Therefore, we used adipose-derived MSCs as the source for MSC-EXO.

3.3. Exosome characterization

Scanning electron microscopy (SEM) was performed to confirm the spherical shape and size of the purified exosomes, as shown in Fig. 4A. They were mostly spherical particles with diameters under 100 nm (Fig. 4A). In addition, to verify the identity of exosomes, the presence of CD63 as the exosome-specific marker was demonstrated by using the dot blot method (Fig. 4C). Furthermore, the size distribution of the exosomes was demonstrated by dynamic light scattering (DLS) (Fig. 4B). DLS results verified that the size distribution of particles is mostly in the expected 30 to 100 nm range for exosome.

3.4. MSC-Exo and TEX decreased the level of Tbx21 and Gata3 expression and increased Foxp3 mRNA levels

In this study, we used splenocytes isolated from EAE mice. All comparisons were performed between the groups of splenocytes that were treated by TEX + MOG or MSC-EXO + MOG with the group of splenocytes that received the MOG (as the control group). Expression of transcription factors corresponding to T_H1 and T_H2 cells were down-regulated and Foxp3 expression was up-regulated after in vitro treatment by TEX and MSC-EXO (Fig. 5). The cells that had been treated by either TEX or MSC-EXO in comparison to the controls that had been stimulated merely by MOG demonstrated lower expression levels of Gata3 and Tbx21. (-1.546 and -1.589 folds, respectively for TEX, $p < 0.05$) and (-1.503 and -1.541 folds respectively for MSC-EXO, $p < 0.05$). We also found that Foxp3 was increased following treatment by TEX (1.391 folds, $p < 0.05$) and MSC-EXO (1.341 folds, $p < 0.05$), respectively. The treatment of splenocytes with TEX + MOG increased the transcription level of Rorc (1.033 times $p < 0.05$), and Elf4 (1.025 times $p < 0.05$). Conversely, Elf-4 and Rorc level were decreased by MSC-EXO + MOG treatment (-1.497 and -1.157 times, respectively, $p < 0.05$).

3.5. The secretion of IL-10 and TGF-β were increased by splenocytes treated with exosomes derived from 4T1 cells, but not MSCs

Previous in vitro and in vivo studies indicated that MSC-EXO inhibits the production of various cytokines by lymphocytes [28,29]. It was our goal to find out if cytokine production could be inhibited in the model, which we were using. Supernatants were collected from cultures 5 days after the initial culture. We analyzed 4 murine cytokines IL-10, IFN-γ, IL-17, and TGF-β by using ELISA. In the group that was co-incubated with TEX and MOG, the mean of IL-10 (425.15 ± 93.4 pg/mL Vs. 193.9 ± 0.34 pg/mL), and TGF-β (1111 ± 57.6 pg/mL Vs. 875.31 ± 66.9 pg/mL) were increased when compared to the cells that were incubated with MOG ($p = 0.020$ and $p = 0.021$, respectively). However, co-incubation of cells using MSC-EXO and MOG in comparison to controls that received MOG did not change the levels of these cytokines (IL-10: 193.24 ± 0.65 vs 193.9 ± 0.34 and TGF-β: 852.93 ± 109.3 vs 875.31 ± 66.9, $p > 0.05$) (Fig. 6A and B).

3.6. Exosomes derived from 4T1 and MSC did not change the IL-17 and IFN-γ production in splenocytes isolated from EAE mice

As shown in Fig. 6C and D, the level of the IL-17 and IFN-γ cytokines in the TEX + MOG group in contrary to the control demonstrated a non-significant decrease from 60.75 ± 11.07 pg/ml to 56.33 ± 3.85 pg/ml for IL-17 and 4600 ± 50.46 pg/ml to 3919.23 ± 207.9 pg/ml for IFN-γ. Similarly, the level of these cytokines did not change for MSC-EXO + MOG treatment from 85.76 ± 17.77 pg/ml to 56.33 ± 3.85 pg/ml and 3976.20 ± 309.5 pg/ml to 3919.23 ± 207.9 pg/ml for IL-17 and IFN-γ, respectively. Nevertheless, these changes were not statistically significant for none of the cytokines ($p > 0.05$).

3.7. Nitric oxide increases by tumor but not MSC derived exosomes

The NO concentration in the supernatant of the group treated by MOG plus TEX compared to the control, which was stimulated with MOG significantly increased (72.9 ± 31 μM Vs. 48.1 ± 29 μM, $p = 0.021$; Fig. 6E). In the group treated by MSC-EXO + MOG, the concentrations were demonstrated a statistically non-significant change (45.1 ± 16.3 μM Vs. 48.1 ± 29 μM, $p > 0.05$).

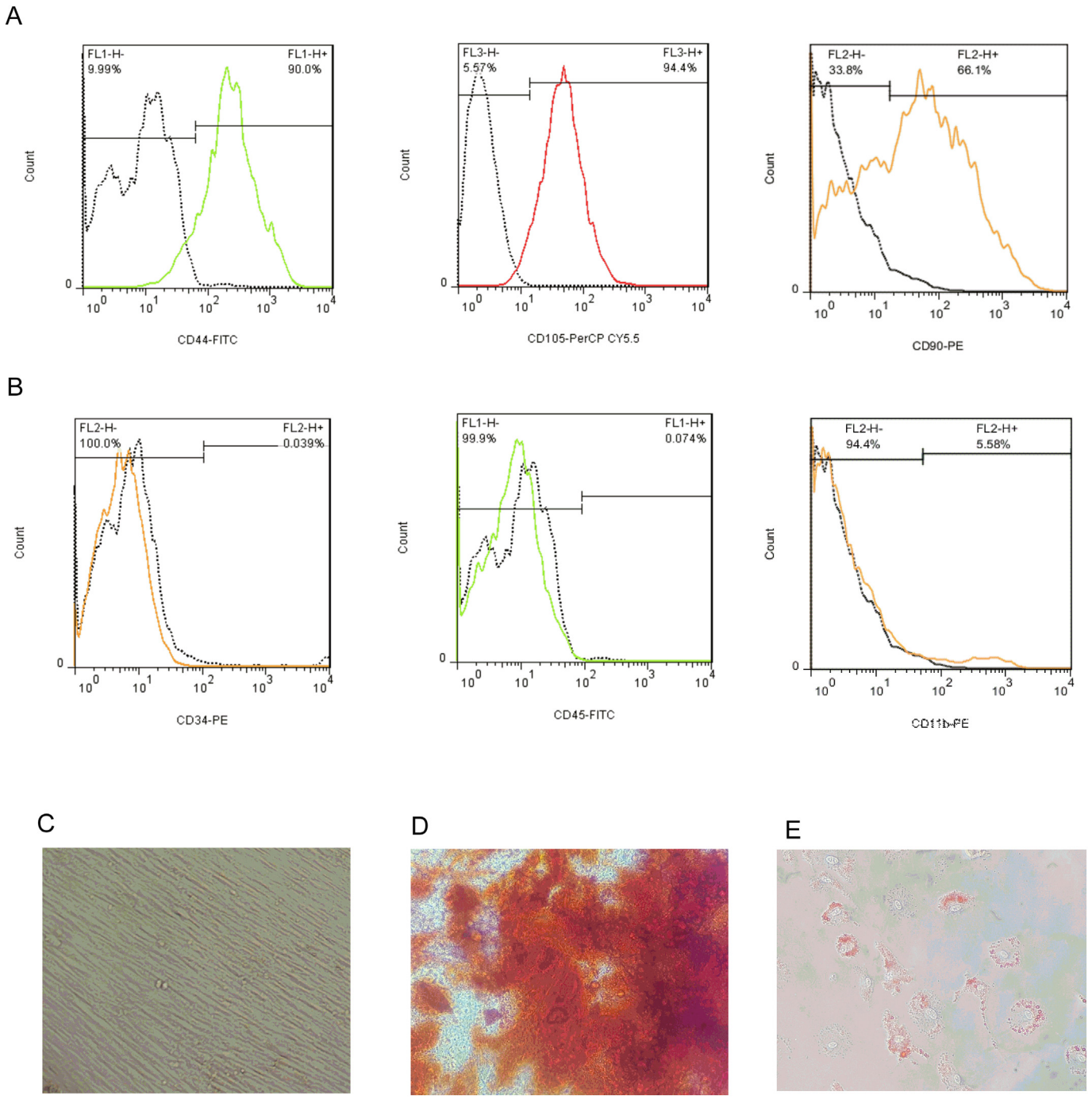

Fig. 3. Characterization of MSCs. The expressions of CD44, CD105, and CD90 as positive markers (A) and CD34, CD45 and CD11b as negative markers (B) on the MSCs were evaluated by flow cytometry to confirm their identity. Normal MSCs in culture were monitored by invert microscopy (**C**). Functional differentiation of MSCs to osteocytes (D) or adipocytes (E) was assessed by using Alizarin red or Oil red staining, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Although increasing evidences suggest that MSC-EXO and TEX could contribute to modulate immune responses, the underlying molecular mechanisms have not been entirely understood. The differentiation of T helper cells into the functional subsets is one of the key aspects of T cell mediated immunity. Indeed, we studied the effect of MSC-EXO and TEX on the expression levels of the master regulators for subtypes of T helper cells. In order to attain this purpose, splenocytes from EAE mice were treated by MSC-EXO or TEX at the time of re-stimulation by MOG peptide.

Incubation of splenocytes with MOG plus TEX or MSC-EXO elicited down-regulation of Tbx21 and Gata3 mRNA levels in comparison to splenocytes that were treated solely with MOG. The expression of Foxp3 transcript was found to be increased in the group treated by MOG + TEX or MOG + MSC-EXO. The effects of MSC-EXO or TEX on transcription levels of Tbx21 and Gata3 as the master regulators of T_H1 and T_H2 have not been studied yet. However, the earlier studies showed that MSC-EXO and TEX suppress immune responses through skewing T cell polarization toward the development of $FOXP3^+$ Treg cells, which co-expressing IL-10 and TGFβ1 [29–31]. In our recent experiment, it was shown that MSC-EXO and conditioned media of MSC exert ameliorative

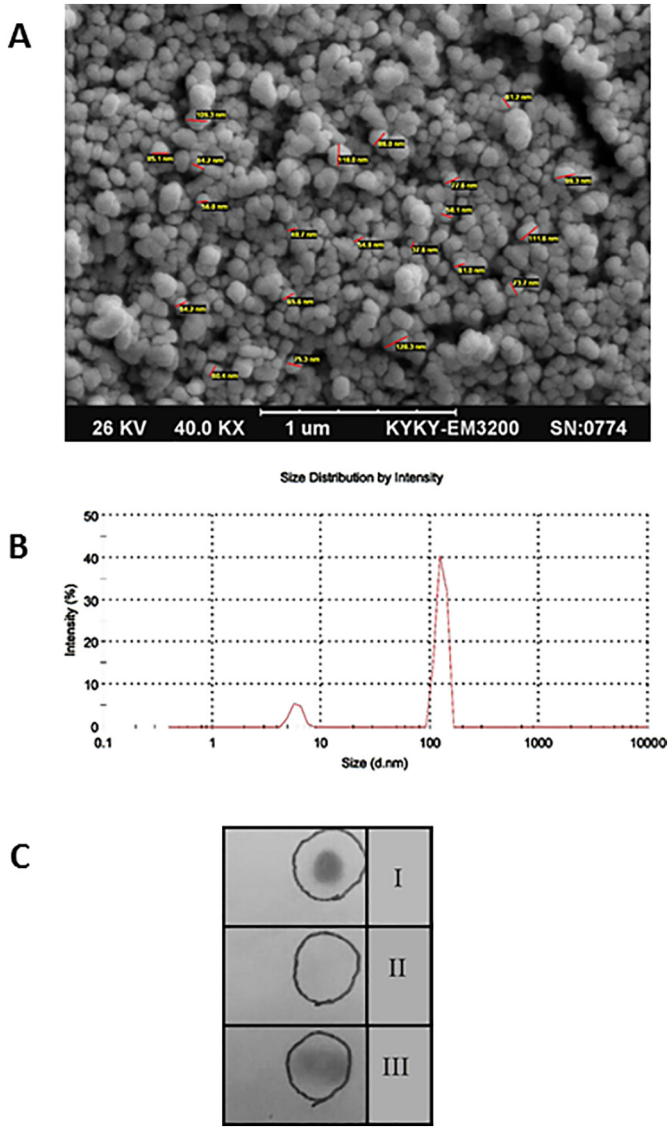

Fig. 4. The scanning electron microscopy of the isolated exosomes. The shape and size of isolated exosomes were evaluated by scanning electron microscopy **(A)**. The results demonstrated that most of the exosomes had a spherical shape with the size of 100 nm or less. The graph demonstrates the distribution of the size of exosome which has been determined by using DLS (**B**). The Dot Blot was used to demonstrate the CD63 protein in the separated exosomes (**C**). I: exosome, II: bovine serum albumin (negative control), III: neutrophils isolated from mouse blood (positive control).

effects on the experimental autoimmune models, through the increasing regulatory T-cell population and their products without a change in the proliferation index of lymphocytes [28,32]. Although in the current study, the frequency of Treg cells was not evaluated by flow cytometry method; the increase that observed in Foxp3 mRNA advocated that some populations of splenocytes may switch into the regulatory phenotype in the presence of exosomes. However, despite the increase in the expression of Foxp3 in splenocytes treated with TEX or MSC-EXO, the increased production of IL-10 and TGF-β were observed merely following the treatment by TEX. Collectively, these findings may imply that TEXs used multiple mechanisms to not only promote the conversion to regulatory phenotype but also increase their function. Additionally, the obtained data from the current study propose that the activated regulatory cells were more responsive to MCS-EXO or TEX-mediated effects than other cells [33]. It should be considered that Foxp3 as well as IL-10 and TGF-β are not exclusively produced by Treg

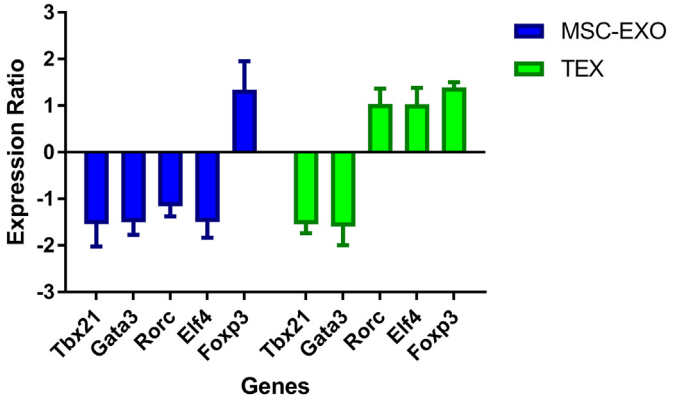

Fig. 5. The changes in the expression levels of transcription factors following the treatment of splenocytes of EAE mice ($n = 3$) the splenocytes (1×10^6) were treated by MOG (control group), MOG + TEX (A) or MOG + MSC-EXO (B). The entire tests were performed three times for accuracy. The graph bar shows "0" on the Y-axis when there would be no change in the expression. In fact, when the gene expression is double, the gene expression bars show one- time increases (+1, on the Y-axis). MSC-EXO: MSC derived exosomes, TEX: 4T1 tumor cell line derived exosomes, MOG: Myelin Oligodendrocyte Glycoprotein, PHA: Phytohaemagglutinin.

cells, but there are other sources for these molecules [34,35].

The reason for the differential activity which was observed between the effects of treatment with MSC-EXO or TEX has not been promptly known, but the difference in the content of the exosomes may explain the observed differences. Regarding the small number of exosomes that were used for cell treatment in the current study, the anti-inflammatory protein cargos such as TGF-β and IL-10 transported by exosomes could not explain the observed differential activity. However, emerging evidences suggest that mRNA and microRNAs are selectively packaged into exosomes [36] and are carriers of information that could be able to reprogram the immune target cells [37]. For example, it has been reported previously that tumor-specific miR-214 can be delivered by microvesicles to the T cells; and induces Treg differentiation through downregulation of phosphatase and tensin homolog (PTEN), which led to the high-level production of IL-10 [18].

Although, the obtained results in the current study are parallel to the previous findings with MSC-EXO and TEX, some of the outcomes in this study are not completely consistent with the earlier reports. For example, we previously showed that intraperitoneal injection of MSC-EXO in the experimental type-1 autoimmune diabetes mice model, led to a significant increase in Treg and regulatory phenotype cytokines; such as, IL-10, TGF-β, and IL-4 and consistently, a decrease was observed in the level of IFN-γ and IL-17 [28]. The obtained data in the current study showed that following the treatment by MSC-EXO, the expression of Rorc and Elf4 was decreased; on the other hand, treatment by TEX increased the transcript level of Rorc and Elf4. Rorc positively regulates the development of IL-17 producing T_H17 subset [38]. Conversely, Elf4 has been recognized as a novel lineage-specific regulator that suppresses the differentiation to the T_H17 subset [39]. In fact, the observed simultaneous and unidirectional changes in Rorc and Elf4 expression, act quite different for controlling the differentiation of T_H17 cells; this could explain the reason that the level of IL-17 did not change after treatment by exosomes. To the best of our knowledge, this is the first report regarding the effects of exosomes on the transcription level of Elf4, as a transcription factor that prevents T_H17 differentiation [39].

In the current experiment, even though the expression level of Tbx-21 was decreased in splenocytes, after treatments with exosomes, we did not attain any significant changes in the production levels of IFN-γ following the treatment of splenocytes by TEX or MSC-EXO. These findings may imply that splenocytes treated by exosomes, to some

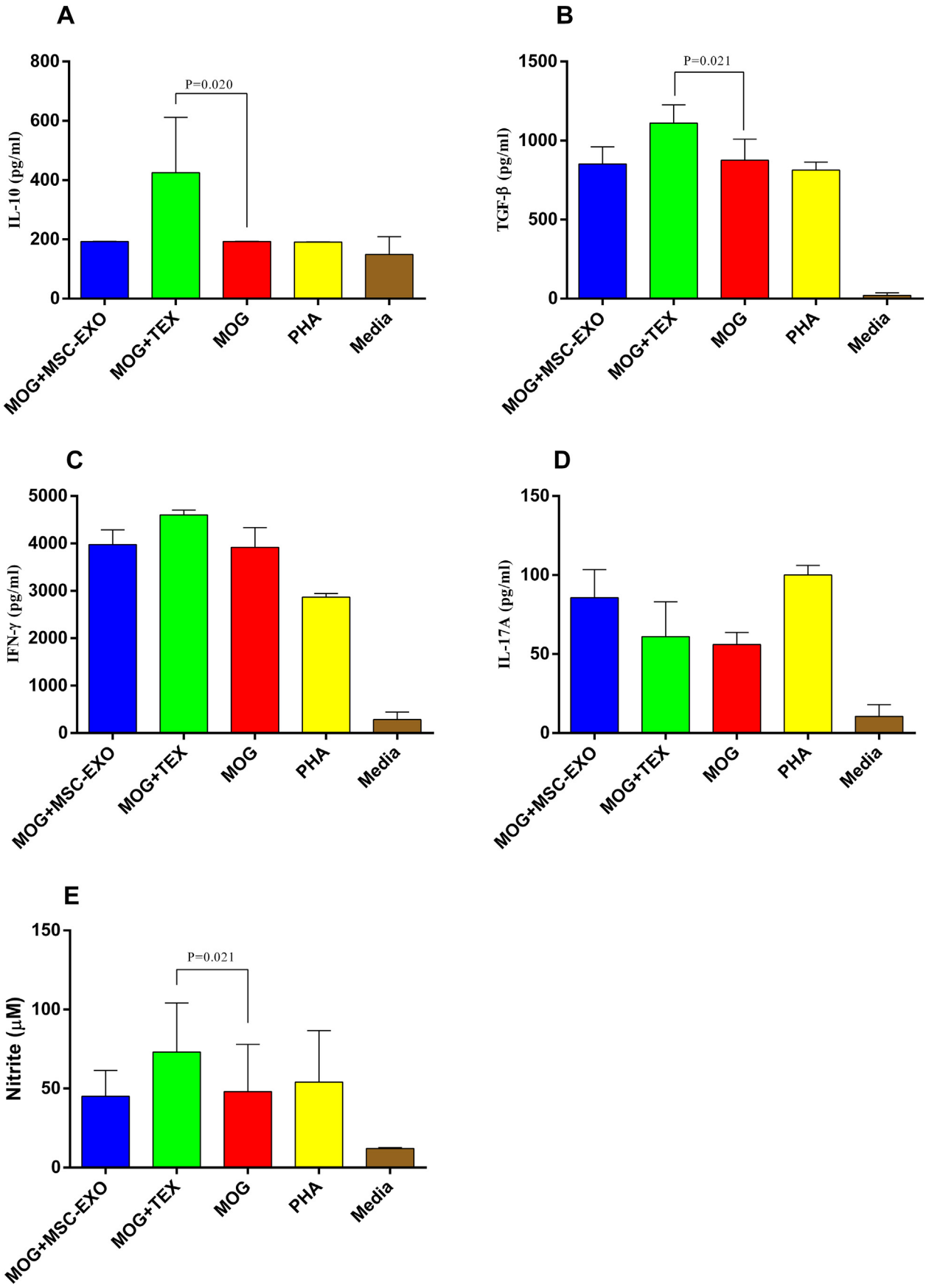

Fig. 6. The effect of TEX or MSC-EXO on the level of IL-10 (**A**), TGF-β (**B**), IFN-γ (**C**), IL-17A (**D**), and Nitric Oxide (E) in the splenocytes of EAE mice (n = 3). The cytokines and Nitric Oxide were measured in the supernatant of 1×10^6 cells, 5 days after treatment by MOG + TEX, MOG + MSC-EXO, MOG, PHA, and media. All the tests were performed three times. TEX: 4T1 tumor cell line derived exosomes, MSC-EXO: MSC derived exosomes, MOG: Myelin Oligodendrocyte Glycoprotein, PHA: Phytohaemagglutinin.

extent showed resistance against the suppression by regulatory mechanisms. Moreover, It has been indicated that various subsets of leukocytes in the splenocyte population respond differently to the treatment by exosomes [33]. Wu et al. reported that in macrophages, TEX treatment led to an increase in IL-6 and TNF-α [40]; that could have roles in the neutralizing of Treg suppression [41,42]. However, a study carried out by Favaro et al. showed that despite a significant IL-6 production, extra-cellular vesicle derived from MSCs inhibited IFN-γ and IL17 secretion in PBMCs of type 1 diabetic patients stimulated by a specific antigen [43].

According to the obtained data, it is hypothesized that the mechanism by which MSC-Exo and TEX exhibit these immunomodulatory properties could be through NO production. NO may influence mitogen-activated protein kinase and nuclear factor κB, which could cause a reduction in the gene expression of pro-inflammatory cytokines [44–46]. The obtained results of the present study showed that NO production was higher in the conditioned media in splenocytes treated by TEX. Apparently, this data is similar to the result of a study carried out by Wang and colleagues. They described that multiple myeloma-derived exosomes can contribute to immunosuppression via upregulation of Nitric Oxide Synthase [47]. In mice, when NO synthesis was inhibited, T cell responsiveness was not susceptible to suppression by MSCs [48]. Thus, NO production is a possible mechanism for the suppression of the inflammatory immune response. Considering the current study with the previously obtained data, it suggests that MSC-EXO is less effective than TEX, since, TEX may act by inducing regulatory phenotype as well as by promoting NO production.

Some discrepancies exist between the results obtained in this study with the earlier reports. This could be due to the differences between the research designs; i.e., in the analysis of the effects of exosomes on fully differentiated splenocytes that have been re-stimulated with specific antigens which have been studied in the present study, compared to the usage of the antibody or mitogen-stimulated resting cells that were reported in some other studies. It has been shown that resting conventional T cells, which were isolated from the peripheral normal blood of the donors when incubated by TEX, increased IL-10; nevertheless, decreased GATA3 mRNA levels [33]. However, when T cells were activated, the expression levels of mRNA of these molecules were not changed. Moreover, the splenocytes, which were used in the current study, was a heterogeneous collection of immune cells that elicited heterogeneous responses to exosomes treatment. Particularly, the use of splenocytes provides more accurate results through cellular interactions that are similar to those of the in vivo. It should also be remarked that most of the immunomodulatory properties which are attributed to the exosomes have been obtained from the studies that used in vivo models; nonetheless, in these studies, during in vitro studies for cytokines assay, the splenocytes were not treated again by exosomes.

Further studies are needed to elucidate the factors that promote the production of the cytokines by T_H1 and T_H17 cells, despite the alterations in the expression of master regulators genes in T cell subsets. Moreover, it needs to be elucidated that the decreases in the transcription levels of Tbx21, Gata3, and Rorc are a direct effect of exosomes or indirect effect which is mediated by Treg suppressor cells. In addition, more investigations should be considered to determine the impact of the interaction between TEX and MSC-EXO on tumor immune escape mechanisms.

Collectively, the findings of the current preliminary study indicate that MSC-EXO and TEX act differentially as reprogramming factors to suppress the immune responses through altering the expression pattern of master regulators genes corresponding in sub-types of CD4+ T cells. The in-vitro treatment of splenocytes of EAE mice with TEX decreased Tbx21 and Gata3; additionally, increased the expression level of Foxp3, which led to IL-10 and TGF-β production. Furthermore, MSC-EXO decreased the gene expression of Tbx-21, Gata3, and Rorc; as well as, decreased Elf4 level. On the other hand, neither TEX nor MSC-EXO could not change the concentration of cytokines that are controlled by those master regulators.

Conflict of interest

The authors declare that there are no financial or other conflicts of interest related to this paper.

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