

Mesenchymal Stem Cells-Secreted TGF- β 1 Restores Treg/Th17 Skewing Induced by Lipopolysaccharide and Hypoxia Challenge via miR-155 Suppression

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Research

Keywords: Lipopolysaccharide, hypoxia, regulatory T cells, T helper 17 cells, mesenchymal stem cells, transforming growth factor- β 1, microRNA-155

Posted Date: September 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-63520/v1>

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Version of Record: A version of this preprint was published at Stem Cells International on March 12th, 2022. See the published version at <https://doi.org/10.1155/2022/5522828>.

Abstract

Background: Regulatory T cells (Treg)/T helper (Th) 17 skewing plays a crucial role in development of acute respiratory distress (ARDS). Mesenchymal stem cells (MSCs)-secreted transforming growth factor (TGF)- β 1 has remarkable immunomodulatory effects on CD4⁺T cells, being environment sensitive and remains lack of discussion in hypoxic and inflammatory condition of ARDS.

Methods: Purified mice splenic CD4⁺ T cells were pre-coated with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml) overnight. RAW264.7 cells were added as antigen presenting cells (APCs). T cells with and without RAW264.7 cells were treated with various LPS concentrations of 0,10,100,1000ng/ml or/and at hypoxia condition of 5% O₂. Based on LPS (100ng/ml) and hypoxia condition (5% O₂) as stimuli, MSCs were set in direct coculture or indirect coculture methods by transwell system. Anti-TGF- β 1 neutralization antibody was added to explore the role of TGF- β 1 among the soluble factors secreted by MSCs; miR-155 overexpression of CD4⁺T cells were performed by transfection and then were added to the MSCs-CD4⁺T cells coculture system in hypoxic and LPS-stimulated condition. After 48 hours, cells or supernatant were collected for detection of frequency of Treg and Th17 subsets, CD4⁺T cells apoptosis and proliferation capacity assay by flowcytometry, secretions of INF- γ , IL-17A, IL-21, TGF- β 1 and IL-10 by ELISA, levels of miR-155, Rorc, Foxp3 and Ptpn2 mRNA expression of CD4⁺T cells by RT-PCR.

Results: MSCs could restore the skewed Treg/Th17 induced by LPS and hypoxia as compared to groups without MSCs with increased secretion of TGF- β 1, IL-10 and IL-17A(P<0.05) and attenuate the increased expression of miR-155 in CD4⁺T cells, which was independent on cell-to-cell contact mechanism while TGF- β 1 neutralization could significantly inhibit the effects of MSCs restoring the skewed Treg/Th17 and abolished its effect on miR-155 expression in CD4⁺T cells.

Conclusions: These findings suggested miR-155 suppression of CD4⁺T cells mediated MSCs-secreted TGF- β 1 modulating the skewed Treg/Th17 induced by LPS-hypoxia challenge, providing evidence when proposing future T lymphocyte-targeted cell therapy in ARDS.

Background

Acute respiratory distress syndrome is a life-threatening clinical syndrome and continues to be a significant burden on society with high morbidity and mortality (1, 2). Progressive hypoxemia and uncontrolled inflammation caused by heterogeneous etiologies interacted and composed the pathological environment (1, 3). MSCs have showed benefit to survival and organ protection in preclinical ARDS animal models while its mechanism involved in modulating immune cells remains to be fully understood (4–6). Cell-based therapy for ARDS requires a detailed understanding of injured cells in response to MSCs taking into account the influence of hypoxic and inflammatory environment during ARDS.

An imbalance between regulatory T cells (Treg) and interleukin (IL)-17-producing T helper cells (Th17) is reported to be characteristic for ARDS development (7, 8). Th17 cells are a population of CD4⁺ T cells that expresses the cytokine IL-17, which drives leukocyte recruitment and activation to bridge innate and adaptive immunity and is in charge of clearance of extracellular pathogens. Excessive generation and activation of Th17 cells attribute to hyperinflammatory damage (9). Recent clinical studies among ARDS patients have identified a significantly closed link between increased Th17 frequency and greater illness severity and showed the levels of IL-17A were increased in bronchoalveolar lavage (10). Accordingly, Tregs, characterized by forkhead box P3(Foxp3) contribute to the resolution of hyperinflammatory response by regulating responses mediated by T helper cells. MSCs have been showed regulating Treg/Th17 balance in Th17 dominance disease such as systemic lupus erythematosus (11). However, effect of MSCs on Treg/Th17 skewing upon hypoxic and inflammatory environment of ARDS remains not completely discussed.

MSC-mediated immunoregulation is primarily via both cell-cell contact or paracrine activity by release of soluble factors under specific condition (12). MSCs secrete a small amount of TGF- β 1 under normal conditions. High TGF- β 1 levels have been detected in MSC medium under inflammatory or hypoxic conditions (13, 14, 15). Meanwhile, TGF- β 1 is critical and an initiative factor for both Th17 and Treg development by inducing the expression of the transcription factors retinoic-acid-receptor related orphan receptor γ t (ROR γ t) and Foxp3, respectively (16, 17). Thus, MSC generated TGF- β 1 could theoretically restore skewed Treg/Th17 induced by ARDS.

MicroRNAs (miRs) are conserved, single-stranded non-coding RNAs, play role in cellular differentiation, and regulate immune system by binding the 3' untranslated region (UTR) of mRNA, leading to the suppression of target gene. MiR-155 and miR-146a is the most commonly reported to be increased in activated immune cells, including T cells (18, 19). Some studies demonstrated miR-155 expression is regulated by TGF- β 1(20) and its level is negatively associated with Treg induction under some inflammatory circumstances, which in other way promoted Th17 cells differentiation (21, 22). Mesenchymal stem cells soluble factors could modulate some specific miRs expression, like those of miR-155 and miR-23b in dendritic cells (23). Whether miRs are involved in underlying mechanism of CD4⁺T cells in response to MSCs upon hypoxic and inflammatory condition during ARDS needs further investigation.

Therefore, in this study, lipopolysaccharide (LPS) with/or hypoxia were used as stimulus to mimic in-vitro ARDS environment. The aim of the present study is to evaluate the generation of CD4⁺T cells to Treg and Th17 upon LPS and/or hypoxia challenge in response to MSCs and discuss the potential mechanism.

Methods

Animals

C57BL/6 male mice, 4–6 weeks of age, were purchased from the Comparative Medicine Centre, Yangzhou University (Yangzhou, China). The animals were housed 5 mice per cage in a laminar air flow room maintained at $22 \pm 2^\circ\text{C}$ with relative humidity of $55 \pm 5\%$. Mice were cared and treated in accordance with the guidelines established by the Committee of Animal Care and Use of Southeast University. The Committee of Animal Care and Use of Southeast University approved this study.

Isolation, purification and identification of mouse spleen derived CD4⁺T cells

CD4⁺ T cells derived from mouse spleen were isolated through positive CD4 selection, using CD4 (L3T4) MicroBeads, MS columns and MiniMACS™ Separator, according to manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The identification was evaluated by immunofluorescence against CD4 by flow cytometry (FCM). CD4⁺T cells were isolated from mice spleen and identified by FCM with purity of 97.01% (Supplemental Fig. 1).

Cell culture with lipopolysaccharide and hypoxia stimulation

CD4⁺T cells isolated from mouse spleen, with addition of RAW264.7 cells provided by Cells Resource Center of Shanghai Institutes for Biological Sciences, the Chinese Academy of Science (Shanghai, China) (14) (10^6 /well) as presenting antigen cells, were seeded in 24-well Petri dishes ($4-5 \times 10^6$) pre-coated with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml) in RPMI1640 (Hyclone, USA) with 10%FBS(Gibco, USA) and were incubated in 37°C in a humidified atmosphere of 5%CO₂ for 24 hours. Then lipopolysaccharide (LPS, Escherichia coli O111:B4, Sigma Aldrich, Munich, Germany)) alone at dose of 10, 100 and 1000ng/ml, hypoxia(5%O₂) alone or combination of LPS at different doses and hypoxia was respectively added as stimuli. After 48 hours, cells were harvested for detection of Treg and Th17 frequency by flow cytometry.

Bone marrow-derived MSCs-treated CD4⁺T cell coculture system

Mouse bone marrow MSCs were purchased from Cyagen Biosciences Inc (Guangzhou, China), which has been identified by detecting cell surface markers and the MSC multipotent potential for differentiation along toward the adipogenic, osteogenic, and chondrogenic lineages (14); and cells were maintained as previously described (14). MSCs (10^6) were directly added to the prepared CD4⁺T cells or planted in the upper chamber of 0.4 um pore size transwell(Millipore, USA) insert to set up a direct or indirect coculture system and then received stimulation of LPS(100ng/ml) combined with hypoxia(5%O₂) at 37°C in a 5% CO₂ incubator.

Reagent treatment

Abti-TGF-β1 blocking by neutralization antibody

To evaluate the effect of TGF-β1 among MSCs-secreted soluble factors, anti-TGF-β1 antibody (ab64715, Abcam, United Kingdom) of 10ug/mL were added into the medium of indirect MSCs-treated CD4⁺T cells

group before LPS (100ng/ml) and hypoxia(5%O₂) stimulation, then followed by Treg/Th17 and miR-155 expression evaluation.

MiR-155 overexpression by miR-155 mimic transfection

To identify the role of miR-155 suppression CD4⁺ T cells on the skewed Treg/Th17 differentiation upon LPS-hypoxia challenge in response to MSCs treatment by transwell. MiR-155 mimic with respective control designed and synthesized by RiboBio Co., Ltd (Guangzhou, China) were pre-transfected respectively to purified CD4⁺ T cells with Lipofectamine 2000 reagent. The expression of miR-155 was tested by real-time polymerase chain reaction (RT-PCR) at 4 hours after transfection. The transfection efficiency of miR-155 overexpression in CD4⁺ T cells was confirmed (Supplemental Figure 2). The CD4⁺T cells pre-transfected with miR-155 mimic or control received MSCs coculture by transwell with/or LPS (100ng/ml)-hypoxia (5%O₂) stimulation for 48 hours. Then frequency of Treg and Th17 population as well as apoptosis and proliferation capacity of CD4⁺ T cells was determined by Flow Cytometry (FCM). The CD4⁺T cells were sorted by CD4 (L3T4) microbeads and gene expression of Ptpn2, Foxp3 and Rorc mRNA of CD4⁺T cells was assayed by RT-PCR.

Detection of Treg and Th17 population by flow cytometry

Cells were harvested, washed extensively and dissociated into single cell suspension. For Th17, IL-17A without CD8 expression lymphocytes were detected as described previously. Cells were stimulated for 4 hours with 2 u/mL leukocyte activation cocktail (BD Pharmingen™, USA) at 37°C and 5% CO₂. Upon harvest, cells were surface stained with antibody Anti-Human CD8a-APC (BD Pharmingen™, USA) at room temperature in the dark and then fixed and permeabilized using intracellular Fixation & Permeabilization (BD Pharmingen™, USA). Following fixing and permeabilization, cells were incubated with PE-conjugated anti IL-17(BD Pharmingen™, USA). For Treg, Foxp3 producing CD25 lymphocytes were detected as described previously. Cells were surface stained with antibody CD25-APC (BD Pharmingen™, USA) at room temperature in the dark and then fixed and permeabilized using intracellular Fixation & Permeabilization (BD Pharmingen™, USA). Following fixing and permeabilization, cells were incubated with PE-conjugated anti-Foxp3 (BD Pharmingen™, USA). Samples were run on a Flow Cytometer (ACEA NovoCyte, USA). Data were analysed using Flowjo software (Flowjo, USA) and NovoExpress software (ACEA Biosciences, USA)

Cytokine quantification

TGF-β1, IL-10, IL-17A, INF-γ and IL-21 in the supernatant was determined via an enzyme-linked immunosorbent assay (ELISA) by using commercially available ELISA sets (ExCellBio, Shanghai, China and Elabscience, Wuhan, China) in accordance with the instructions of the manufacture. All samples were measured in duplicate.

Real-Time PCR detection

Total RNA of cells was obtained by Trizol reagent, then followed by cDNA synthesis. Real-time PCR was performed with the ABI Prism 7000 Sequence Detection System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The average threshold count (Ct) value of 2–3 technical replicates were used in all calculations. The average Ct values of the internal controls was used to calculate Ct values for the samples. Data analysis was performed using the $2^{-\Delta\Delta C_t}$ method. U6 RNA was used as endogenous control for miR detection and β -actin for Foxp3, Rorc and Ptpn2mRNA detection. The primer sequences are as listed in Supplemental file.

Apoptosis and proliferation capacity assay by flow cytometry

CD4⁺T cells isolated from lower chamber in groups of control mimic-CD4⁺ T cells, miR-155 mimic-CD4⁺ T cells and miR-155 inhibitor-CD4⁺ T cells with MSCs treatment were collected and stained with Annexin V/PI kit (BD Pharmingen™, USA) for apoptosis respectively. Cell Trace Violet™ cell proliferation kit (Invitrogen™, USA) was used for proliferation capacity assay with flow cytometry analysis after 72-hours in-vitro stimulation.

Statistical Analysis

Comparisons of variables between groups were performed using unpaired t tests, MannWhitney U tests, or Chi-squared tests, as appropriate by GraphPad PRISM Version 5.3 (San Diego, CA, USA). Statistical significance was set at the level of P<0.05.

Results

Lipopolysaccharide and hypoxia stimulation induced a skewed Treg/Th17

To figure out the impact of hypoxia, LPS or combined stimulation on Treg and Th17 generation, CD4⁺T cells with RAW264.7 cells added as antigen present cells, were cultured in vitro in absence of or with LPS (10, 100, 1000 ng/ml, respectively), hypoxia(5%O₂) or combined stimulation for 48 hours. Compared to control group with no stimulation, frequency of CD25⁺Foxp3⁺ in CD4⁺ T cells was decreased and that of CD8⁻IL17A⁺Th17 cells was increased markedly in CD4⁺ T cells upon LPS stimulation of 100 and 1000 ng/ml (P < 0.05, Fig. 1). Hypoxia alone or combined with LPS challenge significantly reduced frequency of CD25⁺Foxp3⁺ in CD4⁺ T cells in comparison with that of control group (P < 0.05, Fig. 1). Frequency of Th17 population in CD4⁺ T cells was markedly increased upon hypoxia alone or combined with LPS of 100 and 1000 ng/ml (P < 0.05, Fig. 1). No difference in percentages of Treg or Th17 cell populations was detected between group stimulated with LPS at a certain dose alone versus that in combination with hypoxia. Taken together, Treg/Th17 values pronouncedly decreased in groups with LPS alone, hypoxia alone or the combination stimulation compared to that in group with no stimulation (P < 0.0001, Fig. 1). These results showed LPS and(or) hypoxia stimulation in vitro could induce Treg/Th17 skewing, favouring to Th17 rather than Treg populations.

For coexistence of inflammation and hypoxia is common in ARDS and both Treg decrease and Th17 increase appeared when concentration of LPS was above 100 ng/ml, LPS at dose of 100 ng/ml and hypoxia was set as stimuli in the following experiments.

Mesenchymal stem cells attenuated the inflammation and reserved the skewed Treg/Th17 upon LPS and hypoxia challenge in vitro

To discuss the effect of MSCs on Treg/Th17 and potential mechanism in condition of LPS-hypoxia combined stimulation, we introduced a transwell coculture system. CD4⁺ T cells with RAW264.7 cells were planted in the lower chamber, followed by LPS (100 ng/ml)-hypoxia(5%O₂) stimulation, which mimics in-vitro ARDS environment. MSCs were respectively added to the upper and lower chambers for indirect and direct coculture treatment. We found MSCs-treated CD4⁺T cells groups, whether directly or indirectly compared to ARDS group could markedly upregulate CD4⁺CD25⁺Foxp3⁺ Treg frequency and decrease that of CD8⁻ IL-17A⁺ Th17 in CD4⁺T cells with no difference between indirect and direct addition of MSCs (P < 0.05, Fig. 2A). These results indicated that MSCs restored Treg/Th17 imbalance induced by LPS and hypoxia challenge mainly via soluble factors rather than cell-to-cell contact. In addition, MSCs augmented the levels of anti-inflammatory cytokines, including TGF-β1 and IL-10 were markedly increased in supernatant of group receiving MSCs treatment when compared to that of ARDS group (P < 0.05, Fig. 2B).

MSCs restored the skewed Treg/Th17 induced by LPS and hypoxia via TGF-β1 secretion

Among MSCs-secreted soluble factors, TGF-β1 has been reported to be vital for Treg and Th17 differentiation in many disease models (11, 16). In this study, soluble TGF-β1 levels were upregulated in parallel to the reduced Treg/Th17 by MSCs treatment. To clarify the role of TGF-β1 among MSCs-secreted factors on regulating Treg/Th17 in context of in-vitro ARDS environment, TGF-β1 secretion was blocked with TGF-β1 neutralization. Anti-TGF-β1 antibody were added into the medium of indirect MSCs treatment group, followed by detection of Treg and Th17 by FCM. We found that TGF-β1 blocking by neutralization in MSCs-treated CD4⁺T cells caused a reversal effect of MSCs on restoring Treg/Th17 imbalance induced by LPS and hypoxia challenge (P < 0.05, Fig. 3), suggesting that MSCs improved Treg/Th17 exposed LPS and hypoxia stimulation by increased TGF-β1 secretion.

To confirm whether the increased soluble TGF-β1 was originated from MSCs or CD4⁺T cells exposed to LPS and hypoxia stimulation, Tgfb1mRNA expression of CD4⁺T cells sorted from groups of control, ARDS and MSCs treatment was measured by RT-PCR. Tgfb1mRNA levels of CD4⁺T cells were comparable among groups, indicating CD4⁺T cells with LPS and hypoxia stimulation, with or without MSC treatment would not be responsible for the increased TGF-β1 secretion. Moreover, Tgfb1 mRNA expression of MSCs were explored with or without LPS (100 ng/ml) and hypoxia(5%O₂) stimulation, with or without coculturing with CD4⁺T cells by transwell system. When MSCs were cultured alone, Tgfb1mRNA expression of MSCs was markedly increased in group with LPS and hypoxia stimulation

than that without ($P < 0.05$, Fig. 4A). When cocultured with CD4⁺T cells in the transwell insert, the increased Tgfb1mRNA expression of MSCs was also detected in group with LPS-hypoxia stimulation ($P < 0.05$, Fig. 4B), compared to that without. These data confirmed the increased soluble TGF- β 1 modulating the skewed Treg/Th17 induced by LPS and hypoxia challenge was generated from MSCs.

MSCs-secreted TGF- β 1 inhibited miR-155 expression of CD4⁺ T cells upon LPS and hypoxia challenge

MSCs soluble factors was proved to play immunomodulatory effects by regulating miRs expression. Here, we evaluated miR-155 and miR-146a expression, which has been reported to be associated with T cell phenotypic transformation of Treg/Th17 (19)(Adibzadeh et al., 2019), in splenic CD4⁺T cells with or without LPS (100 ng/ml)-hypoxia(5%O₂) stimulation in vitro, with or without MSCs coculture by transwell. The results showed miR-155 and miR-146a expression were significantly increased by in-vitro ARDS environment established by LPS and hypoxia stimulation ($P < 0.05$, Fig. 5). Indirect coculture with MSCs was able to reverse the increase of CD4⁺T cells-expressed miR-155 and miR-146a induced by in-vitro ARDS stimulation of LPS and hypoxia, while effect of MSCs on miR-155 suppression was attenuated by co-treatment with TGF- β 1 neutralization ($P < 0.05$, Fig. 5). These results suggested that miR-155 expression of CD4⁺T cells was suppressed in response to MSCs-secreted TGF- β 1 modulating Treg/Th17 differentiation under LPS-hypoxia induced condition.

MiR-155 mediated MSCs paracrine effects on Treg/Th17 skewing induced by LPS-hypoxia challenge

We next examined whether miR-155 inhibition of CD4⁺T cells was involved in MSCs regulating the skewed Treg/Th17 induced by LPS-hypoxia challenge. MiR-155 overexpression was performed by miR-155 mimic transfection. The CD4⁺T cells pre-transfected with miR-155 mimic and control were stimulated with LPS (100 ng/ml)-hypoxia (5%O₂), followed by MSCs coculture treatment by transwell for 48 hours. Flow cytometric analysis showed compared to CD4⁺T cells pre-transfected with controls with LPS-hypoxia stimulation, MSCs coculture treatment could increase frequency of CD4⁺CD25⁺Foxp3⁺Treg cells and reduced CD8⁻IL-17A⁺Th17 population while miR-155 overexpression by miR-155 mimic transfection reversed these changes (Fig. 6A). Bioinformatic analysis indicated Ptpn2mRNA might be one of potential target with 3'-UTR area binding miR-155(Supplemental file: Figure S3), of which upregulation has been reported to attenuate pro-inflammatory response and promote Treg conversion (24). We observed that, as compared to those of CD4⁺T cells pre-transfected with controls with LPS-hypoxia stimulation, MSCs treatment could significantly enhance expression of Foxp3 and Ptpn2mRNA and reduce Rorc mRNA expression, which was in consistence with the Treg and Th17 phenotypic alteration, while miR-155 overexpression could inhibited the alterations of these genes' expression induced by MSCs (Fig. 6B). These data further confirmed miR-155 suppression in CD4⁺T cells was necessary during MSCs paracrine effect modulating the skewed Treg/Th17 induced by LPS-hypoxia challenge.

Effects of miR-155 expression on proliferation capacity and survival of CD4⁺ T cells

Apart from Treg and Th17 conversion, we further examined the effect of miR-155 overexpression on proliferation and survival of CD4⁺ T cells with MSCs treatment exposed to LPS-hypoxia condition. Annexin V/PI staining results presented no difference in living, early apoptosis, and late apoptosis and dead parts of CD4⁺ T cells among groups (Fig. 7). The CTV staining analysis of CD4⁺ T cells sorted from each group showed miR-155 overexpression did no impact on proliferation capacity of CD4⁺ T cells exposed to MSCs coculture by transwell with/or LPS-hypoxia challenge (Fig. 8).

Discussion

Despite of growing supportive evidence of MSCs in controlling inflammation and protecting organ function (4–6), uncovering its effect and mechanism on different immune cells under specific pathological condition of ARDS will benefit future cell-based therapy and selecting target population. In this study, we investigated the generation of CD4⁺ T cells to Treg and Th17 upon LPS and hypoxia challenge in response to MSCs. We show that in-vitro LPS, hypoxia or their combined stimulation could induce the skewed Treg/Th17, favouring to Th17 cells rather than Treg cells. MSCs could restore the skewed Treg/Th17 while secreting more TGF-β1 and inducing an inhibited effect on miR-155 expression in CD4⁺ T cells upon LPS and hypoxia challenge. The alterations of miR-155 expression in CD4⁺ T cells and Treg/Th17 could be diminished by anti-TGF-β1 blockade. MiR-155 overexpression could abolish MSCs paracrine effects on the skewed Treg/Th17 with reduction in Treg phenotypic gene expression of Ptpn2mRNA and Foxp3mRNA and upregulation of Th17 phenotypic gene expression of Rorc mRNA. Accordingly, we demonstrated that MSCs-secreted TGF-β1 is capable of modulating the skewed Treg/Th17 upon LPS and hypoxia challenge via miR-155 suppression of CD4⁺ T cells.

Several studies have verified that Treg/Th17 skewing plays a crucial role in ARDS (7, 8, 10, 25, 26). LPS, an endotoxin originating from the cell wall of gram-negative bacteria, was most used for a bacterial hyperinflammatory model of experimental ARDS (27). In this study, we utilized LPS and hypoxia stimulation to mimic in-vitro ARDS environment and observed the induction of phenotype skewing to Th17, rather than Treg cells, which was consistent with the results observed in ARDS patients (7). In line with the skewed Treg/Th17 induced by LPS and hypoxia challenge, the levels of pro-inflammatory cytokines, such as IFN-γ, IL-17A and IL-21 was obviously increased. CD4⁺ T cells could be primed by following factors in our experiment settings. First, with the presence of RAW264.7 macrophages, activation of the CD14/TLR4 receptor structure on these cells by LPS complex triggered the production of inflammatory mediators and then drove naive T cells converting into different subset (28, 29). In the presence of soluble IL-6 with/or TGF-β1 detected in LPS-hypoxia stimulated group, IL-17A-expressing Th17 cells and FOXP3-expressing Treg cells could be respectively induced (30). The IL-21 release participates in the differentiation/amplification of Th17 cells (31), of which the remarkably augmentation (Fig. 2B) might contribute to the predominate role of Th17 in the skewed Treg/Th17 upon LPS-hypoxia challenge. Second, LPS is reported to directly regulate Th17 differentiation in vitro in a TLR-4-specific manner though LPS showing modest effect on TLR4 expression was dose-dependently with remarkable effects at a dose of higher than 10 μg/ml (32). In addition, hypoxia exposure could influence the processes of T cell

differentiation and phenotypic stability (33). Previous studies reported that HIF-1, as a cellular production in response to hypoxia, could boost proinflammatory IL-17-producing Th17 cells and suppress the Foxp3-expression Treg cell subsets (34), with which the similar alteration of Treg/Th17 upon hypoxia exposure was observed in current study. Our study was first to show that the combined stimulation of LPS at 10, 100 and 1000 ng/ml and hypoxia(5%O₂) could induce a skewed Treg/Th17, without evident difference detected from those with LPS or hypoxia stimulation alone.

By establishing in-vitro ARDS environment with LPS-hypoxia stimulation on CD4⁺T cells, we found that MSCs coculture restored the skewed Treg/Th17 and significantly augmented the anti-inflammatory cytokine secretion. Upon LPS and hypoxia challenge, MSCs treatment group exhibited a higher frequency of Treg and lower Th17 percentage in comparison with those without. This phenotypic conversion of Treg and Th17 induced by MSCs was partly consistent with previous findings (11, 35) in several disease models while Najjar et al., 2019 study showed adipose tissue-derived MSCs could promote the development of a proinflammatory Th17 phenotype from activated T cells. The discrepancy might be attributed to the distinct environment around MSCs-T cells between different experiment settings, including cell ratio and environmental cytokines release, which needs to be carefully addressed when proposing future cell-based therapy. In keeping with Foxp3-expressing Treg phenotype prevailing induced by MSCs in coculture system stimulated by LPS and hypoxia, the levels of anti-inflammatory cytokines, such as IL-10 and TGF-β1 were remarkably increased.

MSCs exert an immunomodulatory effect mainly via cell contact or paracrine mechanism (6). In our results, MSCs regulating the skewed Treg/Th17 upon LPS and hypoxia challenge was dependent on cell-to-cell contact, suggesting that paracrine is the primary mechanism during this process. Among the soluble factors released by MSCs, TGF-β1 is proved to mediate a therapeutic immunosuppressive effect, switching CD4⁺T cells from a pro-inflammatory phenotype of Th17 to an inflammation-resolving phenotype of Tregs (16) while the secretion of TGF-β1 by MSC is environment sensitive (36). Current literature supported more TGF-β1 production by MSCs under hypoxic or LPS-stimulated condition (13, 14). In the present setting, more TGF-β1 secretion was detected in parallel with reversed Treg/Th17 in MSCs-treated CD4⁺T cells with LPS and hypoxia stimulation. Meanwhile more TGF-β1 release from MSCs, rather than CD4⁺T cells upon LPS and hypoxia challenge, in presence or absence of CD4⁺T cells was confirmed at the mRNA and protein levels by RT-PCR and ELISA. Furthermore, MSCs paracrine effects on restoring the skewed Treg/Th17 induced by LPS and hypoxia challenge could be restrained by anti-TGF-β1 blocking. Based on these observations, we proved that TGF-β1 secreted by MSCs could be responsible for regulating the Treg/Th17 imbalance induced by LPS and hypoxia challenge.

One novel finding of our study was that miR-155 suppression in CD4⁺T cells mediated regulation of MSCs-secreted TGF-β1 on the skewed Treg/Th17 induced by LPS-hypoxia challenge, which was associated with upregulation of Ptpn2mRNA and Foxp3mRNA expression and inhibition of Rorc mRNA. In mice models infected by LPS or Staphylococcal enterotoxin B, miR-155 expression was detected with significant increase with inflammatory cytokine production, indicating its association with

proinflammatory response (37, 38). In addition, miR-155 has been reported to be involved in T cell differentiation, survival and proliferation capacity (22, 39) and could be regulated by TGF- β 1(20). Within our results, LPS and hypoxia stimulation could markedly upregulate miR-155 expression in CD4⁺T cells. In addition to Treg/Th17 skewing, MSCs-secreted TGF- β 1 could induce an inhibited effect on miR-155 expression in CD4⁺T cells stimulated with LPS and hypoxia while miR-155 overexpression in CD4⁺T cells could relieve the regulation of MSCs-secreted TGF- β 1 on Treg/Th17 skewing and showed no impact on cells survival and proliferation capacity. This indicated that miR-155 suppression in CD4⁺T cells was necessary during MSCs-secreted TGF- β 1 regulating the skewed Treg/Th17 upon LPS and hypoxia challenge. According to the bioinformatic prediction, Ptpn2mRNA was one of the potential binding targets of miR-155, which was reported to induce Treg phenotype transformation and inhibit Th1 and Th17 (24). In line with phenotypic alteration of Treg and Th17 upon LPS and hypoxia challenge in response to MSCs, the present study showed Treg phenotypic gene expression of Ptpn2 and Foxp3mRNA were significantly reduced while Rorc mRNA expression of Th17 phenotypic transcription factor was upregulated in CD4⁺T cells with miR-155 mimic pre-transfection when compared to those without. These findings highlighted that MSCs-secreted TGF- β 1 regulated Treg/Th17 skewing in hypoxic and LPS-stimulated condition via miR-155 suppression of CD4⁺T cells.

Limitations should be noted in our study. First, the study was performed in vitro, though RAW264.7 macrophages were added as APC and anti-CD3/CD28 antibodies were pre-coated under hypoxic and LPS-stimulated condition to mimic the physiological and pathological activation of T cells during ARDS. The effect of MSCs-secreted TGF- β 1 on Treg/Th17 should be further verified with in-vivo ARDS models. Second, the coculture of MSCs and CD4⁺T cells was set at 1:5 cell ratio in the present study, which relatively belongs to the high cell ratio coculture (40). In-vitro evidence showed that effects of MSCs on phenotypic generation of CD4⁺T cells are affected by both cell ratio and inflammatory priming (40). The approach to figure out the effective percentage of injected MSCs in vivo on targeted immune cells remains unsolved when it comes to clinical application. Third, MSCs have been reported to exert exosomes or microvesicles (41), which are able to alleviate inflammation and elicit a therapeutic effect. Whether these could work as the origin of soluble TGF- β 1 is worthy to confirm in the further study.

Conclusions

In summary, the study demonstrated that soluble TGF- β 1 from MSCs restored the skewed Treg/Th17 induced by hypoxic and LPS-stimulated condition and dampened the inflammation. Additionally, miR-155 suppression of CD4⁺T cells mediated the regulatory effect of MSCs-secreted TGF- β 1 on the skewed Treg/Th17 upon LPS and hypoxia challenge. These findings may provide evidence of MSCs application for future T lymphocyte-targeted cell therapy in ARDS.

Abbreviations

Ab, antibody; ELISA: enzyme-linked immunosorbent assay; FCM, flow cytometry; FoxP3, fork-head box P3; HGF, hepatocyte growth factor; LPS, lipopolysaccharide; miR, microRNA; MSCs, mesenchymal stem cells; PGE2, prostaglandin E2; ROR γ t, retinoic-acid-receptor related orphan receptor γ t; SLE, systemic lupus erythematosus; TGF- β , transforming growth factor-beta; Th17, T helper 17; Treg, regulatory T; VEGF, vascular endothelial growth factor.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors have given final approval of the version to be published and agree to be accountable for all aspects of this work.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

National Natural Science Foundations of China (Grant Numbers: 81571874, 81930058); Jiangsu Provincial Special Program of Medical Science (Grant Numbers: BE2018743, BE2019749); National Science and Technology Major Project for Control and Prevention of Major Infectious Diseases of China (Grant Number: 2017ZX10103004). The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

X. M. participated in the study design, performed laboratory work and statistical analysis, prepared the drafts of the manuscript and revised it according to advice from the other authors. Z. X. W., C. J. X., L. F. participated in the study design and performed laboratory work. X. J. F., X. J. Y., Y.Y. participated in the study design, and drafting the manuscript. Y. W. P. and Q. H. B. were responsible for the design of the study and coordination and reviewing the intellectual content and conceived of the study, and helped to draft the manuscript. All the authors critically revised the manuscript.

Acknowledgments

Not applicable

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Figures

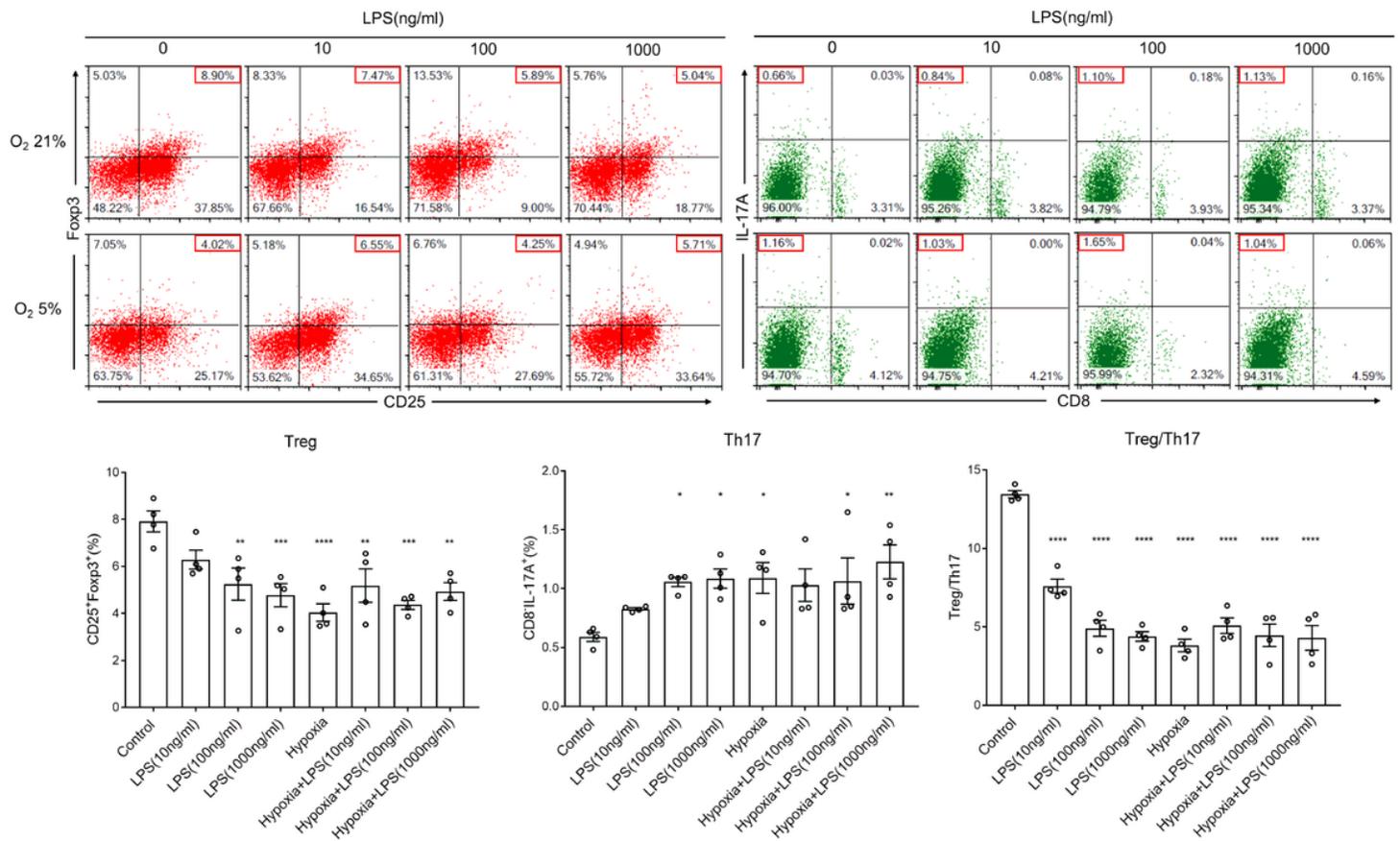


Figure 1

Effects of LPS at 10, 100, 1000ng/ml alone, hypoxia alone or combined stimulation on mouse splenic CD4+T cells differentiation to Treg or Th17 population. The phenotype of Treg and Th17 populations after 48-hours stimulation were assessed by flow cytometry. Treg cells were identified by CD25+Foxp3+ in CD4+T cells and Th17 cells were by CD8-IL-17A+ in CD4+T cells. Representative flow plots were shown. Bar graphs represent mean frequency±SEM of Treg and Th17 and mean values±SEM of Treg/Th17 ratios in the various groups. *P<0.05 vs. control group, **P<0.01 vs. control group, ***P<0.001 vs. control group, ****P<0.0001 vs. control group (n=4). LPS, lipopolysaccharide; SEM, standard error of the mean; Th, T helper; Treg, regulatory T cells

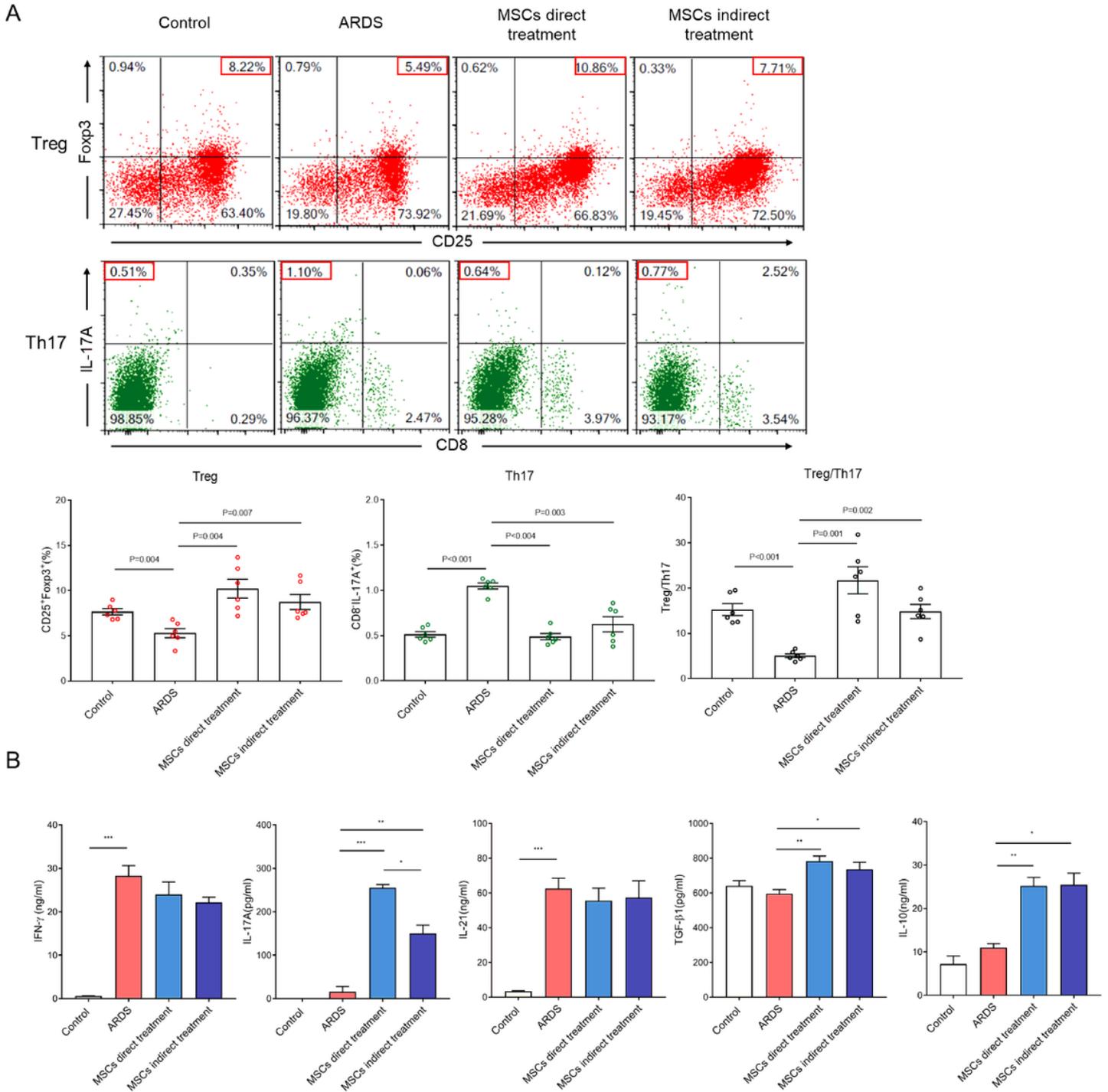


Figure 2

Effects of MSC on the skewed Treg/Th17 in LPS and hypoxia-stimulated CD4⁺T cells and cytokine secretion. CD4⁺T cells alone (Control), CD4⁺T cells with LPS (100ng/ml) -hypoxia (5%O₂) stimulation (ARDS) and MSCs-treated CD4⁺T cells with LPS-hypoxia challenge (MSCs direct treatment and MSCs indirect treatment) were cultured for 48 hours. (A) Then cells were harvest for Treg and Th17 phenotypic identification by flow cytometry. Representative flow plots were shown. Bar graphs represent mean frequency ± SEM of Treg and Th17 and mean values ± SEM of Treg/Th17 ratios in the various groups. (B)

The culture supernatants were collected for IFN- γ , IL-17A, IL-21, IL-10 and TGF- β 1 levels measurement by an enzyme-linked immunosorbent assay, presented with mean \pm SEM in bar graph. *P<0.05 vs. control group, **P<0.01 vs. control group, ***P<0.001 vs. control group (n=3). ARDS, acute respiratory distress syndrome; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MSC, mesenchymal stem cell; SEM, standard error of the mean; TGF, transforming growth factor. Th, T helper; Treg, regulatory T cells

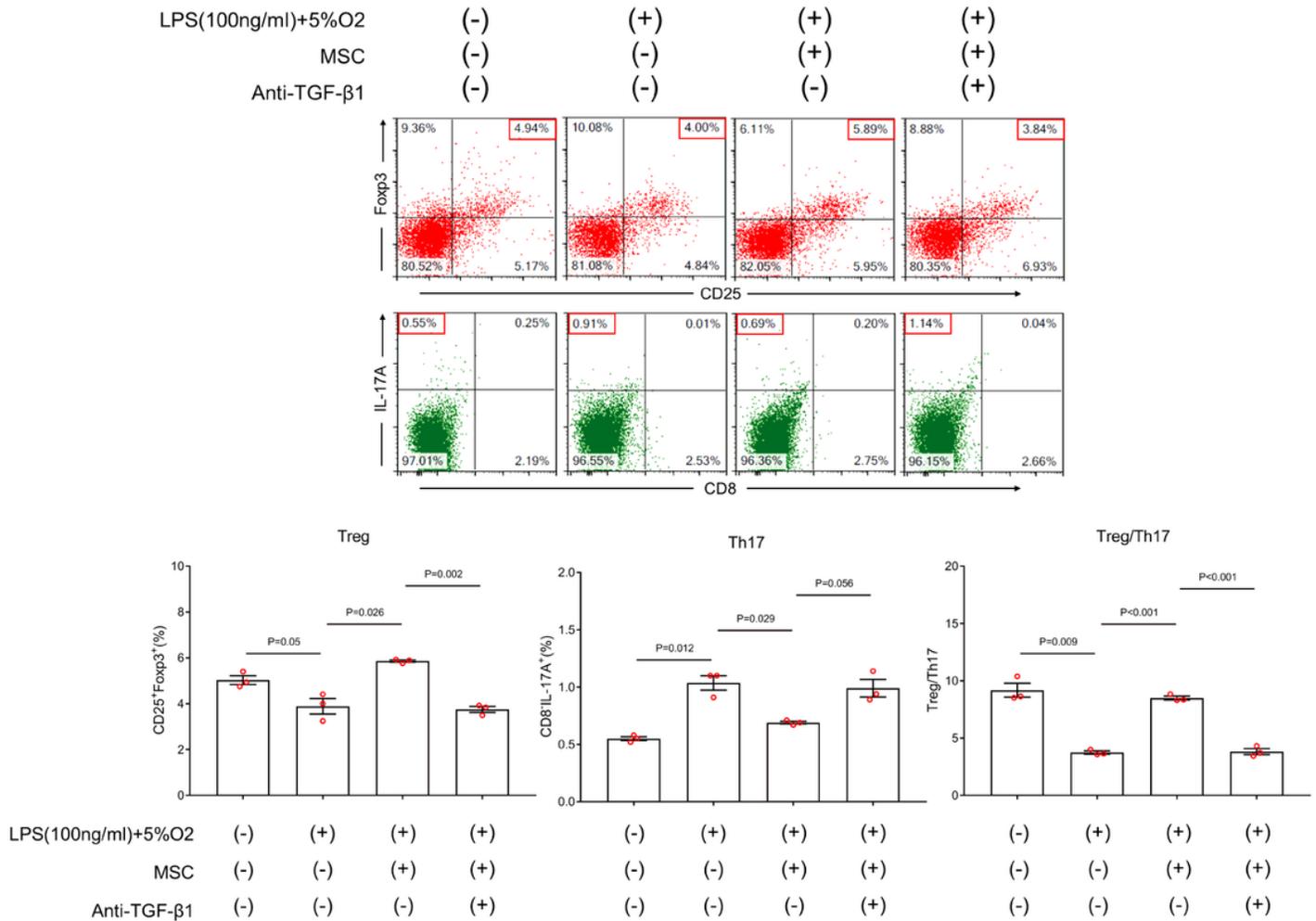


Figure 3

Effects of anti-TGF- β 1 neutralization on LPS and hypoxia-induced Treg/Th17 skewing in response to MSCs treatment. Representative flow plots were shown. Bar graphs represent mean frequency \pm SEM of Treg and Th17 and mean values \pm SEM of Treg/Th17 ratios in the various groups. LPS, lipopolysaccharide; SEM, standard error of the mean; TGF, transforming growth factor; Th, T helper; Treg, regulatory T cells

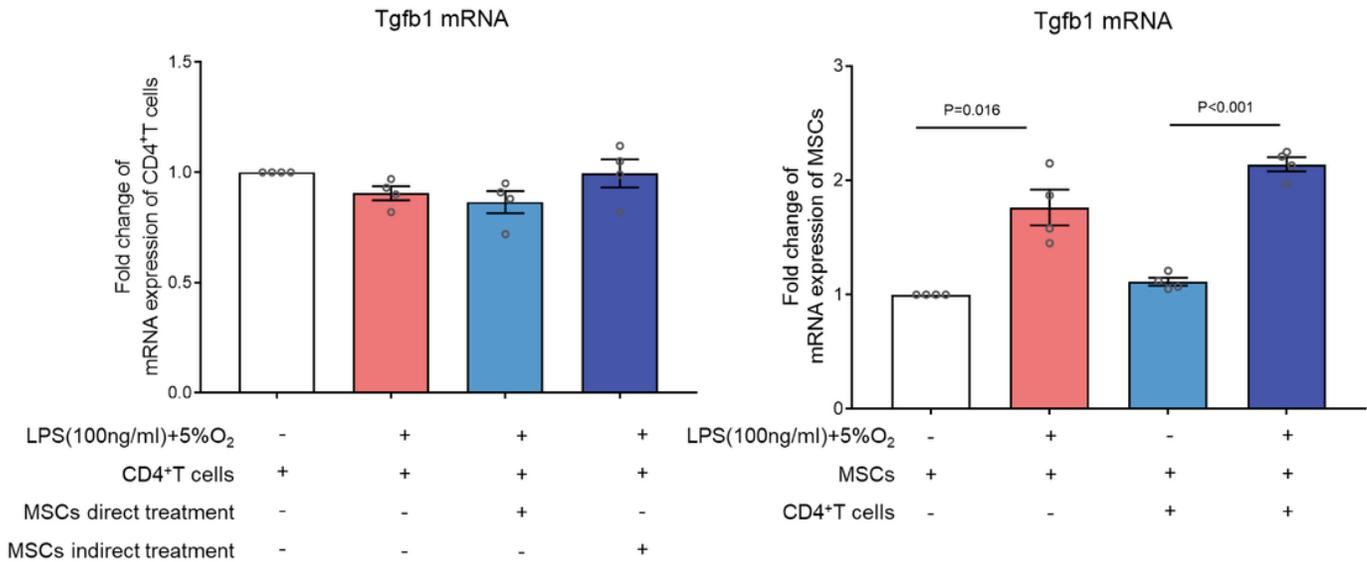


Figure 4

Effects of MSCs-CD4⁺T cells coculture and/or LPS-hypoxia stimulation on TGF-β1 mRNA expression of CD4⁺T cells and MSCs. (A) TGF-β1 mRNA expression of CD4⁺T cells receiving MSCs treatments and/or LPS-hypoxia stimulation was detected by RT-PCR. (B) TGF-β1 mRNA expression of MSCs receiving indirect coculture with CD4⁺T cells and/or LPS-hypoxia stimulation was detected by RT-PCR detection. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; RT-PCR, real-time polymerase chain reaction; TGF, transforming growth factor

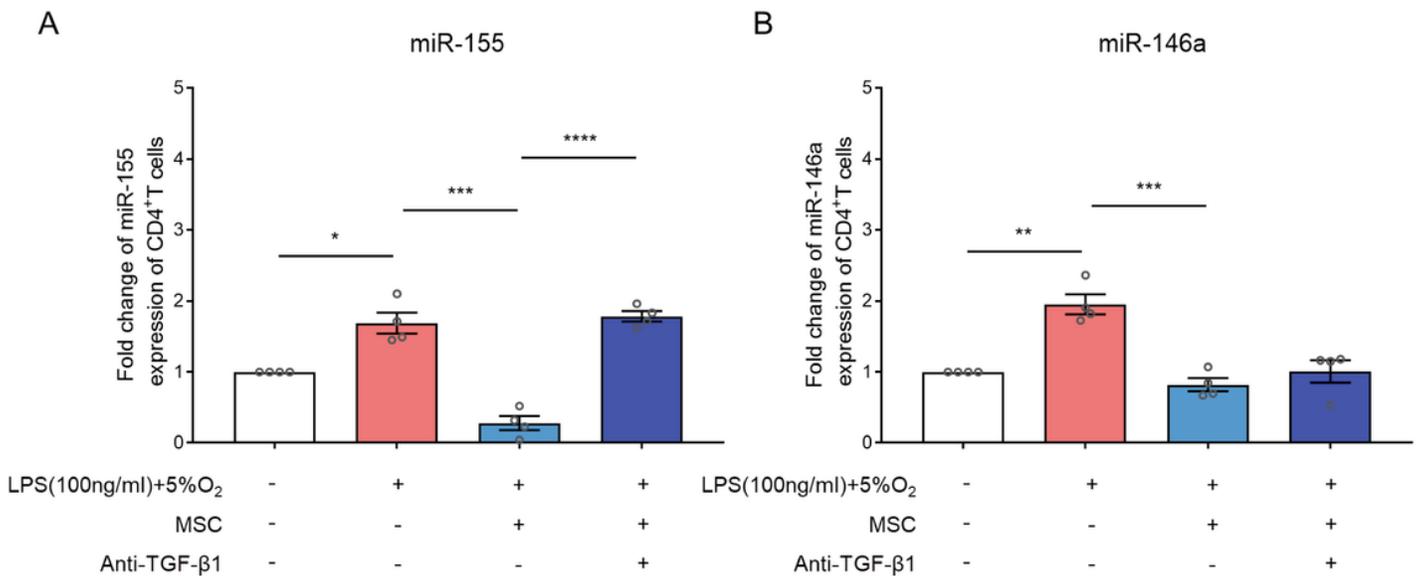


Figure 5

Effects of anti-TGF-β1 neutralization on miR-155 and miR-146a expression of CD4⁺T cells upon LPS and hypoxia challenge in response to MSCs treatment. Bar graphs represent mean±SEM levels of miR-155 (A) and miR-146a (B) expression detected by RT-PCR. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (n=4).

LPS, lipopolysaccharide; MSC, mesenchymal stem cell; RT-PCR, real-time polymerase chain reaction; SEM, standard error of the mean; TGF, transforming growth factor

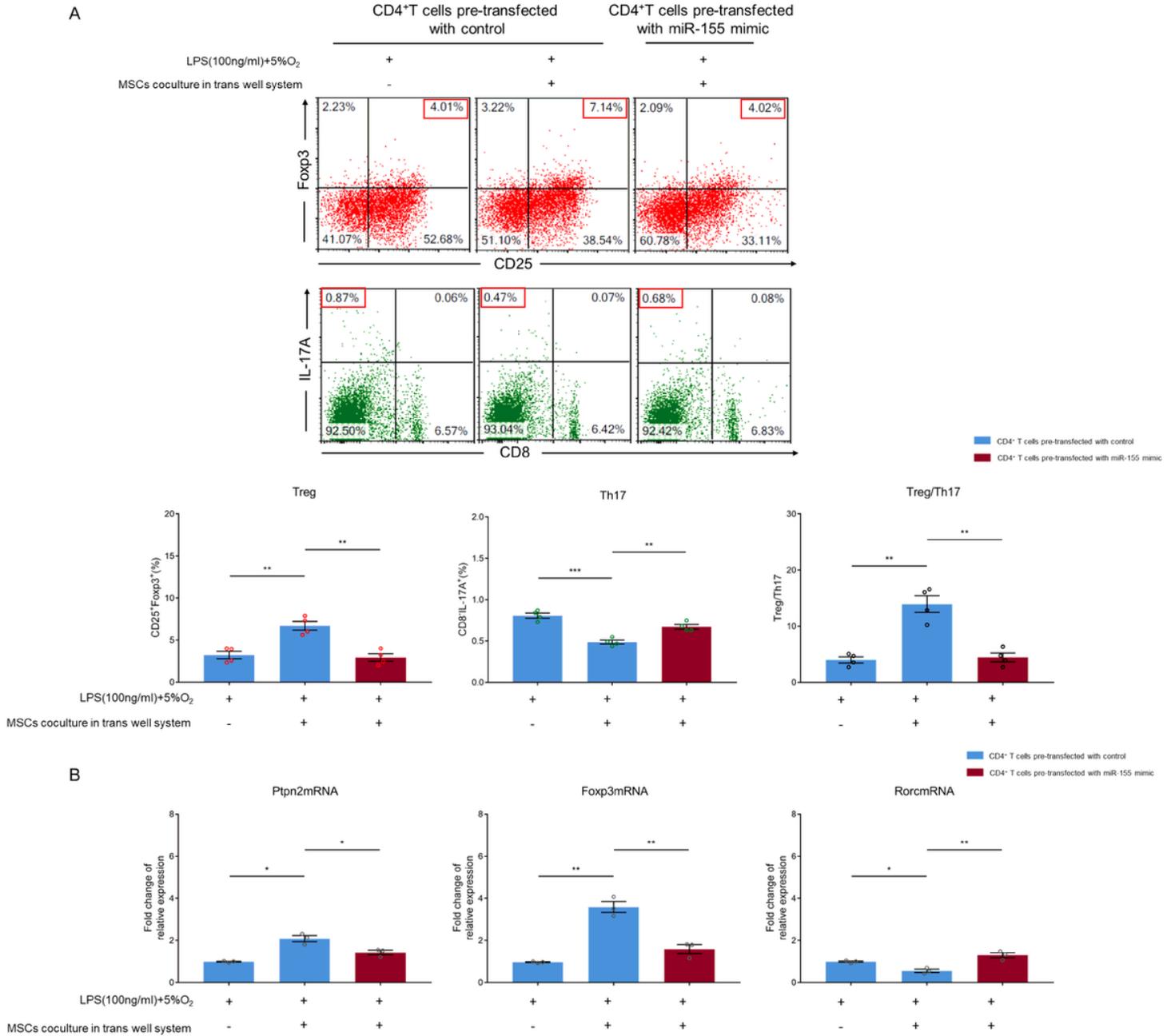


Figure 6

Effects of miR-155 overexpression in CD4⁺T cells on LPS-hypoxia induced Treg/Th17 skewing and related gene expression in response to MSCs treatment. CD4⁺T cells pre-transfected with control/miR-155 mimic received MSCs treatment by trans well system, with/without LPS-hypoxia challenge for 48 hours. Then (A) cells were harvested for Treg and Th17 phenotypic identification by flow cytometry. Representative flow plots were shown. Bar graphs represent mean frequency \pm SEM of Treg and Th17 and mean values \pm SEM of Treg/Th17 ratios in the various groups. (B) The gene expression of Ptpn2, Foxp3 and RorcRNA of CD4⁺T cells were detected by RT-PCR in the various groups. Bar graphs represent mean \pm SEM levels of Ptpn2, Foxp3 and RorcRNA expression. *P<0.05, **P<0.01, ***P<0.001 (n=4). LPS,

lipopolysaccharide; MSC, mesenchymal stem cell; RT-PCR, real-time polymerase chain reaction; SEM, standard error of the mean; Th, T helper; Treg, regulatory T cells

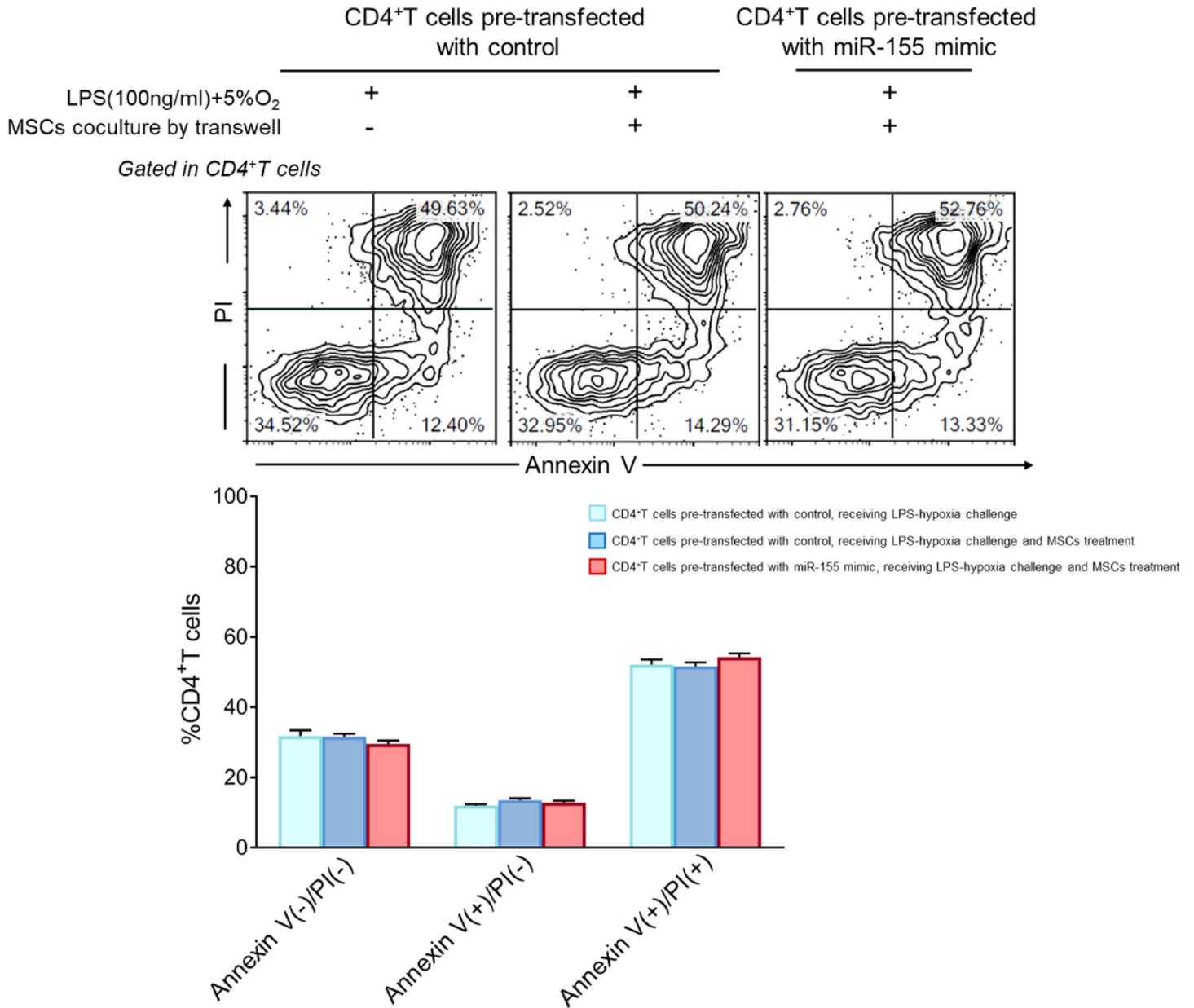


Figure 7

Effects of miR-155 overexpression in CD4⁺T cells on apoptosis upon LPS-hypoxia challenge in response to MSCs treatment. CD4⁺T cells pre-transfected with control/miR-155 mimic received MSCs treatment by trans well system, with/or LPS-hypoxia challenge for 48 hours. Then cells were harvest for apoptosis assay with Annexin V/PI staining by flow cytometry. Representative flow plots were shown. Bar graphs represent mean frequency±SEM of living[(Annexin V(-)/PI(-)], early apoptosis[(Annexin V(+)/PI(-)], late apoptosis and dead[(Annexin V(+)/PI(+)] percentage of CD4⁺T cells in the various groups. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; SEM, standard error of the mean

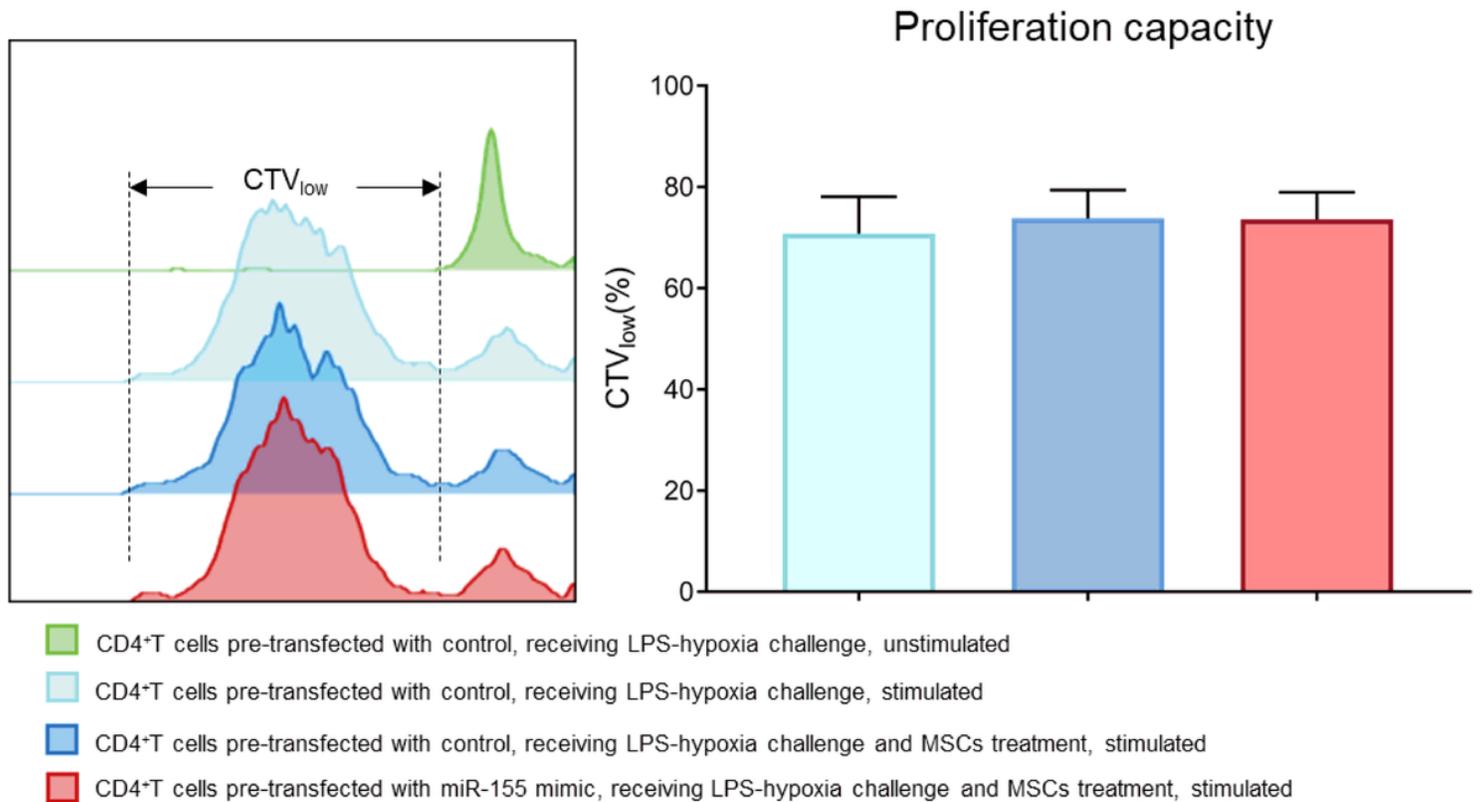


Figure 8

Effects of miR-155 overexpression in CD4⁺T cells on proliferation capacity upon LPS-hypoxia challenge in response to MSCs treatment. CD4⁺T cells pre-transfected with control/miR-155 mimic received MSCs treatment by trans well system, with/or LPS-hypoxia challenge for 48 hours. Then CD4⁺T cells were sorted by positive CD4 selection, using CD4 microbeads and were labelled with CellTrace™ Violet (CTV) reagent prior to stimulation with anti-CD3/CD28 antibodies. Cells unstimulated were set as controls(green). Proliferation capacity of CD4⁺T cells were identified as CVT_{low}% by flow cytometry after 72-hours culture in vitro. Bar graphs represent mean frequency±SEM of proliferation capacity of CD4⁺T cells in the various groups. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; SEM, standard error of the mean

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