Clinical and Experimental Health Sciences

Original Article / *Özgün Araştırma*

Dental Follicle Mesenchymal Stem Cells Enhance CD4+Foxp3+ Regulatory T Cells in the Lymphocytes of Amyotrophic Lateral Sclerosis Patients

Dental Folikül Mezenkimal Kök Hücreleri Amyotrofik Lateral Skleroz Hastalarının Lenfositlerinde CD4+Foxp3+ T-Regülatör Hücreleri Arttırdı

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Abstract

Objective: Amyotrophic lateral sclerosis (ALS) is a disorder that causes the degeneration of motor neurons. Currently, riluzole is the only effective drug used to treat ALS; however, it has limited clinical benefits. Stem cell-based therapy has been studied as a potential novel treatment strategy for ALS and has shown to have an anti-inflammatory effects when treating this disease. In this study, we studied the immunosuppressive effect of dental follicle mesenchymal stem cells (DFSCs) on peripheral blood mononuclear cells (PBMCs) of ALS patients.

Methods: DFSCs were isolated from the third molar teeth of healthy individuals, and cells were seeded in the 48 well plate 48 hours prior to PBMC isolation. PBMCs were isolated from venous blood samples of ALS patients and healthy volunteers and were cultured in the presence or absence of DFSCs. After 72 h of culture period lymphocyte proliferation, apoptosis and CD4+FoxP3+ regulatory T-cell ratios were analyzed.

Results: Analysis revealed an increase in the number of CD4+FoxP3+ regulatory T cells and a decrease in the proliferative responses of lymphocytes with DFSCs. In addition, DFSCs enhanced the apoptotic effect of the lymphocytes of ALS patients, but increased cell survival in healthy individuals.

Conclusion: Our study showed that DFSCs regulate inflammatory responses of lymphocytes in ALS patients and that they can be a novel therapeutic approach for treating neuroinflammatory diseases including ALS.

Keywords: Amyotrophic lateral sclerosis, dental follicle mesenchymal stem cells, immunosuppression

Öz

Amaç: Amyotrofik lateral skleroz (ALS) motor nöronların dejenerasyonuna sebep olan bir hastalıktır. Günümüzde ALS tedavisinde kullanılan tek ilaç riluzoldür; ancak bu ilacın faydası sınırlıdır. Kök hücre temelli tedaviler ALS için yeni bir tedavi seçeneğidir ve bu hastalık üzerin anti-inflamatuar etkisi olduğu gösterilmiştir. Bu çalışmada dental folikül mezenkimal kök hücrelerin (DFSCs) ALS hastalarından izole edilen periferal kan mononükleer hücreler (PBMC) üzerindeki immün baskılayıcı etkisi araştırılmıştır.

Yöntemler: Sağlıklı bireylerin molar dişlerinden izole edilen DFSCs'ler PBMC izolasyonundan 48 saat önce 48 kuyulu hücre kültür plaklarına ekildi. ALS hastalarının ve sağlıklı bireylerin venöz kan örneklerinden PBMC izolasyonu yapıldı ve DFSCs'nin varlığında ve yokluğunda kültürü yapıldı. 72 saatlik kültür süresinin sonunda lenfosit proliferasyonu, apoptoz ve CD4+-FoxP3+ regulator T hücre oranları analiz edildi.

Bulgular: DFSCs ile birlikte kültürü yapılan ALS hastalarının lenfositlerinde CD4+FoxP3+ regulator T hücre oranlarının arttığı ve lenfosit proliferasyonunda azalma olduğu gösterilmiştir. Bunun yanı sıra DFSCs'ler ALS hastalarının lenfositlerinde apoptotik etkiyi arttırırken sağlıklı bireylerin lenfositlerinde hücre canlılığını koruduğu gözlemlenmiştir.

Sonuç: Çalışmamızda DFSCs'in ALS hastalarının lenfositlerinde inflamatuar yanıt üzerinde baskılayıcı etkisi olduğu gösterilmiştir ve ALS dahil diğer nöroinflamatuar hastalıkların tedavisinde yeni bir seçenek olarak kullanılabilir.

Anahtar kelimeler: Amyotrofik lateral skleroz, dental folikül mezenkimal kök hücreler, immün baskılama

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the degeneration of motor neurons. Symptoms include muscle atrophy, weakness, and spasticity (1). Its worldwide incidence is 2-3 per 100,000 individuals, and its prevalence is 4–6 per 100,000 individuals (2). Approximately 90% of ALS cases are sporadic and 10% are familial. Patients generally die 3–5 years after the diagnosis is made because of progressive motor neuron loss and voluntary muscle weakness. The primary cause of death in ALS is weakness of the breathing muscles (3). The pathogenesis of ALS depends on numerous genetic factors. In its familial forms, several genetic mutations have been associated with the disease: mutations in copper superoxide dismutase (SOD1), the C9ORF72 gene, and TAR DNA-binding protein 43 (TDP-43) (4). Recently, studies have revealed that two main processes are responsible for the progression of ALS: autophagy and inflammation. In the sporadic form of ALS, neurodegeneration may result from protein aggregation, glutamate-mediated excitotoxicity, mitochondrial dysfunction, oxidative stress, deficiency in neurotrophic factors that leads to the intracellular accumulation of neurofilaments, and the interaction of multiple cell types (5). Riluzole is the only effective drug; it extends survival by a few months, but it has limited clinical benefits (6).

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Stem cell therapy is a potential option for treating ALS. It has been demonstrated that mesenchymal stem cells (MSCs) could repair degenerated cells of an injured central nervous system (CNS) and reverse inflammation due to their capacity to modulate the immune response (7). MSCs regulate effector functions of adaptive and innate immune cells through the release of soluble factors such as prostaglandin E2, nitric oxide, and indoleamine 2,3- dioxygenase (IDO) (8). Additionally, MSCs can protect axons, improve neuronal survival (9, 10), and have antiapoptotic effects on neurons (11). MSCs are a valuable source of cells for cell replacement therapy. Locally or systemically transplanted MSCs are able to migrate to the inflamed area and exert their desired effect when treating neurodegenerative diseases (10). Recent studies have proposed MSCs, neural stem cells, glial-restricted progenitor cells, embryonic stem cells, and induced pluripotent stem cells for the treatment of ALS (12). Currently, several MSC sources are available and may take place for the replacement and anti-inflammatory treatment in neurodegenerative diseases. Dental follicle MSCs (DFSCs) may be an option for the regenerative and anti-inflammatory treatment of ALS because of their potential to differentiate into neuron-like cells (13) and their high ability in the immunosuppression against inflammatory responses. In this study, we focused on DFSCs and investigated their immunosuppressive effect on peripheral blood mononuclear cells (PBMCs) of ALS patients.

METHODS

Patient Selection

Patients fulfilling the following three inclusion criteria were enrolled in the study: those who 1) met the revised El Escorial criteria for definite or probable ALS, 2) were men or non-pregnant women aged ≥18 years, and 3) had a progressive course not longer than three years prior to the first diagnosis. ALS patients with end-stage organ failure or any other disease that could interfere with the ability to interpret the results, patients with an active infection, and patients with cognitive decline who were unable to understand and sign the informed consent form were excluded. Patients with tracheostomal ventilation or noninvasive ventilation were also excluded. All patients received ordinary medical treatment, including riluzole (50 mg twice daily), for at least three months prior to screening.

Isolation of DFSCs

Dental follicles (DFs) were collected from the Marmara University Faculty of Dentistry. Patients provided informed consent according to the guidelines of the Ethics Committee of Marmara University Ethical Committee. DFs were obtained from three volunteers who had indications for undergoing wisdom tooth extraction, were aged between 19 and 25 years, and had no inflammatory diseases. The protocol for isolating MSCs was performed as previously described (14). Briefly, DF tissue was transferred in phosphate-buffered saline containing 1% penicillin/streptomycin (Gibco™, USA). In the first step, follicles were cut into 0.1–0.5-mm pieces and enzymatically digested in 3 mg/mL collagenase type I (Gibco™, USA) for 45 min at 37°C. The medium was prepared with 5 mL Dulbecco's modified Eagle's medium (DMEM; Gibco™, USA) supplemented with 10% fetal bovine serum (FBS, Gibco™, USA) and 1% penicillin/streptomycin and was named as complete DMEM (CDMEM). After the inactivation of collagenase with CDMEM, the cell suspension was centrifuged and cell pellets were obtained with the aspiration of the supernatant. DFSCs were transferred to T-25 flasks containing 5×10^3 cells/cm^2 and were incubated in a 5% CO_2 atmosphere at 37°C in CDMEM. Fibroblast-like cells were allowed to reach 80–90% confluence by changing the culture medium that was freshly prepared twice a week. The cells were detached with 0.25% trypsin-EDTA (Gibco™, USA) until the third passage. DFSCs were analyzed for positive and negative marker expression that characterized MSCs using flow cytometry (FACSCalibur; BD Biosciences, USA).

Flow Cytometry Analysis

Third-passage DFSCs were incubated with the antibodies of positive markers for MSCs of human CD29 allophycocyanin (APC), CD73 phycoerythrin (PE), CD105 PE, and CD146 fluorescein isothiocyanate (FITC) and with the antibodies of negative markers of CD14 PE, CD34 FITC, CD45 APC, and HLA-DR PE (BD Biosciences, USA) for 15 min at room temperature in the dark. Flow cytometry results were analyzed using FACSCalibur (BD Biosciences).

Differentiation Potential of DFSCs

To induce osteogenic, adipogenic, and chondrogenic differentiation, human MSC stimulatory kits (StemPro) were used. The differentiation assay was performed in six-well plates (5×10^4 cells/well), and differentiation media were prepared according to the manufacturer's instructions and changed three times a week. After 21 days, osteocytes, adipocytes, and chondrocytes were stained with alizarin red, Oil Red O, and alcian blue, respectively, and cell types were evaluated under a microscope.

PBMC Isolation

Peripheral blood samples were collected from five ALS patients aged 25–40 years and five healthy individuals without an autoimmune or inflammatory disease (control group).

Peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare Biosciences) density gradient centrifugation of heparinized peripheral blood samples. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. PBMCs were stimulated with anti-CD3 (0.5 µg/mL; Life Span Biosciences, USA) and anti-CD28 (1 µg/mL; Millipore, California) (CDmix) at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h.

Coculture of PBMCs with DFSCs

Dental follicle mesenchymal stem cells (5×10^4 cells/well in a 48-well plate) were plated 48 h prior to the addition of PBMCs to the culture medium. PBMCs were stimulated using 0.5 µg/mL the CDmix and cultured with PBMCs: DFSCs at a 1:10 ratio and without DFSCs. After a 72-h incubation period, lymphocyte proliferation (carboxyfluorescein succinimidyl ester, CFSE), apoptosis [annexin V/propidium iodide (PI)], and the $CD4^+CD25^+FoxP3^+$ regulatory T-cell (Treg) ratio were analyzed.

Labeling of CFSE and Evaluation of Lymphocyte Proliferation

The proliferation of lymphocytes was quantified using CFSE (Invitrogen, USA). Cells were labeled with 10 µM CFSE and cultured for 72 h unstimulated or stimulated with CDmix in the presence and absence of DFSCs. After the incubation period, lymphocytes were analyzed via flow cytometry (FACSCalibur).

Detection of Apoptosis of Lymphocytes by Annexin V/PI

After 72 h of culture, the apoptosis ratio of lymphocytes was quantified using an annexin V/PI kit (eBiosciences, USA), according to the manufacturer's instructions. The kit included annexin V-FITC and PI.

$CD4^+CD25^+FoxP3^+$ Treg Ratio

$CD4^+CD25^+FoxP3^+$ Tregs were quantified using a human FoxP3 staining kit (eBioscience). The percentage of FoxP3 expressing $CD4^+CD25^+$ Tregs in PBMC cultures was analyzed via flow cytometry. The kit included antihuman CD4 (FITC), antihuman CD25 (APC), and antihuman FoxP3 (PE) (eBioscience).

Statistical Analyses

Differences between the groups were analyzed via one-way ANOVA using GraphPad Prism 6 software. P values less than 0.05 were considered significant.

RESULTS

Isolation, Characterization, and Differentiation of DFSCs

Dental follicle mesenchymal stem cells were attached and formed fibroblast-like colonies during the early days of incubation (P0; Figure 1a) and reached 80% confluency in 5–6 days after being plated. The later passages were continued culturing and formed the same morphology with the increased doubling time (P1, P2, P3; Figure 1a). In the third passage, the immunophenotyping and differentiation potential of cells to osteogenic, chondrogenic, and adipogenic lineages were analyzed.

DFSCs were analyzed by flow cytometry and evaluated with positive markers for CD29, CD73, CD105, and CD146 and were negative for CD14, CD34, CD45, and HLA-DR (Figure 1b).

Dental follicle mesenchymal stem cells were differentiated into osteocytes, adipocytes, and chondrocytes. The potential of osteogenic differentiation was evaluated after 21 days of incubation with the osteogenic stimulants (Stempro®, Thermo Fisher) and stained with alizarin red at the end of the incubation period. Calcium phosphate deposits were formed in the intracellular matrix. The adipogenic differentiation capability was evaluated by culturing the cells in adipogenic induction medium (Stempro®) for 14 days and Oil Red O and hematoxylin-eosin staining. Intracellular oil droplets were observed at the end of the culture period. The chondrogenic differentiation potential was evaluated by culturing with a chondrogenic stimulation kit (Stempro®) in vitro during the culture period, and cell differentiation into chondrocytes and intracellular proteoglycans were observed (Figure 1c).

DFSCs Suppressed Lymphocyte Proliferation in ALS patients

Lymphocyte proliferation was analyzed via flow cytometry. In the CFSE labeling assay, lymphocyte proliferation was evaluated for a 72-h incubation period. The proliferation of CDmix-stimulated lymphocytes was significantly increased in ALS patients compared with healthy individuals ($p<0.05$). DFSCs significantly suppressed the proliferation of lymphocytes in the cocultures of CDmix-stimulated PBMCs in ALS patients ($p<0.05$) (Figure 2).

DFSC-Enhanced Lymphocyte Apoptosis in ALS Patients

Annexin V and PI percent of the lymphocytes was analyzed for apoptosis and cell viability via flow cytometry. The acceleratory effect of DFSCs on the apoptotic signals on the lymphocytes was significant. The apoptosis rate of the lymphocytes stimulated with the CDmix was significantly lower in the PBMCs of ALS patients compared with healthy individuals ($p<0.05$). The apoptosis rate of the lymphocytes stimulated with the CDmix was significantly increased when lymphocytes were cocultured with DFSCs ($p<0.05$). In contrast, DFSCs decreased the apoptotic rate of lymphocytes in healthy individuals (Figure 3).

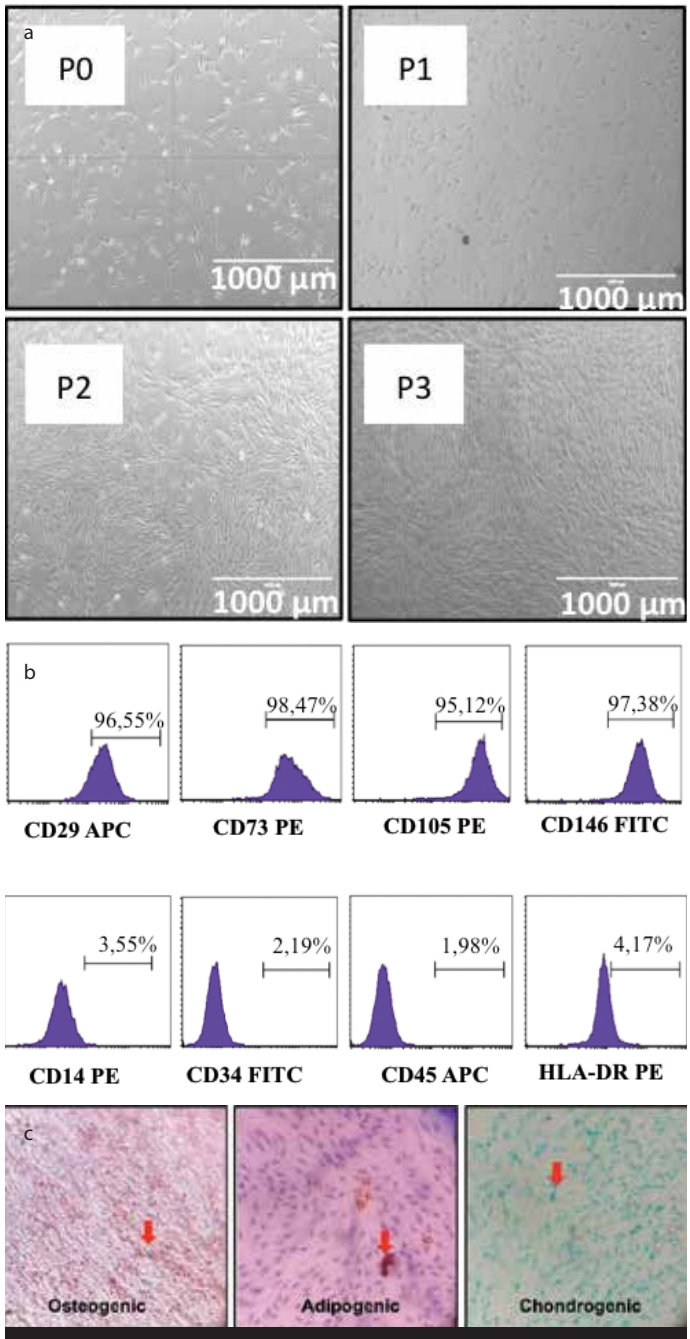

Figure 1. a-c. Isolation, characterization, and differentiation of DFSCs. (a) DFSCs formed fibroblast-like colonies and attached to the plastic surface. P0: initial culture of DFSCs; P1: passage 1; P2: passage 2; P3: passage 3 images of invert microscopy. (b) DFSCs were expressed the positive markers of CD29, CD73, CD105, and CD146 and lacked the expression of negative markers CD14, CD34, CD45, and HLA-DR. (c) DFSCs differentiated into osteoblasts and formed intracellular calcium phosphate deposits. Adipocytes were formed, and oil droplets were seen with adipogenic induction after 14 days. Chondrocytes and proteoglycans were seen by staining the cell layers with alcian blue
DFSCs: dental follicle mesenchymal stem cells

DFSC Increased the $CD4^+CD25^+FoxP3^+$ Treg Ratio in ALS Patients

We studied the effects of DFSCs on the Treg ratio in the PBMCs of ALS patients The $CD4^+CD25^+FoxP3^+$ Treg ratio was lower in the PBMC

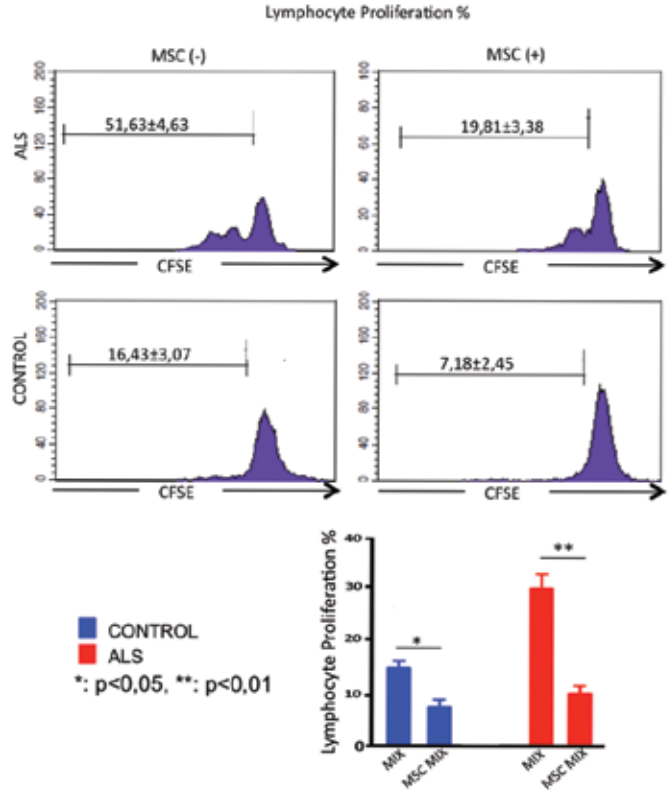

Figure 2. Lymphocyte proliferation. DFSCs significantly suppressed the proliferation of lymphocytes isolated from the peripheral blood of ALS patients compared with PBMC cultures without stem cells ($p<0.05$)
DFSCs: dental follicle mesenchymal stem cells; ALS: amyotrophic lateral sclerosis

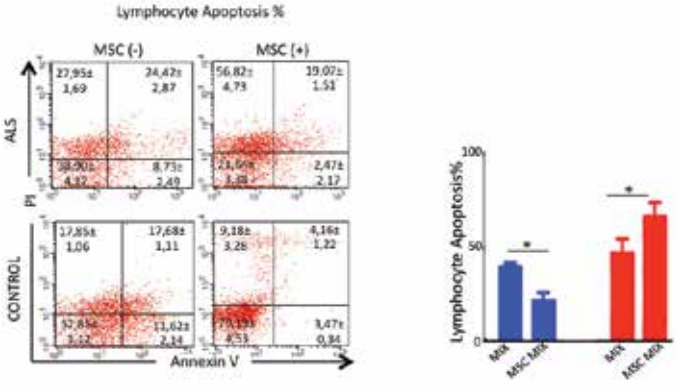

Figure 3. Lymphocyte apoptosis. DFSCs significantly enhanced the apoptosis of lymphocytes in ALS patients compared to PBMC cultures without DFSCs ($p<0.05$), whereas they increased viability in healthy individuals
DFSCs: dental follicle mesenchymal stem cells; ALS: amyotrophic lateral sclerosis; PBMC: peripheral blood mononuclear cell

cultures of ALS patients than those of healthy individuals. DFSCs increased the $CD4^+CD25^+FoxP3^+$ Treg ratio significantly compared with the PBMC cultures of ALS patients ($p<0.05$). There was a difference between ALS patients and healthy individuals in the cocultures of PBMCs and DFSCs. DFSCs significantly increased the FoxP3-expressing Treg ratio in ALS patients compared with the healthy group ($p<0.05$) (Figure 4).

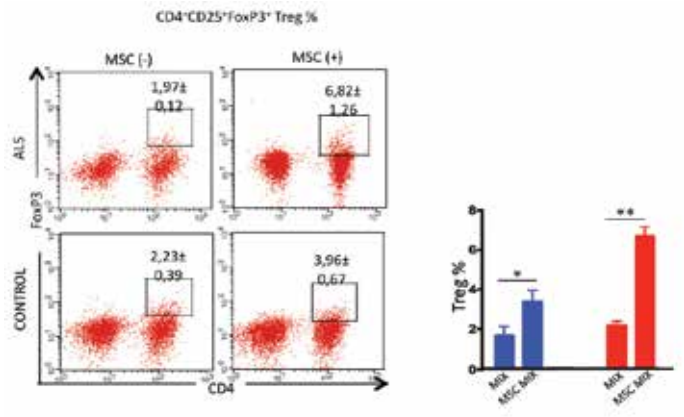

Figure 4. CD4+CD25+FoxP3+ Treg ratio. The CD4+CD25+FoxP3+ Treg ratio was lower in ALS patients than in healthy individuals in PBMCs cultures without stem cells. DFSCs significantly increased the CD4+CD25+FoxP3+ Treg ratio in the PBMCs of ALS patients ($p<0.05$).
DFSCs: dental follicle mesenchymal stem cells; ALS: amyotrophic lateral sclerosis; PBMC: peripheral blood mononuclear cell

DISCUSSION

Neurodegenerative diseases including Parkinson's disease, Alzheimer's disease AD, and ALS are affected by the neuroinflammatory response that results in microglial activation and the death of motor neurons (15). In the spinal cords of ALS patients, T-cell infiltrates were found in the corticospinal tract (16, 17). The interest in cellular therapy from various sources is increasing in the treatment of ALS (18). MSCs respond to activated immune cells by secreting high levels of soluble factors such as IDO and prostaglandin E2, which induces Tregs to suppress T-cell proliferation (19). DFSCs are advantageous; they can be easily isolated and coming from neural crest, which makes them ideal candidates for cell-based therapy for neurodegenerative diseases (20). The immunosuppressive effects of DFSCs (DPSCs, SHED, and DFSCs) have been reported with our previous studies (14). In this study, we isolated and used DFSCs because of their potential immunosuppressive effects. We investigated the effects of DFSCs on PBMCs isolated from ALS patients.

According to the minimal criteria for MSCs, cells should adhere to plastic surfaces in standard culture conditions and must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14, and HLA-DR. MSCs should be able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* (21). We have isolated DFSCs, and they remained adherent to plastic surface and seem as fibroblast-like cells. The expression of CD29, CD73, CD105, and CD146 was over 95%, and CD14, CD34, CD20, and HLA-DR were not expressed. Attached cells were differentiated into osteogenic, chondrogenic and adipogenic lineages in vitro.

Activated T cells are able to exceed into the CNS where they perform immune surveillance, but a healthy CNS is the immune privilege region and the cerebrospinal fluid contains few innate immune cells (22). Once T cells are present within the extracellular space of the CNS, they secrete inflammatory mediators to contribute to neuroinflammatory responses (23, 24). In ALS, secreted SOD1 is presented by MHC II by antigen-presenting cells, causing the activation and proliferation of $CD4^+$ T cells (25). In this study, we studied the effects of DFSCs on the proliferative response of $CD4^+$ T cells in the PBMCs of ALS patients. Compared with the PBMC cultures of healthy indi-

viduals. lymphocyte proliferation increased in those of ALS patients with. DFSCs significantly decreased lymphocyte proliferation in the cocultures of the PBMCs of ALS patients. These data demonstrate that DFSCs have antiproliferative effects on activated T cells.

Recently, studies have shown that $CD4^+CD25^+$ Tregs have important roles in the progression of ALS (26). The Treg ratio correlates with the disease progression rates. A study has suggested that increased levels of Tregs in ALS patients at the early phase of the disease aids patients for longer periods of time (27). In the present study, we investigated the Treg ratio in order to determine the immunosuppressive effect of DFSCs. The FoxP3-expressing $CD4^+$ Treg percent was significantly lower in ALS patients than in healthy individuals in the PBMC cultures. The number of Tregs in PBMCs significantly increased when cultured with DFSCs. Additionally, there was a difference between the cocultures of ALS patients and healthy individuals. The $CD4^+CD25^+FoxP3^+$ Treg ratio was significantly higher in the PBMCs of ALS patients when cultured with DFSCs. These results indicate that DFSCs have immunosuppressive effects on activated T lymphocytes by increasing the number of Tregs in PBMCs.

There are several pathways that MSCs mediate the immune responses during variable stimuli of immune activation. MSCs lead to the upregulation of regulatory T cells and suppress proliferation, which results in immune tolerance and promotes cell survival (24). Conversely, MSCs can suppress lymphocyte proliferation by inducing activated or self-reactive T-cell apoptosis through the FAS ligand/FAS-mediated death pathway (16, 17) but do not induce apoptosis in resting lymphocytes. These data indicate that MSCs regulate or suppress immune activation according to the type of stimuli. In this study, we analyzed the effect of DFSCs on the apoptosis of T lymphocytes in PBMCs isolated from ALS patients. The results showed that there was a significant difference in the apoptosis ratio between ALS patients and healthy individuals. DFSCs significantly increased lymphocyte apoptosis in ALS patients, whereas they decrease the apoptosis ratio and increase cell viability in healthy individuals. The heterogeneity of immune responses in different individuals and different types of immune-related diseases and the interplay between lymphocytes and MSCs are influenced by mediators in the microenvironment. A study has demonstrated the involvement of $CD4^+$ T cells against CNS antigens and the response against these antigens with the activation of self-reactive lymphocytes (18). On the basis of these data, we showed that DFSCs inhibited the immune response by increasing the apoptosis of T lymphocytes in ALS patients.

CONCLUSION

In the present study, we clarified that DFSCs have an immunosuppressive effect on the activated T lymphocytes of ALS patients by decreasing proliferative responses, increasing the number of Tregs, and increasing the apoptosis of lymphocytes. We conclude that DFSCs can be a useful cell-based therapeutic tool for treating neuroinflammatory diseases including ALS.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Marmara University (09.2015.317/70737436-050.06.04).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – T.A.; Design – D.G.; Supervision – T.A.; Resources – D.G.; Materials – N.Z., K.U.; Data Collection and/or Processing – D.G.; Analysis and/or Interpretation – T.A.; Literature Search – N.Z., M.G.; Writing Manuscript – D.G.; Critical Review – P.K.K., T.T.

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