In vitro treatment of Murine splenocyte with mesenchymal stem cell derived extracellular vesicles altered the mRNA levels of master regulators gene of T cell subsets

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Abstract

Background

The purpose of the current study was to evaluate the effect of mesenchymal stem cells-derived extracellular vesicles (MSC-EVs) on the production of cytokines and expression of genes, which are corresponded to the subsets of T helper cells.

Materials and methods

The supernatant of the second passage of MSCs that had been isolated from C57BL/6 mice abdominal adipose tissue was used to collect the MSC-EV. Splenocytes of healthy mice were activated using anti-CD3 and anti-CD28 antibodies and simultaneously were treated using the MSC-EVs. The proliferation rate of lymphocytes and the frequency of regulatory T cells were measured using flow cytometry. In addition, the expressions of T helper cell subset-specific transcription factors were evaluated using a real-time PCR assay. To appraise the effects of MSC-EV on splenocytes, the levels of IFN-γ, IL-17A, IL-10, and TGF-β were measured using ELISA.

Results

The results showed that the treatment of the CD3/CD28-activated splenocytes with MSC-EV did not statistically change the proliferation of CD3 + splenocytes. However, after the treatment, the mRNA levels of FoxP3 and Elf4 as well as the frequency of regulatory T cells was significantly higher when compared to the control group. The expression levels of Gata3, Rorc, and Tbx21 were down-regulated while, the corresponding cytokines levels did not altered.

Conclusion

The results revealed that the *in vitro* treatment of MSC-EV was associated with the increase in the frequency of CD4 + CD25 + Foxp3 + T cells and upregulation of Foxp3 mRNA level.

Introduction

Adipose tissue-derived mesenchymal stem cells (MSCs) are multipotent stem cells that have been reported to participate in repairing tissue or organ injury mainly through their proliferation and differentiation and their paracrine effects to attenuate inflammation [1]. Paracrine secretion of extracellular vesicles (EVs) including exosomes and microvesicles by MSCs is among the most attractive properties of these cells [2]. EVs are nanovesicles bilayer lipid secreted by almost all cell types [3]. Recent studies have shown that EVs participate in intercellular communication, which are essential in various physiological processes, including tissue homeostasis, immune response and anti-inflammatory

functions [4]. MSC-EVs' effect on target cells is mediated mainly via transferring macromolecules including DNA, mRNA, microRNA, lipids, proteins, and surface receptors from MSC to specific recipient cells [5].MSC-EVs participate in regulating the immune responses by reducing proliferation, conducting differentiation and function of immune cells, especially T cells, and reducing the production of pro-inflammatory factors by them [6].

The effects of MSC-EVs on immune responses are affected by many pivotal factors such as the source of MSC, the size of isolated EVs, and the state of target cells [7]. The state of lymphocytes i.e. naive, effector, or memory is among the variables that may affect the interaction between MSC-EVs and lymphocytes. The effect of MSC-EVs on the recall antigen-specific responses has been reported recently [8]. The *in vitro* study demonstrated that MSC-EVs can significantly increase the level of IL-10 and TGF-β immunomodulatory cytokines in splenocytes co-cultured with the MSC-EVs. Furthermore, the master regulator transcription factors explicitly expressed by effector T cell sub-populations including Tbx21 (T_H1), Gata3 (T_H2), Rorc (T_H17) Elf4 (suppressor for T_H17) and FoxP3 (Treg), were down-regulated after the co-culture [8]. Moreover, in another *in vivo* study, the MSC-EV treatment suppressed the immune responses mediated by effector cells through increasing the Treg cells' frequency and TGF-β level [9].

In the current *in vitro* study, we explored cytokines production and gene expression of transcription factors corresponding to T helper subsets in the splenocytes treated by anti-CD3, anti-CD28, and MSC-EV. The results showed that MSC-EVs are capable to increase the Foxp3 level as well as the frequency of CD4 + CD25 + regulatory T cells.

Materials And Methods

Animals

All protocols used in the current study were approved by the Ethics and Research Committee of Shahid Beheshti University of Medical Sciences (Ethical approval code: IR.SBMU.MSP.REC.1400.142). C57BL/6 male mice (6–8 weeks old) were purchased from the Pasteur Institute of Iran.

Splenocytes Culture And Msc-ev Toxicity Assay

The MSC-EVs used in the present study had been isolated and characterized during a previous study [8]. Splenocytes were isolated from healthy C57BL/6 mice and were cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). To remove the macrophages, the cells were first cultured for 24h in a treated flask (SPL, Korea). Consequently, the cells were incubated in plates coated with 2 µg/mL anti-CD3e antibody (eBioscience) and treated using 4 µg/m soluble anti-CD28 antibody (eBioscience).

To evaluate the probable cytotoxic effect of MSC-EV, the splenocytes were treated with 5, 15, 50, and 100 µg/mL of MSC-EV for 24h. Splenocytes with no MSC-EV treatment were used as a negative control. Moreover, a plate of cultured splenocytes was treated for 30 min using UV irradiation and incubated for 4 h at 37◦C as a positive control of apoptosis.

Flow Cytometry

To evaluate the viability of splenocytes, they were stained with annexin V-FITC (Biolegend,) and Propidium iodide (PI) (Biolegend). After 15 min incubation, the cells were evaluated using flow cytometry and the results were analyzed using FlowJo™ 7.6.1 software. The cells which were only positive for annexin V-FITC staining were considered apoptotic cells; the PI-positive cells were considered necrotic cells. Double negative cells represented live cells.

To estimate the effects of MSC-EV on the proliferation of splenocytes, the cells were first stained using CFSE (eBioscience). Afterward, the cells were treated with 1% PHA (Gibco, Thermo Fisher Scientific, Inc.) and 5, 15, and 50 µg/mL of MSC-EV; then were incubated for 5 days at 37◦ C. After the incubation time, the cells were stained using an anti-CD3 antibody conjugated with PE (Miltenyi Biotech) and evaluated using flow cytometry. Furthermore, splenocytes were activated using anti-CD3 and anti-CD28 antibodies and treated with 15 µg/mL of MCS-EV for 5 days; finally, the proliferation rate was evaluated using the protocol mentioned previously.

Treg cell's frequency among splenocytes was analyzed using a Treg detection kit (Miltenyi Biotech) and a BD FACSCalibur® instrument. The results were analyzed using the FlowJo™ 7.6.1 software.

Treg cells frequency among splenocytes was analyzed using Treg detection kit (CD4/CD25/FoxP3 (PE) (Miltenyi Biotech) according to the manufacturer’s instructions. The BD FACSCalibur® instrument was used to measure the regulatory T cells. The results were analyzed using FlowJo™ 7.6.1 software.

Cytokine Assay

The IFN-γ, IL-17A, and IL-10 cytokines levels were measured using the ELISA method (Mabtech, Sweden) according to the manufacturer's instructions. In addition, TGF-β level was measured following activation by 1 N HCl using the DuoSet ELISA kit according to the manufacturer's instructions (R&D Systems, UK).

Real-time PCR

The study of relative expression of genes was performed according to the previously reported study [8]. Briefly, the total RNA of the splenocytes was extracted (Favergen, Taiwan, then converted to cDNA using random primers (Yekta Tajhiz, Iran). The relative mRNA levels of Tbx21, Gata3, Foxp3, Rorc, Elf4 and β-actin as a housekeeping gene were evaluated using SYBR® Green Real-Time PCR (BioFact, Korea).

Statistical analysis

Statistical analyses were performed using SPSS (SPSS, Inc., Chicago, IL, USA, version 22). The data were expressed as mean ± standard error of the mean (SEM). The significant threshold was considered as P-value smaller than 0.05. The graphs were generated by Graph-Pad Prism (GraphPad Software, Inc., San Diego, CA version; 6).

Results

In vitro, **Treatment of splenocytes using MSC-EV did not suppress their proliferation.**

Initially, to evaluate the cytotoxicity of MSC-EV on the splenocytes, the cells were isolated from C57BL/6 mice; then had been activated using either anti-CD3 + CD28 antibodies or 1% PHA and treated for 5 days with, 5, 15, 50, and 100 μg/mL of MSC-EV. The results showed that none of these concentrations of MSC-EV demon strated a cytotoxic effect on splenocytes. The proliferation index of CD3 + cells in the group treated with MSC-EV was 2.77 ± 0.4 and for the control group was 2.83 ± 0.35, ($P > 0.05$, Fig. 1a & b).

MSC-EV increased the mRNA level of transcription factor corresponding to regulatory T cells differentiation and function

To evaluate the MSC-EV effect on the T cell subsets, the mRNA expression level of T cell master regulator transcription factors i.e. FoxP3 (Treg), Tbx21 (T_H1), Gata3 (T_H2), Rorc (T_H17), and Elf4 (suppressor for T_H17) were measured using real-time RT-PCR assay. The results showed that after treatment with MSC-EV, the FoxP3 transcription factor, which is the master regulator of regulatory T cells was up-regulated (1.254 ± 0.478 times; Fig. 2). Furthermore, Elf4 which is known to restrain the differentiation of T_H17 [10] was also up-regulated (1.197 ± 1.344 times). However, Gata3, Rorc, and T-bet transcription factors were down-regulated (0.945 ± 0.295, 0.904 ± 0.333, 0.854 ± 0.149, times respectively).

MSC-EV increased the frequency of CD4 + CD25 + FoxP3 + regulatory T cells in vitro

To evaluate the *in vitro* effects of MSC-EV on the frequency of Treg cells, the splenocytes were activated using anti-CD3 and anti-CD28 antibodies. The rate of CD4 + CD25 + FoxP3 + regulatory T cells was measured using flow cytometry. The results showed that the frequency of regulatory T cells in the splenocytes treated by MSC-EV was significantly higher compared to the control group (9.15 ± 0.95% and 5.22 ± 1% respectively, $P = 0.043$, Fig. 3).

Treatment of CD3-CD28-activated splenocytes by MSC-EV did not change the cytokine concentration in vitro

To investigate the effect of MSC-EV treatment on cytokines that are mainly produced in responses mediated by subsets of helper T cells, the level of TGF-β, IL-10, IFN-γ, and IL17A were measured using ELISA. The results showed that the levels of immunomodulatory cytokines i.e. TGF-β and IL-10 in

splenocytes that were activated using anti CD3 + CD28 antibodies and treated with MSC-EV were not significantly different from the control group (1038.5 ± 73 and 258 ± 27 vs. 1037.9 ± 87 and 387 ± 84, respectively, Fig. 4). Likewise, the production of inflammatory cytokines IFN-γ and IL-17A did not significantly change following MSC-EV treatment compared to the control group (4164 ± 179 and 2012 ± 212 vs. 4037 ± 128 and 2475 ± 162, respectively).

Discussion

In the present study, we evaluated the effects of MSC-EV on the splenocytes that were activated *in vitro* by anti-CD3 and anti-CD28 antibodies. The results showed that, although treatment with MSC-EV has no significant effect on the proliferation index of splenocytes activated with anti-CD3 and anti-CD-28 compared to the control group, however, it caused a significant increase in the frequency of CD4 + CD25 + FOXP3 + Tregs compared to the control group. At the molecular level, treatment of activated splenocytes with MSC-EV increased the expression of Foxp3 and Elf4 and decreased the expression of Gata3, Tbx-21, and Rorc. However, these changes did not significantly affect the production of inhibitory (i.e. IL-10 and TGF-β) and pro-inflammatory (i.e. IL-17 and IFN-γ) cytokines by these cells.

One of the main characteristics of adaptive immune responses is clonal proliferation, therefore we measured the changes in the proliferation rate of CD3 + cells following treatment with MSC-EV. Results showed that the MSC-EV treatment did not change the proliferation rate of cells that were activated by anti-CD3 and anti-CD28 stimulation or different concentrations of PHA. This is in line with the finding by Bai et al. which implies that MSC-EV has no effect on the proliferation of Murine lymphocytes activated by a specific antigen or ConA [11]. However, there are other studies including Blazquez et al. study which reported the inhibitory effects of MSC-EV on proliferation. However, it should be noted that Blazquez et al. used human cells in their study [12]. One simple explanation for our observation may be MSC-EV may not have a direct inhibitory effect on lymphocyte proliferation specifically in vitro study, in which the stimulation is so artificially strong, that it may not be easily harnessed.

Instead, the MSC-EV effects can be observed by changes in T cell subpopulation, including regulatory T cells. The results of the present study showed that consistent with the increased frequency of Foxp3 + Treg cells, the expression level of Foxp3 mRNA was also increased in the MSC-EV treated study group. In line with these findings, Fattore et al. and Fujii et al. both reported increased Treg after treatment with MSC-EV in PBMCs activated by anti-CD3 and anti-CD28 [13, 14]. In contrast, Zhang et al. demonstrated that the Treg ratio was not increased in purified CD4 + T cells activated by anti-CD3 and anti-CD28 [15]. It should be noted that this discrepancy may be due to the fact that Zhang et al. used purified CD4 + T cells, while Tregs require the presence of other myeloid and lymphoid cells not only for their optimal function but also for efficient induction of their regulatory phenotype [13].

Despite the observed increased Treg cells, there was no increase in the production of immunomodulatory cytokines i.e. IL-10 and TGF-β. MSC-EV did not decrease the level of pro-inflammatory cytokines following treatment. However, it should be noted that the production of immunomodulatory cytokines including IL-

10 and TGF-β is only one of the different mechanisms of Tregs to modulate immune responses [16]. Interestingly, previous studies showed that the main immunomodulatory mechanism used by Tregs induced in the presence of anti-CD3 and anti-CD28 antibodies is through cell-to-cell contact, and they do not use the IL-10 and TGF-β cytokines mechanism [17]. Because we removed myeloid cells to a large extent in the designing of the *in vitro* study and the fact that one mechanism used by Tregs is indirectly by induction of immature DCs [16], one can hypothesize that the Tregs have no access to all of their arsenal depositories, therefore the production of pro-inflammatory cytokines did not suppress efficiently. As previously discussed, the responses which are induced by anti-CD3 and anti-CD28 antibodies are so artificially powerful and also remain sustainable for a long-time, therefore may not be an appropriate model to reflect the potential of immunomodulatory treatments of MSC-EV.

In a previous study [9], we evaluated the effects of MSC-EV on the splenocyte of EAE mice after re-stimulation with a specific antigen, i.e. MOG. Results showed that the expression of Tbx21, Gata3, Rorc, and Elf4 were downregulated, and Foxp3 expression was up-regulated after in vitro treatment using MSC-EXO. In the present study, the FoxP3 and Elf4 expression was up-regulated; while Gata3, Rorc, and Tbx21 transcription factors were down-regulated. In the previous study, the levels of IL-10 and TGF-β in splenocytes after incubation with MSC-EXO and MOG did not change when compared to controls that received merely MOG. Similarly, the level of IL-17 and IFN-γ did not change after MSC-EXO + MOG treatment. The findings of our previous study and the present study on cytokine production are completely consistent.

Elf4 has been recognized as a lineage-specific regulator that suppresses the differentiation of T helper cells to the TH17 subset [10]. In fact, the observed simultaneous and unidirectional changes in Rorc and Elf4 expression levels, act oppositely with each other in controlling the differentiation of TH17 cells; this could explain the reason that the level of IL-17 did not change after treatment by exosomes.

Collectively, in the current study, the MSC-EV potential to induce Tregs was shown. However, results revealed that MSC-EV treatment of splenocytes that were stimulated by anti-CD3 and anti-CD28 antibodies was not able to change the levels of cytokines.

Declarations

Acknowledgments

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Conflict of interest

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

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Figures

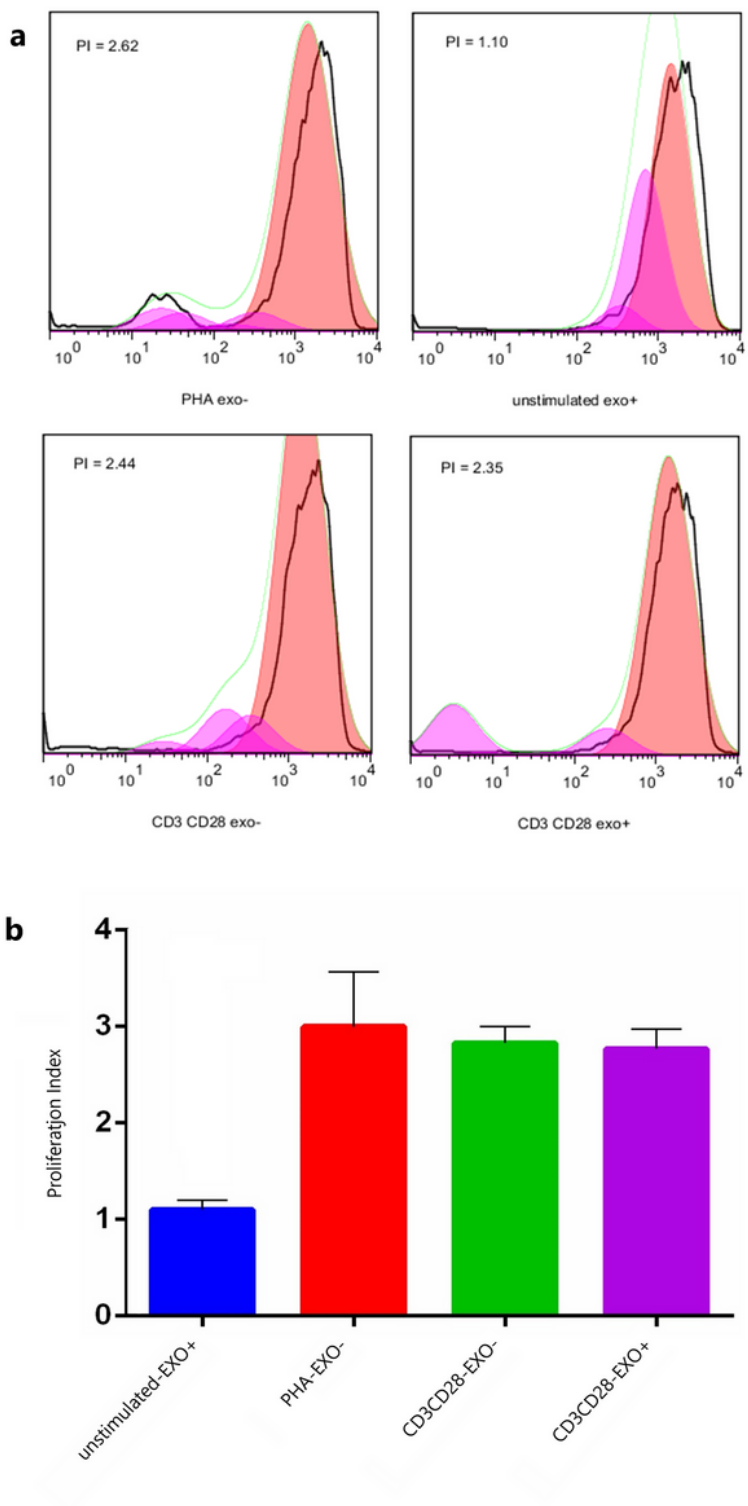

Figure 1

Investigating the effect of MSC-EXO on the proliferation of splenocytes stimulated with Anti-CD3/Anti-CD28. (a) The cells were first stained with CFSE dye and after proliferation, immediately before reading with the device, they were stained using anti-CD3 antibody conjugated with PerCp to determine the population of lymphocytes. Black line peaks show undivided cells (G0 control). The amount of fluorescent CFSE probe in the main samples (coral peak) decreased with each division time (pink peak).

(b). Comparison of splenocyte proliferation stimulated with Anti-CD3/Anti-CD28 in the presence and absence of MSC-EXO. As it is clear in the picture, the proliferation index (PI) of splenocytes did not change under the influence of MSC-EXO treatment (statistical analyzes was performed using Mann Whitney; n = 4).

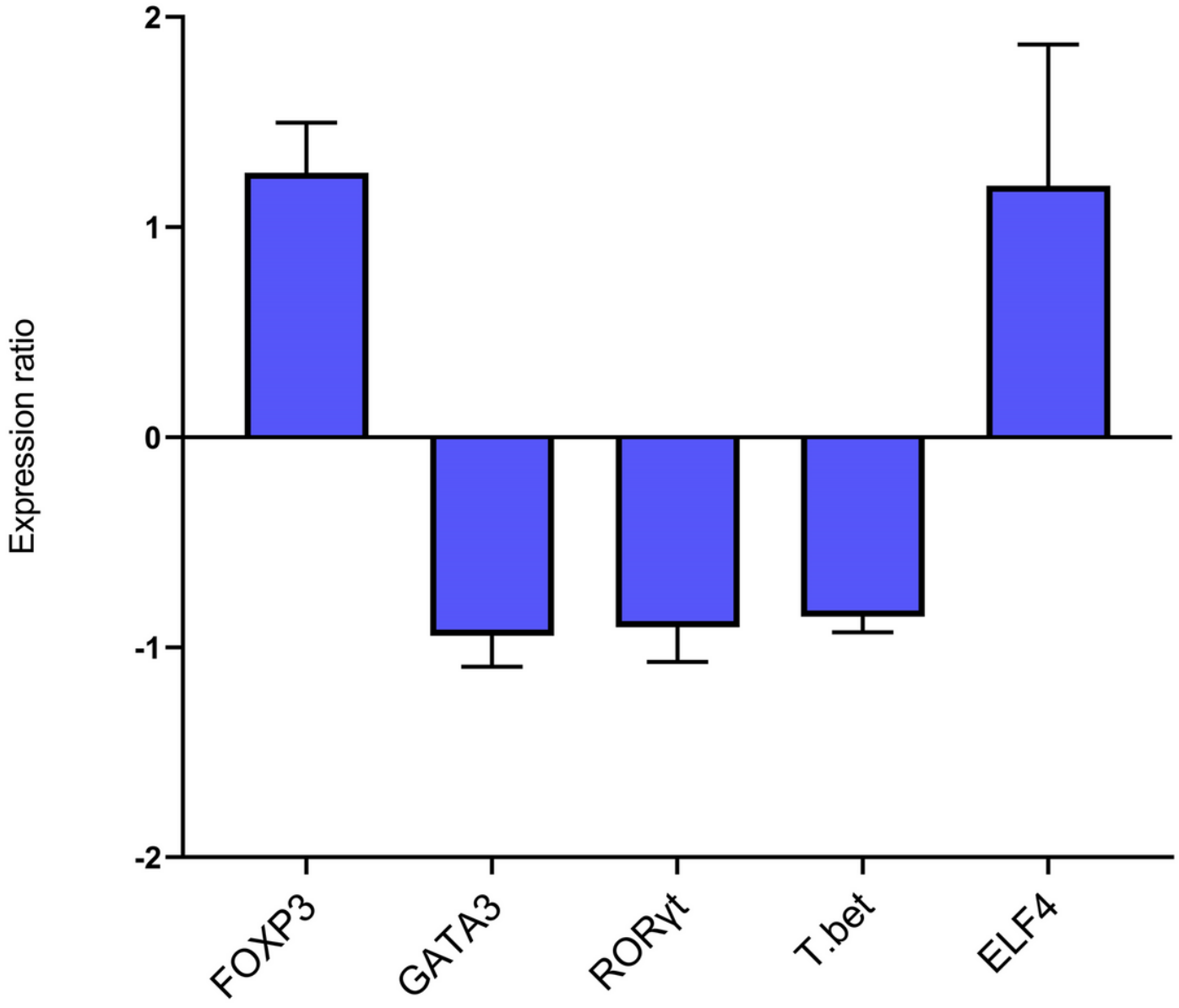

Figure 2

The effect of MSC-EXO on the expression of transcription factors FOXP3, GATA3, RORγt, T.bet and ELF4 in splenocytes stimulated with Anti-CD3/Anti-CD28. It should be noted that when there would be no change in the relative gene expression, the height of the bar on the Y-axis is “0”. In fact, when the gene expression doubled, the gene expression bar shows one-time increases (+1 on the Y-axis). (Statistical analyzes were performed using REST; n = 4).

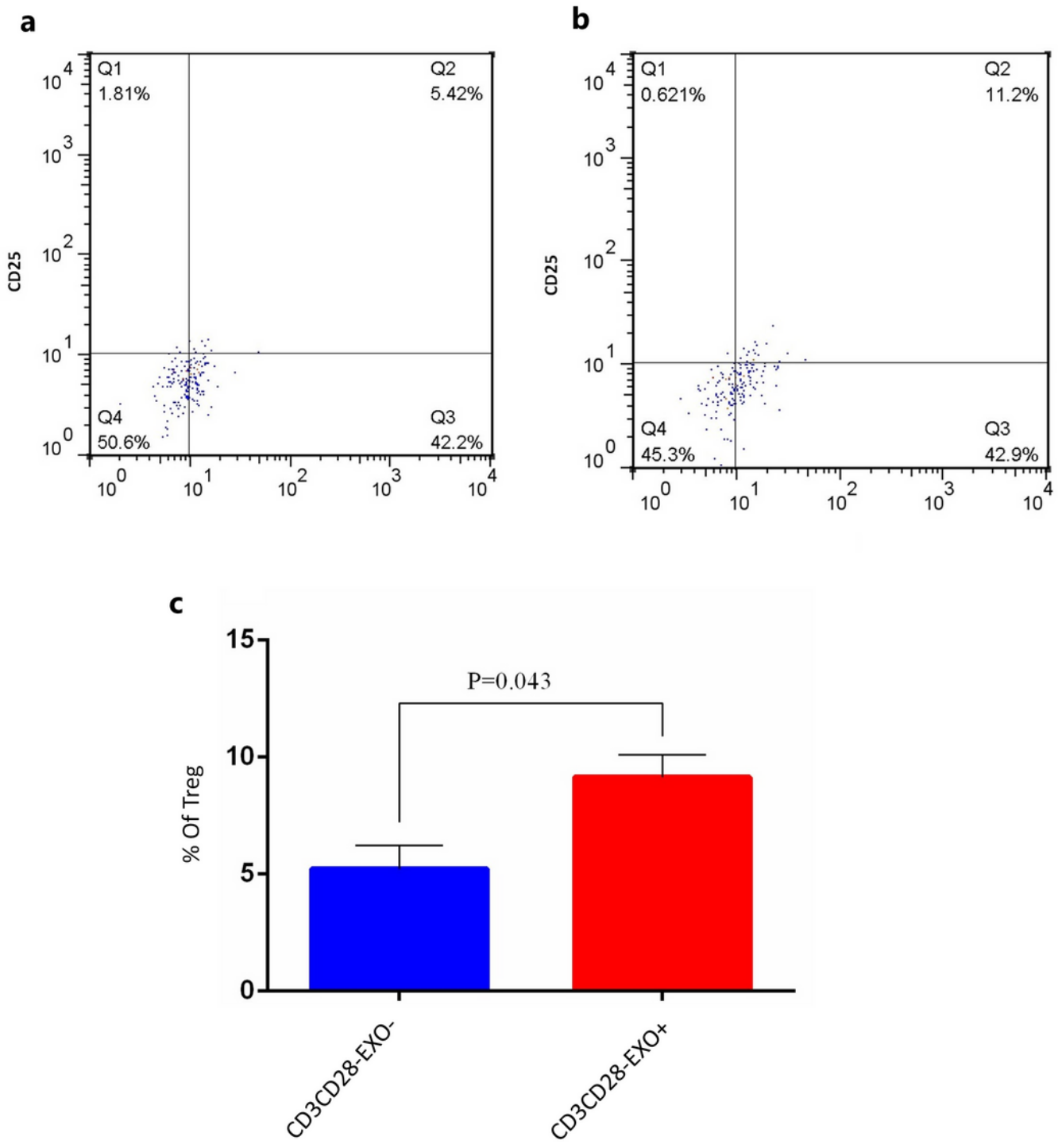

Figure 3

Investigating the effect of MSCs-EV on the frequency of Treg cells. Dot plot diagram showing the abundance of Treg cells in splenocytes stimulated with Anti-CD3/Anti-CD28. (a) Untreated and (b) treated with MSC-EXO. The effect of MSC-EXO on the percentage of Treg cells in splenocytes stimulated with Anti-CD3/Anti-CD28 (c) (statistical analyzes were performed using Mann Whitney; n = 4).

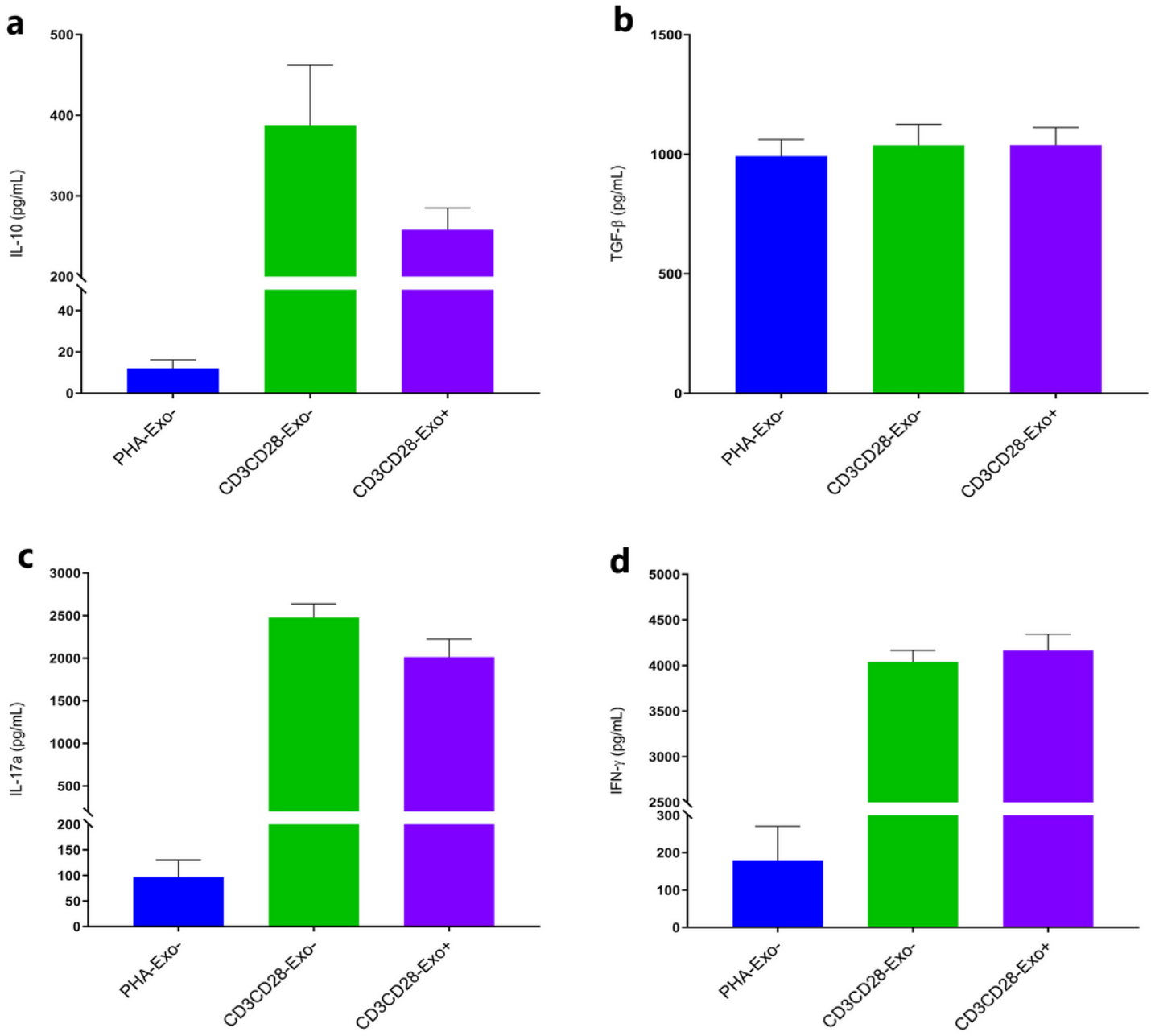

Figure 4

The effect of MSC-EV on the level of IL-10 (a), TGF-β (b), IL-17a (c), IFN-γ (d) in the splenocytes stimulated with Anti-CD3/Anti-CD28. (Statistical analyzes were performed using Kruskal-Wallis, n = 4).