










Effect of Dental Follicle Mesenchymal Stem Cells on Th1 and Th2 Derived Naive T Cells in Atopic Dermatitis Patients

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ABSTRACT

Objective: The purpose of our study is to investigate the immunomodulatory effects of Dental Follicle Mesenchymal Stem Cells (DF-MSCs) on lymphocytes isolated from peripheral blood of Atopic Dermatitis (AD) patients, a Th2 disease and psoriasis, a Th1 / Th17 disease and compare them with healthy individuals in vitro.

Methods: Patients with the AD (n = 9) and psoriasis (n = 6) who are followed up in Marmara University Pediatric Allergy and Immunology and Dermatology outpatient clinics and healthy subjects (n = 6) were included. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from 20 ml of venous blood of all participants. Cells were cultured for 72 hours in the absence and presence of DF-MSCs with anti-CD3/anti-CD28 stimulation or without stimulation. At the end of this period, CD4+ and CD8+ T lymphocyte proliferation and cytokine levels from the culture supernatants were analyzed by flow cytometry.

Results: In the presence of DF-MSCs, proliferation ratio was suppressed in both CD4+ and CD8+ cells in AD and psoriasis patients (p<0,05). IFN- γ levels significantly increased in AD patients in the presence of DF-MSCs (p<0,05) whereas decreased significantly in psoriasis patients in the presence of DF-MSCs (p<0,05). IL-4 levels significantly decreased in AD patients in the presence of DF-MSCs (p<0,05) but remained unchanged in psoriasis patients (p>0,05). IL-10 increased significantly in both groups in the presence of DF-MSCs (p<0,05).

Conclusion: Our results support immunoregulatory effects of DF-MSCs on both AD and psoriasis which are Th2 and Th1 / Th17 dominant diseases respectively. Our evidence-based results demonstrated that DF-MSCs could have a beneficial therapeutic implication for inflammatory skin diseases.

Keywords: Mesenchymal Stem Cells, Immunoregulation, Soluble factors, Atopic Dermatitis, Psoriasis

1. INTRODUCTION

Atopic Dermatitis (AD) is a chronic inflammatory skin disease in which immune responses are mediated by Th2 (T helper 2) cells (1-3). It is one of the most common skin disorders with an estimated prevalence of up to 20% of children and 3% of adults (4,5). It is characterized by xerosis, eczematous lesions, and severe pruritus (6,7). There is a complex interplay between genetic, environmental and immunological factors in the pathogenesis of AD (8). Psoriasis is, in contrast, a skin disease mediated mainly by Th1 and Th17 cells that produce IFN- γ and IL-17 (9-11).

Several first-line treatments are available for anti-inflammatory response to reduce the clinical symptoms of the AD and Psoriasis. Topical corticosteroids and systemic immunosuppressants and cytokine antagonists biologic therapies are used for treatment. However, none of these agents can provide a cure and have multiple side effects (12-15). Accordingly there is an unmet need for more effective and safe therapeutic options in AD management. Mesenchymal Stem Cells (MSCs) can be a promising candidate because they can regulate multiple factors simultaneously in response to the inflammatory conditions (16,17).

MSCs are the non-haematopoietic, multipotent stem cells with the capacity to differentiate into mesodermal lineage such as osteocytes, adipocytes and chondrocytes. MSCs express cell surface markers like cluster of differentiation CD29, CD44, CD73, CD90, CD105 and lack the expression of CD14, CD34, CD45 and HLA (Human Leucocyte Antigen)-DR (18). MSCs regulate the functions of various immune cells, including T cells, B cells, natural killer cells, monocyte/macrophages, dendritic cells, and neutrophils. T lymphocytes are the central mediators of many autoimmune and inflammatory diseases as well as of transplant rejection and graft-versus-host disease (19). Dental tissues provide a readily accessible source of MSCs (20). Among the dental origin MSCs, Dental follicle MSCs (DF-MSCs) show potent immunomodulatory properties which make them attractive approach for suppression of inflammatory conditions (21). Immunomodulation by MSCs is mediated by both direct cell-cell contact and release of soluble factors such as prostaglandin E2 (PGE2), indoleamine 2,3 dioxygenase (IDO), Transforming Growth Factor β (TGF- β), released in response to stimulation by inflammatory cytokines (22-24).

Several recent studies have investigated the effects of Umbilical Cord-MSCs ve Bone Marrow-MSCs on antibody

secreted B cell and mast cell degranulation in AD patients. Current data supports different and robust modulatory effects of MSCs, particularly on T cell response (25). Furthermore, the preventive or therapeutic potency and the mechanisms of action can be altered by changing the sources of MSCs.

The purpose of our study is to investigate the regulatory potential of DF-MSCs on CD4⁺T helper (Th) and CD8⁺ T cytotoxic (Tc) cell proliferation, inflammatory and anti-inflammatory cytokine levels in AD patients and compare them with psoriatics and healthy subjects.

2. METHODS

2.1. Study Subjects

Nine patients with an AD (mean age, 15,6 ±1,62 years) who fulfilled the criteria of Hanifin and Rajka (R) are included in

the study. All patients showed positive cutaneous reaction to house dust mite. Patients showed total IgE of >400 IU/mL (mean, 2650±506,99 IU/mL). None of the patients had any systemic immunosuppressive treatment for at least 2 months before testing and don't have any other chronic disease. Six Psoriasis patients (mean age, 22,33±4,68 years) are included. Their mean serum IgE level was 19,5 ±6,09 IU/mL. None of the patients had any systemic immunosuppressive treatment for at least 2 months before testing. Six healthy controls (mean age, 24,5±3,03 years) with no history of atopy are included in the study as controls. Their mean serum IgE level was 17 ±1,2 IU/mL. (Table 1).

This study is approved by Ethics Committee of the Marmara University Medical Faculty in Istanbul, Turkey (Protocol No: 09.2016.196/70737436-050.06.04). Written informed consent was obtained from all patients.

Table 1. Demographic data and clinical characteristics of the Patients. AD; Atopic Dermatitis patients (new diagnosed and non-treated), Ps; Psoriasis patients (new diagnosed and non-treated), C; Control group including HC. F: Female; M: Male; DF: Dermatophagoides farinae; DP: Dermatophagoides pteronyssinus, N: Negative.

Patients	Age	Gender	Total IgE U/ml	SPT(mm)	Topical Therapy
AD1	18	M	2423	DF: 5*6, DP : 6*6	N
AD2	14	M	2748	DF: 6*5, DP:6*6	N
AD3	15	F	2105	DF: 3*3, DP:4*3	N
AD4	14	M	2867	DF: 5*5, DP: 4*4	Y
AD5	14	M	2374	DF: 6*6, DP: 5*6	N
AD6	13	F	3715	DF: 4*5, DP: 5*5	Y
AD7	22	M	2604	DF: 6*6, DP: 5*6	N
AD8	17	M	2031	DF: 4*5, DP: 6*6	N
AD9	18	M	2987	DF: 5*5, DP: 5*4	N
Ps1	28	F	28	N	N
Ps2	22	F	15	N	N
Ps3	17	M	11	N	N
Ps4	20	M	24	N	N
Ps5	19	M	20	N	N
Ps6	28	F	19	N	N
HC1	22	F	25	N	N
HC2	23	F	15	N	N
HC3	25	M	23	N	N
HC4	20	M	10	N	N
HC5	27	M	28	N	N
HC6	30	F	17	N	N

2.2. Isolation and culture of DF-MSCs

Dental follicle tissues were collected from 4 healthy volunteers aged between 19-25 years who have no abscess or inflammatory diseases. Third molar teeth were surgically removed before its eruption, and the dental follicle was extracted under sterile conditions. Dental follicle was cut into approximately 0,5 mm of diameter pieces and digested

with 3 mg/mL collagenase type I (Sigma) in PBS (Invitrogen, USA) containing penicillin-streptomycin (Gibco, USA) for 45 min at 37°C. The obtained cell suspensions were washed for two times and resuspended in DMEM containing %10 Fetal Bovine Serum (FBS) and 1% P/S (referred as complete DMEM) and cultured in 25 cm² culture flasks. Non-adherent cells were removed by changing the cultivation medium.

2.3. Characterization and Determination of Multipotency of DF-MSCs

DF-MSCs were characterized and differentiated into osteogenic, chondrogenic and adipogenic lineages in the third passage, in order to determine the multipotency of these cells. To evaluate the expression of surface markers, DF-MSCs were trypsinized with 0,25% trypsin EDTA and washed in phosphate-buffered saline (PBS; Gibco, Gaithersburg, MD). After 15 minutes of incubation period with FITC or PE-conjugated mouse anti-human antibodies specific to CD34, CD45, CD14, CD29, CD44, CD73, CD90, CD105, or HLA-DR, cells were analyzed *via* flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) with CellQuest software. The mouse IgG served as isotype control.

DF-MSCs were stimulated with StemPro® Osteogenesis Differentiation (Gibco, Grand Island, NY), StemPro® Adipogenesis Differentiation Kit (Gibco) and StemPro® Chondrogenesis Differentiation Kit (Gibco) according to manufacturer's protocol to differentiate into osteocytes, adipocytes, and chondrocytes. Briefly, cells (1×10^5 /well) were seeded in 6-well plates and 3 days after seeding the cells were replaced with differentiation mediums. The cells were grown for 3 weeks, with medium replacement twice a week. Osteogenesis was detected by staining with Alizarin Red to determine extracellular calcium deposits. Adipogenesis was determined by Oil Red O to stain oil droplets produced by adipocytes. Chondrogenesis was assessed by Alcian Blue staining to determine extracellular proteoglycans. All Images of stained cells were captured by using a light microscope.

2.4. Isolation of PBMCs from Whole Blood Samples

Twenty milliliters of peripheral blood was collected in heparinized tubes prior to isolation procedure. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation for 20 min at 2000 rpm. PBMCs were resuspended in complete culture medium (RPMI-1640, supplemented with 1% P/S, 10% FBS, all from Invitrogen) after cell counting by hemocytometer and adjusted to a final concentration of 5×10^5 cells/well in 48 well plates (26).

2.5. Co-culture of DF-MSCs with PBMC

In co-cultures, DF-MSCs (5×10^4 / well in a 48-well plate) were plated 48 h before the addition of 5×10^5 of PBMCs (in the ratio of 1:10) with complete RPMI-1640 medium (RPMI 1640 containing %10 FBS and 1% P/S) and were co-cultured for 3 days. T lymphocytes were stimulated using 0,5µg/ml anti-CD3 (eBioscience, San Diego, CA) and 0,5µg/ml anti-CD28 (eBioscience, San Diego, CA) antibodies (21).

2.6. Lymphocyte Proliferation Assay

In order to determine the effect of DF-MSCs on proliferative response of CD4⁺ or CD8⁺ T lymphocytes, PBMC was labeled with Carboxyfluorescein succinimidyl ester (CFSE) prior to culturing. Briefly, each $1-2 \times 10^6$ PBMC was diluted in 1 mL of

PBS with 18 mM of CFSE and incubated for 6 minutes at 4°C in dark conditions. After incubation period cells were washed twice with culture medium (completeRPMI), supernatant was discharged and remaining cell pellet was resuspended in culture medium before culturing. Cells were analyzed for CFSE (FITC) signaling *via* flow cytometry after 3 days of culture period (27).

2.7. Analysis of Cytokine Expression Profiles

Supernatant from cultures was collected and stored at -80°C until assayed. Samples were measured and analyzed for IFN-γ, IL-4 and IL-10 cytokine levels by Cytokine Bead Array (CBA) kit (BD Biosciences, USA) according to the manufacturer's protocol. Briefly, all the CBA kit contents and samples should be at room temperature at least 15 minutes. fifty microliters of culture supernatants, fifty microliters of capture beads and fifty microliters of detection reagent were added and incubated for 3 hours. After incubation, samples were washed for two times with cold PBS. Samples were acquired in a FACS Calibur flow cytometer (BD Biosciences) and analyzed using the FCAP Array v1.0.1 software (Soft Flow Inc.). Results were expressed as picograms per milliliter.

2.8. Down Regulation of IDO, PGE-2 and TGF-β

To explore the crucial factors responsible for the suppressive effect of DF-MSCs on T cytokine secretion, we inhibited the synthesis or the action of crucial factors using selective inhibitors. The crucial molecules or signals such as transforming growth factor (TGF)-β1, indoleamine-2, 3-dioxygenase-1 (IDO-1), and cyclooxygenase-2 (COX-2) were down-regulated with anti-TGF-β1 (0.5µg/ml) neutralizing antibody, 1-methyl tryptophan (MDT, 0,1 Mm,) 1-Methyl-D-tryptophan (1-MDT) and SC-58125 (50 µM,) respectively. After culture period, supernatants were analyzed for the cytokines levels *via* flow cytometry.

2.9. Statistical analysis

The statistical analysis was achieved by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). One way analysis of variance (ANOVA) with Tukey's multiple comparisons was used for multi-group comparisons, and a two-tailed unpaired Student's t-test was used for comparisons between two groups, and a p-value of <0.05 was considered statistically significant.

3. RESULTS

3.1. Isolation, Characterization and Differentiation of DF-MSCs

The MSCs were isolated from dental follicle tissues. Their proliferation gradually formed small colonies in 3 days. The MSCs reached 70% confluency in the primary culture 7 days after plating for the first passage. Most of the MSCs exhibited fibroblast-like morphology at the P3 passage (Figure 1A).

The MSCs were analyzed *via* flow cytometry. These cells exhibited positive staining for CD29, CD90, CD 146, CD73 and

CD106 but were negative for CD34, CD45, CD14, CD28 and CD25 (Figure 1B).

The MSCs differentiated into osteocytes, adipocytes, and chondrocytes. First, the osteogenic differentiation capability was investigated *in vitro* during a twenty-eight-day culture period in osteogenic induction medium. The MSCs were stained with Alizarin red and the cells formed calcified bone nodule structures. Next, *in vitro* adipogenic

differentiation capability was assessed by culturing the cells in adipogenic induction medium and staining with Oil Red O. Intracellular lipid droplets was observed in these cells. Chondrogenic differentiation capability was investigated *in vitro* following fourteen-day culture period in chondrogenic induction medium and cell differentiation into chondrocytes was confirmed with Alcian blue staining which exhibited intracellular proteoglycans in those cells (Figure 1C).

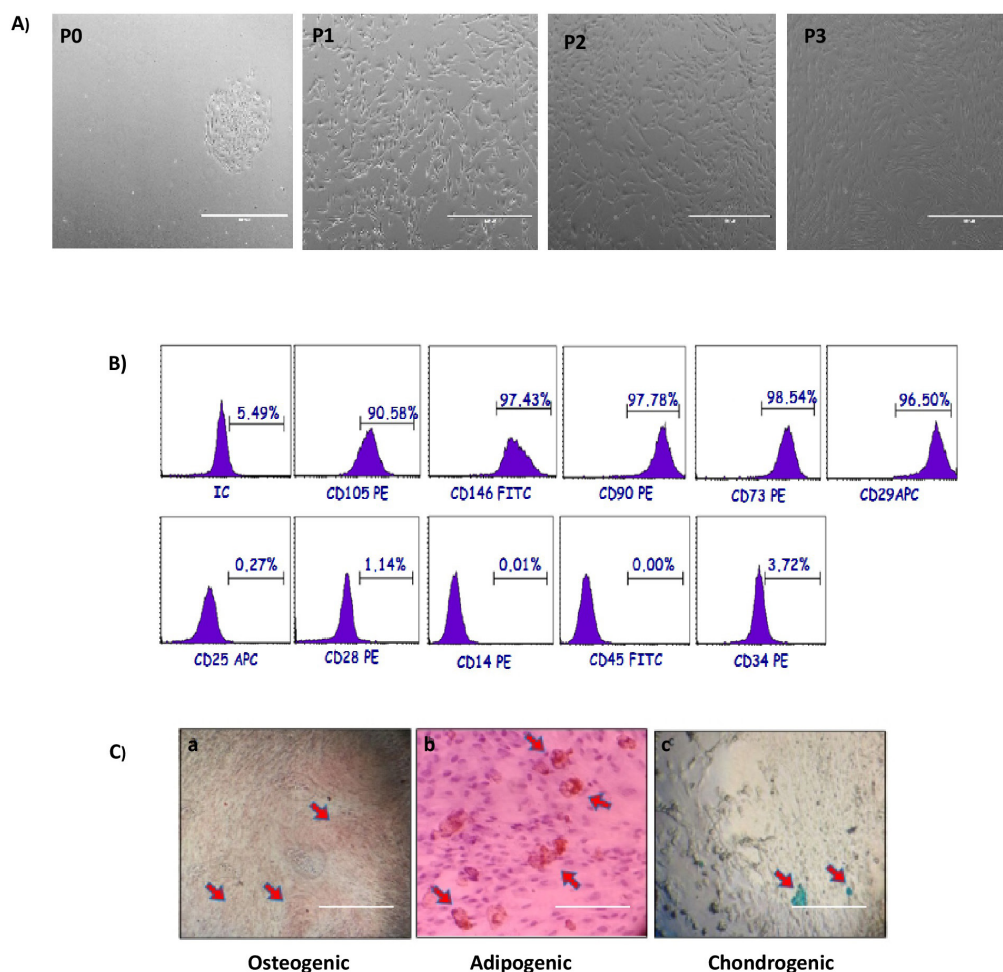


Figure 1. Morphological appearance, characterization, and differentiation of DFSCs. A). Morphology of DFSCs in P0, P1, P2, and P3 (magnification = 10 \times). B). Representative flow cytometry analysis of positive surface markers CD105, CD146, CD90, CD73, CD29 and negative surface marker CD25, CD28, CD14, CD45, CD34 for DFSCs at the third passages. C). (a) Alizarin red staining of osteogenic induced DFSCs (b) Oil Red staining of adipogenic induced DFSCs (c) Alcian blue staining of chondrogenic induced DFSCs, scale bar = 1000 μ m.

3.2. DF-MSCs Decreased Proliferative Response of Lymphocytes in AD Patients

We investigated the immunomodulatory effect of DF-MSCs on CD4⁺Th and CD8⁺ T cell phenotypes in AD and Psoriasis Patients by CFSE cell labeling. According to our results, CD4⁺Th proliferation capacity was significantly higher in AD and Psoriasis compared to healthy controls ($p < 0.01$, $p < 0.05$, respectively). CD4⁺Th cells proliferation was decreased in the presence of DF-MSCs in both AD and Psoriasis group, but it

was not shown a significant difference in healthy controls ($p < 0.01$, $p < 0.05$, $p > 0.05$, respectively). CD8⁺Tc proliferation capacity tended to increase in AD and Psoriasis compared to healthy controls but it is not statistically significant ($p > 0.05$, $p > 0.05$, respectively).

CD8⁺Th cells proliferation was decreased in the presence of DF-MSCs in both AD and Psoriasis group, but it was not shown a significant difference in healthy controls ($p < 0.001$, $p < 0.05$, $p > 0.05$, respectively) (Figure 2).

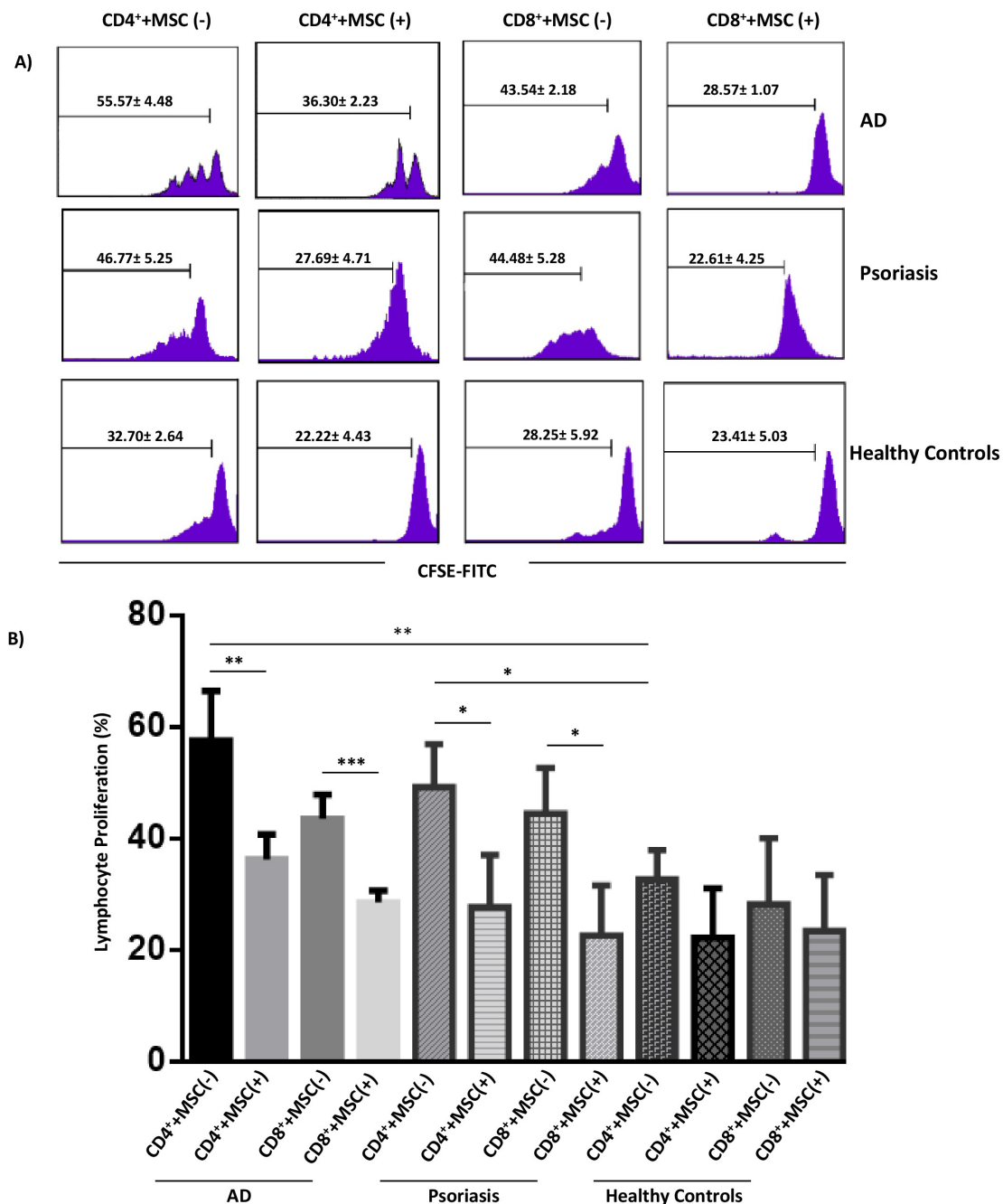


Figure 2. Inhibitory effect of DF-MSCs on the proliferation of lymphocytes as detected by CFSE. A) Inhibitory effect of DF-MSCs on the proliferation of lymphocytes displayed by flow cytometry. B) Inhibitory effect of DF-MSCs on the proliferation of lymphocytes displayed statistically. * $P < 0.05$. Results are shown as mean \pm SD.

3.3. DF-MSCs Regulate the Inflammatory and Anti-Inflammatory Cytokine Production in CDmix-Stimulated PBMCs in AD Patients.

We investigated the immunomodulatory effect of DF-MSCs on Th cell phenotypes by evaluating IFN- γ levels for Th1 and IL-4 levels for Th2 cells, and IL-10 levels as an anti-inflammatory cytokine mainly produced by T regulatory cells. Culture supernatants were collected on 3 day of culture period and analyzed with CBA kit *via* flow cytometry. IL-4 levels were significantly high and IFN- γ levels were low in AD patients' PBMC cultures compared to healthy subjects

and PS patients ($p < 0,05$). DF-MSCs significantly decreased IL-4 levels and increased IFN- γ levels in co-cultures of AD patients ($p < 0,05$), while decreased IFN- γ levels in co-cultures of PS patients ($p < 0,05$). There was no significant difference between DF-MSC (-) and DF-MSC (+) groups in healthy controls ($p > 0,05$). IL-10 levels were significantly increased with DF-MSCs in AD patients and PS patients ($p < 0,005$ and $p < 0,01$, respectively), and tend to increase in healthy controls but it was not significant. There is not significant difference in IFN- γ , IL-4 an IL-10 cytokine levels between PGE-2, TGF- β and IDO blockade culture supernatant in AD, Psoriasis and healthy controls (Figure 3).

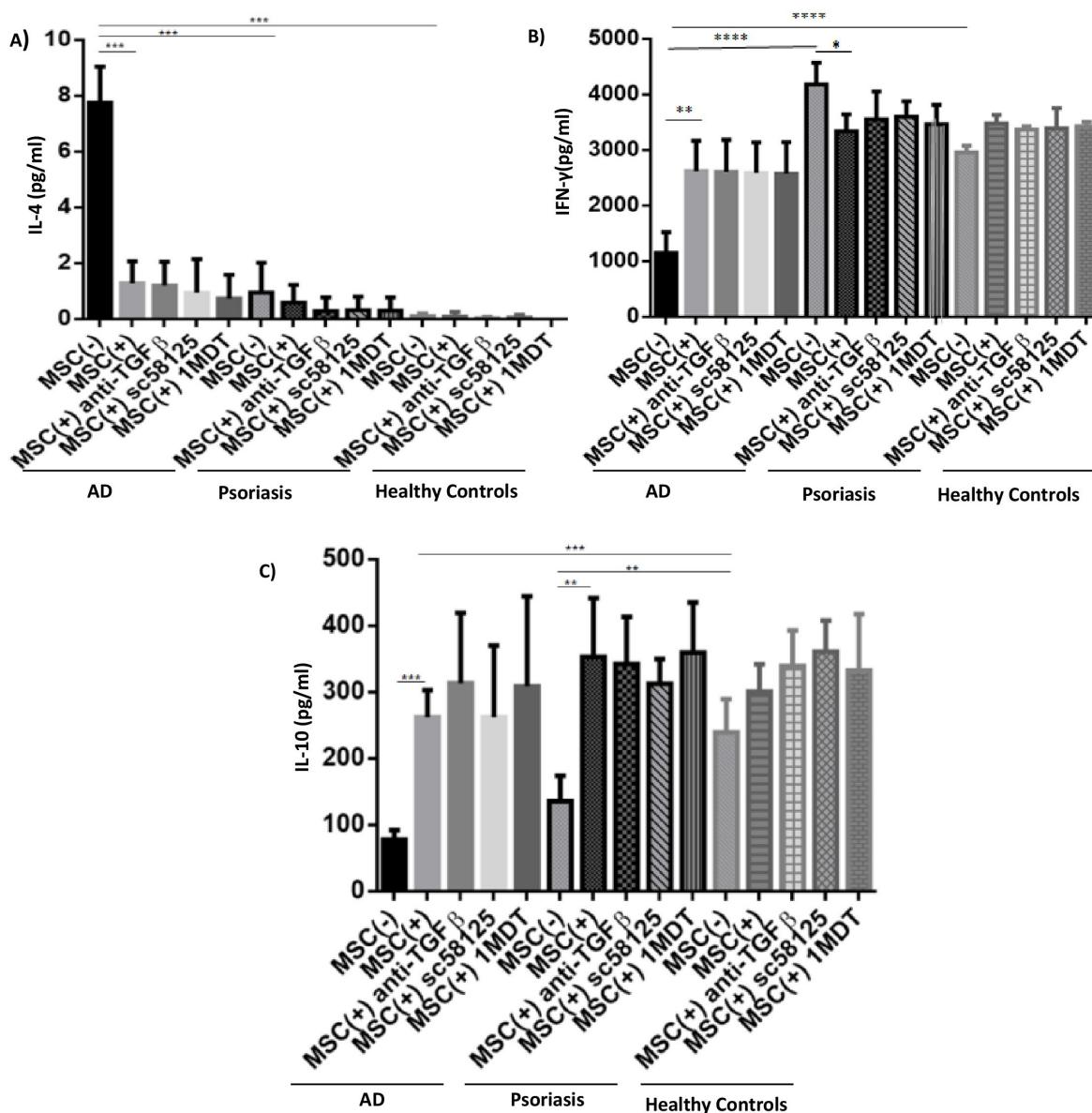


Figure 3. DF-MSCs-modulated cytokine levels in the supernatants of CDmix-stimulated PBMCs. PBMCs (5×10^5 cells/well) collected from patients with the AD, Psoriasis, and HC were cultured in a 48-well plate following CDmix ($0.5 \mu\text{g/ml}$, $0.5 \mu\text{g/ml}$) stimulation in the presence or absence of DF-MSCs (5×10^4 /well) for 3 days. The levels of IFN- γ , IL-4, and IL-10 in the supernatants of CDmix-stimulated PBMCs were determined using flow cytometry. DF-MSCs significantly decreased IL-4(A) ($p < 0.001$) but significantly increase IFN- γ (B) and IL-10(E) levels ($p < 0.01$, $p < 0.001$, respectively) in AD patients. DF-MSCs significantly decreased IFN- γ (B) ($p < 0.05$) but significantly increase IL-10 levels (E) ($p < 0.01$) in Psoriasis patients. IL-4 was not shown a significant difference in presence of DF-MSCs in Psoriasis patients. * $P < 0.05$. Results are shown as mean \pm SD.

4. DISCUSSION

AD is a common chronic skin disease. The currently available therapeutics are limited, and AD management becomes challenging in most cases. To develop better therapeutics for the treatment of AD, many studies have focused on the pathogenesis of AD. Historically, AD was considered a Th2 cell-mediated disease. Experimental and clinical evidence suggests that Th2 cells and their related cytokines like IL-4 and chemokines are critical for the pathogenesis of AD (1-3). Therefore, many researchers have targeted Th2 cells or its signature cytokine IL-4 to treat AD. Indeed, human anti-IL-4

antibody (Dupilumap) effectively treats AD (28). Recently, MSCs have been shown to have immunosuppressive effects. Furthermore, it has been proposed that MSCs could inhibit Th2 cell differentiation by reducing IL-4 cytokine level (29). These discoveries of immunosuppressive function and the ability to inhibit lymphocytes have driven scientists to test the possibility that MSCs can be used for the treatment of allergic inflammation diseases because Th2 cells are significant targets of allergic inflammation diseases. In this study, we demonstrated that DF-MSCs could inhibit T cells proliferation capacity and reduce IL-4 cytokine levels in AD patients.

Immunomodulatory effects of MSCs make them new candidates as a cellular therapy for the prevention and treatment of various inflammatory diseases (30). Mesenchymal stem cells can be isolated from many different tissues which exhibit remarkable tissue regenerative and immunosuppressive properties (31). Among these cells, dental tissue MSCs represent a source that is easily accessible and have the potential to differentiate into other tissue cell lines (32). Yildirim and Colleagues show that DF-MSCs reduced inflammatory response compared to other dental sources (21). We investigate DF-MSCs immunomodulatory effect in inflammatory skin diseases for the first time.

MSCs suppress the response of T cells to stimulation factors. In a previous study, Bone marrow-derived stem cells (BM-MSCs) suppress T cell proliferation in OVA albumin induced atopic dermatitis mouse model (17). Although Th2-type CD4⁺ Th cells appear to be significant in AD pathogenesis, CD8⁺ Tc cells represent the dominant effector cell responses in the development of the allergic skin inflammation (33). To examine the CD4⁺Th and CD8⁺Tc cell in AD pathogenesis, we investigated the proliferative response of both T cell types in the presence of DF-MSCs. According to our results, DF-MSCs decrease CD4⁺Th and CD8⁺ Tc cell response in AD patients. This suppression was seen in the CD4⁺Th and CD8⁺ Tc cells of Th1/Th17 –type psoriasis patients. For the first time, we investigate the role of DF-MSCs on the response of CD4⁺Th and CD8⁺ Tc in two different inflammatory skin disease.

Na and colleagues showed that BM-MSCs suppress both IL-4 and IFN – γ through decreasing T-bet and GATA-3 expression (17). According to Fu and colleagues, BM-MSCs increased IFN – γ cytokine levels in allergic rhinitis with Th2-skewed eosinophilic inflammation but reduce IL-4 levels (25). After of Lymphocytes with DF-MSCs, the supernatants were collected, and cytokines analysis were performed. In our study, DF-MSCs suppressed the expression of IL-4 whereas the expression of IFN – γ and IL-10 was increased in AD patients. DF-MSCs increased IFN – γ levels in Th1/Th17 skewed Psoriasis patients, but IL-4 levels were not changed. According to our results, DF-MSCs provides immunomodulation according to the characteristics of lymphocyte in the culture.

5. CONCLUSION

These inhibitory actions might contribute to the therapeutic effects of DF-MSCs on both AD and Psoriasis patients. To our knowledge, these findings provide the new perspective that MSCs manipulation is a potential novel strategy for the treatment of inflammatory skin disease.

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