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Repeated intravenous injection of adipose tissue derived mesenchymal stem cells enhances Th1 immune responses in *Leishmania major*-infected BALB/c mice

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ABSTRACT

Mesenchymal stem cell (MSCs) therapy are among new strategies that are used to combat infections through immunomodulation. Cell number, route and frequency of injection and the duration of exposure to the infectious agent are of the main factors to determine the effectiveness of cell therapy. The current study was aimed to assess the effect of multiple intravenous (i.v.) injection of adipose tissue derived (AD)-MSCs on immune response of *Leishmania* (*L.*) *major*-infected BALB/c mice. Therefore, infected mice received AD-MSCs four times during the early phase of infection through i.v. route. They were then monitored weekly for footpad swelling and lesion development. Parasite burden, nitric oxide (NO) and cytokine production were measured in the spleen and lymph node 90 days post-infection. Delayed lesion development, significant reduction in footpad swelling and lower parasite burden in the spleen of AD-MSCs-treated mice showed the relative effect of AD-MSCs therapy in the control of *L. major* dissemination. In addition, MSCs were able to manage direct cytokine responses toward T-helper 1 (Th1). Although the level of interleukin (IL)-10 was still higher than the associated level of tumor necrosis factor (TNF)-α, a shift towards higher level of TNF-α was also observed.

1. Introduction

Mesenchymal stem cells (MSCs) known as non-hematopoietic fibroblastic cells with self-renewal and multi-lineage differentiation potential, are isolated from almost every connective tissue type with perinatal or adult origins [1,2]. Recently, MSCs have received a lot of attention in the field of infectious disease in terms of their bilateral interaction with pathogens and immune system. MSCs indicate different microbial infectivity potential, and also express diverse types of pathogen recognition receptors, and could respond to almost all of microbial stimuli throughout altering its migration, proliferation, and differentiation pattern [3–5]. The MSCs protective or destructive role in infection control are associated to the outcome of their interaction with pathogens and immune system. One of the MSCs destructive role is creating a desirable niche for pathogen proliferation and hiding. Accordingly, MSCs are identified as a habitat for *Mycobacterium tuberculosis* and can support their maintenance in the infection site [6]. However, antimicrobial peptides (AMPs) secretion by MSCs disrupt the pathogenic targets and restrict the microbial infections directly [7]. MSCs also could help to overcome the infections in sepsis, acute respiratory distress syndrome, and cystic fibrosis-related infections [8,9]. In addition, MSCs are the best candidate to interact with immune system in order to fight effectively against pathogen. MSCs could help the immune system by reducing regulatory T cells (Tregs) and induction of inflammation to combat malaria infection [10].

Based on the evaluations of the MSCs role in infectious diseases, parasitic infections are considered as one of the real MSC therapy targets [11]. Leishmaniasis is an infection, which caused by different *Leishmania* (*L.*) species that are obligatory intracellular parasites [12,13]. With respect to difficulties in the parasite clearance from insect and animal reservoirs, multiple transmission routes, re-emerging disease appearance, as well as relative efficacy of vaccination, various non-immunotherapy methods are required to be considered for exploring new strategies in order to overcome disease [14,15]. In this study, the effect of repeated intravenous (i.v.) injection of adipose tissue derived (AD)-MSCs was assessed in *L. major* infected BALB/c mice. In order to achieve this goal, infected mice were received AD-MSCs four times during the early infection phase by i.v. route. After that, the parasite burden and the induced immune responses were investigated in the spleen and lymph node. Induction of cytokine production to the benefit of Th1 immune response and lower parasite dissemination in the spleen indicated the effectiveness of AD-MSCs therapy.

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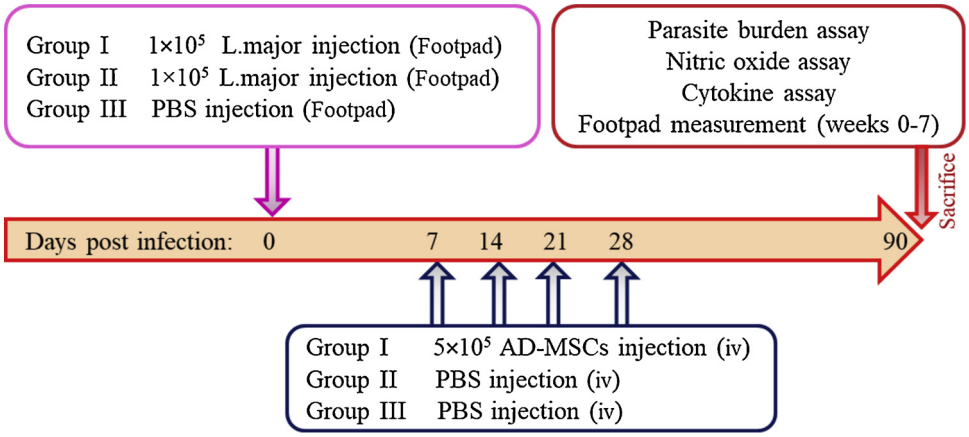

Fig. 1. Schematic representation of experimental groups and their treatment schedule.

2. Materials and methods

2.1. Animals

Thirty five female BALB/c mice (Six to eight weeks old) were purchased from Pasteur Institute, Tehran, Iran. Five mice were used as L. *major* reservoir for fresh parasite preparation, and those remaining mice (N = 30) were distributed as different experimental groups into separate cages (N = 10/each cage). The animals were kept and handled under the standard laboratory conditions in terms of the guidelines of the ethical committee of Tarbiat Modares University. This study was approved by the Ethics Committee with the code of IR.TMU.REC.1394.180.

2.2. AD-MSCs isolation and culture

Abdominal fat tissues of BALB/c mice were minced, and also were washed in the phosphate-buffered saline (PBS). The fat Cell content was harvested after the tissue digestion with 0.075% type I collagenase for 20 min at 37 °C. Digested tissues were centrifuged at 500 × *g* for 5 min. The pellet was cultured in DMEM (Biowest,France) containing 10% FBS (Gibco, Germany), and supplemented with 2 mM L-glutamine and incubated in humidified air containing 5% CO2 at 37 °C. Non-adherent cells were removed by passing 24 h. After the adherent cells reached to confluency, they were trypsinized and also expanded. All of the experiments were accomplished using AD-MSCs at passages three.

2.3. Characterization of AD-MSCs

Immunophenotyping of AD-MSCs was performed by flow-cytometry analysis of CD44, CD45, CD 29, and CD105 cell surface markers. In order to achieve this goal, 1×10^5 cells of each isolated AD-MSCs sample were stained with monoclonal antibodies against the mentioned cell surface markers, and the related isotype control antibodies (all of them were purchased from eBioscience, USA). In addition, FACSCalibur flow-cytometer (BD Biosciences, USA) and Cyflogic software (CyFlo Ltd., Finland) were applied for data analysis.

The ability of AD-MSCs osteogenic and adipogenic differentiation was determined throughout culturing these cells in the presence of related inducer media for 21 days. DMEM containing 10% FBS, 250 nM dexamethasone (Sigma-Aldrich), 5 mM insulin (Sigma-Aldrich), 0.5 nM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 100 mM indomethacin (Sigma-Aldrich) were considered for adipogenic differentiation, while DMEM was supplemented with 10% FBS, 50 mg/ml ascorbic acid-2- phosphate (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), and 10 mM beta-glycerophosphate (Merck, UK) were applied for osteogenic differentiation. At the end of the incubation time, those treated AD-MSCs were stained using Oil Red O (ORO) and Alizarin Red (AR) in order to determine their adipogenic and osteogenic potential, respectively.

2.4. L. major preparation

Fresh *L. major* (MRHO/IR/75/ER) parasite was isolated from spleen and lymph node of infected (reservoir) BALB/c mice. Briefly, spleen and lymph node of infected mice were minced and transferred to the liquid phase of Novy-MaccNeal-Nicolle (NNN) medium. Released promastigotes were cultured in RPMI (Biowest, France) medium containing 5% heat-inactivated FBS at 26 °C. Stationary phase promastigotes were induced by starving the parasite at 2×10^7 *L. major*/ml of RPMI medium for 5 days. BALB/c mice were infected by footpad injection of 1×10^6 stationary phase promastigotes.

2.5. Experimental groups and AD-MSCs therapy

Thirty mice were categorized into three study groups (N = 10 mice per cage). Group I and II were infected using footpad injection of 50 µL of PBS containing 1×10^5 *L. major* promastigotes. Group III was only received 50 µL of PBS. At 7, 14, 21 and 28 days after infection, 600 µL of PBS containing 5×10^5 of AD-MSCs at passage 3 was intravenously injected to the animals of Group I. The same volume (600 µL) of PBS was also intravenously injected to other two study groups at the same intervals. Treated mice were kept under the standard conditions, and also were monitored to the end of the experiment (Fig. 1).

2.6. Footpad thickness measurement

Footpad of *L. major* infected BALB/c mice were daily checked in order to determine the start point of swelling time. Thickness was measured weekly using digital caliper, after observing the first swelling footpad symptom.

2.7. Parasite burden

Three mice from each group were sacrificed at 90 days post-infection, and their spleens were removed under the aseptic condition. Approximately one-third of each spleen was cut and weighed, and that was followed by being homogenized in 2 ml of Schneider's Drosophila medium (Gibco, Germany) containing 10% FBS and 1% penicillin/streptomycin. Therefore, a serial dilution (10^{-1} to 10^{-40}) was prepared and 200 µl of each dilution was transferred into the wells of a 96-wells plate in triplicate. Plates were examined with an inverted microscope in order to find the last well, which contained at least a motile promastigote 15 days after incubation at 26°C [14,16]. The parasite burden

was calculated using the following formula: Parasite burden = -log10 (parasite dilution/tissue weight).

2.8. Leishmania antigen preparation

Soluble leishmania antigen (SLA) was prepared for *in vitro* stimulation of immune cells. To this end, promastigotes of *L. major* were harvested by centrifugation, and then washed with sterile PBS three times. Parasites at 10^9 cell/ml were undergone freezing and thawing cycles ten times. Suspension was centrifuged at 8000g for 15 min at 4 °C in order to remove derbies and cell particles. Obtained supernatant containing SLA was collected and kept at -70 °C. The amount of soluble antigen protein was measured using Bradford assay.

2.9. NO measurement

In order to assess the MSCs therapy effect on NO production, the total cells of the spleen and inguinal lymph node of five mice were harvested from each group. After the red blood cell (RBC) lysis, total cells were counted and cultured at 2×10^6 cell/ well of 6-well plate in RPMI medium containing 10% FBS. Each experimental group were distributed into three sub-groups, which were treated with SLA (10 μg/ml), lipopolysaccharide (LPS) (1 μg/ml) and not-treated with any stimulator. The Supernatant were collected and examined for NO production by Griess method 72 h post *in vitro* stimulation. The absorbance of developed color was read at 540 nm and was converted into NO amount (μM) by the use of sodium nitrite (Merck, Germany) standard concentrations. All of the *in vitro* treatments were performed in triplicate.

2.10. Cytokine measurement

Five mice from each group were sacrificed by passing 90 days after L. *major* infection. Inguinal lymph node cells and splenocytes were isolated and were cultured at 2×10^6 cell/ well of 6-well plate in RPMI medium containing 10% FBS. The cells of each experimental group were treated with media, 10 μg/mL of SLA, and 1 μg/mL of PHA in triplicate. The cells were incubated for 72 h; and the supernatants were collected and analyzed for the presence of interleukin (IL)-10, IL-4, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) after incubation, using enzyme-linked immunosorbent assay (ELISA) method by Kits from R&D systems (Minneapolis, USA) regarding the manufacturer's instruction [17].

2.11. Analysis of T lymphocyte population

In order to accomplishing further analysis about the AD-MSC therapy effect on lymphocyte populations, T lymphocytes of the spleen of different study groups were isolated by MACS method using the Pan T Cell Isolation Kit II (Miltenyi Biotec 130-095-130) at 50 days post infection. After that, the percentage of CD4, CD8 and regulatory T cell population was determined by flow cytometry using anti-mouse CD4-FITC (11-0042-82), anti-mouse CD8-PE (12-0081-82) and Regulatory T Cell Staining Kit (88-8118-40) antibodies all from eBioscience.

In addition, total RNA was extracted from freshly isolated lymphocytes using TRIzol (Invitrogen, USA). After Dnase treatment, cDNA synthesis and qRT-PCR was accomplished using Prime ScriptTM RT reagent Kit (TaKaRa, RR037A), and RT2 SYBR Green High ROX Mastermix, respectively. All PCRs were performed in triplicates and run in at least 3 independent experiments. The $2-\Delta\Delta Ct$ algorithm was considered in order to report the relative expression level of each gene. The nucleotide sequence of the related primers is displayed in Table 1.

2.12. Statistical analysis

To determine the statistical differences between test groups, Statistical Package for the Social Sciences [(SPSS), SPSS Inc., USA] version 13.0 were applied. Mann-Whitney assay and one-way analysis of variance (ANOVA) were also applied for non-parametric and parametric assays, respectively. The value of $p < 0.05$ was considered as statistically significant. The results are indicated as mean ± standard deviation (SD).

Table 1
Designed primers for PCR assay.

Primer	Sequence (5'-3')
IFN-Y	F : GGCTGTTTCTGGCTGTTACTGC R : CCATCCTTTTGCCAGTTCCTC
TGF-β	F: CGCAACAACGCCATCTATGAG R: CACATGTTGCTCCACACTTGA
IL-4	F: CTCATGGAGCTGCAGAGACTCTT R: CATTCATGGTGCATCTTATCGA
IL-10	F: GCTGTCATCGATTTCTCCCCT R: CCAGCAGACTCAATACACACT
T-bet	F:GGTGGACATATAAGCGGTTC R:AGCAAGGACGGCGAATG
GATA-3	F:TGACGGAAGAGGTGGACG R:CTGGCTCCCGTGGTGG
FOXP3	F:AGGCAGGCTGGATAAGG R:GGTATTGAGGGTGGGTGTC
GAPDH	F: CCTGGAGAAACCTGCCAAGTA R: GGCATCGAAGGTGGAAGAGT

3. Result

3.1. Characterization of AD-MSCs

A homogeneous population of AD-MSCs with fibroblast like morphology is shown in Fig. 2A. Multipotent differentiation potential of AD-MSCs (at passage three) was confirmed by presence of lipid and calcium deposits after 21 days from culture using adipogenic or osteogenic differentiation media. Lipid droplets and calcium-rich area are displayed in Fig. 2B and C, respectively. Flow-cytometry analysis indicated that isolated and expanded AD-MSCs were negative for CD45 and CD34 cell surface marker, but CD29, CD73, CD90 and CD105 surface markers were expressed at mean percent of 87, 48, 63 and 62%, respectively (Fig. 2D).

3.2. Footpad thickness measurement

After *L. major* infection, footpad swelling of mice was monitored weekly and measured by digital caliper. As presented in Fig. 3A, the mice footpad average thickness is 2.2 mm that significantly increased to about 4 mm in both of *L. major* infected groups. This increase continued to the end of the study (the seventh week after the infection). However, at the fourth week of infection, footpad swelling in mice treated with MSCs (Group I) showed a significant decrease in comparison with the untreated infected mice (Group II). By passing eight weeks from the infection, footpad swelling was converted into lesion, and tissue necrosis was also appeared. Therefore, swelling measurement was stopped, and was replaced by spleen parasite burden assay.

3.3. Spleen parasite burden

Spleen parasite burden was assessed 90 days post-infection in order to monitor *L. major* dissemination in the body. As indicated in Fig. 3B, AD-MSCs-treated mice showed significantly ($p \leq 0.05$) lower parasite load in comparison with non-treated infected mice (Group II).

3.4. NO production

At 90 days post-infection, nitric oxide production were assessed in the splenocyte and lymph node isolated cells using Griess method. As

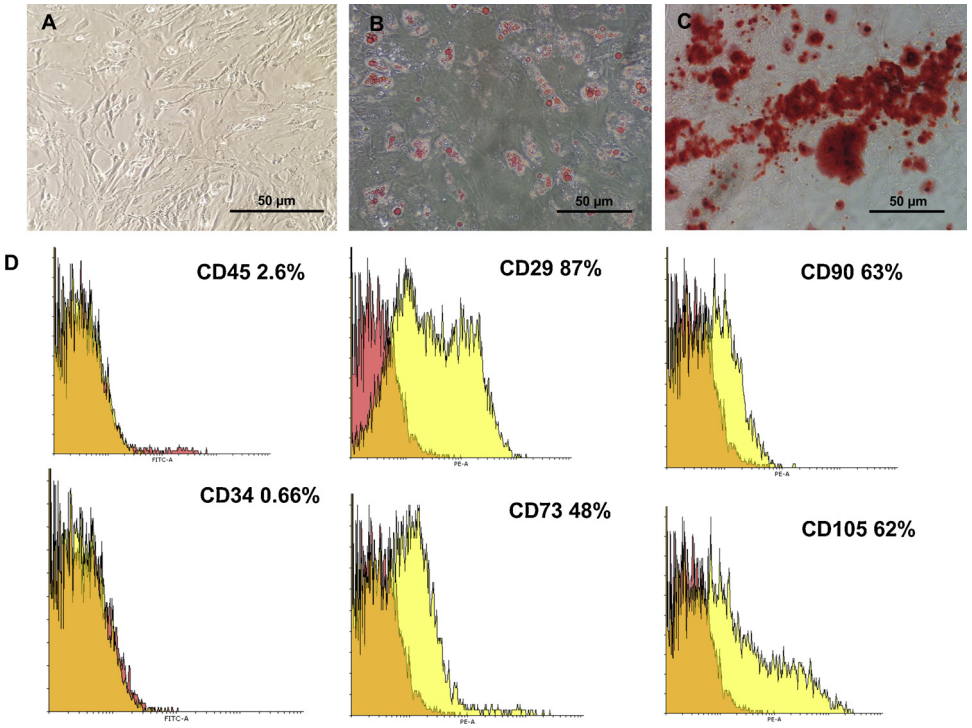

Fig. 2. AD-MSCs characterization. Fibroblastic morphology of AD-MSCs (A). Lipid droplets in AD-MSCs differentiating to adipocyte and calcium deposits in AD-MSCs differentiating to osteocytes (B and C respectively). The histograms of AD-MSCs immunophenotyping (D).

indicated in Fig. 4A, NO production was significantly induced in the splenocytes of AD-MSCs-treated mice stimulated with LPS or SLA ($p \leq 0.05$). Additionally, lymph node cells of AD-MSCs-treated group significantly produced ($p \leq 0.05$) higher level of NO in response to stimulation with LPS or SLA (4B).

3.5. Evaluation of Lymph node cytokine production

At 90 days post-infection, the cytokine production of the lymph node isolated cells were assessed by ELISA method. As indicated in Fig. 5A and B, lymph node isolated cells of the AD-MSCs-treated group, produced higher INF-γ and IL-4 cytokines level in comparison with non-treated one ($p \leq 0.05$). In the case of IL-10 cytokine, a significant ($p \leq 0.05$) decrease was observed in the AD-MSCs-treated group in comparison with the untreated one (Fig. 5C). TNF-α production was significantly increased in the lymph node isolated cells of AD-MSCs-treated group ($p \leq 0.05$) (Fig. 5D). Fig. 7 presented the ratio levels of INF-γ/IL-4 and TNF-α/IL-10 in the lymph node isolated-cells stimulated with SLA. The result explained the re-direction of immune system to Th1 immune responses and also inflammation induction in the AD-MSCs-treated group.

3.6. Evaluation of splenocyte cytokine production

At 90 days post infection, the cytokine production of splenocytes was assessed by ELISA method. As indicated in Fig. 6A and D, both INF-γ and TNF-α production were significantly induced ($p \leq 0.05$) in AD-MSCs-treated group in comparison with non-treated ones. There was no statistically significant difference in the IL-4 production (6B) level. *In vitro* stimulation of splenocytes with SLA, induces a significant reduction ($p \leq 0.05$) in IL-10 cytokine production of AD-MSCs-treated group (Fig. 6C). Fig. 7 represents the ratio of INF-γ/IL-4 and TNF-α/IL-10 cytokine production in the SLA stimulated splenocytes of the experimental groups. The result explained the re-direction of immune system to Th1 immune responses and induction of inflammation in AD-MSCs-treated group.

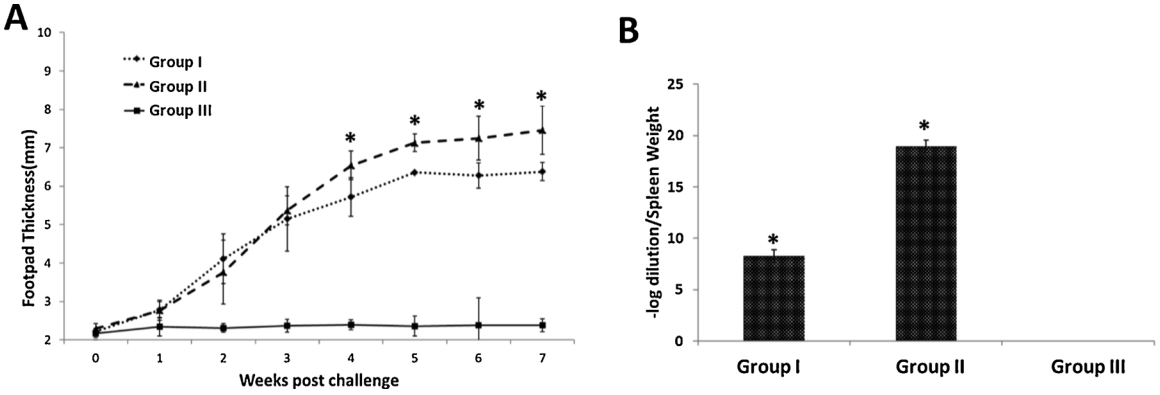

Fig. 3. Footpad thickness of ten mice in each experimental group was measured during 7 weeks post-infection and represented as mean ± SD. (A). The mean values of parasite burden of the spleen of study groups 90 days post-*L. major* infection. (B) *; Indicates that there is a statistically significant difference among groups ($P < 0.05$). Group I: AD-MSCs-treated *L. major*-infected mice, Group II: *L. major* infected mice, and Group III: non-treated mice.

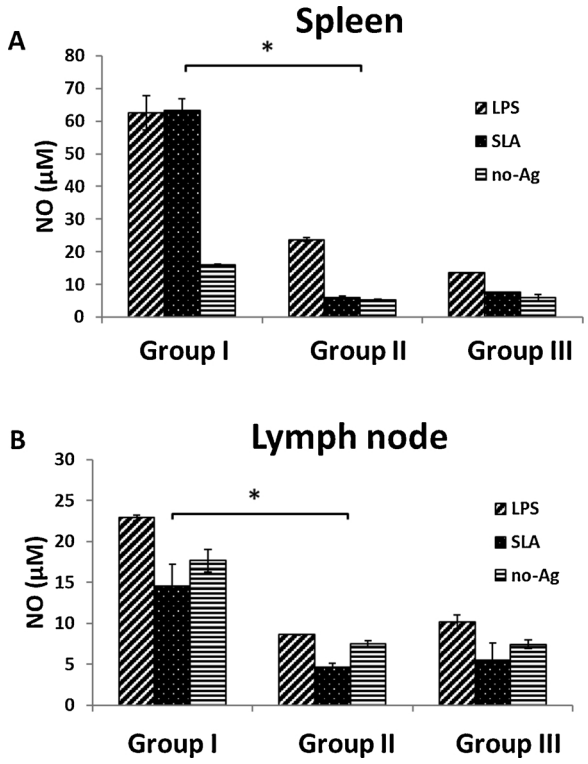

Fig. 4. The amount of nitric oxide production in the supernatants of splenocytes (A) and lymph node isolated-cells (B) of each experimental group, after *in vitro* stimulation with LPS or SLA or no-Ag. Data represented the mean ± SD of the results obtained from 5 mice in each group.* Indicated groups are significantly different from each other ($P < 0.05$). Group I: AD-MSCs-treated *L. major*-infected mice, Group II: *L. major*-infected mice, and Group III: non-treated mice.

3.7. Analysis of T lymphocyte population

In order to achieve this goal, the percent of CD4+, CD8+ and CD4+CD25+FOXP3+ T lymphocyte of the spleen of the study groups was determined by flowcytometry method. As indicated in Fig. 8, the mean percent of CD8 + T lymphocyte in *L.major* infected group was 9.2%, which was significantly increased ($p \leq 0.05$) to 36.5% in AD-MSCs-treated group. While the mean percent of CD4 + T lymphocyte in *L.major* infected group was 50.8%, which was significantly decreased ($p \leq 0.05$) to 25% in AD-MSCs-treated group. Regulatory T cell staining indicated the significant decrease ($p \leq 0.05$) in AD-MSCs-treated group in comparison with *L.major* infected non-treated group. Moreover, the gene expression of the cytokine and transcription factors that was associated to TH1, TH2 and Treg subsets was investigated by real time PCR. As indicated in Fig. 9, the Tbet and IFN-γ expression level (7.6 and 3.2-fold change) was significantly induced ($p \leq 0.05$) in AD-MSCs treated group in comparison with non-treated group. While the expression fold change of GATA-3 and IL-4 genes was 2 and 2.6 in *L.major* infected group, which was significantly reduced in AD-MSCs treated group. Significant ($p \leq 0.05$) induction was observed in FOXP3 and TGF-β gene expression in T lymphocyte of *L.major* infected group, which has a reduction in AD-MSCs treated group (9C).

4. Discussion

Recently, stem cell therapy was introduced as a new intervention strategy for controlling and also treating an wide range of infections [3,18]. The use of new therapies for parasitic infections has a specific importance in terms of the complexity of their interaction with the host, including viability, metabolism, and cell signaling that could sabotage the host immune responses [19]. Although, vaccination is still the best and most popular immunotherapy method for parasitic infections, but the use of cells in the way of enhancing the immune system is also considered as a hopeful immunotherapy approach [20]. However, according to contradictory results, the efficacy of stem cell therapy to combat parasitic infections is unclear [11,20]. Some of these results indicated the MSC therapy effectiveness by prolonging the survival of infected mice through a decrease in the serum level of TGF- β1in *Schistosoma* (*S.*) *japonicum* infection, suppression of IL-10, induction of IL-12 production in malaria and recovery of heart failure in Chagas disease [10,21–23]. While another experiments showed that i.v injection of MSCs enhanced parasite burden and IL-10 producing T cells in the spleen of BALB/c mice infected with *L. amazonensis* [21]. However, a review of the presented works demonstrated that most of these contradictory results were in association with the mesenchymal stem cell activity status, the frequency of injection, as well as dosing and timing of stem cell application during infection phase. In the study done by

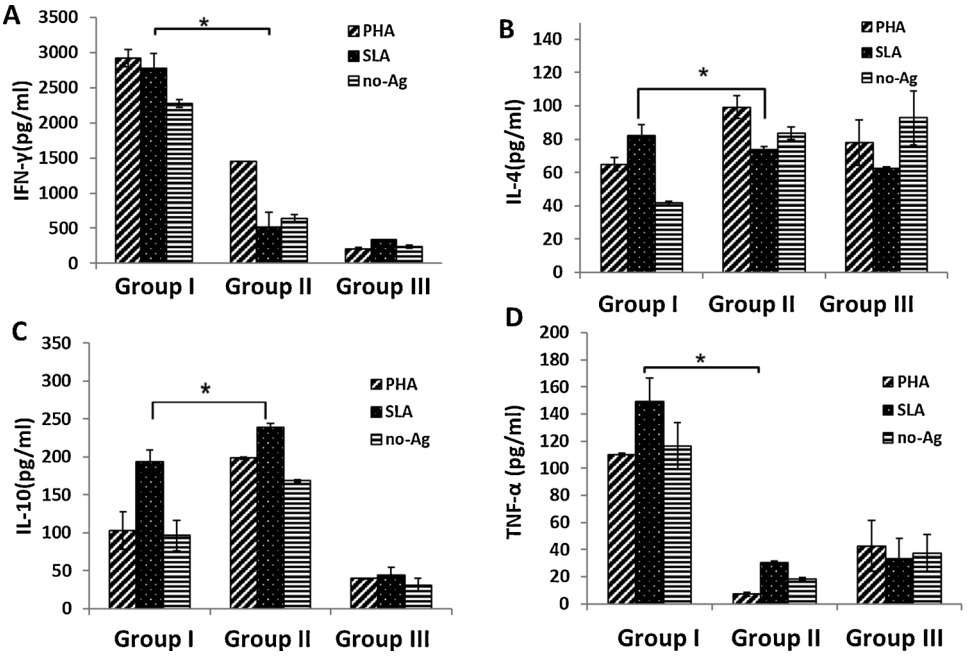

Fig. 5. IFN-γ (A), IL-4 (B), IL-10(C) and TNF-α(D) production in the supernatants of lymph node isolated cells of each experimental group after *in vitro* stimulation with PHA, SLA and no-Ag. Data represented the mean ± SD of the results obtained from 5 mice in each group. * Indicated groups are significantly different from each other ($P < 0.05$). Group I: AD-MSCs-treated *L. major*-infected mice, Group II: *L. major*-infected mice, and Group III: non-treated mice.

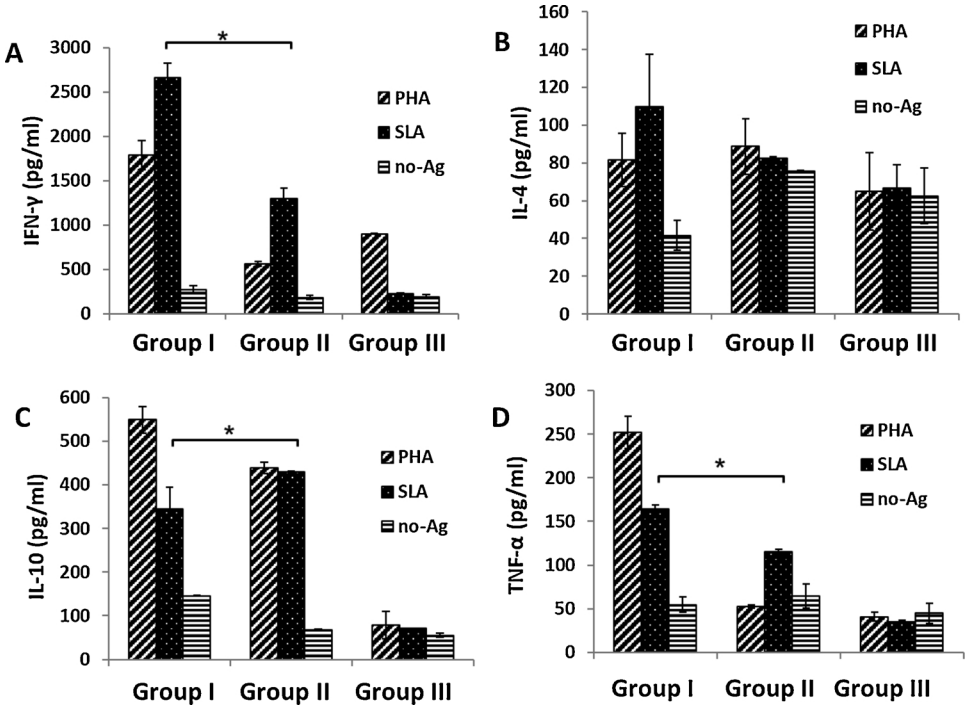

Fig. 6. IFN-γ (A), IL-4 (B), IL-10(C) and TNF-α(D) production in the supernatants of splenocytes of each experimental group after *in vitro* stimulation with PHA, SLA and no-Ag. Data represented the mean ± SD of the results obtained from 5 mice in each group. * Indicated groups are significantly different from each other ($P < 0.05$). Group I: AD-MSCs-treated *L. major*-infected mice, Group II: *L. major*-infected mice, and Group III: non-treated mice.

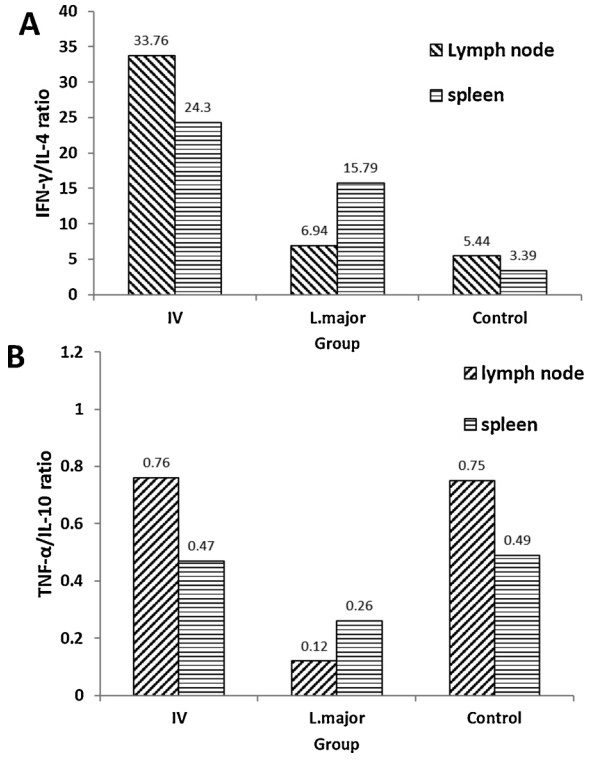

Fig. 7. IFN-γ/ IL-4(A) and TNF-α /IL-10(**B**) Ratio of *in vitro* SLA- stimulated groups in splenocytes and lymph node-isolated cells of experimental groups. Group I: AD-MSCs treated *L. major*-infected mice, Group II: *L. major*-infected mice, Group III: non-treated mice.

Valerie Johnson et al., it was indicated that repeated i.v. administration of activated MSCs can overcome drug-resistant bacterial infections [24]. In another study, El-Shennawy et al., proved the effectiveness of MSCs transplantation in improvement of liver failure in acute infection in comparison with chronic infection that was caused by hepatic schistosomiasis [25]. For this reason, repeated injection of AD-MSCs at early phase of infection was applied in the current study for treatment of *L. major*-induced cutaneous lesions. As it was indicated in the results, the process of wound formation and footpad swelling of the mice showed a significant reduction starting from the fourth to seventh weeks after infection in the AD-MSCs-treated group in comparison with the untreated group. This finding along with a significant reduction in parasite load in the spleen at the day of 90 after the infection, established the AD-MSCs role in reducing inflammation and controlling the spread of parasites from the initial site of the infection. Control of pathogen spreading by MSCs had been previously reported in both parasitic and bacterial infections. In a study by Mello et al., they have demonstrated the role of AD-MSCs in the control of Chagas disease caused by *Trypanosoma* (*T.*) *cruzi* throughout protective immune responses induction in the lymphoid organs [26]. Other researches have also confirmed the MSCs inhibitory effect on bacterial dissemination by antimicrobial peptides secretion [7,26,27].

NO production is the most important defensive activity of macrophage against *L. major* [28]. Macrophages Activation by IFN-γ and TNF-α are required in order to produce sufficient amounts of NO inhibiting the intracellular parasites proliferation [29,30]. As it was mentioned in result, i.v. treatment of infected BALB/c mice could enhance NO production in both of the splenocytes and lymph node-isolated cells. LPS and SLA -stimulated splenocytes produced higher amount of NO in comparison with lymph node-isolated cells that indicated the more potential of the spleen for NO production, due to more macrophages in the spleen or more NO production per macrophage in response to the stimulation. The findings of two other studies have indicated that both of non-activated AD-MSCs and pre-activated AD-MSCs by leishmania antigens could reduce the NO production from macrophages in a cell contact manner [31–33]. However, the current study indicated the induction of NO in *in vivo* AD-MSCs-treated group, which might be the result of the AD-MSCs indirect effect on macrophage function or the AD-MSCs direct effect on other NO producers [34]. The findings of different studies on L. *major* infection, demonstrated that innate immune cells activation in the resistance mice, are in a way that direct development of Th1 immune responses in order to control parasite dissemination and wound formation [35]. Another study has also showed appearance of Th2-type immune response with a significant increase in IL-4, transformation of growth factor-beta (TGF-β), and IL-

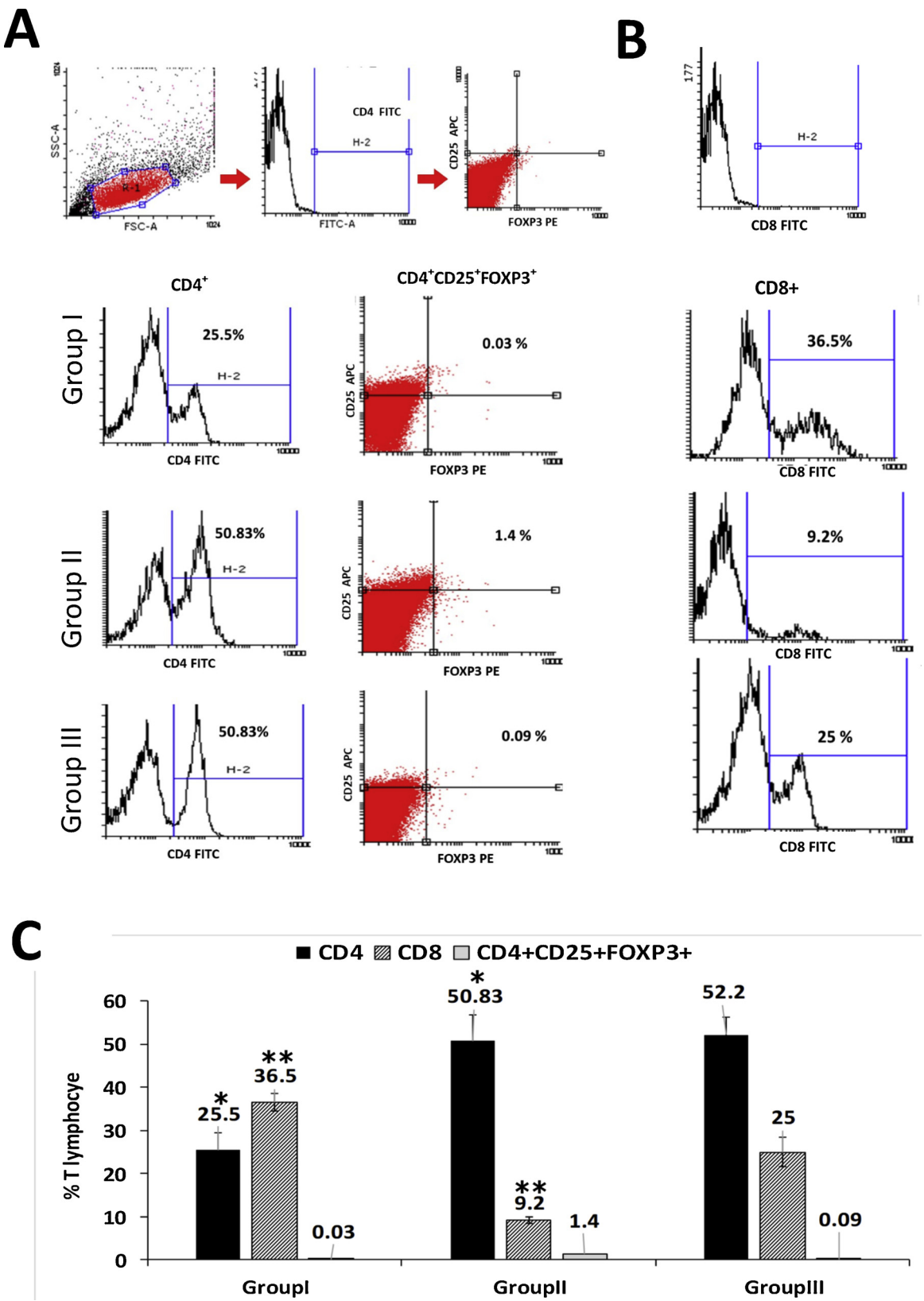

Fig. 8. Flow cytometry analysis of spleen isolated T lymphocyte for the expression of CD4, and CD4/CD25/FOXP3(A) and CD8 (B) markers. Comparison of CD4, CD8 and CD4/CD25/FOXP3 cell counts (expressed as % of positive T lymphocytes of 10,000 cells counted) (C). *, ** Indicated groups are significantly different from each other ($P < 0.05$). Group I: AD-MSCs treated *L. major*-infected mice, Group II: *L. major*-infected mice, Group III: non-treated mice.

10 production, could enhance the progression of non-healing and chronic form of leishmaniasis [15]. Analysis of T cell population of the spleen of experimental groups, demonstrated that MSCs redirect immune responses to the CD8+ cells induction and reduction in regulatory T cells, which terminated to an increase in IFN-y/ IL-4 ratio in both RNA and protein level. Earlier studies demonstrated the protective

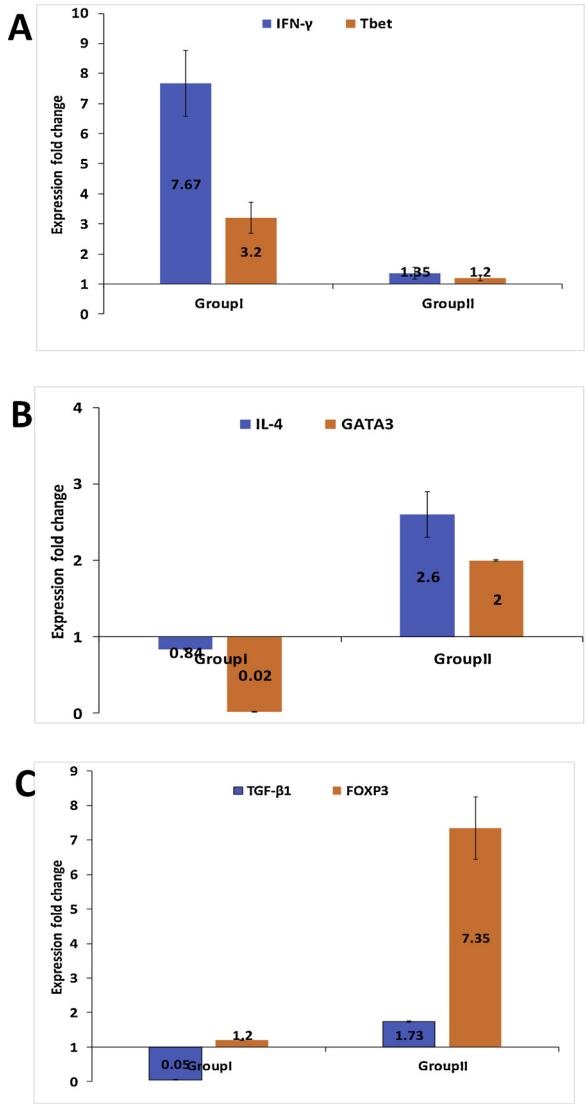

Fig. 9. Expression pattern of IFN-γ, Tbet (A), IL-4, GATA3 (B) and TGF-β1, FOXP3 (C) in the spleen isolated T lymphocytes of the experimental groups by real time (qRT-PCR) method. Group I: AD-MSCs treated *L. major*-infected mice, Group II: *L. major*-infected mice.

role of CD8 + T cells against leishmaniasis by enhancing the TH1 immune responses and empowering the macrophages [36]. However, Okwor et.al indicated that CD8+ and CD4 + T cells are differentially activated in *L.major* infection depending on the infection dose [37]. The higher ratio of CD8+/CD4 + T lymphocyte in AD-MSCs treated group, confirm the MSCs indirect effect on *L.major* dose throughout the induction of macrophage killing activity and restriction of parasite dissemination.

Increasing and reducing levels of IL-10 and TNF-α, in both lymph node and spleen of the *L. major*-infected mice reflected the immune suppressive environment for killing activity of macrophage and Th1 development, respectively. Increased ratio of TNF-α/IL-10 in repeated i.v. MSC-treated mice changes the inflammatory condition balance, which is beneficial for Th1 immune response. It is noteworthy to state that an increase in TNF-α/IL-10 ratio is not as much as the associated ratio of IFN-γ/ IL-4, while IL-10 is still a dominant cytokine in the repeated i.v. MSCs-treated mice. In a study by Pereira et al., they have showed an increase in IL-10 level leading to production of T cells and parasite load in the spleen after i.v. injection of MSCs in *L. amazonensis*-infected mice [20]. Our findings indicated the effectiveness of repeated i.v. injection of AD-MSCs in *L. major* infection, however, this effectiveness had some limitations. This method has the ability to direct the immune response towards Th1, leading to an increase in NO, and also a decrease in parasite load, while it was unable to completely inhibit the spread *of L. major* from infection site that terminated to delayed death of susceptible mice. MSC therapy, may be beneficial in increasing the efficacy of vaccination or other immunotherapy methods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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