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ORIGINAL ARTICLE

Immunomodulatory Properties of Bone Marrow Mesenchymal Stem Cells from Patients with Amyotrophic Lateral Sclerosis and Healthy Donors

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Abstract

Pathogenesis of amyotrophic lateral sclerosis (ALS) involves several mechanisms resulting in a shift from a neuroprotective to a neurotoxic immune reaction. A promising tool for ALS treatment is represented by mesenchymal stem cells (MSCs), which possess both regenerative potential and immunomodulatory properties. In this study, we aimed to compare the immunomodulatory properties of MSCs isolated from the bone marrow of patients suffering from ALS and healthy donors. Moreover, the influence of proinflammatory cytokines on the immunoregulatory functions of MSCs was also evaluated. We found that MSCs from ALS patients and healthy donors comparably affected mitogen-stimulated peripheral blood mononuclear cells and reduced the percentage of T helper (Th)1, Th17 and $CD8^+CD25^+$ lymphocytes. These MSCs also equally increased the percentage of Th2 and $CD4^+FOXP3^+$ T lymphocytes. On the other hand, MSCs from ALS patients decreased more strongly the production of tumour necrosis factor-α than MSCs from healthy donors, but this difference was abrogated in the case of MSCs stimulated with cytokines. Significant differences between cytokine-treated MSCs from ALS patients and healthy donors were detected in the effects on the percentage of $CD8^+CD25^+$ and $CD4^+FOXP3^+$ T lymphocytes. In general, treatment of MSCs with cytokines results in a potentiation of their effects, but in the case of MSCs from ALS patients, it causes stagnation or even restriction of some of their immunomodulatory properties. We conclude that MSCs from ALS patients exert comparable immunomodulatory effects to MSCs from healthy donors, but respond differently to stimulation with proinflammatory cytokines.

Keywords Mesenchymal stem cells · Amyotrophic lateral sclerosis · Immunomodulation · Helper T lymphocytes · $CD4^+FOXP3^+$ T lymphocytes · Proinflammatory cytokines

Abbreviations

ALS	amyotrophic lateral sclerosis
APC	allophycocyanin
BM	bone marrow
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
HC	healthy control
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MS	multiple sclerosis
MSCs	mesenchymal stem cells
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD-L1	programmed death-ligand 1
PE	phycoerythrin
PHA	phytohemagglutinin
RA	rheumatoid arthritis
Th	T helper
TNF	tumour necrosis factor
Treg	T regulatory

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Introduction

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease affecting motor neurons and causing paralysis, muscle atrophy and finally respiratory failure and death within 2 to 4 years from diagnosis (Chiò et al. 2013). Although precise pathophysiological mechanisms causing ALS have not yet been elucidated, aberrant immune reactions represent one of the major factors which are supposed to be implicated in neurodegeneration. Inflammatory markers and infiltration by macrophages, mast cells and T lymphocytes were found in the affected neural tissue of patients suffering from ALS (Graves et al. 2004). During the early phase of ALS progression, the neuroprotective immune response including T helper (Th)2 lymphocytes, M2 microglia and T regulatory (Treg) lymphocytes predominates. These cells participate in the formation of the anti-inflammatory environment, which supports motor neuron viability. In the later phase, rapid disease progression and an increase of a number of damaged motor neurons are associated with the formation of the neurotoxic immune response including M1 microglia and Th1 and Th17 lymphocytes (Hooten et al. 2015). The only currently available therapy for this so far lethal disease is the anti-glutamate agent riluzole which slows down the progression of ALS (Bensimon et al. 1994). Presently, various new approaches in the treatment of ALS including anti-inflammatory, anti-glutamatergic and stem cell-based therapy have been proposed and tested. Among them, mesenchymal stem cells (MSCs) represent promising candidates for cell-based therapy due to their versatility and effectiveness in the treatment of other diseases (Karussis et al. 2010; Bonab et al. 2012).

MSCs are multipotent stem cells characterized by an adherence to plastic surfaces, the ability to differentiate into osteoblasts, adipocytes and chondrocytes and by the expression of markers including CD73, CD90 and CD105, and the absence of surface molecules CD11b, CD14, CD19, CD34, CD45, CD79α and HLA-DR (Dominici et al. 2006). Furthermore, MSCs produce numerous growth and trophic factors and possess a broad range of immunomodulatory properties, influencing cells of the innate as well as adaptive immune system. MSCs are able to modulate the functions and subsets of macrophage (Kim and Hematti 2009) and T lymphocyte (Aggarwal and Pittenger 2005; Svobodova et al. 2012) populations, which represent the cells involved in the pathogenesis of ALS. Immunoregulatory properties of MSCs exert plasticity, and can be modulated in dependence on the local environment (Holan et al. 2016). The expression of some immunomodulatory molecules can be induced in MSCs by treatment with proinflammatory cytokines such as interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α) (English et al. 2007; Javorkova et al. 2014).

So far, several studies comparing the phenotype and differentiation ability of MSCs from patients suffering from ALS and healthy donors have been published (Ferrero et al. 2008; Cho et al. 2010; Koh et al. 2012), but there is a lack of studies focused on the functional characterization of MSCs from patients with ALS. It has been shown, that neuroinflammation significantly participates in neurodegeneration during ALS progression, and that the immunomodulatory properties of MSCs represent a potential therapeutic tool to reduce inflammation in ALS (Vercelli et al. 2008). Since some properties of MSCs can be influenced by the disease, studies evaluating the immunomodulatory properties of MSCs obtained from ALS patients are desirable to determine the convenience of autologous MSCs for the therapy. Therefore, we compared the immunomodulatory properties of MSCs from patients suffering from ALS and healthy donors. In addition, the effects of proinflammatory cytokines IFN-γ, TNF-α and interleukin (IL)-6, which are involved in the pathogenesis of ALS, on the functional properties of MSCs from ALS patients and healthy donors were also evaluated.

Materials and Methods

Preparation of MSCs

MSCs were isolated from the bone marrow (BM) of 14 patients suffering from ALS (mean age 47.7 years, 2 females and 12 males) and, as controls, 14 donors undergoing orthopaedic surgery (mean age 49.5 years, 3 females and 11 males). The BM was collected from iliac crest. The control group included patients with anterior cruciate ligament injury, rotator cuff rupture and knee or hip arthrosis. All ALS patients included to this study were ambulatory, at early phase of disease and none of the patients suffered from malnutrition or needed ventilation of the lungs. All MSC donors signed informed consent concerning the use of excessive material for experimental purposes. Diagnosis of definite ALS was established on the basis of El Escorial Revised criteria (Brooks et al. 2000), detailed neurological observations, the ALS functional rating scale, the Norris scale, forced vital capacity and brain and spinal cord magnetic resonance imaging. MSCs were cultured from nucleated cell fraction, isolated from BM, and settled on gelofusine (B. Braun Melsungen AG, Melsungen, Germany). Nucleated cells were seeded at

a concentration of 1×10^6 cells/ml in 10 ml of Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St. Louis, MO), supplemented with 5% of platelet lysate (IKEM, Prague, Czech Republic), antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) and 10 mM HEPES buffer (hereafter referred to as complete DMEM) in 75-cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland). Nonadherent cells were washed away after a 72-h cultivation period, and adherent cells were cultured with a regular exchange of medium and passaging to maintain optimal cell density. MSCs were harvested using trypsin-like enzyme (CTS TrypLE SELECT, Gibco, Waltham, MA) within the 3rd passage, and were stored in a complete DMEM supplemented with 10% of dimethyl sulfoxide (DMSO, Serva Electrophoresis, Heidelberg, Germany) in liquid nitrogen. For the experiments, the cells were thawed and cultured in 75-cm^2 tissue culture flasks until confluence. Half of MSCs were treated for 24 h before use by incubation at 37 °C with 10 ng/ml of human recombinant IFN-γ, TNF-α and IL-6 (all purchased from PeproTech, Rocky Hill, NJ). Treated MSCs were washed two times with RPMI 1640 medium (Sigma Aldrich), supplemented with 10% of fetal calf serum (Gibco), antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) and 10 mM HEPES buffer (hereafter referred to as complete RPMI medium) to remove added cytokines.

Phenotypic Characterization of MSCs by Flow Cytometry

Untreated MSCs or MSCs treated with cytokines were stained for 30 min in phosphate-buffered saline (PBS) at 4 °C with the following monoclonal antibodies (mAb) (all purchased from BioLegend, San Diego, CA): fluorescein isothiocyanate (FITC)-labeled anti-CD45 (clone HI30), FITC-labeled anti-CD90 (clone 5E10), FITC-labeled anti-CD105 (clone 43A3), allophycocyanin (APC)-labeled anti-CD11b (clone M1/70), APC-labeled anti-CD44 (clone IM7), APC-labeled anti-programmed death-ligand 1 (PD-L1) (clone 29E.2A3), phycoerythrin (PE)-labeled anti-CD11a (clone HI111), PE-labeled anti-CD86 (clone IT2.2) and PE-labeled anti-CD106 (clone STA). Dead cells were stained with Hoechst 33258 dye (Invitrogen, Carlsbad, CA), data were collected using LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed by FlowJo software 9.9.3 (LLC, Ashland, OR).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from the buffy coat obtained from the peripheral blood of healthy donors. A mixture of buffy coat and PBS was layered over Ficoll Paque Plus (GE Healthcare, Chicago, IL) and centrifuged (400 x g for 35 min) according to the manufacturer's instructions. The layer with PBMCs was collected and washed three times in complete RPMI medium. PBMCs were stored at aliquots in complete RPMI medium supplemented with 10% of DMSO in liquid nitrogen.

Cultivation of PBMCs with MSCs for Flow Cytometry Analysis

To determine the influence of MSCs on the proportion of selected leukocyte populations, PBMCs were cultured at a final concentration of 1×10^6 cells/ml in a volume of 1 ml of complete RPMI medium in 24-well plates (TPP) with MSCs at ratios from 20:1 to 320:1 for 72 h unstimulated or stimulated with 10 μg/ml of phytohemagglutinin (PHA, Pharmacia, New Jersey, NJ). Cells were then harvested in PBS and labeled for determination either of changes in proportion of activated $CD25^+$ $CD4^+$ and $CD8^+$ T lymphocytes or of changes in the percentage of Th1, Th2, Th17 or $CD4^+FOXP3^+$ T lymphocytes.

Flow Cytometry Analysis of Activated $CD4^+$ and $CD8^+$ T Lymphocyte Populations

PBMCs cultured with MSCs and stimulated with PHA for 72 h (see above) were collected in PBS and stained for 30 min at 4 °C with the following mAb: FITC-labeled anti-CD4 (clone RPA-T4, BioLegend), PE-labeled anti-CD8a (clone HIT8a, BioLegend) and APC-labeled anti-CD25 (clone MEM-181, Exbio, Prague, CR). Dead cells were stained with Hoechst 33258 dye, and data were collected using LSRII flow cytometer and analyzed by FlowJo software 9.9.3.

Intracellular Staining of Transcription Factors FOXP3, RORγt, T-BET and GATA-3

PBMCs cultured with MSCs and stimulated with PHA for 72 h (see above) were collected in PBS, and before intracellular staining incubated for 30 min at 4 °C with FITC-labeled anti-CD4 mAb and Live/Dead Fixable Violet Dead Cell Stain Kit (Invitrogen) for staining of dead cells. Samples were washed in PBS, fixed and permeabilized using Foxp3 Staining Buffer Set (eBioscience, San Diego, CA) according to the manufacturer's instructions. Samples for intracellular detection of transcription factors were stained for 30 min at 4 °C with the following mAb: Alexa Fluor 647-labeled anti-FOXP3 (clone 150D, BioLegend), PE-labeled anti-RORγt (clone AFKJS-9, eBioscience), PE-labeled anti-

GATA-3 (clone TWAJ, eBioscience) and Alexa Fuor 647-labeled anti-T-BET (clone 4B10, BioLegend). Data were collected using LSRII flow cytometer and analyzed by FlowJo software 9.9.3.

Cultivation of MSCs with PBMCs for Enzyme-Linked Immunosorbent Assay (ELISA) and Real-Time PCR

PBMCs were cultured at a final concentration of 0.6×10^6 cells/ml in a volume of 1 ml of complete RPMI medium in 48-well plates (TPP) with MSCs at ratios from 20:1 to 160:1 unstimulated or stimulated for 48 h with 10 μg/ml of PHA or 5 μg/ml of lipopolysaccharide (LPS, Sigma Aldrich). Culture supernatants were harvested and cells were collected into TRI Reagent (Molecular Research Center, Cincinnati, OH) and stored at −80 °C.

Cytokine Detection by ELISA

Supernatants were collected after a 48-h cultivation of PBMCs with MSCs (see above). The production of IL-10, IFN-γ and TNF-α was quantified by ELISA kits purchased from R&D Systems (Minneapolis, MN).

Detection of IFN-γ, IL-10 Ad IL-17 Gene Expression by Real-Time PCR

The total RNA was extracted from samples of PBMCs cultured with MSCs and stimulated with PHA using TRI Reagent according to the manufacturer's instructions. One μg of total RNA was treated with deoxyribonuclease I (Promega, Madison, WI) and used for reverse transcription. The first-strand of cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 μl using M-MLV Reverse Transcriptase (Promega). Real-time PCR was performed using Power SYBR Green PCR master MIX (Applied Biosystems, Carlsbad, CA) on cycler StepOne Plus Real-Time PCR System (Applied Biosystem). The primers used for amplification were as follows (all from Generi Biotech, Hradec Kralove, CR): GAPDH: 5′-AGCCACATCGCTCAGACAC-3′ (sense), 5′-GCCCAATACGACCAAATCC-3′ (antisense); IFN-γ: 5′-ATATTGCAGGCAGGACAACC-3′ (sense), 5′-TCATCCAAGTGATGGCTGAA-3′ (antisense); IL-10: 5′-GGTGATGCCCCAAGCTGA-3′ (sense), 5′-TCCCCCAG GGAGTTCACA-3′ (antisense); IL-17F: 5′- CCGTTCCC ATCCAGCAAGAG -3′ (sense), 5′- ACAGTCAC CAGCACCTTCTC -3′ (antisense). The PCR parameters included denaturation at 95 °C for 3 min, 40 cycles at 95 °C for 20 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80 °C for 5 s, and were analyzed using StepOne Software version 2.2.2 (Applied Biosystems).

Statistical Analysis

A total of 4 groups of MSCs including MSCs isolated from ALS patients (group ALS) and from healthy controls (group HC), and MSCs of both groups treated for 24 h with proinflammatory cytokines (group ALSc and HCc) were tested.

The results are expressed as the mean ± SD. Comparison between two groups (ALS and HC or ALSc and HCc) was performed by Student's t test. A value of $p < 0.05$ was considered as statistically significant. The effects of MSCs on PBMC functions were statistically evaluated using ANOVA and Dunnett's test.

Results

Characterization of MSCs

MSCs isolated from the BM of patients suffering from ALS and healthy donors, which were untreated or treated with cytokines, were characterized using flow cytometry. As demonstrated in Fig. 1, no significant differences between the groups in the expression of positive (CD44, CD90, CD105 and CD106) and negative (CD11a, CD11b, CD45 and CD86) MSC markers were detected. In addition, the expression of immunomodulatory molecule PD-L1 which is absent in unstimulated MSCs, was induced comparably in MSCs treated with cytokines. MSCs from ALS patients and HC were also able to differentiate into adipocytes and osteoblasts, and exerted comparable metabolic activity (data not shown). Analysis of gene expression of selected immunomodulatory molecules (indoleamine-2,3-dioxygenase, cyclooxygenase-2, TNF-stimulated gene 6, programmed death ligand 1, transforming growth factor-β, vascular endothelial growth factor) did not show any differences between ALS and HC groups. Only MSCs from ALS and ALSc group expressed increased levels of gene for IL-6, but this difference was not confirmed on protein level by ELISA. MSCs reacted to stimulation with proinflammatory cytokines by increase in the expression of inducible molecules (data not shown).

MSCs Modulate IL-10 and TNF-α Production by PBMCs Stimulated with LPS

Since innate immunity can participate in the pathogenesis of ALS, we assessed the effects of MSCs on the production of IL-10 and TNF-α by PBMCs stimulated

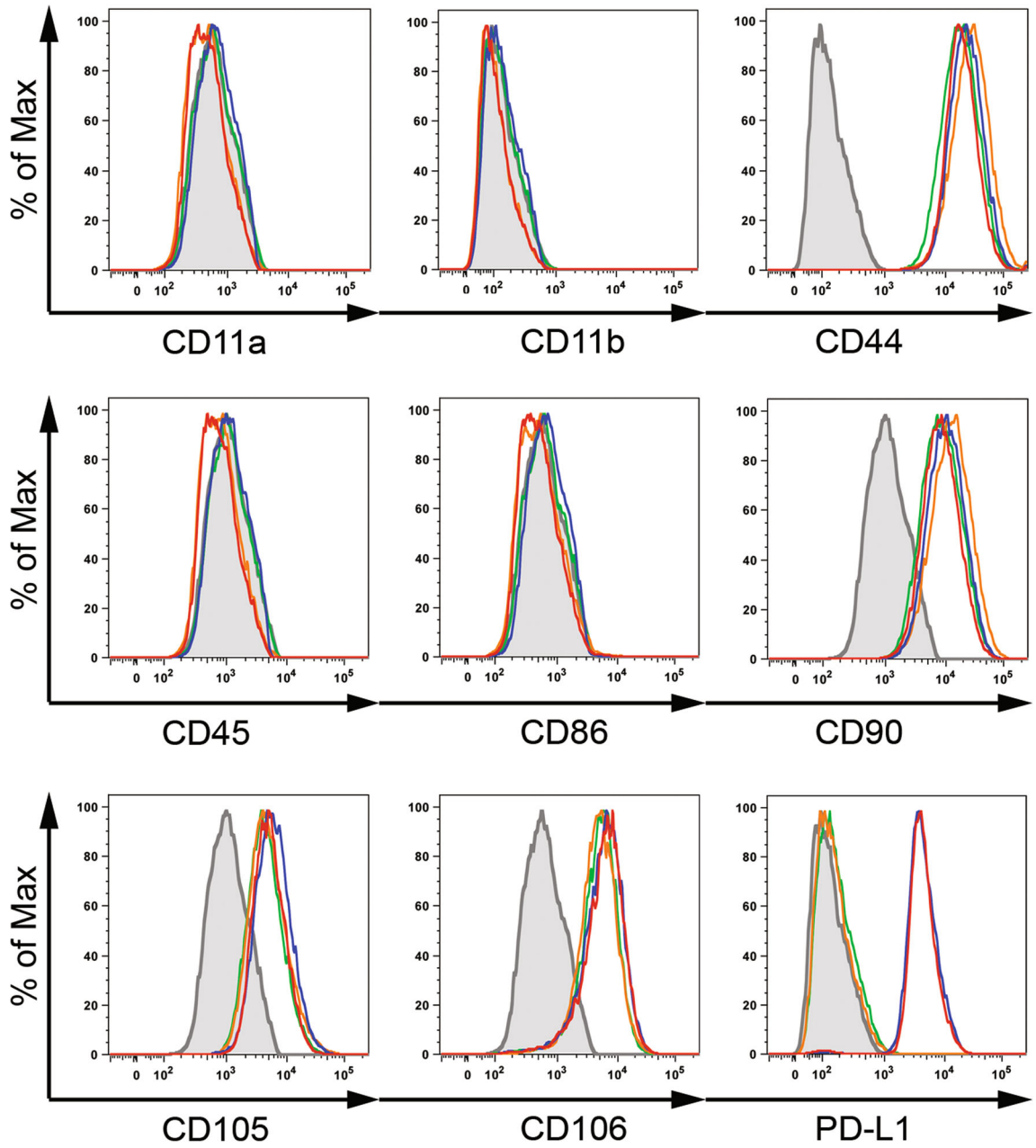

Fig. 1 Characterization of unstimulated MSCs (ALS and HC) and MSCs treated with proinflammatory cytokines (ALSc and HCc). Flow cytometry analysis of CD11a, CD11b, CD44, CD45, CD86, CD90, CD105, CD106 and PD-L1 molecules expressed by MSCs from ALS (orange curve), HC (green curve), ALSc (red curve) or HCc (blue curve) groups in comparison with control unlabeled MSCs (grey tinted curve). Histograms of one representative MSC sample for each group are shown

with LPS. Untreated MSCs and MSCs treated with cytokines significantly increased the levels of IL-10 and decreased production of TNF-α (Fig. 2). However, no differences in the effect on production of IL-10 were detected between the ALS and HC group (Fig. 2a). On the other hand, statistically significant differences in the effect on production of TNF-α were observed between the ALS and HC group, but not between the ALSc and HCc group. MSCs from ALS group decreased production of TNF-α more strongly than MSCs from the HC group (Fig. 2b). Unstimulated and LPS-treated MSCs from ALS and HC groups produced only very low levels of IL-10 (up to 29 pg/ml) and TNF-α (up to 12 pg/ml).

Effects of MSCs on the Proportion of Activated $CD4^+$ and $CD8^+$ T Lymphocytes

Molecule CD25 is an important marker of T lymphocyte activation. Using flow cytometry analysis, we observed a

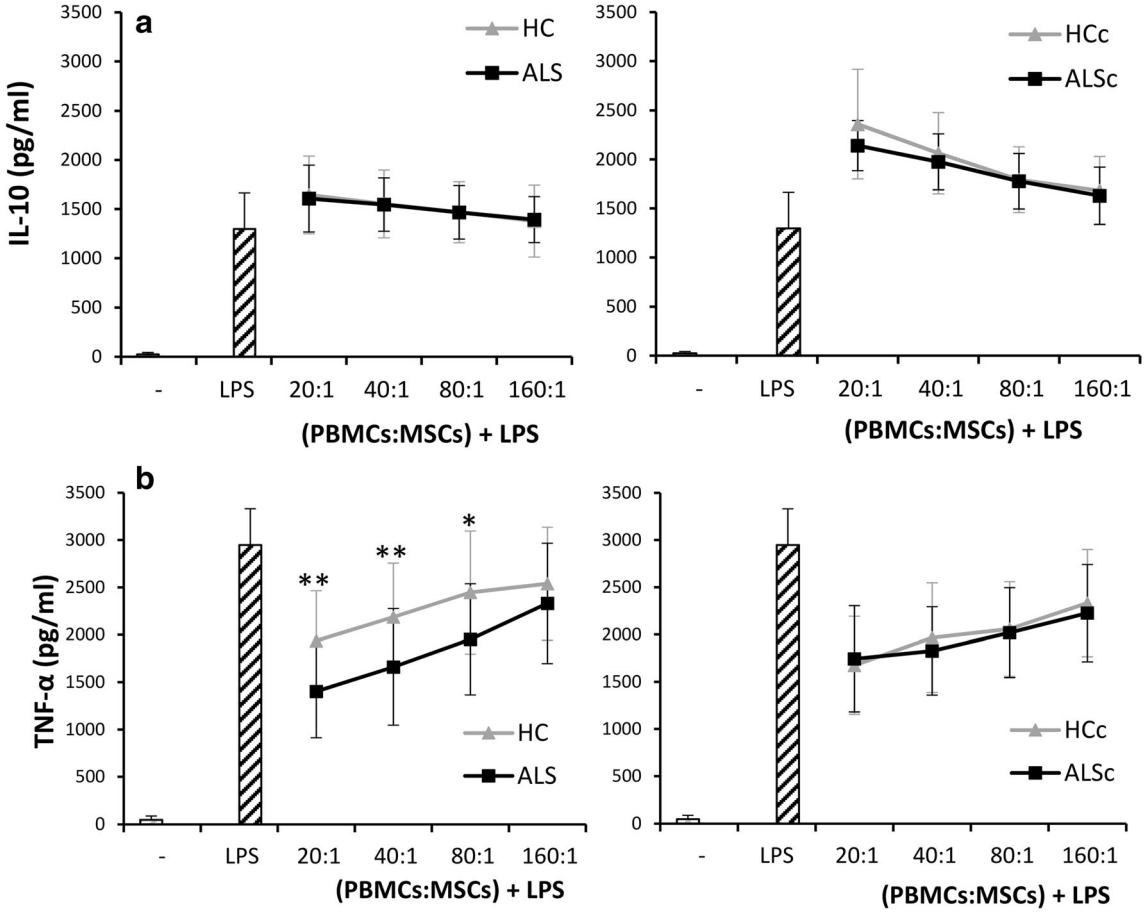

Fig. 2 Effect of MSCs on IL-10 and TNF-α production by PBMCs stimulated with LPS. PBMCs were cultured for 48 h unstimulated (−), stimulated with LPS (LPS) or stimulated with LPS in the presence of MSCs at ratios 20:1, 40:1, 80:1 and 160:1. The production of IL-10 **(a)** and TNF-α **(b)** was tested by ELISA in PBMC cultures containing MSCs from ALS groups (ALS and ALSc) and HC groups (HC and HCc). Values with asterisks show significant difference (*$p < 0.05$, **$p < 0.01$) between the effect of MSCs from ALS ($n = 14$) and HC ($n = 14$) groups, and each point or bar represents the mean ± SD

statistically significant decrease in a proportion of $CD4^+$ and $CD8^+$ T lymphocytes expressing CD25 within PBMCs stimulated with PHA and cultured with MSCs. No significant differences were detected between the effect of MSCs from the ALS and HC group on the percentage of activated $CD4^+$ T lymphocytes (Fig. 3a). On the other hand, statistically significant differences between the ALSc and HCc groups but not between the ALS and HC groups were observed in the effect of MSCs on the percentage of $CD8^+CD25^+$ T lymphocytes. In this respect, MSCs from the ALS groups were less inhibitory than MSCs from the HC groups (Fig. 3b).

MSCs Decrease the Percentage of Th1 Lymphocytes and Production of IFN-γ

Since Th lymphocytes are involved in ALS pathogenesis, the effects of MSCs on the balance between Th1, Th2 and Th17 lymphocytes were tested. MSCs from both the ALS and HC group significantly decreased the percentage of T-BET^+ Th1 lymphocytes, but significant differences between the ALS and HC groups were not detected (Fig. 4a). Moreover, MSCs from both the ALS and HC groups significantly decreased production of IFN-γ by PBMCs stimulated with PHA, but no significant differences between the effect of MSCs from the ALS and HC groups were observed (Fig. 4b and c).

Effect of MSCs on the Percentage of Th2 Lymphocytes and Production of IL-10

MSCs from both the ALS and HC group significantly increased the percentage of GATA-3^+ Th2 lymphocytes, but no significant differences in the effects of MSCs were detected between the ALS and HC groups (Fig. 5a). In accordance with these findings, MSCs from the ALS and HC groups increased production of IL-10 by PBMCs stimulated with PHA, but no significant differences between the ALS and HC groups were found (Fig. 5b and c).

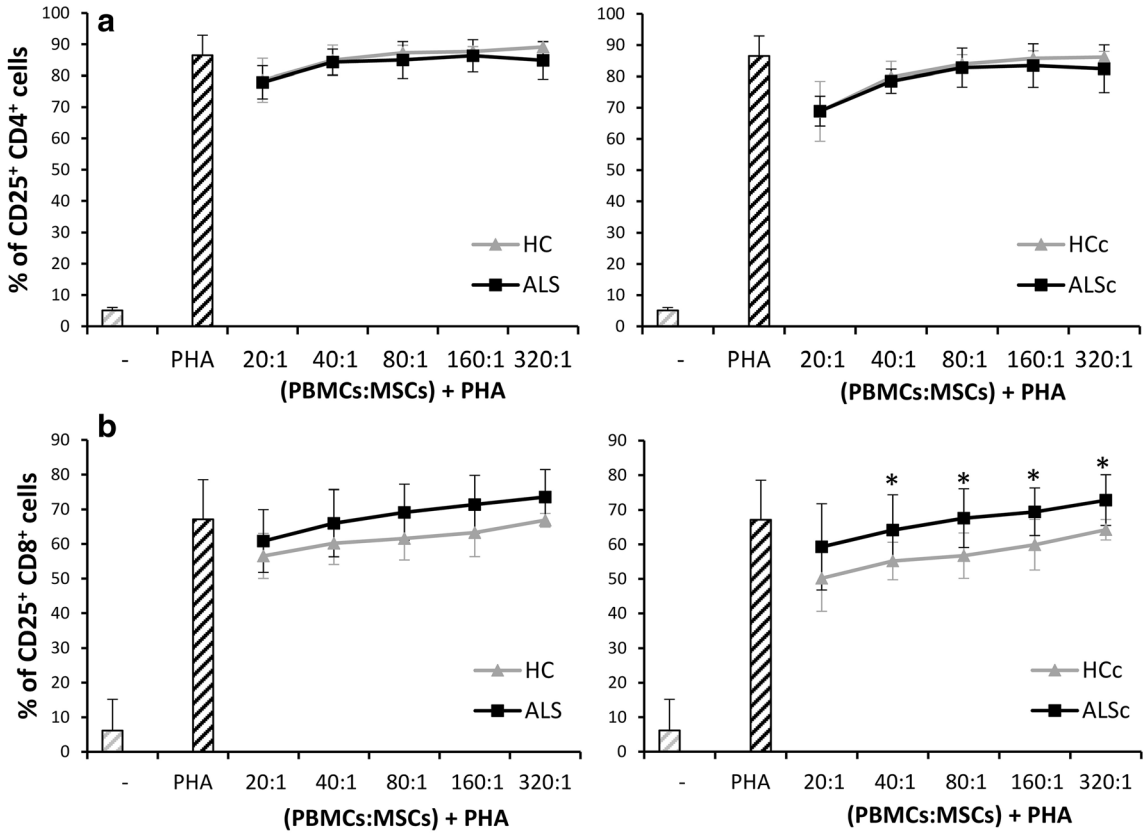

Fig. 3 Effect of MSCs on the percentage of activated $CD4^+$ and $CD8^+$ T lymphocytes. PBMCs were cultured for 72 h unstimulated (−), stimulated with PHA (PHA) or stimulated with PHA in the presence of MSCs at ratios 20:1, 40:1, 80:1, 160:1 and 320:1. Effect of MSCs from ALS groups (ALS and ALSc) and HC groups (HC and HCc) on the percentage of $CD25^+$ $CD4^+$ **(a)** or $CD25^+$ $CD8^+$ **(b)** T lymphocytes was tested using flow cytometry (expressed as a percentage of $CD25^+$ cells gated on $CD4^+$ or $CD8^+$ cells). Values with an asterisk show significant difference ($*p < 0.05$) between the effect of MSCs from ALSc ($n = 14$) and HCc ($n = 14$) groups, and each point or bar represents the mean ± SD

Effect of MSCs on the Percentage of $CD4^+FOXP3^+$ T Lymphocytes

MSCs from ALS, HC and HCc groups significantly increased the percentage of $FOXP3^+$ Treg lymphocytes in cultures of PBMCs stimulated with PHA. Statistically significant differences in the effects of MSCs on the percentage of $CD4^+FOXP3^+$ T lymphocytes were found between the ALSc and HCc groups. MSCs from the ALSc group did not increase the percentage of $CD4^+FOXP3^+$ T lymphocytes (Fig. 6a).

The Effects of MSCs on the Percentage of Th17 Lymphocytes and Expression of Gene for IL-17

The percentage of $ROR\gamma t^+$ Th17 lymphocytes was significantly decreased only in cultures containing MSCs from groups treated with cytokines (ALSc and HCc), but no significant differences were detected between the ALSc and HCc groups (Fig. 6b). Moreover, neither a significant decrease of expression of gene for IL-17 nor a significant difference between ALS and HC groups in the effect of MSCs on IL-17 expression by stimulated PBMCs, were observed (Fig. 6c).

Discussion

Autologous BM-derived MSCs represent one of the most often considered cell types for potential cell-based therapy of ALS. The application of MSCs possessing sufficient therapeutic potential is a crucial factor for effective therapy. However, some previous studies of MSCs isolated from ALS patients have demonstrated diminished pluripotency and decreased capacity to produce trophic factors (Cho et al. 2010; Koh et al. 2012) and on the other hand unchanged karyotype, phenotype and growth properties (Ferrero et al. 2008). The results of these studies indicated, that ALS could influence some properties or functions of MSCs. Since aberrant immune reactions play an important role in ALS progression and immunomodulatory properties of MSCs could also be modified by ALS, the present study was designed to evaluate the immunomodulatory

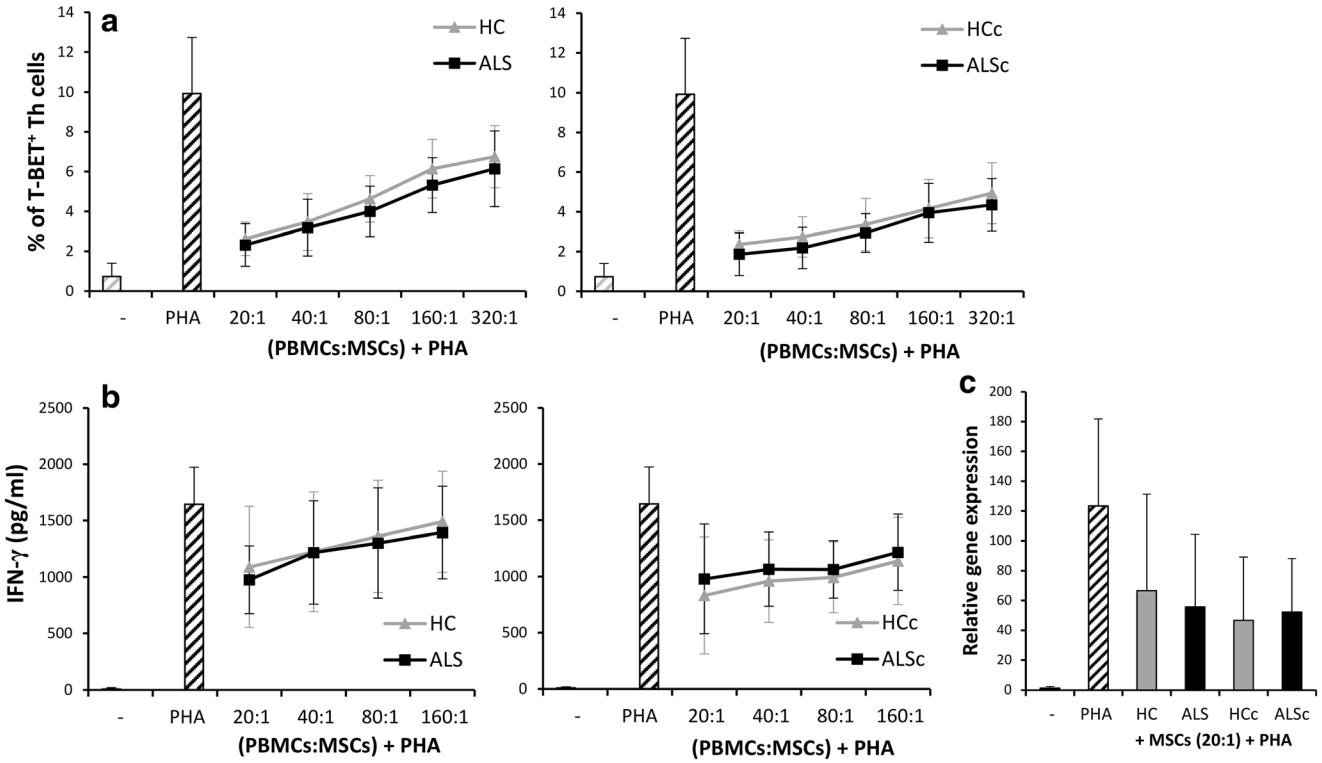

Fig. 4 Influence of MSCs on the percentage of Th1 lymphocytes and production or expression of IFN-γ. PBMCs were cultured for 72 h unstimulated (−), stimulated with PHA (PHA) or stimulated with PHA in the presence of MSCs at ratios 20:1, 40:1, 80:1, 160:1 and 320:1. **a** Effect of MSCs from ALS groups (ALS and ALSc) and HC groups (HC and HCc) on the percentage of Th1 lymphocytes (expressed as a percentage of T-BET$^+$ cells gated on CD4$^+$ cells) was tested using flow cytometry. **b** Effect of MSCs on the production of IFN-γ by PBMCs was tested by ELISA. **C** Influence of MSCs on the expression of gene for IFN-γ by PBMCs was tested by real-time PCR. Each point or bar represents the mean ± SD ($n = 14$)

capacity of MSCs from patients suffering from ALS and healthy donors.

Since the properties of MSCs can be influenced by the inflammatory microenvironment (English et al. 2007; Javorkova et al. 2014), we also assessed MSCs treated with IFN-γ, TNF-α and IL-6, which are the main cytokines produced during the later neurotoxic phase of ALS (Hooten et al. 2015). The regulatory effects of MSCs were tested on mitogen-activated PBMCs isolated from healthy donors to mimic the conditions occurring during the neurotoxic phase of ALS.

Initially, we tested the effect of MSCs on LPS-stimulated PBMCs, because a shift from the M2 to M1 microglial response causes transposition to the neurotoxic microenvironment during ALS progression (Hooten et al. 2015). We tested the effects of MSCs on the production of IL-10 and TNF-α by PBMCs, which represent cytokines characteristic for M2 and M1 macrophages respectively (Mantovani et al. 2013). Untreated MSCs and MSCs treated with cytokines significantly increased level of IL-10, and on the other hand reduced production of TNF-α by PBMCs stimulated with LPS. These findings are in agreement with the observation that MSCs are able to turn activated macrophages into a regulatory-like profile (Maggini et al. 2010). However, significant differences between ALS and HC groups were not detected in the effect of MSCs on the production of IL-10. However, statistically significant differences between ALS and HC groups, but not between ALSc and HCc groups were found in the effect on production of TNF-α. MSCs from the ALS group decreased production of TNF-α more effectively than MSCs from the HC group. Interestingly, MSCs from the ALS, HCc and ALSc groups decreased production of TNF-α comparably. This finding suggests that MSCs from the ALS group could already be stimulated in the organism of the patient as a reaction to the inflammatory environment. On the other hand, de Oliveira and colleagues did not detect significant differences in TNF-α levels in supernatants derived from cocultures of PBMCs with MSCs isolated from healthy donors and patients suffering from multiple sclerosis (MS). These authors rather observed in MSCs from MS patients a trend to increase TNF-α production and significantly decrease production of anti-inflammatory cytokines IL-10 and transforming growth factor-β (de Oliveira et al. 2015).

Next, we evaluated the changes in a proportion of activated CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T lymphocytes in a population

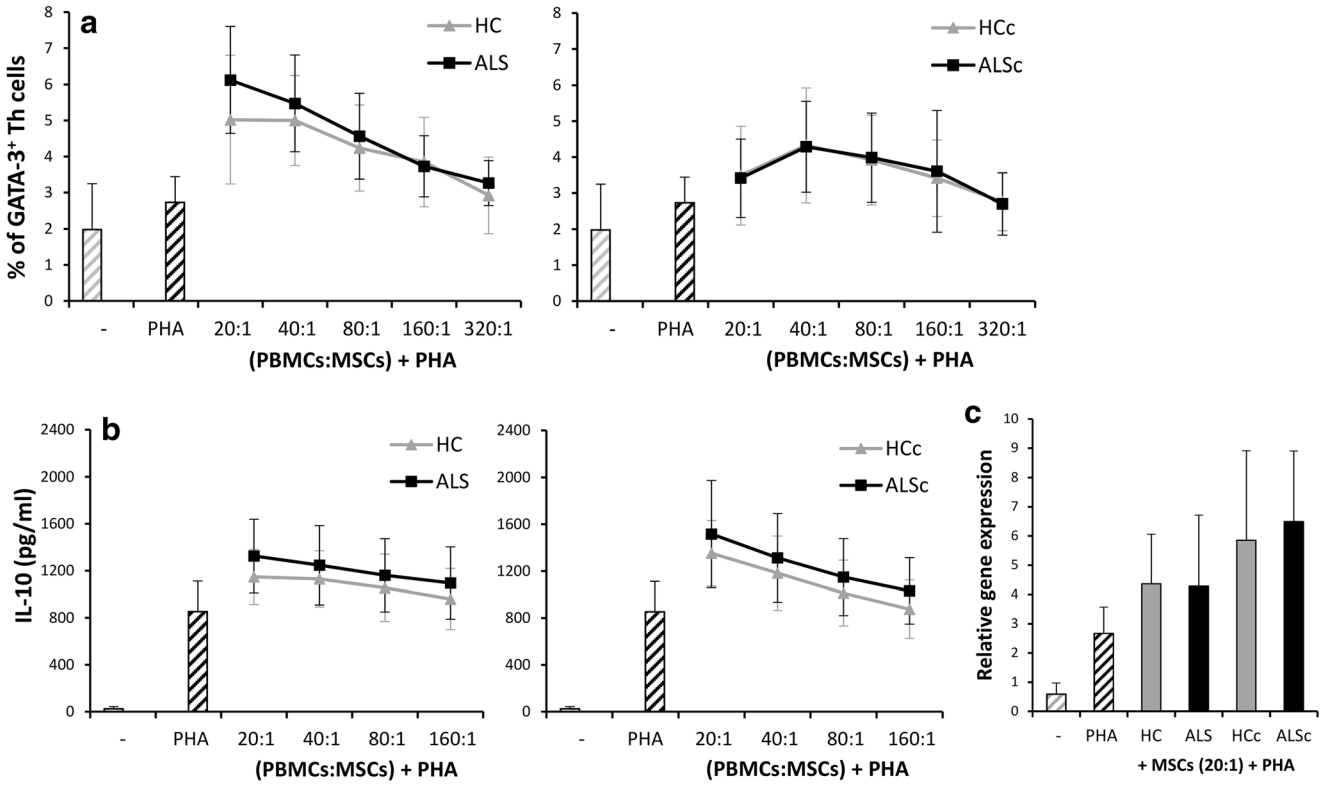

Fig. 5 Effect of MSCs on the percentage of Th2 lymphocytes and production or expression of IL-10. PBMCs were cultured for 72 h unstimulated (−), stimulated with PHA (PHA) or stimulated with PHA in the presence of MSCs at ratios 20:1, 40:1, 80:1, 160:1 and 320:1. **a** Effect of MSCs from ALS groups (ALS and ALSc) and HC groups (HC and HCc) on the percentage of Th2 lymphocytes (expressed as percentage of GATA-3$^+$ cells gated on CD4$^+$ cells) was tested using flow cytometry. **b** Effect of MSCs on production of IL-10 by PBMCs was tested by ELISA. **C** Influence of MSCs on the expression of gene for IL-10 by PBMCs stimulated with PHA was tested by real-time PCR. Each point or bar represents the mean ± SD (n = 14)

of PBMCs stimulated with PHA in the presence of MSCs. The molecule CD25 is an α-subunit of IL-2 receptor and represents an important marker of T lymphocyte activation (Schuurman et al. 1989). Activated CD8$^+$ cytotoxic T lymphocytes belong to immune cell types participating in the late neurotoxic phase of ALS (Hooten et al. 2015). Statistically significant differences in the effects of MSCs on the proportion of activated CD8$^+$ T lymphocytes were found between the ALSc and HCc group, but not between the ALS and HC group. MSCs from the HCc group decreased the percentage of activated CD8$^+$ T lymphocytes more effectively than MSCs from the ALSc group. On the other hand, no significant differences between the effects of MSCs from the ALS and HC group on the proportion of CD4$^+$CD25$^+$ T lymphocytes were found. The ability of MSCs to inhibit T lymphocyte activation has been well documented (Le Blanc et al. 2004). Our results are in agreement with the findings of Skalska and Kontny, who showed that MSCs from patients suffering from rheumatoid arthritis (RA) and control MSCs from osteoarthritis patients comparably affected the percentage of CD25$^+$ T lymphocytes (Skalska and Kontny 2016).

Finally, the effects of MSCs on the percentage of Th1, Th2, Th17 and CD4$^+$FOXP3$^+$ T lymphocytes, and on the production of IFN-γ, IL-10 and IL-17 by mitogen-stimulated PBMCs were assessed. Since in ALS pathogenesis, Th2 and Treg lymphocytes mediate neuroprotective, and Th1 and Th17 lymphocytes neurotoxic immune reactions (Hooten et al. 2015), the effects of MSCs on the balance between these CD4$^+$ lymphocyte populations represent an important way to slow-down the progression of this disease. MSCs from both the ALS and HC groups significantly decreased the percentage of T-BET$^+$ Th1 and RORγt$^+$ Th17 lymphocytes, and elevated the percentage of GATA-3$^+$ Th2 and CD4$^+$FOXP3$^+$ T lymphocytes. These findings are in agreement with the described ability of MSCs to support anti-inflammatory responses mediated by the Th2 and Treg lymphocytes, and to suppress proinflammatory Th1 and Th17 responses (Aggarwal and Pittenger 2005; Ghannam et al. 2010). We did not find any significant differences between the effects of MSCs from the ALS and HC group on the percentage of Th1, Th2 and Th17 lymphocytes and production of IFN-γ, IL-10 and IL-17. These results are in agreement with the findings of Skalska and Contny, who showed that MSCs from patients suffering from RA and control MSCs from patients with osteoarthritis had comparable effects on the production of IFN-γ, IL-10 and IL-17 (Skalska and Kontny 2016).

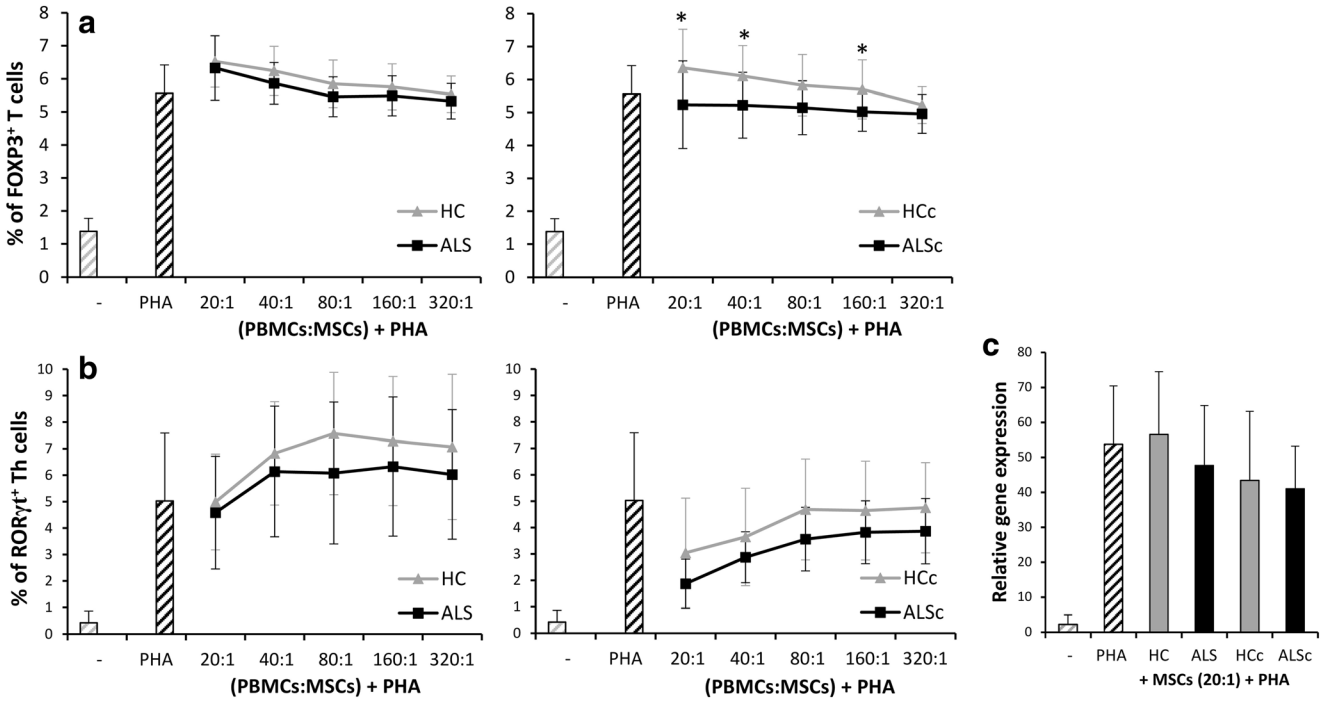

Fig. 6 Effect of MSCs on the percentage of $CD4^+FOXP3^+$ T cells and Th17 lymphocytes and expression of *IL-17* gene. PBMCs were cultured for 72 h unstimulated (−), stimulated with PHA (PHA) or stimulated with PHA in the presence of MSCs at ratios 20:1, 40:1, 80:1, 160:1 and 320:1. The effect of MSCs from ALS groups (ALS and ALSc) and HC groups (HC and HCc) on the percentage of $CD4^+FOXP3^+$ lymphocytes (expressed as a percentage of $FOXP3^+$ cells gated on $CD4^+$ cells) **(a)** or percentage of Th17 lymphocytes (expressed as a percentage of $ROR\gamma t^+$ cells gated on $CD4^+$ cells) **(b)** was tested using flow cytometry. **C** Effect of MSCs on expression of gene for IL-17 by PBMCs stimulated with PHA was tested by real-time PCR. Values with an asterisk represent significant difference ($*p < 0.05$) between the effect of MSCs from ALSc ($n = 14$) and HCc ($n = 14$) groups. Each point or bar represents the mean ± SD

However, impaired capacity to inhibit Th17 lymphocyte polarization was described in MSCs from RA patients (Sun et al. 2015). Nevertheless, we detected significant differences between the effects of MSCs from the ALSc and HCc group on the percentage of $CD4^+FOXP3^+$ T lymphocytes, and only MSCs from the HCc group significantly increased the percentage of $CD4^+FOXP3^+$ T lymphocytes. Another study testing the effect of MSCs from MS and RA patients on the development of Treg lymphocytes showed comparable results (de Oliveira et al. 2015; Sun et al. 2015).

In this study, we demonstrated that MSCs from patients suffering from ALS exerted comparable immunomodulatory effects to MSCs from healthy donors. Nevertheless, we detected in MSCs from the ALSc group an impaired capability to decrease the percentage of activated $CD8^+$ T lymphocytes, and even an inability to upregulate a proportion of $CD4^+FOXP3^+$ T lymphocytes. This suggests that proinflammatory cytokines could negatively influence at least some immunomodulatory properties of a patient's MSCs.

Conclusion

Our findings demonstrate that MSCs from patients suffering from ALS exert comparable immunoregulatory properties to MSCs from healthy donors. Nevertheless, the treatment of MSCs from ALS patients with proinflammatory cytokines reduced some of their immunomodulatory effects. On the basis of these results we conclude, that MSCs from ALS patients possess comparable beneficial potential for therapy of ALS to MSCs from healthy donors, however additional studies investigating more precise impacts of the proinflammatory microenvironment on the effectiveness of therapy with autologous MSCs from ALS patients would be desirable.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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