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In-Vitro Evaluation of Immunomodulation Effects of Mesenchymal Stem Cell-Derived Exosomes in Refractory Chronic Spontaneous Urticaria

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ABSTRACT

Objective: Approximately half of chronic spontaneous urticaria (CSU) patients are thought to have an autoimmune pathology, and they are resistant to current treatment approaches. Mesenchymal stem cells (MSCs) are adult cells that have been shown to be useful in many autoimmune pathologies due to their immunomodulation properties. This study aimed to investigate the immunomodulatory effects of MSCs, and exosomes isolated from refractory CSU patients.

Materials and Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from 5 refractory CSU patients and 5 healthy volunteers. The effects of MSCs isolated from CSU patients and healthy MSCs were compared. Co-culture experiments were performed to evaluate the efficacy of Mesenchymal Stem Cells and exosomes on PBMCs of CSU patients and healthy volunteers. To compare the resulting effects, changes in IFN-γ, IL-4, IL-10, IL-17a, and TGF-β cytokines were detected by the ELISA method. Cell proliferations were detected with the CCK-8 kit.

Results: The effects of autologous and allogeneic MSCs on IFN- γ expressions were similar, both providing significant suppression at all cell ratios. However, IL-4 and IL-10 expression of PBMCs co-cultured with allogeneic MSCs significantly decreased while IL-17a and TGF-β expression increased significantly. In addition, our findings indicated that exosomes were capable of significant suppression at low PBMC ratios, regardless of autologous or allogeneic origin, but MSCs were more effective as the number of PBMCs increased.

Conclusion: These preliminary findings from in-vitro experiments suggested that allogeneic MSC, or high-dose exosome administration may be a potential approach for treatment in CSU patients, most of whom are regarded as suffering from an autoimmune disease and resistant to current treatments. However, our findings need to be supported by clinical studies.

Keywords: Chronic spontaneous urticaria, mesenchymal stem cells, exosome, immunomodulation

INTRODUCTION

Urticaria is a skin disease characterized by itchy, edematous, round, or oval shaped, red, and raised lesions on the skin ranging in size from a few millimeters to centimeters. The disease occurs in the form of episodes and if this condition lasts longer than 6 weeks it is called chronic urticaria (CU). Skin findings may be caused by certain stimuli such as cold, pressure, sun exposure and this condition is called inducible urticaria. However, in most cases, there is no identifiable trigger, and this group of patients is defined as chronic spontaneous urticaria (CSU) (1). The interaction of allergen-IgE complexes with the FcεRI receptor on the surface of mast cells causes degranulation, and the resulting mediators such as histamine, tryptase and leu-

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kotrienes cause skin manifestations. Although there is no history of allergy, mast cell activation and skin symptoms are the main findings in CSU cases. It has been reported that mast cells are activated either by an autoantibodies of type IgG against FcεRI (type IIb autoimmune CSU) or by auto-IgEs that complex with the auto-allergen (type I autoimmune CSU) (2,3). Specifically, autoantibodies to IgE or IgE receptors are thought to be present in 30-40% of CSU cases (3,4). Omalizumab is a biological therapy agent that blocks the interaction of IgE and FcεRI and has been shown to be effective in many CSU patients (5). However, there is a significant group of CSU patients who do not even respond to omalizumab therapy, and autoantibodies to FceRI may be the cause of this resistance. Although there are studies showing that tumor necrosis factor alpha inhibitors and intravenous immunoglobulin treatments can be beneficial, apart from the treatment algorithm recommended in the current guideline, these costly treatments may not provide a complete cure and may cause serious side effects (6,7). The skin symptoms of CSU have a direct impact on patients' quality of life, and the chronicity of the disease results in the perception that the patient will never recover, resulting in anxiety and serious damage to social relationships and the emotional state (8). For this reason, the search for an effective treatment alternative for resistant CSU patients continues intensively. One of these is Mesenchymal stem cells (MSCs), which have been shown to be beneficial in autoimmune diseases.

MSCs are multipotent adult cells, and they can be isolated from various tissues such as adipose tissue, bone marrow, and dental pulp. Since they are a heterogeneous group of cells, some criteria have been determined to define them. MSCs are cells that adhere to plastic surfaces; they can differentiate into cell types of mesenchymal origin such as chondrocytes, adipocyte, and osteocytes; and in terms of surface markers they must express CD73, CD90, and CD105 molecules, but not express CD11b, CD14, CD19, CD34, CD45, and HLA-DR (9). MSCs are immunomodulatory cells that suppress immune cells like T cells (10), B cells, natural killer (NK) cells (11), and antigen-presenting cells such as macrophages and dendritic cells (12), but activate regulatory T and regulatory B cells (10,13). The roles of indolamine 2.3 dioxygenase (IDO), prostaglandin E2 (PGE2), interleukin (IL)-10, hepatocyte growth factor, human leukocyte antigen (HLA)-G, and transforming growth factor (TGF) -β on the immunomodulation effects of MSCs are well elucidated (14). Therefore, MSCs have become an attractive research area for immune-related pathological conditions such as Crohn's disease, systemic lupus erythematosus, multiple sclerosis, and even graft-versus-host disease (15). Recently, it has been reported that not only MSCs but also extracellular vesicles obtained from MSCs can provide similar immunomodulation. Exosomes originating from MSCs provide a paracrine effect in the form of the delivery of their cytoplasmic contents consisting of DNA, RNA, and proteins to target cells (16). Considering the potential effects of exosomes that can be as effective as MSCs, it is understood that they are of great importance in the development of cell-free and effective treatment approaches. MSCs may be a potential option in the treatment of refractory CSU cases considered to have an autoimmune background. In a clinical trial with a small study group, we showed the positive effects of MSCs on refractory CSU cases (17). However, there is no study showing the effects of MSC-derived exosomes on refractory CSU. Therefore, we investigated the immunomodulatory effects of MSCs and MSC-derived exosomes on immune cells belonging to refractory CSU cases in this study. We aimed to obtain preliminary and comparative data on the cell-free treatment approaches for CSU in this way.

MATERIALS and METHODS

Patients and control subjects:

The protocol and content of this project was reviewed and approved by the Manisa Celal Bayar University Faculty of Medicine Ethics Committee (Approval number: 27-09-2017 / 20.478.486). In this study, adipose tissue-derived MSCs isolated from patients with refractory CSU in our previous study, and the ATCC adipose-derived MSC cell line were used (17). Venous blood samples were taken from three female and two male CSU patients whose MSCs were used for co-culture experiments and PBMC isolation was performed. All patients were diagnosed with CSU for at least one year (according to the EAACI/GA²LEN/EDF/WAO guidelines) (18), and they were those who could not experience disease control despite using omalizumab and/or cyclosporine for six months or longer. Since the patients were unresponsive to omalizumab/cyclosporine, their treatment was continued with high-dose antihistamine drugs, and yet the weekly urticaria activity score (UAS7) was greater than 20. Patients with a history of chronic inducible urticaria, atopic dermatitis, another underlying itchy skin disease, parasitic infection,

and malignancy were excluded from the study. Five age- and sex-matched healthy subjects without any allergies, urticaria, itchy skin disease, autoimmune disease, parasitic infection, or a history of malignancy were included in the study as a control group.

Cell culture

To evaluate autologous interactions, adipose tissue-derived MSCs isolated from refractory CSU patients in our previous study were used (urticaria patient-derived MSC; uMSC). Human adipose-derived MSCs (ATCC® PCS-500-011 ™) were purchased from the American Type Culture Collection to evaluate allogeneic interactions (healthy donor MSC; hMSC). All MSCs were cultured by using Dulbecco's Modified Eagle's medium-F12 (Biosera, USA) medium that including 10% fetal bovine serum (FBS) (Biosera, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Biosera, USA) and 1% 2 mM L-glutamate (Biosera, USA) at 37 °C and 5% CO_2 incubator. Cells were controlled daily, and sub-cultured when they reached 70-80 % confluence. The 3rd passage of MSCs was used to perform exosome isolation and co-culture experiments. Venous blood samples of all patients were taken into BD Vacutainer® Plus Plastic K2 EDTA tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using Lymphosep lymphocyte separation medium (density: 1.077, Biosera, USA). PBMCs were cultivated in a culture medium composed of Roswell Park Memorial Institute -1640 medium (Biosera, USA), 10% FBS (Biosera, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Biosera, USA) and 1% 2 mM L-glutamate (Biosera, USA) at 37 °C and 5% CO_2 incubator.

Exosome isolation

MSCs were grown to 90% confluency, the old medium was removed, fresh medium containing exosome-depleted FBS (Exo-FBS, System Biosciences, USA) was added, and then cultured for another 3 days. Medium supernatants were collected at the end of the incubation, and centrifugation was performed for 30 minutes at speeds of 300g, 1.200g and 10.000g, respectively, in order to remove cells and apoptotic residues in the medium supernatants. After each centrifugation, the medium on the top was carefully collected and transferred to another tube. Subsequently, exosome isolation of each case was performed using the Exo-Prep One-step Exosome Isolation kit and according to the manufacturer's protocol (Hansa Biomed, Estonya). The protein content of the exosomes was extracted using RIPA lysis buffer (ThermoFisher Scientific, USA), followed by determining the amount of protein using the BCA protein assay (ThermoFisher Scientific, USA). To verify the exosomes, the Exo-FACS kit (Hansa Biomed, Estonya) was used, and the exosomes loaded into the latex-beads were labeled with CD63 Alexa 488 (Hansa Biomed, Estonya), CD9 FITC (Clone: MEM-61, Exbio, Czech Republic), and CD105 (Clone: MEM-226, Exbio, Czech Republic) antibodies, and then analyzed with the Accuri C5 Flow cytometry device (BD Biosciences, USA).

Co-Culture

To evaluate immunomodulatory interactions, $1x10^4$ cells/well MSCs were seeded in appropriate positions of a 96-well plate and incubated for 24 hours to attach. CSU patients (uP) and healthy control PBMCs (hP) were activated with v/v 2% phytohemagglutinin (PHA) M (Gibco, USA) for 48 hours before the co-culture, and then $1x10^4$ (1:1), $5x10^4$ (5:1) and $1x10^5$ (10:1) cells/well were added to appropriate positions. To evaluate the effects of exosomes, $1x10^4$, $5x10^4$, and $1x10^5$ activated PBMCs were added to the appropriate wells of another 96-well culture dish and 10 µg/mL of exosomes isolated from each patient's own MSCs (uExo) or ATCC-MSCs (hExo) were added; the cells were then incubated for 24 hours. The same number of PHA-activated and unstimulated PBMCs was determined as positive and negative controls, and then all cells were incubated for 24 hours. The co-culture experiment plan is summarized in Figure 1.

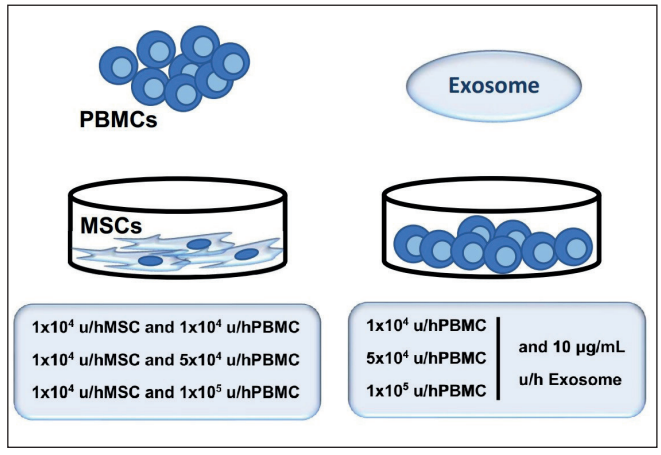

Figure 1. Schematic summary of co-culture experiments.
*(**u:** CSU patient, **h:** Healthy control, **MSC:** Mesenchymal stem cell, **PBMC:** Peripheral blood mononuclear cell)*

Cell proliferation assays

The effects of MSCs and exosomes on the proliferation of PBMCs were determined by the Cell Counting Kit-8 (CCK-8) (Abbkine Scientific Co. China). For this, the number of cells used in the co-culture experimental setup was used and after 24 hours of incubation, 10 µL of CCK-8 reagent was added to each well and the cells were re-incubated for 4 hours. Absorbances were measured at 450 nm using the ELX800 microplate reader.

ELISA

ELISA analyzes were performed to evaluate the effects of MSCs and exosomes on inflammatory cytokine expressions. The collected cell culture supernatants were centrifuged at 4000 rpm for 10 minutes and cell debris was removed. ELISA kits capable of measuring human IFN-γ, IL-4, IL-6, IL-10, IL-17a, and TGF-β levels were purchased from Diaclone, France. Cytokine changes were measured in accordance with the manufacturer's protocol. Absorbances were determined using the Multiskan™ FC Microplate Photometer (Thermo Scientific™, USA) instrument. The PRISM v8 (GraphPad San Diego, USA) software was used for the creation of standard curves and calculations.

Statistical Analysis

The data obtained were analyzed using the PRISM v8 (GraphPad San Diego, USA) statistical software. Normality and lognormality tests were performed to determine whether the groups were in normal distribution and nonparametric calculation methods were applied for the groups with Shapiro-Wilk normality test results $p<0.05$. The one-way ANOVA (Kruskal-Wallis for nonparametric groups) method was used to compare groups with each other. Comparison data found as $p<0.05$ were considered statistically significant.

RESULTS

Cell culture

Cultured cells were plastic adherent and had spindle morphology. In flow cytometry analysis of the isolated exosomes, CD9, CD63, and CD105 markers were found to be positive (Figure 2). In PBMCs cultured with MSCs at 1:1 and 1:5 ratios, clustering, which is the indicator of activation, was not evident, but was present in the culture with a ratio of 1:10. On the contrary, clusters were observed at all cell ratios in PBMCs cultured with exosomes (Figure 3).

Cytokine alterations

First, we compared the cytokine levels of CSU patients and healthy subjects (Figure 4). We observed that the IFN-γ and IL-4 levels of hP cells at $1x10^4$ cell ratios were significantly higher than uPs ($p = 0.01$ and $p= 0.02$ respectively), but there was no significant difference for $5x10^4$ ($p = 0.46$ and $p = 0.91$ respectively) and $1x10^5$ cell ratios ($p = 0.98$ and $p= 0.86$ respectively). For all cell ratios, IL-10 and TGF-β levels of uPs were significantly lower than hPs (for $1x10^4$: $p<0.001$ and $p<0.001$; for $5x10^4$: $p= 0.005$ and $p<0.001$; for $1x10^5$: $p<0.001$ and $p<0.001$ respectively), but IL-17a levels were significantly higher (for cell ratios $p<0.001$).

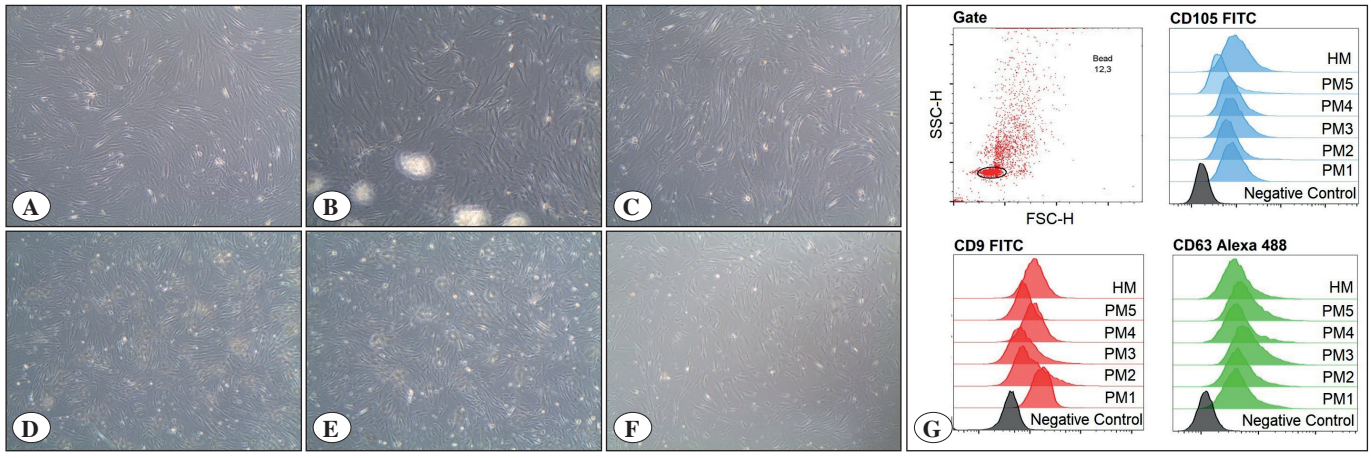

Figure 2. Microscopic images of mesenchymal stem cells of chronic spontaneous urticaria patients **(A-E)** and ATCC cell line **(F)**. Flow cytometry histogram graphics of exosomes isolated from mesenchymal stem cells **(G)**.
*(**PM:** CSU patient mesenchymal stem cells, **HM:** Healthy mesenchymal stem cells)*

We then compared the effects of MSCs and exosomes on cytokine expressions. At 1:1 cell ratios, IFN-γ expression of uP and hP was significantly reduced by both u/hMSCs and u/hExos. At 1:5 cell ratios, IFN-γ expression of hP was significantly reduced by u/hMSCs and u/hExos, but only u/hMSCs for uP led to significant reduction. As for the 1:10 cell ratios, significant reduction was achieved by u/hMSCs only. At all cell ratios, hPs' IL-4 expressions were significantly reduced by u/hMSCs and u/hExos. In contrast, IL-4 expressions of uP were significantly reduced at 1:1 and 1:5 cell ratios and only by hMSCs. IL-10 expressions of uP were significantly reduced by u/hMSCs and u/hExos at 1:1 cell ratios. However, while a significant reduction was achieved with hMSCs and uMSCs at 1:5

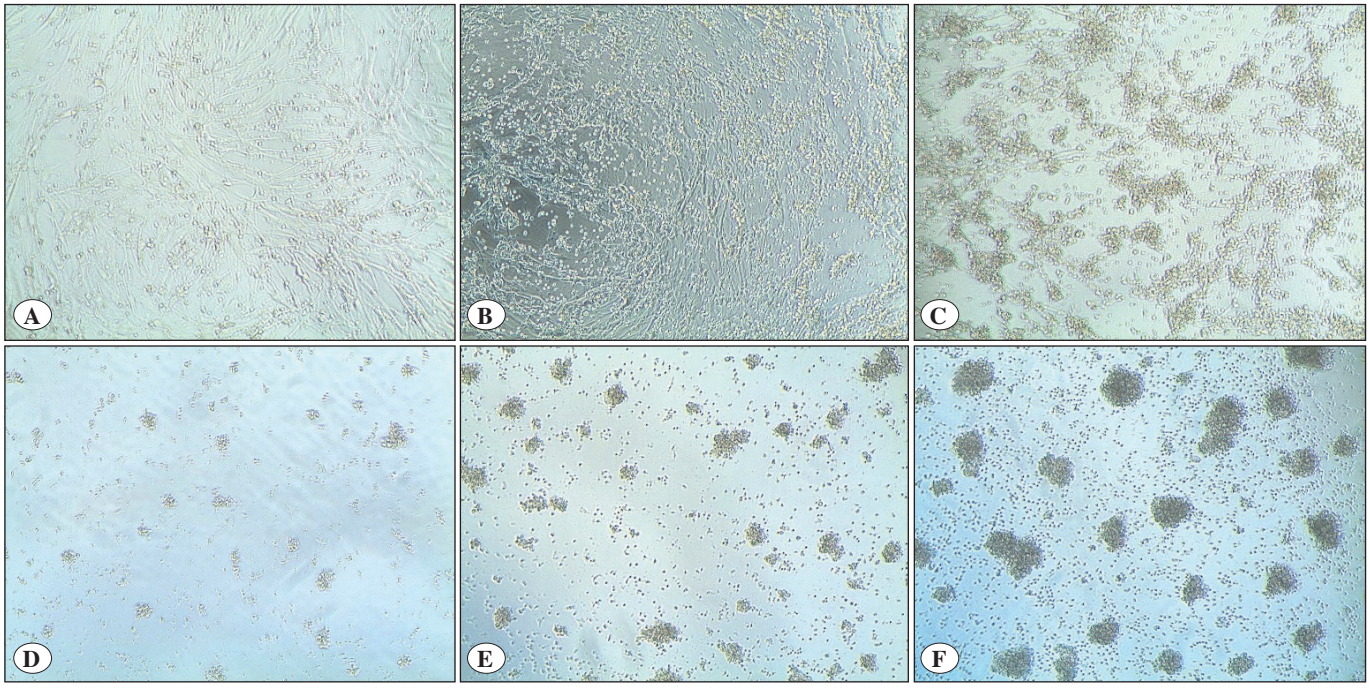

Figure 3. MRepresentative microscopy images of co-cultured mesenchymal stem cells and PBMCs at 1:1 **(A)**, 1:5 **(B)** and 1:10 **(C)** cell ratios, and PBMCs at 1x104 **(D)**, 5x104 **(E)** and 1x105 **(F)** numbers cultured by adding 10 µg/mL exosome.

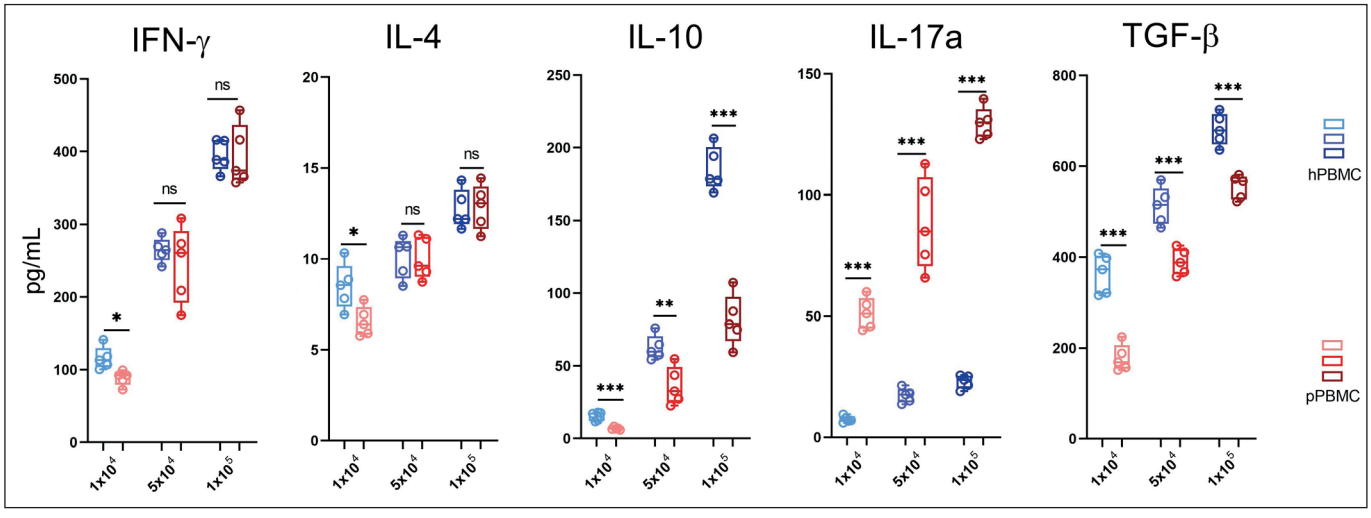

Figure 4. Comparison graphs of cytokine levels of activated peripheral blood mononuclear cell (PBMC) at different cell ratios of chronic spontaneous urticaria patients (uPBMC) and healthy subjects (hPBMC). Data are presented as mean and standard deviation.
(: p<0.05 statistically significant)*

cell ratios, only hMSCs led to a significant reduction at 1:10 ratios. Interestingly, we found that the resulting significant increase in IL-17a and TGF-β expressions of uP were only caused by hMSCs across all cell ratios. However, both hMSCs and uMSCs led to a significant increase in IL-17 expression of hP. At all cell proportions, TGF-β expressions of hPs increased significantly with hMSCs, but uMSCs only provided a significant increase at 1:1 and 1:5 cell ratios. Exosomes did not cause a significant change in IL-17a and TGF-β expression of uP or hP. The "p" values of the statistical comparison of the groups are given in Table I, and comparison charts of the groups are presented in Figure 5.

Cell proliferations

The optical density changes determined by the CCK-8 assay are presented in Figure 5. Proliferations of PHA-activated uP cells at 1:1 cell ratios were significantly

Table I: Table of "p" values obtained from statistical analysis.

Cytokine	Groups	hMSCs	uMSCs	hEXOs	uEXOs
IFN-γ	uPBMCs (1:1)	p<0.001	p= 0.001	p= 0.01	p= 0.04
	hPBMCs (1:1)	p<0.001	p<0.001	p= 0.001	p= 0.001
	uPBMCs (1:5)	p= 0.03	p= 0.02	p>0.99	p>0.99
	hPBMCs (1:5)	p<0.001	p<0.001	p<0.001	p<0.001
	uPBMCs (1:10)	p= 0.007	p= 0.003	p= 0.93	p= 0.89
	hPBMCs (1:10)	p<0.001	p<0.001	p= 0.14	p= 0.72
IL-4	uPBMCs (1:1)	p=0.004	p= 0.94	p>0.99	p= 0.98
	hPBMCs (1:1)	p= 0.001	p= 0.001	p= 0.01	p= 0.007
	uPBMCs (1:5)	p=0.03	p= 0.96	p= 0.95	p>0.99
	hPBMCs (1:5)	p= 0.002	p= 0.007	p= 0.02	p= 0.01
	uPBMCs (1:10)	p>0.99	p= 0.90	p= 0.37	p= 0.51
	hPBMCs (1:10)	p<0.001	p<0.001	p= 0.005	p= 0.01
IL-10	uPBMCs (1:1)	p<0.001	p<0.001	p= 0.002	p<0.001
	hPBMCs (1:1)	p<0.001	p<0.001	p<0.001	p<0.001
	uPBMCs (1:5)	p= 0.003	p= 0.02	p= 0.80	p= 0.81
	hPBMCs (1:5)	p<0.001	p= 0.005	p= 0.19	p= 0.22
	uPBMCs (1:10)	p= 0.02	p= 0.27	p>0.99	p>0.99
	hPBMCs (1:10)	p<0.001	P= 0.006	p= 0.14	p= 0.34
IL-17	uPBMCs (1:1)	p= 0.02	p= 0.54	p= 0.48	p= 0.39
	hPBMCs (1:1)	p<0.001	p<0.001	p>0.99	p>0.99
	uPBMCs (1:5)	p= 0.02	p= 0.90	p= 0.98	p>0.99
	hPBMCs (1:5)	p<0.001	p<0.001	p= 0.19	p= 0.22
	uPBMCs (1:10)	p<0.001	p= 0.15	p= 0.14	p= 0.28
	hPBMCs (1:10)	p<0.001	p<0.001	p>0.99	p>0.99
TGF-β	uPBMCs (1:1)	p= 0.003	p>0.99	p>0.99	p>0.99
	hPBMCs (1:1)	p= 0.006	p= 0.01	p>0.99	p>0.99
	uPBMCs (1:5)	p<0.001	p= 0.36	p= 0.82	p= 0.93
	hPBMCs (1:5)	p<0.001	p=0.04	p>0.99	p>0.99
	uPBMCs (1:10)	p<0.001	p= 0.94	p= 0.83	p= 0.52
	hPBMCs (1:10)	p= 0.04	p= 0.80	p>0.99	p>0.99

u: Chronic spontaneous urticaria patients, **h:** Healthy control, **PBMC:** Peripheral blood mononuclear cells, **MSC:** Mesenchymal stem cell, **EXO:** Exosome

suppressed by hMSCs (p<0.001), uMSCs (p<0.001), hExo (p<0.001), and uExo (p<0.001). The suppression created by hMSCs and uMSCs was significantly higher compared to hExo (p<0.001 and p<0.001 respectively) and uExo (p<0.001 and p<0.001 respectively). At the 1:5 cell ratios, proliferations of uP cells were significantly suppressed with both hMSCs (p= 0.004) and uMSCs (p= 0.02), but at a 1:10 cell ratio, only hMSCs produced significant suppression (p= 0.005). Proliferations of PHA-activated hP cells at 1:1 and 1:5 cell ratios were significantly suppressed by hMSCs (p<0.001 and p<0.001 respectively), uMSCs (p<0.001 and p<0.001 respectively), hExo (p<0.001 and p<0.001 respectively), and uExo (p<0.001 and p<0.001 respectively). Like uP cells, suppression formed by hMSCs and uMSCs was significantly higher compared to hExo (for 1:1 ratio p<0.001 and p<0.001; for 1:5 ratio p<0.001 and p<0.001 respectively) and uExo (for 1:1 ratio p<0.001 and p<0.001; for 1:5 ratio p<0.001 and p<0.001 respectively). At the 1:10 cell ratio, the proliferation of hPs was significantly suppressed with both hMSCs (p<0.001) and uMSCs (p<0.001), and the suppression induced by hMSCs was significantly higher than for uMSCs (p= 0.02).

DISCUSSION

In this study, we investigated the in vitro immunomodulation effects of MSCs isolated from adipose tissues of refractory CSU patients and exosomes obtained from these cells. As a control, we used allogeneic adipose tissue MSCs, and exosomes obtained from a healthy individual. We evaluated how these interactions changed at different MSC:PBMC or exosome:PBMC ratios. IFN-γ is a pro-inflammatory cytokine that enhances the activation of immune cells, major histocompatibility complex (MHC) expressions, immunoglobulin productions, and production of reactive oxygen derivatives (19). In studies performed with serum samples of CSU cases, it was reported that IFN-γ levels were significantly higher than in healthy controls, and this elevation decreased significantly

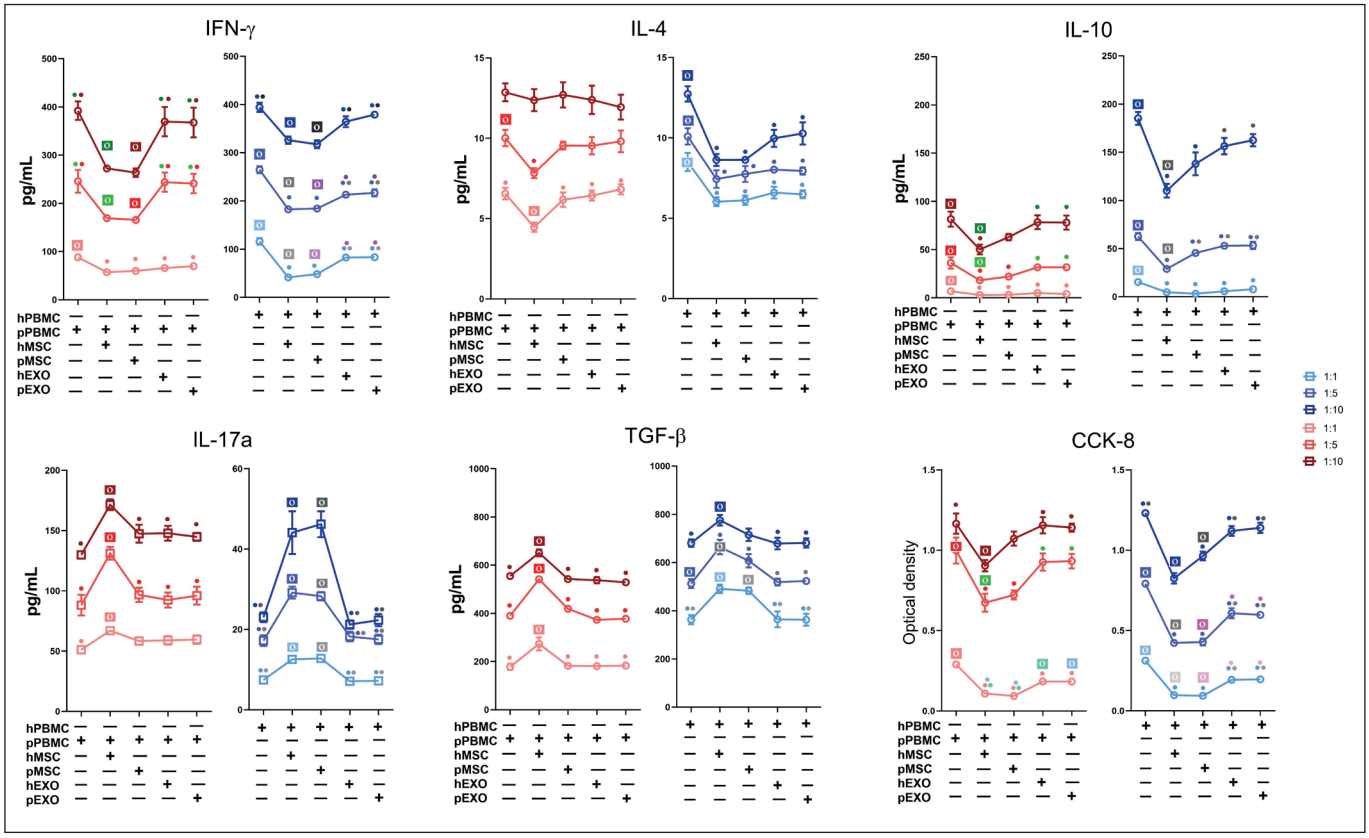

Figure 5. Comparison graphics of cytokine measurements obtained from co-culture experiments. Data are presented as mean and standard deviation. Which cell or exosome is used in co-culture groups is indicated with a (+) sign. There is a statistically significant difference (p<0.05) between the "o" symbols and points having the same color.
(p: patient, h: healthy)

after the use of omalizumab, a human anti-IgE antibody (20,21). However, in our previous study with refractory CSU cases, we reported that IFN-γ levels were not affected by the use of omalizumab (17). In other words, it is understood that there may be aberrant differences in cytokine levels between omalizumab-responsive and resistant CSU patients. In this study, we observed that IFN-γ secretions of PHA-activated uPs were significantly suppressed by both autologous and allogeneic MSCs, but the same effect could not be produced by exosomes, except for a 1:1 ratio. As for the IFN-γ expression of hPs, we found that both u/h MSCs and u/h exosomes (except 1:10 ratio) were capable of significant suppression at all ratios. However, the effect formed by u/hMSCs was significantly superior to exosomes. It has been shown in many studies that both MSCs and MSC-derived exosomes can effectively suppress IFN-γ expression of PBMCs (10,12,22-24). However, our findings suggested that the suppression caused by the direct interaction of MSCs with immune cells could be superior.

In studies performed for IL-4, a cytokine closely associated with allergic diseases, it has been reported that the IL-4 expressions of the PBMCs of CSU patients are significantly lower and this situation is more pronounced in cases with positive ASST (25-27). There are many studies showing that IL-4 expressions of PBMCs from both healthy and allergic patients are effectively suppressed by MSCs and also exosomes (10,12,23,28,29). We observed that IL-4 expression of hPs was significantly suppressed by both u/hMSCs and u/h exosomes at all cell ratios. However, we found that this suppression in uPs occurs at 1:1 and 1:5 cell ratios and only by hMSCs. This finding suggested that interaction with autologous MSCs may not influence IL-4 expression of uPs, and this may also apply to autologous and allogeneic exosomes.

Approximately half of the refractory CSU cases are thought to be an autoimmune pathology, and Th17 cells and its cytokine IL-17 are prominent in autoimmune diseases (3). There are studies revealing the presence of high Th17/IL-17 in both serum and skin biopsies of CSU patients and its correlation with disease severity (17,30,31). Consistent with the literature, we also observed that IL-17 levels of uPs were significantly higher than hPs. In our experiments with MSCs and exosomes, we found that hPs' IL-17a expression was significantly increased by both hMSCs and uMSCs, but in uPs this increase was caused only by hMSCs. We observed that u/h exosomes did not cause a significant change in both hPs and uPs. It has been reported that the interaction of MSCs with monocytes/macrophages increases IL-17 expression of T cells, and suppression of IL-17a levels occurs with the removal of monocytes from the environment (10,12,32). In addition, the increase in IL-17a that occurs in the presence of MSCs has been shown to potentiate the effect of the resulting immune suppression (33). In our study, the significant increase in IL-17a in hPs interacting with both hMSCs and uMSCs was consistent with the data in the literature. However, this increase in uPs was only provided by hMSCs. These data lead to two different interpretations. One of them is that allogeneic interaction may be an important factor in increases in IL-17a expression of PBMCs. The other is that MSCs of CSU patients may not have an effect on the IL-17a expression of own PBMCs. IL-6 stands out as an important cytokine in differentiation of naive T cells to Th17. However, simultaneous TGF-β stimulation with IL-6 causes these cells to differentiate from the proinflammatory Th17 type to anti-inflammatory Treg cells. Interestingly, it has been shown that there is an increase in IL-17 and IL-10 expression simultaneously in Th17 cells (34,35). As with IL-17a expressions, we detected a similar interaction in TGF-β expressions. Considering the effect of TGF-β on Th17/Treg plasticity, our findings revealed that allogeneic MSCs may have especially contributed to the immunosuppressive differentiation of Th17 cells. However, there was no significant change induced by exosomes for both IL-17a and TGF-β.

IL-10 is a strong immunosuppressive cytokine. Degirmenci et al. have reported in their study that the IL-10 expressions of PBMCs of CSU patients were significantly lower than healthy individuals (26). Another study has shown that the use of omalizumab causes a decrease in IL-10-producing T cell frequencies in CSU patients (36). We also observed that PBMCs of CSU patients had significantly lower IL-10 expressions than healthy subjects. However, the responses of PBMCs of patients and healthy subjects to MSCs and exosomes for IL-10 were similar. We observed that at 1:1 cell ratios, IL-10 expression of uPs and hPs were significantly suppressed by both MSCs and exosomes, but the effect of exosomes disappeared as the cell ratios increased. Another important finding we observed was that IL-10 expressions of hPs were suppressed by hMSCs and uMSCs at all cell ratios, whereas in uPs this was only provided by hMSCs. These findings suggested that the allogeneic interaction could play an important role in the manifestation of the effects of MSCs. We detected a sup-

pression profile in cell proliferations that we assessed with CCK-8 assays, typically compatible with IL-10 and IFN-γ curves. Proliferations of hPs at 1:1 and 1:5 cell ratios were significantly suppressed by both MSCs and exosomes, but only by MSCs at 1:10 ratios. In uPs, exosomes were only able to suppress 1:1 cell ratio. However, only hMSCs produced significant suppression at all cell ratios.

There are several prominent limitations of our study. First, our results with a very limited number of cases need to be confirmed with larger cohorts. Second, although we used the CSU patients' own PBMCs, MSCs, and exosomes in the experiments, we unfortunately used allogeneic MSCs and exosomes in control subjects. Finally, the accepted gold standard method for exosome isolation is the use of ultracentrifugation. Since we do not have this instrument in our laboratory, we used commercially available exosome isolation kits.

In this study, we evaluated the responses of PBMCs of refractory CSU patients to autologous and allogeneic MSC and exosome applications based on changes in cytokine expressions. Our findings revealed that the effect of MSCs and exosomes at low PBMC numbers was similar, but MSCs were more effective as the number of PBMCs increased. Another important point was that allogeneic interaction was superior to autologous interaction. In particular, we observed that allogeneic MSCs formed typical alterations in IL-4, IL-10, IL-17a and TGF-β expressions and in this way could provide a superior suppression. These preliminary findings from in-vitro experiments suggested that allogeneic MSCs may be a more appropriate option for treatment in refractory CSU patients. In addition, our findings indicated that exosomes were capable of significant suppression at low PBMC ratios, regardless of autologous or allogeneic origin. In conclusion, our study supports that allogeneic MSC, or high-dose exosome administration may be a potential approach for disease treatment in CSU patients, most of whom are regarded as having an autoimmune disease and resistant to current treatments. However, our findings need to be supported by clinical studies.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authorship Contributions

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