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ORIGINAL ARTICLE

Asthma and Rhinitis

Dental follicle mesenchymal stem cells down-regulate Th2-mediated immune response in asthmatic patients mononuclear cells

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Summary

Background: Asthma is a chronic inflammatory disease in which inflammatory responses have the polarisation of $CD4^+$ T cells to Th2 cells. Dental follicle mesenchymal stem cells (DFSCs) have strong anti-inflammatory properties comparable to other mesenchymal stem cells.

Objective: We investigated the immunomodulatory effects of DFSCs on $CD4^+$ T helper cell responses of asthmatic patients and compared the results with those obtained with asthmatic subjects on immunotherapy and with healthy individuals.

Method: Peripheral blood mononuclear cells (PBMC) were isolated from immunotherapy naïve asthmatics, asthmatics on subcutaneous Der p1 immunotherapy and from healthy individuals. PBMC were pre-conditioned with anti-CD3/anti-CD28 mAbs, Der p1 or IFN-γ in the presence and absence of DFSCs and analysed for T cell viability and proliferation, $CD4^+CD25^+FOXP3^+$ regulatory T cell frequencies, cytokine expression, and GATA3, T bet and FoxP3 expressions. Neutralisation of TGF-β and blockade of IDO and PGE2 pathways were performed to determine suppressive signalling pathways of DFSCs.

Results: Dental follicle mesenchymal stem cells suppressed proliferative responses of $CD4^+$ T lymphocytes and increased the frequency of Treg cells. DFSCs decreased effector and effector memory $CD4^+$ T cell phenotypes in favour of naïve T cell markers. DFSCs decreased IL-4 and GATA3 expression and increased IFN-γ, T-bet and IL-10 expression in asthmatics. Costimulatory molecules were suppressed in monocytes with DFSCs in the cocultures. DFSCs down-regulated inflammatory responses via IDO and TGF-β pathways in asthmatic patients.

Conclusion: Dental follicle mesenchymal stem cells suppressed allergen-induced Th2-cell polarisation in favour of Th1 responses and attenuated antigen-presenting cell co-stimulatory activities. These studies suggest that DFSC-based cell therapy may provide pro-tolerogenic immunomodulation relevant to allergic diseases such as asthma.

KEYWORDS
asthma, dental follicle mesenchymal stem cells, house dust mite, immunomodulation

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1 | INTRODUCTION

Asthma is a Th2-mediated inflammatory disease with increased levels of IgE and Th2 signature cytokines.[1] It is often associated with structural remodelling of the airways characterised by airway epithelial damage, wall thickening and sub-epithelial changes.[2] Recent studies reveal that asthma is linked to many biological mechanisms with complex interplay between genecity, environmental factors and immune interaction that influence the progression of asthma.[3]

Novel therapeutics and management strategies are being developed in the treatment of asthma such as pharmacotherapeutic approach with steroids or anti-inflammatory drugs.[4] These drugs both help to control and prevent asthma attacks. However, long-term use of high-dose corticosteroids or glucocorticoids has systemic side-effects such as decreased bone mineral density, skin thinning and bruising.[5] Another option in the treatment of asthma is allergen-specific immunotherapy, which the administration of a specific antigen is injected subcutaneously with the increasing doses. The most striking feature of this therapy is the decreased IL-4 levels due to the enhanced IL-12 and IFN gamma levels.[6] The subcutaneous immunotherapy (SCIT) manipulates the generation of regulatory T cells are and IgG1 levels in allergic diseases.[7] However, allergen-specific immunotherapy is a long-term approach and may have unwanted contraindications in individual cases such as anaphylaxis.[8] Therefore, safety, short-term and effective treatment strategies should be developed for the treatment of allergic diseases including asthma.

Mesenchymal stem cells (MSCs) are multipotent cells and can be isolated from several sources mainly from bone marrow, adipose and dental tissues and further they have ability to differentiate into various lineages of cell types.[9] Recently, several types of MSCs reserved in dental and oral tissues have been identified. MSCs originated from orafacial tissues are easy to obtain and can be easily isolated.[10] In addition to their differentiation potential and tissue regeneration, dental MSCs have been reported to regulate immune responses in autoimmune and inflammatory diseases.[11,12] MSCs are able to regulate both innate and adaptive immunity by secreting large variety of soluble factors, including transforming growth factor-β1 (TGF-β1), indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2), which induce development of regulatory T cells from naïve $CD4^+$ T lymphocytes.[13] Our previous studies showed that dental follicle is one of the sources of MSCs, which can be isolated in high amounts and has potential immunosuppressive effects on proliferating $CD4^+$ T lymphocytes by increasing regulatory T cell amounts.[14] Additionally, Interferon-γ (IFN-γ) is the signature cytokine of Th1 effector cells, which is known to be effective for down-regulating Th2-mediated responses in allergic diseases. Moreover, IFN-γ pretreated MSCs have an enhanced ability to modulate T cell over-activities with direct or indirect contact by inducing MSC inhibitory factors.[15] In this study, we therefore additionally evaluated the modulatory effect of DFSCs on T cell responses with the simultaneous stimulation of IFN-γ.

Currently, the role of MSCs in allergic asthma has also been reported. However, the regulatory potential of DFSCs in asthma remains to be elucidated. In this study, we aimed to evaluate the immunomodulatory effect of DFSCs on house dust mite sensitive asthma patients PBMC in vitro. We investigated the proliferative response, T cell viability, frequency of $CD4^+CD25^+FOXP3^+$ regulatory T cells, $CD4^+$T lymphocyte ratios in distinct compartments, costimulatory molecule activations of antigen-presenting cells (APCs), soluble cytokine levels and protein expressions of Th1, Th2 and Treg transcription factors isolated from peripheral blood samples of untreated asthma patients in the presence and absence of DFSCs and compared results with immunotherapy subjects. The immunoregulatory effect of DFSCs on $CD4^+$ T lymphocytes was evaluated with anti-CD3/anti-CD28, Der p1 or with the simultaneous stimulation of IFN-γ. In addition, we conducted neutralisation experiments to determine, which of the soluble mediators produced by DFSCs have an immunomodulatory effect on immune responses of asthmatic patients.

2 | MATERIAL AND METHOD

2.1 | Study Subjects

New diagnosed and non-treated nineteen asthma patients (age between 5-14 years), and six patients treated with subcutaneous Derp1 immunotherapy for more than 3 years (age between 11-17 years) and eight non-asthmatic healthy individuals are included in the study within 4 months of clinical reviews. All of the procedures were performed in accordance with Marmara University Clinical Ethical Committee standards (70737436-050.06.04). All of the participants were subjected to skin prick test (SPT), forced expiratory volume (FEV%), physical examination and serum IgE levels were recorded for the evaluation of patient selection criteria (Figure S1 and Figure S2).

Dental follicle tissues for mesenchymal stem cell isolation were collected in aseptic conditions from patients with indications of wisdom teeth extraction from Marmara University Faculty of Dentistry (age between 19-25).

2.2 | Isolation, characterisation and multipotency of Dental follicle mesenchymal stem cells

Dental follicle mesenchymal stem cells were isolated from dental follicle tissues as previously described.[14] DFSCs were isolated from extracted dental follicle tissues of healthy adults. Briefly, dental follicle tissues mechanically cut approximately into 0.5 mm of diameter pieces, and digested enzymatically with 3 mg/mL collagenase type I in sterile PBS solution for 45 minutes. After, incubation period enzyme inactivation was performed by adding 5 mL of complete DMEM (DMEM containing %10 FBS and 1% penicillin/streptomycin), and centrifugated at 1500 rpm for 5 minutes. Then, supernatant was discarded, and remaining cell pellet was resuspended with 5 mL of

complete DMEM and incubated in T25 culture flask at 37°C 5% CO_2 chamber until cells reach 70%-80% confluency. Cells were trypsinised with 0.25% trypsin EDTA and washed two times with complete DMEM for the next passage. Cells were characterised via flow cytometry, and differentiated into osteogenic, chondrogenic and adipogenic lineages to determine the multipotency as described in the Supplementary text (Appendix S1).

2.3 | Isolation of mononuclear cells from peripheral blood

Peripheral blood samples were collected into heparinised tubes from patients for PBMC isolation. Blood samples were diluted 1/1 (v/v) with sterile PBS and transferred over ficoll solution in 15 mL tubes for density gradient separation. After centrifugation at 2000 rpm for 20 minutes, the buffy coat was collected, washed twice with complete RPMI 1640 (cRPMI) medium (RPMI supplemented with 10% FBS and 1% penicilline/streptomycin) by centrifugation at 1500 rpm for 5 minutes. Supernatant discarded, and remaining cell pellet was resuspended with cRPMI medium for culturing. 1×10^6 cells/mL was counted in the culture media.

2.4 | Culture conditions

Dental follicle mesenchymal stem cells were seeded in 48 well plates 48 hours ago PBMC isolation and incubated at 37°C 5% CO_2 in humidified chamber. PBMC were divided into two groups and cultured in the presence and absence of DFSCs by stimulating with anti-CD3/anti-CD28 (CD mix) or Der p1 or IFN-γ, separately. 5×10^5 PBMC were cultured in 500 μL of cRPMI medium for each well. DFSCs:PBMC ratio was 1:10 in cocultures with same conditions and incubated for 72 hours.

2.5 | CFSE labelling for proliferation assay

Peripheral blood mononuclear cells were labelled with CFSE dye, before culturing, according to the manufacturers' instructions. Briefly, CFSE vial concentration was adjusted to 18 mmol L^{-1} to resuspend the cells in 1 mL PBS for 7 minutes. After the incubation period cells were washed with 5 mL of cRPMI medium and centrifugated at 1500 rpm for 5 minutes. Supernatant was discarded, and remaining cell pellet was resuspended in cRPMI medium at adequate volume and transferred into culture plates.

2.6 | Detection of cell viability and apoptosis

Peripheral blood mononuclear cells were stained with Annexin V (PE) and 7 AAD to analyse the cell viability and apoptosis after 72 hours of culturing. Cultured cells were washed with PBS and centrifuged at 1500 rpm for 5 minutes. Cell pellet was resuspended with 100 μL of PBS and incubated with 5 μL of Annexin V (PE) and 10 μL of 7AAD antibodies for 15 minutes at room temperature. Stained cells were analysed via flow cytometry (FACS Calibur).

2.7 | Phenotypic analysis of $CD4^+CD25^+FOXP3^+$ Treg cells

Treg cells were analysed in PBMC cultures after 72 hours of culture period. For this purpose, cells were washed with PBS and cell pellet was labelled with human FOXP3 kit (eBioscience) according to the manufacturer's instructions. Briefly, cells were incubated with anti-CD4 (FITC) and anti-CD25 (APC) antibodies for 20 minutes at room temperature in dark. After incubation cells were washed with 100 μL of 1XPBS and incubated with permeabilisation buffer, and then permeabilised cells were incubated with anti-FOXP3 antibody for 45 minutes at room temperature by avoiding cells from light. After washing step, supernatant was discarded, and cell lysate was analysed via flow cytometer (FACS Calibur).

2.8 | Determining $CD4^+$ T lymphocyte subsets in distinct compartments

Cultured lymphocytes were analysed for naïve, effector, central memory and effector memory subsets to determine the effect of DFSCs on distinct compartments of T lymphocytes. After 72 hours of culture period, cells were labelled with anti-CD3 (PerCp), anti-CD45RA (FITC) and anti-CCR7 (PE) antibodies and analysed via flow cytometer. The gated cells were determined as following; $CCR7^+CD45RA^+$ (naïve), $CCR7^+CD45RA^-$ (central memory), $CCR7^-CD45RA^+$ (effector) and $CCR7^-CD45RA^-$ (effector memory) T cell subsets.

2.9 | Analysis of activation of costimulatory molecules on monocytes

Antigen-presenting cell types were analysed for CD80, CD83 and CD86 costimulatory molecule expressions after 72 hours of culture period. PBMC were stained with anti-CD14 (APC) antibody to determine monocytes. Additionally, cells were stained with anti-CD80 (FITC), anti-CD83 (PerCp) and anti-CD86 (PE) and analysed via flow cytometry.

2.10 | Detection of cytokine levels in culture supernatants

IL-4, IL-10 and IFN-γ were measured simultaneously in the culture supernatants by cytokine bead array kit (BD Biosciences) via flow cytometry. Fifty microlitres of culture supernatant were collected and coated with capturing antibodies. After incubation period, washing and acquisition of fluorescence data, the results were generated in graphical format using the BD CBA software. The concentrations of cytokine IL-4, IL-10 and IFN-γ were measured.

2.11 | Western blot analysis of transcription factors

Cell lysates were prepared from cultured PBMC. Protein extraction was performed with lysis and extraction buffer (RIPA, Thermo Scientific). Total protein was separated using 10% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis. The primary antibody dilutions in the ratio of 1:500 were used for GATA3, Tbet1, FOXP3 and β-actin and secondary antibodies were diluted in the ratio of 1:2000. Protein levels were normalised to β-actin and differences were determined.

2.12 | Blockade of indoleamine 2,3-dioxygenase and prostaglandin E2 pathways of dental follicle mesenchymal stem cells and neutralisation of TGF-β

To determine the modulatory mechanism of DFSCs mediated through regulatory T cells, PBMC were treated with 1-Methyl-D-tryptophan (1-MDT) for the inhibition of IDO pathway or SC-58125 for the inhibition of PGE2 pathway through the selective inhibition of cyclooxygenase 2 (COX-2) or anti-TGFβ for the neutralisation of soluble TGF-β in the presence and absence of DFSCs. Briefly, 0.1 mmol L^{-1} 1-MDT or 50 μmol L^{-1} SC-58125 or anti-TGFβ (0.5 μg/mL) was added in each culture well of asthmatic or healthy PBMC cultured with and without DFSCs and cultured for 72 hours. After culture period, PBMC were analysed for the frequency of $CD4^+CD25^+FOXP3^+$ regulatory T cells via flow cytometry.

The gating strategy of flow cytometric analysis is given in the supplement (Figure S4).

3 | RESULTS

3.1 | Dental follicle mesenchymal stem cells suppressed the proliferative response of $CD4^+$ T lymphocytes in asthma patients

We first assessed the inhibitory effect of DFSCs on the proliferation of $CD4^+$ T lymphocytes of asthma patients. PBMC of all groups were proliferated with CD mix stimulation but the proliferative response to the specific antigen (Der p1) of house dust mite was significant in asthma patients (46.2 ± 7.6) while PBMC of healthy individuals (8.6 ± 1.9) and immunotherapy subjects (6.8 ± 1.7) lack the proliferation to Der p1 challenge. DFSCs inhibited the proliferation of lymphocytes in Der p1 stimulated cultures of lymphocytes of asthmatic patients significantly when compared with PBMC cultures alone ($P < .005$). IFN-γ stimulation increased the suppressive effect of DFSCs on $CD4^+$ T lymphocytes in all groups. In summary, DFSCs reduced the lymphocyte proliferation of $CD4^+$ T lymphocytes of asthma patients to values close to the immunotherapy subjects PBMC cultures without stem cells in CD mix and Der p1 stimulated cultures. (Figure 1)

3.2 | Dental follicle mesenchymal stem cells increased the cell viability of lymphocytes in asthmatic patients

We assessed the anti-apoptotic effect of DFSCs on the stimulated lymphocytes of patients. The cell viability of CD mix, Der p1 and IFN-γ stimulated lymphocytes were significantly lower in asthmatic patients (61.9 ± 5.6, 59.4 ± 9.5, 70.5 ± 1.6). DFSCs increased the cell viability of $CD4^+$ T lymphocytes in all groups, but the increase in CD mix (90.7 ± 2.1) and Der p1 (89.4 ± 2.8) stimulated cultures with stem cells was remarkable in asthma patients. (Figure 2)

3.3 | Dental follicle mesenchymal stem cells enhanced regulatory T cell population

Mesenchymal stem cells are known to promote the induction of $CD4^+CD25^+FOXP3^+$ Treg cells.[16] We therefore studied the regulatory T cell per cent in the PBMC cultures in the presence and absence of DFSCs. The frequency of $CD4^+CD25^+FOXP3^+$ Treg cells was notably higher in CD mix (8.3 ± 0.4), Der p1 (7.1 ± 0.1) and IFN-γ (7.5 ± 0.1) stimulated PBMC cultures of immunotherapy subjects. DFSCs did not change Treg cell frequency in immunotherapy subjects but significantly enhanced the frequency of $CD4^+CD25^+FOXP3^+$ Treg cells in CD mix (4.3 ± 0.1), Der p1 (3.7 ± 0.2) and IFN-γ (6.7 ± 0.1) stimulated co-cultures in asthmatic patients compared to PBMC cultures alone ($P < .01$). As a result, Treg frequency in IFN-γ stimulated co-cultures in asthmatic patients was close to immunotherapy subjects PBMC cultures alone. (Figure 3)

3.4 | Dental follicle mesenchymal stem cells reduced the costimulatory activation of monocytes

The costimulatory pathway CD28/CD80/CD86 plays an important role in allergic immune responses. While the initial signal by MHC-II provides antigen specificity, the secondary signal of B7 family molecules activates T lymphocytes.[17] We studied the effect of DFSCs on the costimulation of monocytes. The gated $CD14^+$ cells in PBMC were analysed for the expressions of CD80, CD83 and CD86 molecules in the presence and absence of DFSCs.

Costimulatory molecules CD80 and CD86, expressed on $CD14^+$ cells were increased with CD mix stimulated PBMC of asthmatic patients (CD80;18.6 ± 2.8, CD86;25.4 ± 3.9), but the expression of these molecules was significantly elevated in asthmatic patients with Der p1 stimulation (CD80;29.1 ± 4.5, CD86;26.5 ± 2.2), which indicates the allergen-specific response of antigen-presenting cells of house dust mite sensitive asthmatic patients. IFN-γ did not significantly change costimulation of $CD14^+$ cells in PBMC cultures alone in asthmatic patients. DFSCs down-regulated the expression of CD80 and CD86 on $CD14^+$ cells significantly in CD mix co-cultures of all groups, but the reduction of these molecules in cocultures of asthma patients was noteworthy ($CD80_{Derp1}$;6.3 ± 1.9, $CD86_{Derp1}$;12.4 ± 1.0), whereas there was no significant change in cocultures of Der p1 stimulated PBMC of immunotherapy and healthy individuals, which may be an evidence of immunoregulatory effect of DFSCs on the specific response of antigen-presenting cells. (Figure 4)

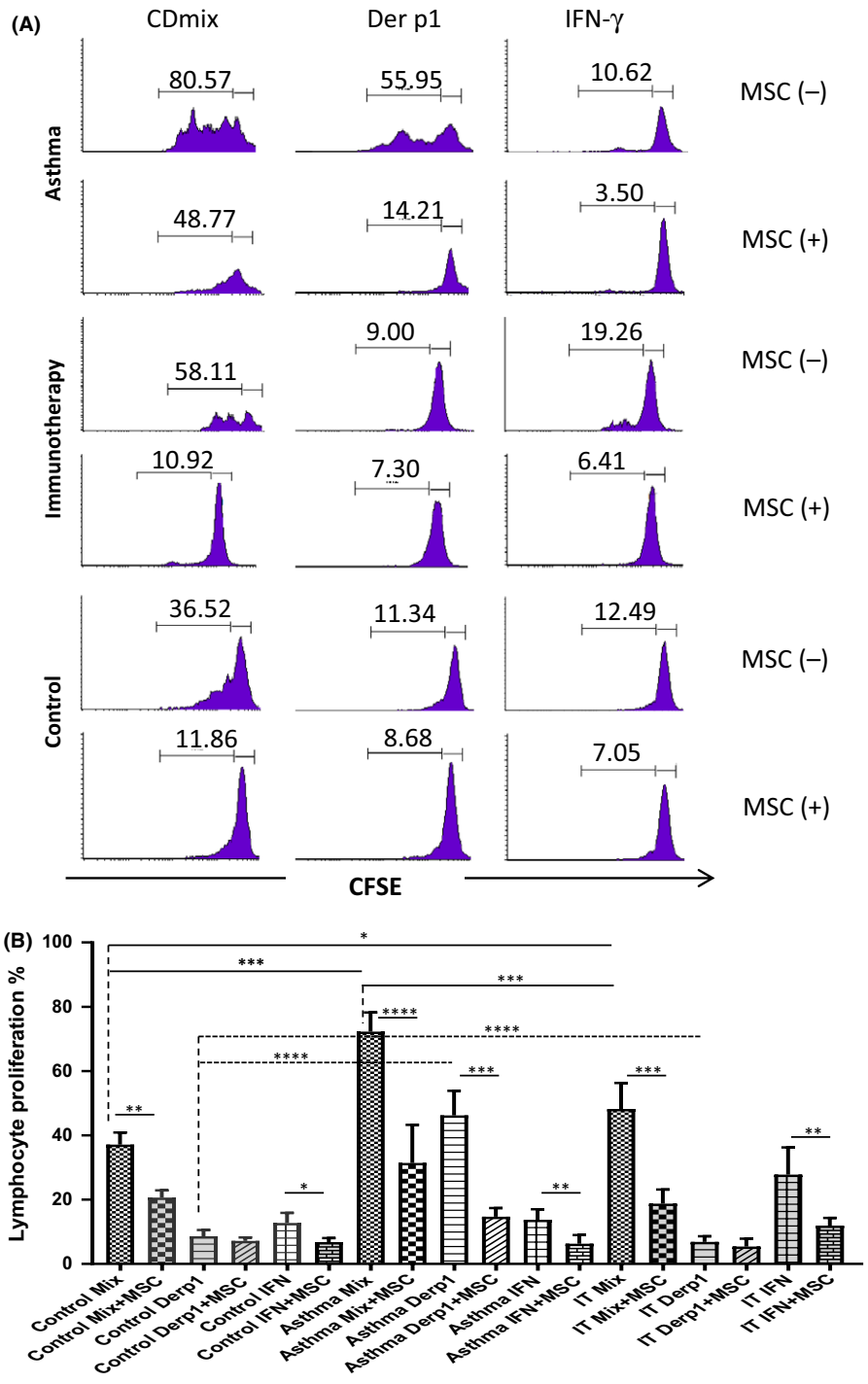


FIGURE 1 Proliferation analysis of CD4 + T lymphocytes. A, Asthma patients showed high proliferative response to CDmix and Der p1 stimulation, whereas no response was seen in immunotherapy subjects in Der p1 stimulated Peripheral blood mononuclear cells (PBMC) cultures. Interestingly, there was a slight response with IFN-γ stimulation. Healthy individuals showed moderate response to CDmix stimulation in PBMC cultures, but no response occurred in Der p1 stimulated PBMC. B, In statistical data there was significant difference between groups in CDmix and Der p1 stimulated PBMC cultures ($P < .05$). Statistical data demonstrated that DFSCs significantly decreased proliferation ratio after 72 h of culture period in all groups in CDmix stimulated and IFN-γ stimulated co-cultures ($P < .05$). In addition, DFSCs significantly decreased proliferative response of CD4 + T lymphocytes in asthma patients with Der p1 stimulation ($P < .005$), whereas no significant change seen in immunotherapy subjects and healthy individuals with Der p1 stimulated co-cultures compared to PBMC cultures alone ($P > .05$)

3.5 | Dental follicle mesenchymal stem cells reduced effector and effector memory T cell subsets in asthmatic patients

After the demonstration of suppressive activity of DFSCs on the proliferating T lymphocytes and inducing FOXP3 expressing regulatory T cell population, we questioned the effect of DFSCs on the T cell subsets in distinct compartments.

In CD mix and Der p1 stimulated PBMC cultures the per cent of naïve T cells was significantly lower ($P < .05$) and effector and effector memory T cells were significantly higher in asthmatic patients compared to immunotherapy and healthy groups ($P < .01$ and $P < .001$, respectively). DFSCs preferentially decreased the per cent of effector and effector memory T cell subsets. In CD mix cocultures, DFSCs significantly decreased the effector and effector memory subsets of T cells in all groups, but selectively reduced these lymphocyte subsets in Der p1 stimulated cocultures of asthma patients ($P < .05$ and $P < .01$, respectively). In summary, DFSCs was observed to reduce the proportion of effector cells that develop in antigen-specific responses in asthmatic patients.

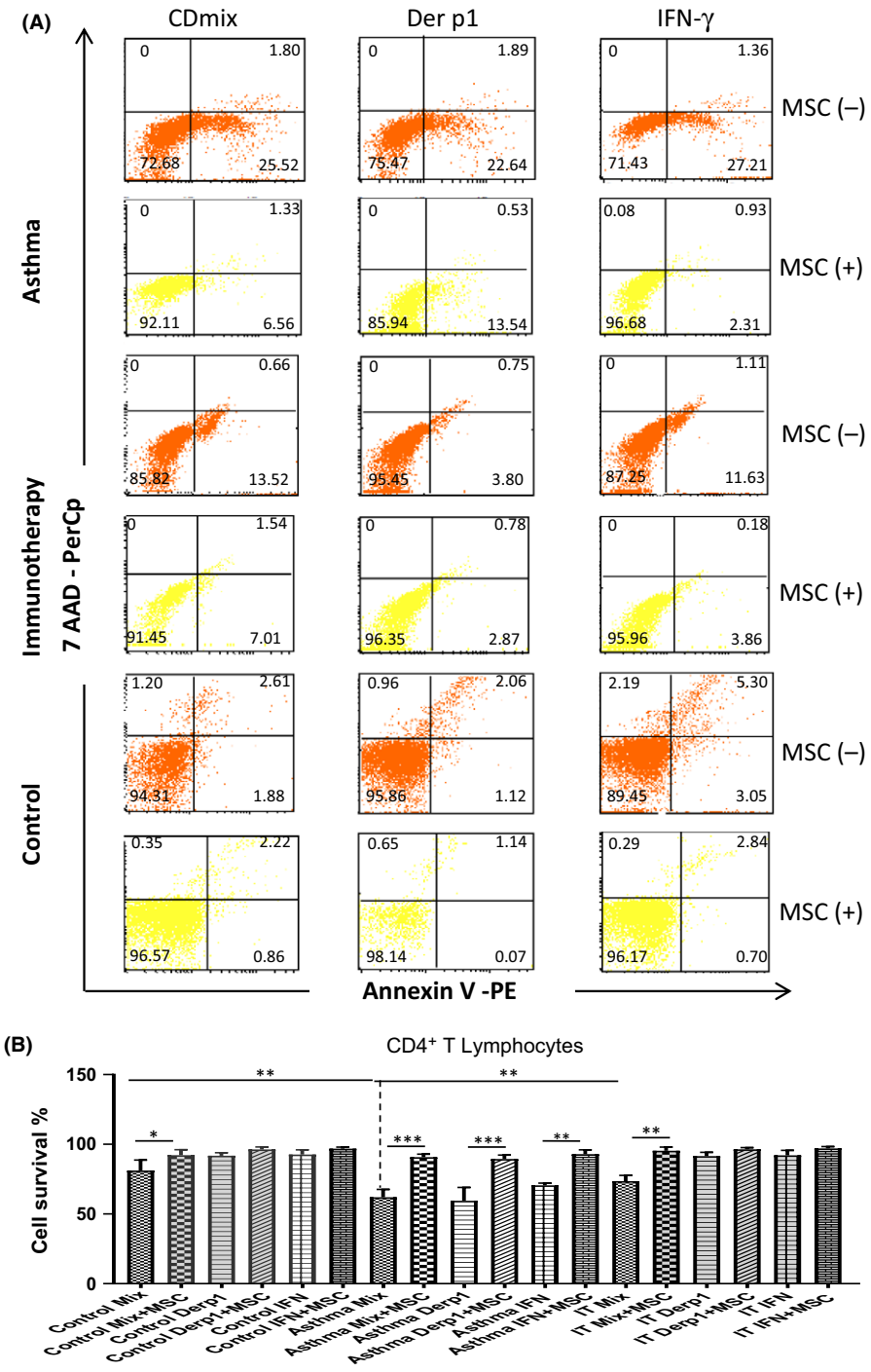


FIGURE 2 Cell survival analysis of CD4 + T lymphocytes. A, Lymphocytes were stained with anti-CD3, anti-CD4, Annexin V and 7AAD to analyse cell survival and apoptotic ratio of CD4 + T lymphocytes. Lower left quadrants were evaluated for cell survival ratio. B, In the statistical analysis cell survival ratio of Peripheral blood mononuclear cells increased with DFSCs in all groups significantly ($P < .05$). In addition, DFSCs significantly increased lymphocyte survival ratio in asthmatic patients in Der p1 stimulated co-cultures ($P < .005$), while no significant change seen in healthy individuals and immunotherapy subjects in Der p1 stimulated co-cultures ($P > .05$)

In addition, DFSCs remarkably increased the relative per cent of naïve T cells in CD mix and IFN-γ stimulated ($P < .01$) and notably Der p1 stimulated cocultures of asthmatic patients ($P < .005$), while no significant change observed in immunotherapy and healthy groups. These data indicate that DFSCs may be using one of two different mechanisms, the first of which is the inhibitory effect on the activation signals for the differentiation of T lymphocytes into the effector stage, and the second is the relatively increase in naïve T cell subsets due to the reduction of effector or effector memory T lymphocytes. (Figure 5)

3.6 | Dental follicle mesenchymal stem cells reduced GATA3 and increased Tbet1 and FOXP3 expressions in asthmatic patients

Two main transcription factors GATA3 and Tbet1 act as the main regulators of Th2 and Th1 differentiation, respectively. We analysed GATA3 and Tbet1 protein expressions by western blotting to confirm the analysis of cytokine expressions. FoxP3 analysis was performed for regulatory T lymphocytes' protein expressions. Lanes were compared and normalised with beta-actin protein for each test sample.

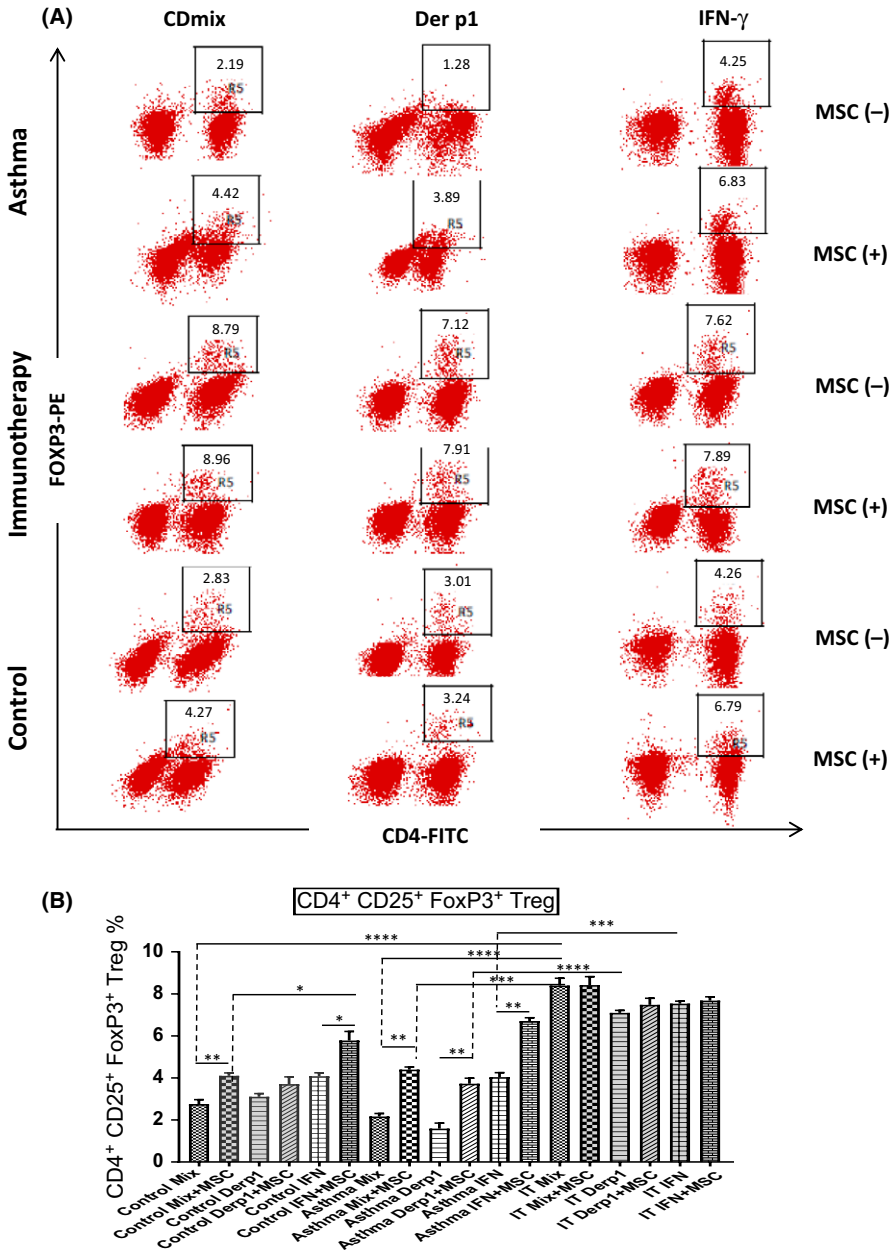


FIGURE 3 $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells. A, T regulatory cell population was significantly high in immunotherapy patients compared to asthmatic patients and healthy individuals in Peripheral blood mononuclear cells (PBMC) cultures. Asthmatic patients showed low frequency of Tregulatory cells compared to other groups. B, In the statistical data, it was shown that DFSCs significantly increased $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cell frequency in all stimulated co-cultures compared to PBMC cultures alone ($P < .05$) whereas no significant change occured in Der p1 stimulated co-cultures of healthy individuals and immunotherapy subjects ($P > .05$). On the other hand, DFSCs did not significantly change T regulatory cell frequency in co-cultures in immunotherapy subjects compared to PBMC cultures ($P > .05$)

GATA3 was highly expressed in CD mix and Der p1 stimulated PBMC cultures, while reduced Tbet1 and FoxP3 protein expression were observed in asthmatic patients compared to immunotherapy and healthy subjects. DFSCs down-regulated expression of GATA3 in CD mix and Der p1 stimulated cocultures and up-regulated expression of Tbet1 in CD mix and IFN-γ stimulated cocultures significantly ($P < .05$) and remarkably increased FoxP3 expressions in CD mix, Der p1 and IFN-γ stimulated cocultures ($P < .01$, $P < .005$ and $P < .005$, respectively) in asthmatic patients. In addition, Tbet1 was dominantly expressed and FoxP3 expression was significantly higher in PBMC cultures of immunotherapy patients, and lack the expression of GATA3, which may be associated with the reduction of GATA3 expression by increasing FoxP3 expressing Treg cells. And there was no significant change observed in FoxP3 expressions between PBMC cultures and cocultures with DFSCs, but over-expression of Tbet1 notably down-regulated with CD mix stimulated cocultures with DFSCs in immunotherapy group ($P < .05$). These results indicate that DFSCs selectively induces $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells to combat with Th2-mediated responses in asthmatic patients while decreasing high amounts of Th1 cells in immunotherapy subjects, which suggests the mechanism of immunosuppression. (Figure 6)

3.7 | Dental follicle mesenchymal stem cells enhanced IFN-γ and IL-10 and reduced IL-4 levels in asthma patients

To demonstrate the change in the cytokine profile, we performed cytokine analysis in the supernatants of PBMC cultures in the

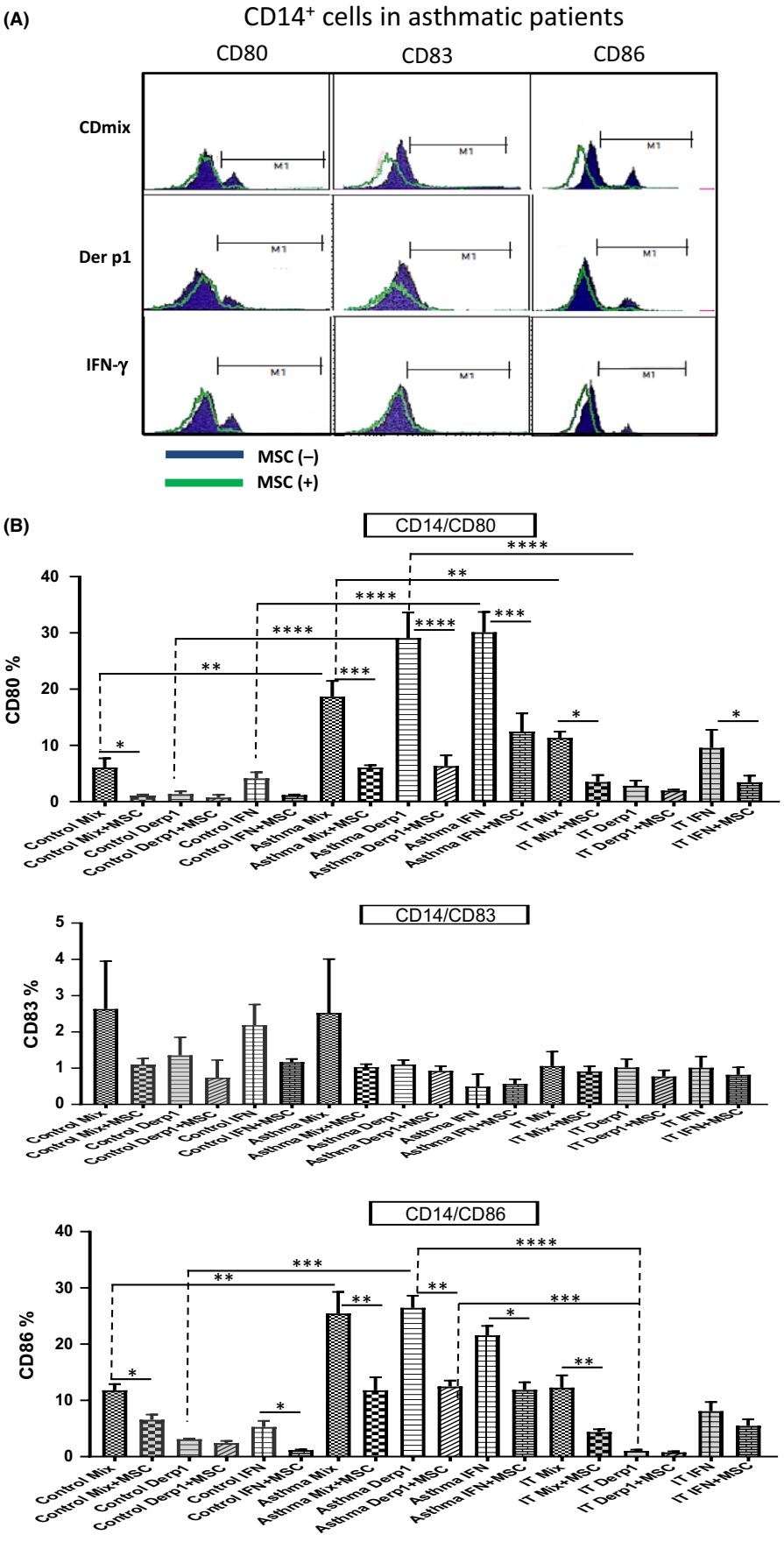


FIGURE 4 Costimulatory molecules expression on CD14+ cells. A, CD80 and CD86 expressions were high in CD14 + cells in Peripheral blood mononuclear cells (PBMC) cultures of asthmatic patients. DFSCs lowered the expression of CD80 and CD86 costimulatory molecules on monocytes of asthmatic patients. Dark blue colour filled histograms show PBMC cultures without stem cells and green coloured lines show PBMC cocultures with DFSCs. B, In the statistical data, CD80 and CD86 increased with the stimulation of CDmix and Der p1 in PBMC cultures of asthma patients. It was shown that DFSCs significantly reduced CD80 and CD86 expressions on monocytes of asthma patients in CDmix (CD80; $P < .005$, $P < .001$ and $P < .005$, and CD86; $P < .01$, $P < .01$ and $P < .05$, respectively)

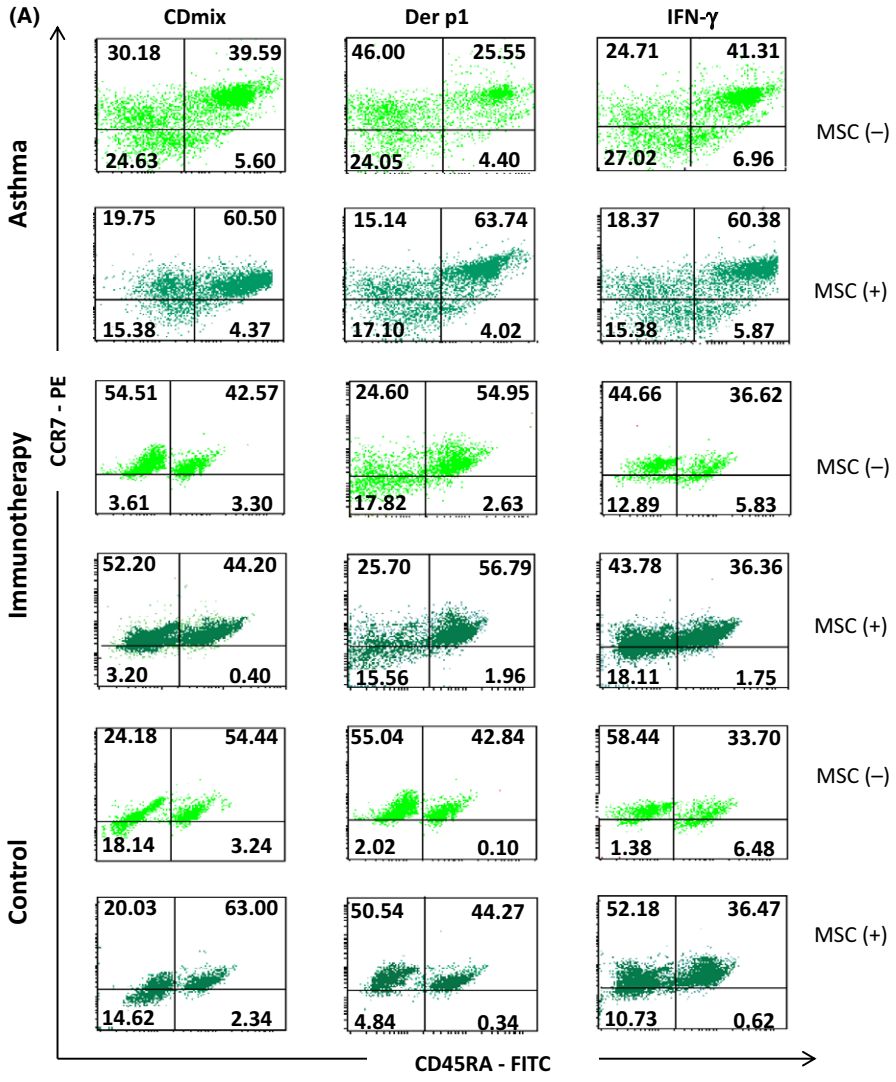


FIGURE 5 $CD3^+$ T lymphocyte populations in distinct compartments. A, $CD3^+$ Lymphocytes were gated for CCR7 and CD45RA expressions to determine naïve, effector and memory populations. Quadrants were evaluated as LL; Effector memory, LR; Effector, UR; Naïve and UL; Central memory CD3 + T cells. B, In the statistical data, it was shown that naïve CD3 + T cells were lower and effector cells were higher in asthmatic patients than in immunotherapy subjects and healthy individuals. DFSCs significantly increased naïve CD3 + T lymphocyte population relatively compared to Peripheral blood mononuclear cells (PBMCs) alone in all stimulated cultures ($P < .05$), especially in Der p1 stimulated co-cultures ($P < .005$). In addition, DFSCs did not significantly change naïve CD3 + T cells in healthy individuals and immunotherapy subjects significantly in CDmix or Der p1 stimulated co-cultures compared to PBMC cultures alone ($P > .05$)

presence and absence of DFSCs. IL-4 levels were significantly high, and IFN-γ and IL-10 levels were low in the CD mix, Der p1 and IFN-γ stimulated PBMC cultures of asthmatic patients compared to healthy individuals and immunotherapy patients ($P < .001$). DFSCs significantly decreased IL-4 levels in CD mix ($P < .001$), Der p1 ($P < .01$) and IFN-γ stimulated cocultures ($P < .005$) and increased IL-10 levels in all stimulated cocultures in asthma patients ($P < .01$) when compared with PBMC cultures alone. Interestingly, IFN-γ levels in PBMC were significantly increased in CD mix, Der p1 and IFN-γ stimulated PBMC cocultures with DFSCs ($P < .05$, $P < .05$ and $P < .01$, respectively) while decreased levels were observed in the cocultures of immunotherapy patients and healthy individuals compared with PBMC cultures alone. Increase in the IL-10 levels indicates the suppressive effect of DFSCs by inducing regulatory T cells, which is the main mechanism thought for immunosuppression. Additionally, decrease in the IL-4 levels and increase in the IFN-γ levels are considered as the suppressive effect of DFSCs on the activated Th2 cells, which supports the differentiation of the T helper lymphocytes towards Th1 cells. (Figure 7)

3.8 | TGF-β and indoleamine 2,3-dioxygenase blockade down-regulated the generation of $CD4^+CD25^+FOXP3^+$ regulatory T cells in asthmatic patients

We investigated the mechanism underlying the effects on regulatory T cell frequency, which was enhanced by DFSCs in asthma, by neutralising TGF-β and blocking the pathways of IDO and PGE2. We blocked IDO and PGE2 pathways and soluble TGF-β in PBMC cultures in the presence or absence of DFSCs to determine the regulatory T cell enhancing mediators expressed by DFSCs in asthmatic patients. DFSCs significantly increased $CD4^+CD25^+FOXP3^+$ T regulatory cell amount compared to CD mix and Der p1 stimulated PBMC cultures in asthmatics, and the increase in asthmatics was higher than in healthy subjects. 1-MDT and anti-TGFβ reduced the $CD4^+CD25^+FOXP3^+$ T regulatory cell frequency in cocultures of asthmatic patients significantly when compared with DFSCs cocultures without blockade, but the decrease with anti-TGFβ was less than in 1-MDT (1-MDT: $P < .01$; anti-TGFβ: $P < .05$). In contrast, 1-MDT was not changed T regulatory cell frequency in cocultures of healthy individuals

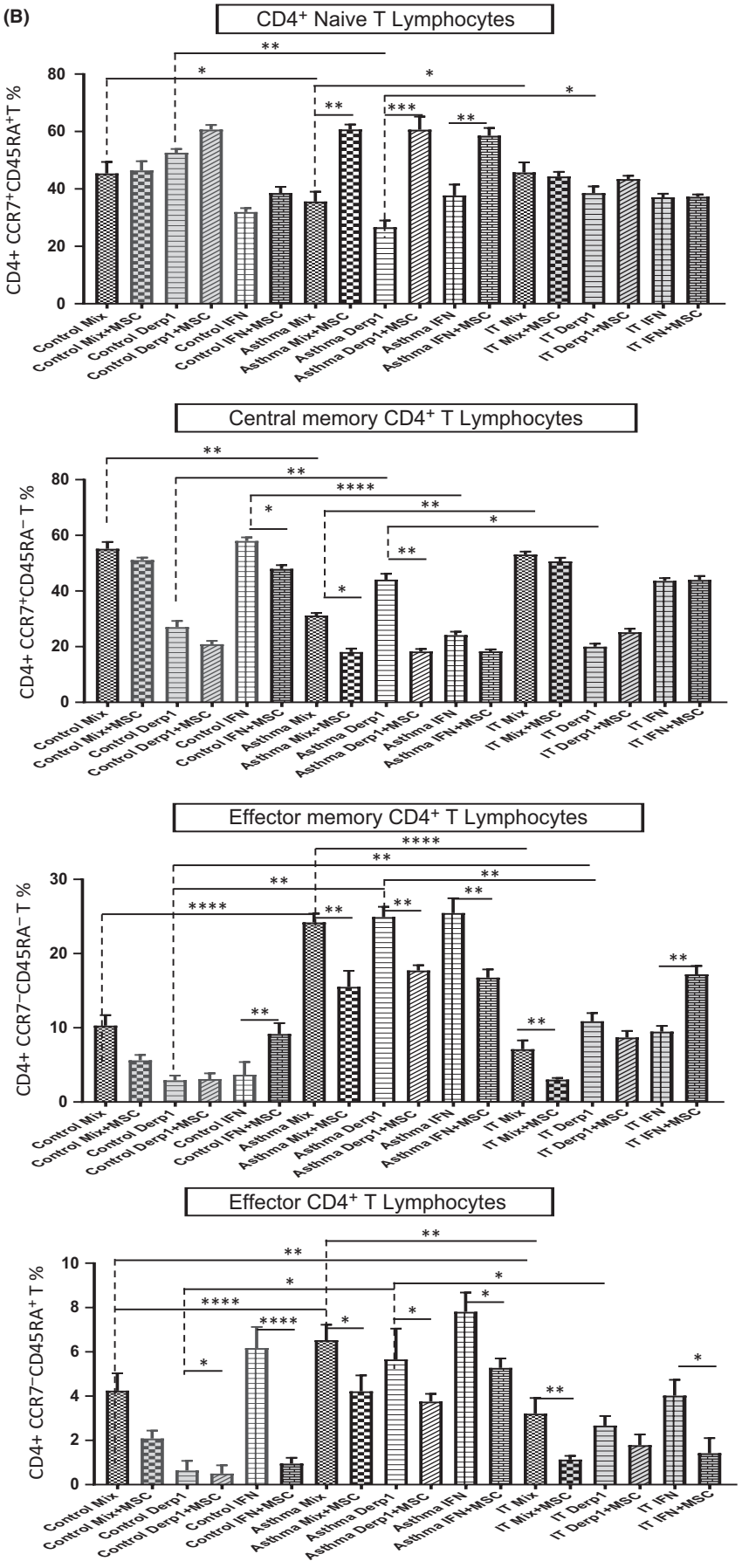


FIGURE 5 Continued

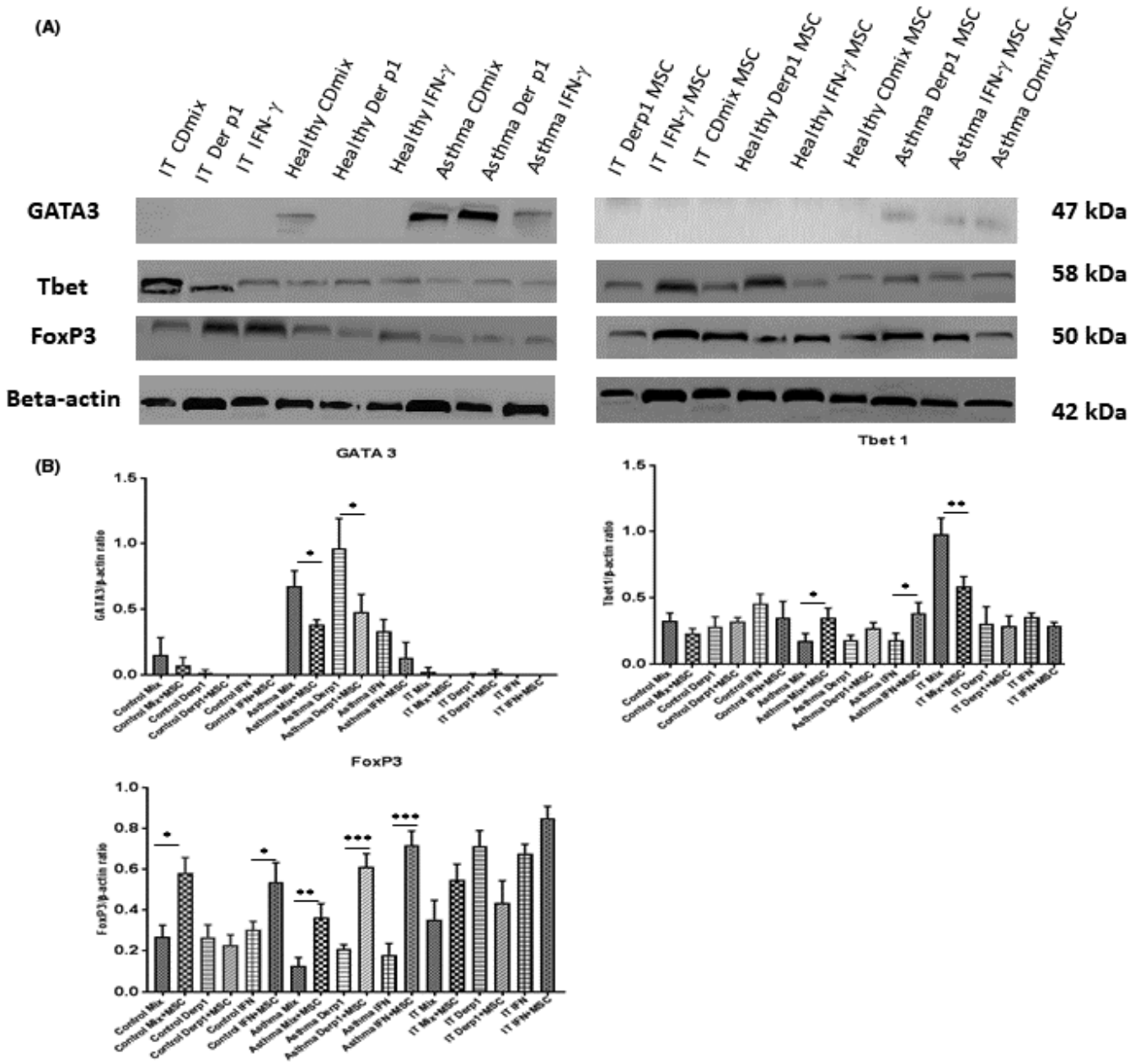


FIGURE 6 Protein expression analysis of Peripheral blood mononuclear cells (PBMCs). A, Protein isolation was performed from PBMCs after 72 h of incubation period and analysed by western blotting for GATA3, Tbet1 and FoxP3 expressions. Protein levels were normalised to β-actin for each lane. B, Statistical data showed that GATA3 expression was high in asthma patients' PBMCs compared to healthy individuals and immunotherapy subjects in CDmix and Der p1 stimulated cultures. DFSCs significantly decreased GATA3 expression in CDmix and Der p1 stimulated co-cultures in asthmatic patients compared to PBMC cultures alone ($P < .05$). Tbet1 expression was high in immunotherapy subjects and healthy individuals compared to asthmatic patients in PBMC cultures. DFSCs significantly increased Tbet1 expression in PBMCs of asthmatic patients in CDmix and IFN-γ stimulated co-cultures cocultures ($P < .05$) and tend to increase in Der p1 stimulated cocultures, whereas significant decrease was observed in CDmix cocultures of immunotherapy subjects compared with the same stimulant in PBMC cultures alone ($P < .05$). FoxP3 expression was significantly increased in CDmix, Der p1 and IFN-γ stimulated PBMCs of asthmatic patients when cultured with DFSCs compared to PBMC cultures alone ($P < .01$, $P < .005$ and $P < .005$, respectively)

significantly ($P > .05$), but significantly reduced with anti-TGFβ when compared with cocultures without blockade ($P < .05$) both with CD mix and Der p1 stimulation. Blockade of PGE2 with SC-582125 was not significantly changed the T regulatory cell frequency compared to DFSCs cocultures without blockade both in asthmatics and healthy subjects ($P > .05$). (Figure 8)

4 | DISCUSSION

Asthma is a chronic inflammatory disease that results in reduced respiratory function and remodelling of airways.[18] House dust mite (HDM) is one of the main risk factor underlying allergic asthma.[19] The link between exposure to allergen and the molecular factors in

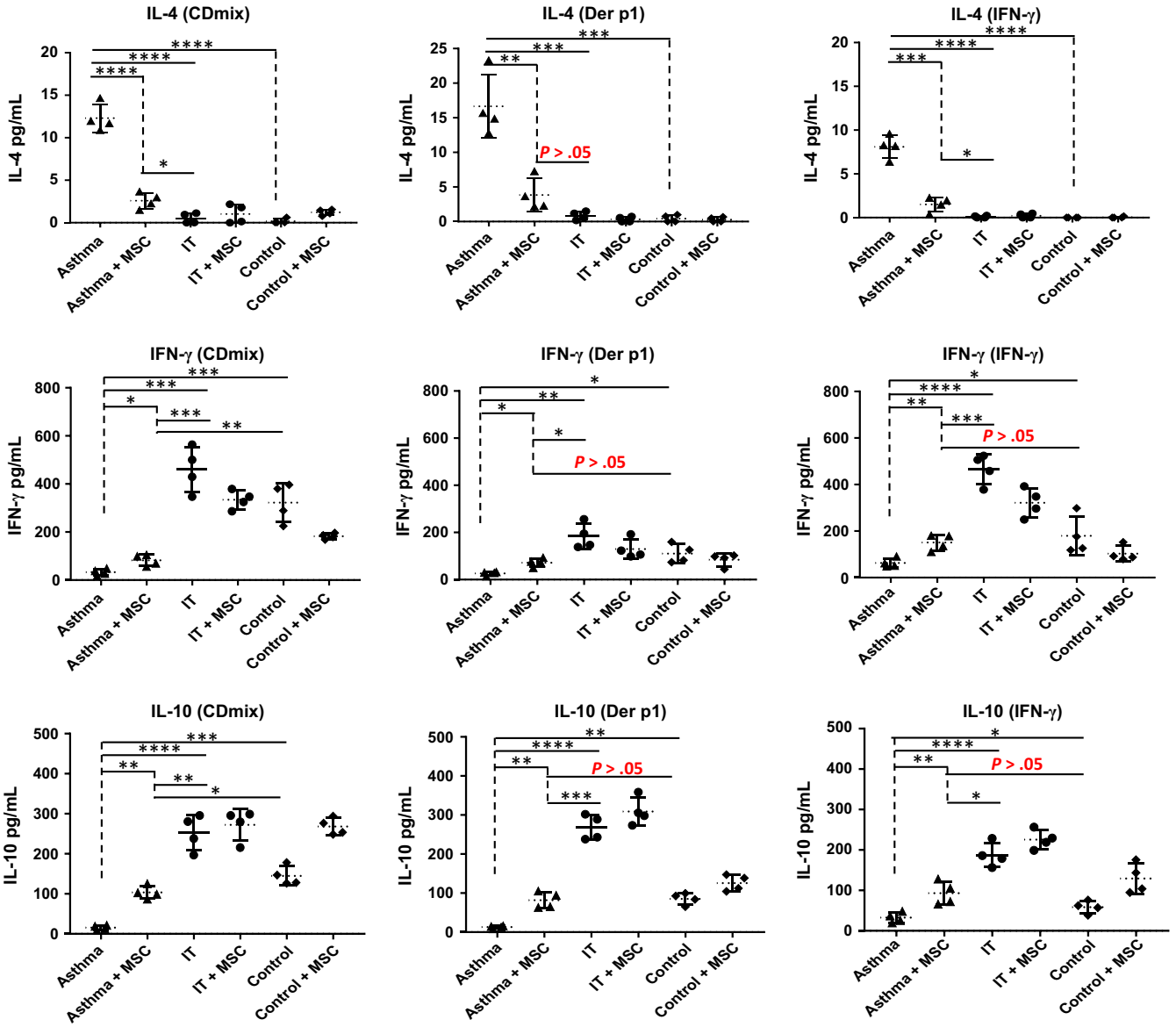


FIGURE 7 Cytokine profiles in culture supernatants. Supernatants were collected from cultures after 72 h of incubation period and analysed via flow cytometry for IL-4, IFN-γ and IL-10. Statistical data showed that IL-4 levels were was high in asthma patients' Peripheral blood mononuclear cells (PBMCs) compared to healthy individuals and immunotherapy subjects in CDmix, Der p1 and IFN-γ stimulated cultures. DFSCs significantly decreased IL-4 levels in CDmix, Der p1 and IFN-γ stimulated co-cultures in asthmatic patients compared to PBMC cultures alone ($P < .001$, $P < .01$ and .005, respectively). IFN-γ levels were lower in asthmatic patients compared to immunotherapy subjects and healthy individuals. DFSCs significantly increased IFN-γ levels in PBMCs of asthmatic patients in CDmix, Der p1 and IFN-γ stimulated co-cultures ($P < .05$, $P < .05$ and $P < .01$, respectively). IL-10 levels were significantly increased in CDmix, Der p1 and IFN-γ stimulated PBMCs of asthmatic patients when cultured with DFSCs compared to PBMC cultures alone ($P < .005$, $P < .01$ and $P < .01$, respectively)

the initiation and progression of asthma aggravates the complexity of this disease.[20] A wide variety of immune cells involved in the allergen-mediated inflammatory responses. The response is initiated with the presentation of antigen by dendritic cells to naïve T lymphocytes and triggered with Th2 differentiated lymphocyte activations. B cells involved in this process by producing IgE antibodies, which then binds on the FcεR on mast cells, by the effect of Th2 mediators (IL-4, IL-5 and IL-13). Re-exposure to allergen then activates mast cells to recruit other immune cells in airways.[21,22] In the present study, we focused on the immunomodulatory effect of DFSCs on the costimulation of monocytes and regulatory effects on $CD4^+$ T cells in the inflammatory response in asthma patients.

Allergen-specific immunotherapy (ASIT) is the only modifying curative option by inducing tolerance of immune cells to specific allergen in allergic diseases.[1] The basic principle of ASIT is the administration of specific allergen to patients in repeated certain doses by

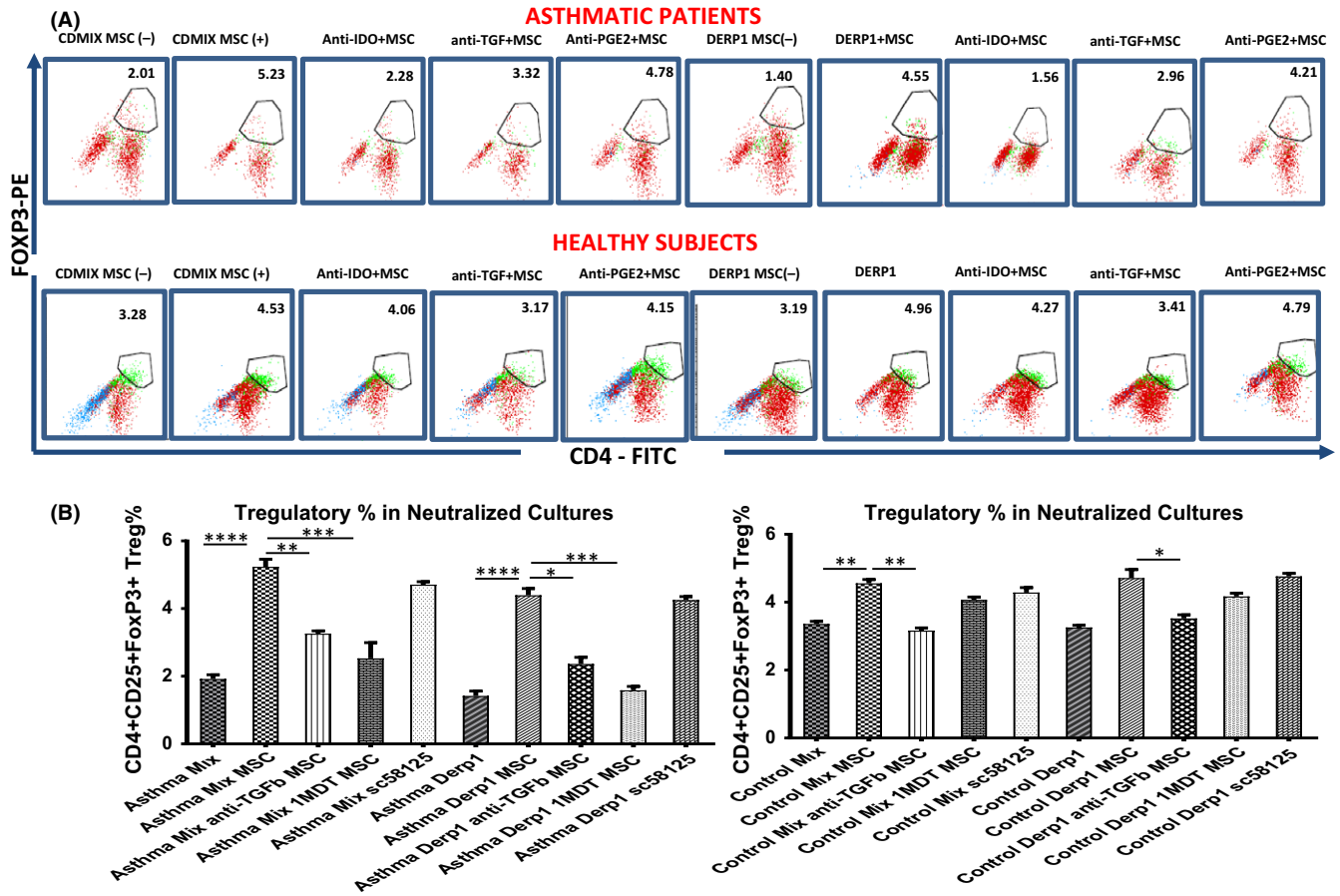


FIGURE 8 Neutralisation of TGF-β and blockade of IDO and PGE2 pathways of DFSCs. A, Peripheral blood mononuclear cells (PBMCs) of asthmatic patients and healthy individuals were cultured in the presence and absence of DFSCs with unstimulated or anti-TGFβ or 1-MDT (anti-IDO) or sc-58125 (anti-PGE2) to determine the pathways included in suppressive mechanism of DFSCs. B, T regulatory cell frequency was low in CDmix and Der p1 stimulated PBMC cultures in asthmatic patients. Unstimulated DFSCs significantly increased T regulatory cell amounts in CDmix and Der p1 stimulated co-cultures compared to PBMC cultures alone ($P < .001$). T regulatory cell frequency significantly decreased when DFSCs neutralised with anti-TGFβ and blockade of IDO pathway compared to unstimulated DFSCs (CDmix; $P < .01$ and $P < .005$, Der p1; $P < .05$ and $P < .005$, respectively). In healthy subjects neutralisation of DFSCs with anti-TGFβ significantly decreased T regulatory cell ratio in CDmix and Der p1 stimulated co-cultures compared to unstimulated DFSCs co-cultures ($P < .01$ and $P < .05$, respectively), while blockade of IDO or PGE2 did not significantly change T regulatory cell frequency ($P > .05$)

subcutaneous or sublingual way.[21,23] ASIT is successful intolerance to allergen, however have disadvantages, such as its inconvenience, invasiveness and potential systemic reactions.[24] House dust mites (HDM) belong to Pyroglyphidae family, consisting of *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, which are the most common factors in allergic asthma.[25] In this study, we compared the immunoregulatory effect of DFSCs with allergen-specific immunotherapy. Thus, we included ASIT patients treated with subcutaneous Der p1 and observed successful tolerance to Der p1 stimulation with non-proliferative response of lymphocytes. At the same time, $CD4^+CD25^+FOXP3^+$ regulatory T cell population was significantly higher in ASIT patients' PBMC cultures compared to asthmatic patients and healthy individuals. This data shows the immune tolerance to specific allergen in immunotherapy subjects. Additionally, protein expressions in Tbet1 and FOXP3 were higher in immunotherapy subjects than in asthmatic patients, and lack the GATA3 expression in PBMC cultures, which indicates the increase in Th1 and Treg and decrease in Th2 lymphocyte subsets. Additional data was obtained from soluble cytokine levels from culture supernatants, which supports protein expression levels and flow cytometric results of regulatory T cells. IL-10 levels of PBMC cultures were significantly higher in immunotherapy subjects than in asthmatic patients. In contrast to asthmatic patients, IFN-γ levels were high and IL-4 levels were low in PBMC cultures of immunotherapy patients. Overall, PBMC cultures of immunotherapy group show that Th1 cells are more intense than Th2 cells, and increased amounts of regulatory T cell population provides immunosuppression. There is also a plasticity in helper T cells, which environmental factors effect during inflammatory responses, result with the generation or differentiation of distinct T lymphocyte phenotypes due to inflammatory or anti-inflammatory stimuli.[13,26,27] In the current study, IL-4 levels were significantly reduced with DFSCs in asthma patients to the level of PBMC cultures of immunotherapy and

healthy individuals. IFN-γ levels were increased in the cocultures with DFSCs in asthma patients, in contrast, there was a decrease in the level of IFN-γ in immunotherapy and healthy subjects. We supported these data with the change in the protein expression levels in PBMC and PBMC/DFSCs cocultures. GATA3 levels were decreased and Tbet1 levels were increased with DFSCs in asthma patients while no significant change observed in immunotherapy group. We interpret these results as the suppressive effect of DFSCs on Th2 lymphocytes and supporting the differentiation of T lymphocytes towards Th1 cells. In a recent study performed on murine model of asthma, it was shown that bone marrow-derived mesenchymal stem cells reduced the production of IL-4, IL-5 and IL-13 cytokine levels in mediastinal lymph nodes and slightly increased IFN-γ levels.[28] We observed that these results are compatible with previous studies, which demonstrated the inhibition of Th2 based inflammatory responses due to IFN-γ production by Th1 cells.[28-30] In addition, one of the studies on immunosuppressive activity of dental pulp MSCs on $CD4^+$ T lymphocytes of healthy individuals it was reported that dental pulp MSCs decreased the frequency of Tbet and down-regulated IFN-γ expression of T lymphocytes by increasing FoxP3 expression and GATA3 expression was decreased in the cocultures.[31] Similarly, we demonstrated that DFSCs down-regulated Tbet expression and IFN-γ secretion in $CD4^+$ T cells in healthy subjects and down-regulated GATA3 expression and IL-4 secretion in asthmatic patients, but in contrast Tbet expression and IFN-γ secretion was up-regulated in asthmatic patients in cocultures. The difference in the results may be due to variations in the immunoregulatory action of DFSCs on Th1- and Th2-mediated responses.

Naïve $CD4^+$ T lymphocytes are differentiated into Th2 cells by the activation of CD3 and CD28 receptors and in IL-4 cytokine environment.[32] Although there are several studies investigating the immunosuppressive properties of mesenchymal stem cells, very few studies have focused on the effects of T cells in distinct compartments. Therefore, in the present study, we evaluated the effect of DFSCs on naïve, central memory, effector memory and effector compartments of T lymphocytes with the expression profile of CD45RA and CCR7. Our results showed that DFSCs down-regulated Der p1 stimulated effector, effector memory and central memory T cell subsets in asthma patients while there was no significant change in immunotherapy and healthy individuals. In addition, naïve T lymphocyte population relatively increased due to the reduction of effector and memory T cell subsets. At the same time, results were evaluated with apoptotic effects of DFSCs in the cocultures to determine whether effector T cell subsets were either undergo apoptosis or directly suppressed by DFSCs. Results indicated that the anti-apoptotic effect of DFSCs in the cocultures suggests the suppressive effect of these cells on effector T lymphocyte subsets rather than apoptosis. Previous studies revealed that soluble and cell contact-dependent mediators produced by MSCs suppress T-cell activations and effector functions.[13,33] In concordance with these studies, our results support the protective mechanism of DFSCs on naïve T lymphocyte population by suppressing antigen-specific T lymphocyte activation during inflammatory responses.

In the current study, we observed potent anti-proliferative effect of DFSCs on $CD4^+$ T lymphocytes in asthma patients, while less value of suppressive activity was observed in healthy individuals. Another remarkable result was obtained from specific allergen (Der p1) stimulated cocultures that DFSCs suppressed the lymphocyte proliferation in asthma patients whereas there was no significant change observed in immunotherapy and healthy subjects with the same stimulation. This data supports the suppressive effect of DFSCs on the activated $CD4^+$ T lymphocytes. IFN-γ has been found to be decrease the proliferative response of $CD4^+$ T lymphocytes when used alone, but the increase in the apoptotic effect was observed with this stimulation, while high suppressive and anti-apoptotic effect was seen in the cocultures of DFSCs simultaneously stimulated with IFN-γ.

Several studies discuss whether MSCs induce the inducible T regulatory (iTreg) cell generation during inflammatory responses.[16,26] Inducible T regulatory cells are generated by the influence of TGF-β on $CD4^+CD25^+$ precursors during inflammatory responses in the periphery.[34] In addition, the immunosuppressive ability of MSCs are mediated by TGF-β, prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO), which are main inducers of regulatory T cell subsets during the differentiation process of T helper lymphocytes.[35,36] In this study, low levels of $CD4^+CD25^+FOXP3^+$ regulatory T lymphocyte population was increased with DFSCs in asthma patients especially with IFN-γ stimulation, however no significant change observed in the high levels of T regulatory cells in the immunotherapy group. At the same time, protein expression of FoxP3 supports this data that two folds of increase was seen in the cocultures of asthma patients while no significant change observed in immunotherapy subjects in the protein expression with DFSCs. The increase in IL-10 levels in the cocultures of asthma patients was significant, however, there was no significant difference was observed in IL-10 levels with DFSCs compared to PBMC cultures of immunotherapy patients. In a recent study, compatible with our results, it was demonstrated that secretion of IFN-γ by T lymphocytes enhances the suppressive activity of dental pulp MSCs, which further produce TGF-β.[32] Similarly, one of the studies suggests that dental pulp MSCs enhanced $CD4^+CD25^+FoxP3^+$ T regulatory cell frequency and reduced proinflammatory cytokine levels in $CD3^+$ T lymphocytes by the paracrine effect of TGF-β and IL-10 secretion.[37] These results may be an evidence of immunomodulation through the activation of inducible T regulatory cells by DFSCs during inflammatory responses.

A recent study demonstrated that the administration of adipose tissue-derived mesenchymal stem cells (ASCs) in murine asthma model increased IDO, TGF-β and PGE2 levels in lungs and improved lung function through the induction of regulatory T cell expansion.[38] In another study it was shown that blocking PGE2 and neutralisation of TGF-β eliminated the immunosuppressive effect of ASCs.[39] In a recent study it was stated that human dental pulp MSCs enhanced $CD4^+CD25^+FoxP3^+$ Treg frequency. The mechanism of immunosuppression of dental pulp MSCs was determined by the neutralisation of TGF-β and IL-10, and demonstrated that blockade of TGF-β and

IL-10 resulted with decreased T regulatory cell frequency, which shows that TGF-β involves in immune tolerance action by enhancing IL-10 secreting T regulatory cell frequency.[40] We therefore analysed $CD4^+CD25^+FOXP3^+$ regulatory T cell frequency by neutralising soluble TGF-β or the inhibition of IDO or PGE2 pathways, to determine which of the suppressive mediators produced by DFSCs has a triggering effect on the generation of regulatory T cell population. The inhibition of IDO pathway by 1-methyltryptphan reduced $CD4^+CD25^+FOXP3^+$ regulatory T cell amounts in asthmatics, which demonstrated that IDO is involved in DFSCs-mediated T regulatory cell induction. In asthmatic patients, TGF-β was found to be less effective than IDO in the induction of T regulatory cells when stimulated with the specific antigen Der p1. The variation of immunosuppressive action of MSCs may vary with different sources and immunomodulatory mechanism may be affected from diverse types of diseases due to inflammatory cell types. In contrast with previous studies, blockage of PGE2 did not significantly changed the frequency of T regulatory cells in both asthmatics and healthy subjects, which indicates the suppressive mechanism of DFSCs is not mediated through PGE2. These results suggest that the suppressive mechanism of DFSCs varies from other mesenchymal stem cells with the mediators it use and involved molecular mechanisms may further be investigated.

The crucial factor in inflammatory responses is the costimulatory activation of antigen-presenting cells. Conventional and monocyte-derived dendritic cells initiate and determine the fate of helper T lymphocyte whether it will differentiate into Th1 or Th2 effector phenotype, and there is a plasticity in T helper cell phenotypes during inflammatory responses.[41,42] The initiation of signalling mechanism begins with the presentation of antigens by MHC-class II and activation of costimulatory molecules (CD80, CD83 and CD86), which CD86 induced with allergen-specific responses.[43] In a study, it was stated that un-stimulated monocytes have the ability to modulate T cell activation in the culture wells, because monocytes can undergo slight differentiation after plating into culture wells.[44] A current study suggested that resting monocytes of non-atopic asthmatic patients showed low expression of CD80 and high expression of CD86 when stimulated with LPS in the culture.[45] In this study, we investigated the effect of DFSCs on the activation of costimulatory molecules on monocytes in PBMC. Asthmatic patients showed high expression of CD80 and CD86 on monocytes while immunotherapy patients lack the expression of these costimulatory molecules in antigen-specific stimulation with Der p1. This data indicates that there was a tolerance in monocytes to the specific antigen in immunotherapy subjects. Our results showed that DFSCs down-regulated the activation of CD80 and CD86 on monocytes. These results suggest the inhibitory effect of DFSCs on the costimulatory activation of APCs during antigen challenge.

5 | CONCLUSION

In the present study, we demonstrated immunomodulatory effects of DFSCs, which suppressed proliferative response of $CD4^+$ T lymphocytes by increasing T regulatory cell frequency, altering helper T lymphocyte phenotype by decreasing IL-4 cytokine levels and increasing IFN-γ levels in the favour of Th1 cells, reducing effector T lymphocyte ratios and by down-regulating the costimulatory activation of monocytes. We conclude that DFSCs can modulate Th2-mediated responses in allergic asthma and further in vivo studies on allergic diseases including asthma can be achieved.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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