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Adjustment of a Fibrosis Marker, Pro-Inflammatory Cytokines, and IgE in Asthmatic Animals

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Abstract:
Background: A lot of patients are suffering from asthma. For decreasing the asthma symptoms, we studied the effects of conditioned medium (CM) of human amniotic membrane mesenchymal stem cells (hAM-MSCs) as a source of anti-inflammatory cytokines on splenocyte and lung tissue of asthmatic Balb/c mice.
Methods: Forty mice were categorized into four groups; ovalbumin (OVA)-induced asthma, CM-treated asthma, DMEM (Dulbecco's Modified Eagle Medium)-treated asthma, and saline control. Each group received related treatment. The lung alpha-smooth muscle actin (α-SMA) and splenocyte inflammatory cytokines and IgE were examined through Western blot analysis.
Results: Western blot showed α-SMA overexpression in the OVA and DMEM groups compared with the saline group. CM therapy could significantly reverse it compared with OVA and OVA+DMEM categories by elevating IL-10 and IFN-γ and reducing IL-4, IgE, and TGF-β .
Conclusion: CM treatment could improve asthma symptoms by adjusting α-SMA in lung tissue and pro-inflammatory cytokines and IgE in splenocytes.

INTRODUCTION

More than 300,000 patients worldwide are suffering from asthma (1) as an uncomfortable respiratory inflammation of the airways (2) with a high mortality rate (3). Several cells including eosinophils, mast cells, B lymphocytes, activated T lymphocytes, neutrophils, airway epithelial cells, as well as airway smooth muscle (ASM) can develop remodeling and narrowing of the airway, bronchial hyper-reactivity (BHR), and fibrosis (4); and T helper (Th)1/Th2 imbalance and increased inflammatory cytokines are essential in its pathogenesis (5). Thus, asthma is characterized by infiltration of inflammatory cells and overproduction of various cytokines like Interleukin (IL)-4, IL-5, and IL-13, as well as Immunoglobulin E (IgE) (3). Among imbalanced cytokines, IL-4, as a Th2 cytokine, plays the most important role in inflammation and airway remodeling and leads to airway hyper-responsiveness (AHR), infiltration of inflammatory cells, and secretion of mucus into the lungs (6).

Inflammation of the airways causes tissue remodeling and structural changes such as the thickness, increase of the basement membrane, and elevation of collagen density (7). Proliferation of the airway smooth muscles is the most important factor related to asthma exacerbation and decreased lung physiological function. This thickening is due to hypertrophy, hyperplasia, as well as increased extracellular matrix (ECM) protein deposition. In addition, airway smooth muscle in asthmatic patients is associated with the infiltration of inflammatory

cells like mast cells, eosinophils, and lymphocytes (8). Airway smooth muscle remodeling is considered an asthma hallmark and could be detected by the deregulation of proteins such as alpha-smooth muscle actin (α-SMA) (9). α-SMA is considered a smooth muscle cell marker and demonstrates myofibroblast differentiation. As α-SMA is expressed by Myofibroblasts as a differentiated form of fibroblasts, it plays a role in fibrogenesis (10), and its high expression indicates more differentiation of fibroblasts to myofibroblasts (11).

On the other hand, the spleen plays an important role in allergic response induction and pathogenesis of allergic conditions. Studies show that in inflammatory conditions such as allergies, spleen-derived mast cells produce IL-4, IL-6, and Tumor Necrosis Factor-alpha (TNF-α) in response to receptor-bound IgE. In allergic inflammation, a decrease in Interferon-gamma (IFN-γ) intensifies the Th2 response (12). In addition, IFN-γ is classified as a prototype of Th1 cytokine (5). Fluctuation of these cytokines in the spleen is an indicator of immune system activities (13).

For many years, high-dose corticosteroids, along with beta-agonists, were used to treat asthma, but they failed. So, some novel therapies exert their influence by focusing on the control of lymphocyte functionality and cytokine release; in this regard, stem cells have shown promising applications in the therapy of various human disorders (1). Among different kinds of stem cells, stem cells derived from the Human amniotic membrane (hAM) have advantages because hAM can be obtained easily after birth, and in normal situations it is not used, a small piece of this membrane has a lot of single stem cells, and hAM causes less immunogenic reactions (14).

Although stem cells have some disadvantages, for example, some shortages may occur in the tissue banks and they are not cost-effective (15),

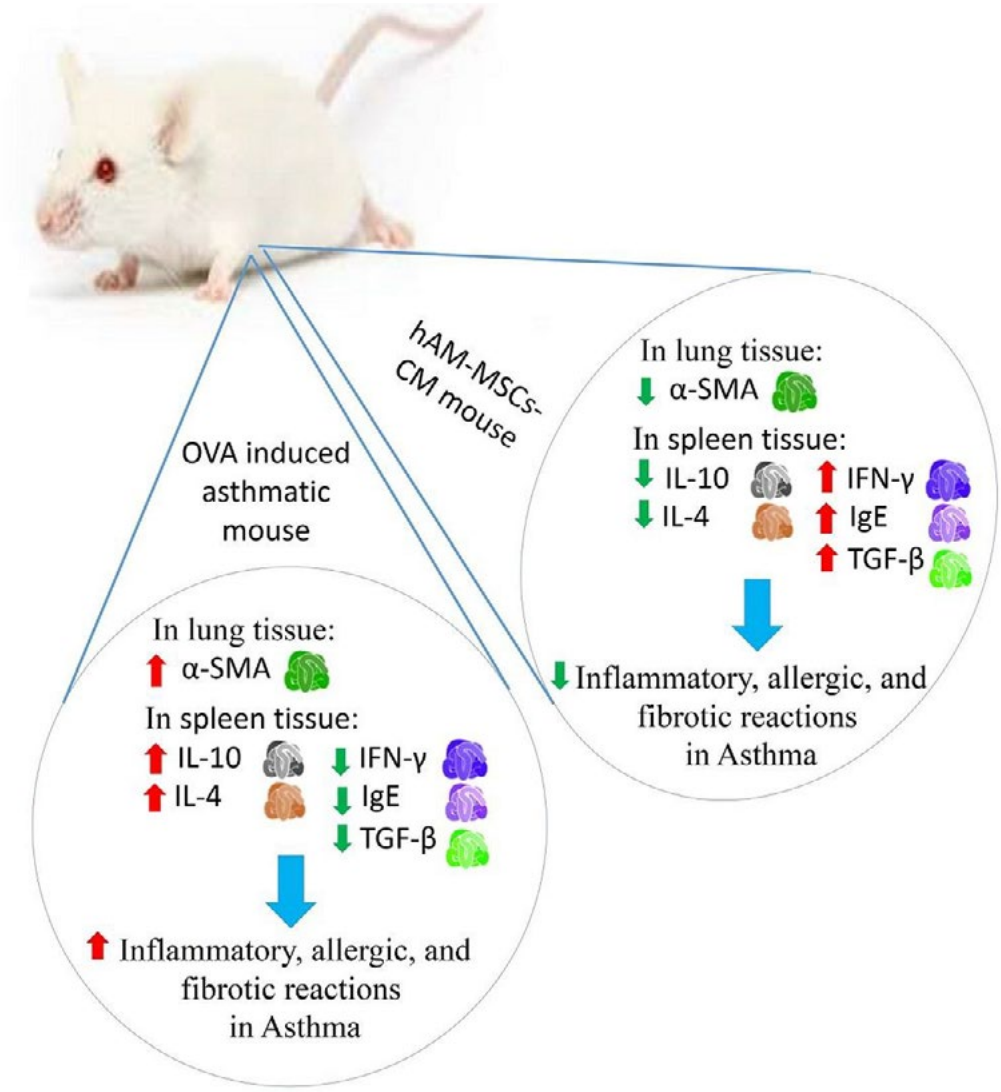

Fig 1. A graphical abstract of our study. hAMSCs-CM improved asthma symptoms by adjusting α-SMA in lung tissue and IL-10, IFN-γ, IgE, IL-4, and TGF-β in splenocytes in an OVA-induced asthmatic mouse.

the age and health status of donors and recipients affect them, they need invasive techniques, they are easily contaminated, in some cases, they show poor homing ability, low transplantation efficiency, and uncontrolled viability in vivo (16).

Researchers are exploring hAM-MSCs as a viable research and treatment option due to their high cytokine secretion and fewer ethical concerns. To address their limitations, they have introduced conditioned medium (CM), a cell culture medium that contains secretory factors from these cells, which has demonstrated therapeutic effects similar to stem cells (17, 18). CM derived from different stem cells can improve health problems like acute and chronic hind limb ischemia and myocardial infarction (18).

This study aims to overcome the current practical challenges in asthma treatment by using hAM-MSCs-CM to alleviate lung fibrosis and spleen inflammation in asthmatic mouse models for the first time.

This research is innovative in two different aspects; it is assessing the effect of hAM-MSCs-CM on asthma improvement via adjusting spleen secretion in animal models for the first time. Furthermore, as most asthma treatments are based on relieving the inflammatory and fibrotic symptoms of airways (19), it evaluates several immune responses, as well as inflammatory and fibrotic factors to assess the effectiveness of our innovative treatments.

We hypothesized with the results of this investigation, we will fill the gap of knowledge about the therapeutic effect of hAM-MSCs-CM on inflammatory factors of spleen culture supernatant and α-SMA expression in ovalbumin (OVA)-associated asthma in vivo. A summary of this study has been presented in Fig. 1.

MATERIALS AND METHODS

Preparation of the hAMSCs-CM

The hAM-MSCs and their CM were obtained and processed using established methods (17, 20). Briefly, the amniotic membrane was obtained from women with natural childbirth or elective cesarean. All donors of hAM-MSCs signed informed consent before donation. The clear vascular-free membrane was separated, washed with phosphate-buffered saline (PBS), fragmented into smaller pieces, and treated with 0.05% trypsin-EDTA. After centrifugation, the cells were washed with PBS and passed through 100µM disposable mesh. The collected cells were cultured in flasks containing high glucose DMEM, 10% FBS, Penicillin/ Streptomycin, and 10 ng/mL EGF (Royan Institute, Tehran, Iran), and then, incubated in a CO2 incubator at 37°C. After 24 h, non-adherent cells were removed, and a fresh medium was added to the culture and incubated for 48 h. Subsequently, the culture was treated with 0.25% trypsin-EDTA, and the third passage was supplemented with serum-free α-MEM medium to obtain CM. After 48 hours of incubation, the cell-free CM was obtained by filtering the supernatant through a 0.22µM filter (21).

Animal grouping and sensitization protocol

Forty male Balb/c mice (25-30 g) were purchased from Iran University of Medical Sciences (IUMS), and were categorized into four groups (10 in each group); in three groups, respiratory allergic asthma was induced by intraperitoneal injection of ovalbumin (OVA), followed by daily respiratory challenge with OVA (Sigma-Aldrich, St. Louis, MO, USA). Mice were intraperitoneally sensitized with OVA (20 µg) combined with aluminum hydroxide (2 mg) (Sigma-Aldrich) in normal saline (1ml) on days 1, 8, and 14. Then, they were daily exposed to inhalation of 3% OVA with a nebulizer (Omron CX3, Japan) in the plexiglass box from day 21 up to one week (30 minutes a day). The controls were undergone the same challenge with normal saline instead of OVA (22). Then, one of the asthma-induced groups was kept untreated (OVA) and the other groups were treated either with hAM-MSCs-CM (OVA + CM) or fresh cell culture medium (OVA + DMEM). The two treatment categories were administered two doses of either CM or DMEM intravenously (i.v), each consisting of 50 µl, on days 28 and 29.

Measurement of α-smooth muscle actin (α-SMA)

On day 30, the animals underwent surgical anesthesia. The lungs were lysed in RIPA to evaluate the α-SMA level. Approximately 20 µg of the lysates were run on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA, USA). Then, the membranes were blocked with 5% BSA (Sigma Aldrich, MO, USA) in 0.1% Tween 20 for 1 h and were incubated with rabbit polyclonal anti-α-SMA (Abcam, ab5694), as well as rabbit polyclonal anti-beta actin antibodies (Abcam, ab8227) for 1h at 25^{O}C. Then, membranes were washed three times with PBS containing 0.05% tween 20, and incubated with HRP-labeled goat anti-rabbit IgG (Abcam, ab6721) secondary antibody. The membranes were incubated with enhanced chemiluminescence (ECL) substrate for 1–2 min and evaluated by a chemo-documentation system. Protein bands were normalized to β-actin bands. Bands' densitometry was measured by the gel analyzer Version 2010a software (NIH, USA), and the percentage of the area under the curve (AUC) of each α-SMA was divided by the related β-actin band, and subsequently, calculated values were compared between groups.

Spleenocyte culture and cytokine analysis

The removed spleens were placed in RPMI and

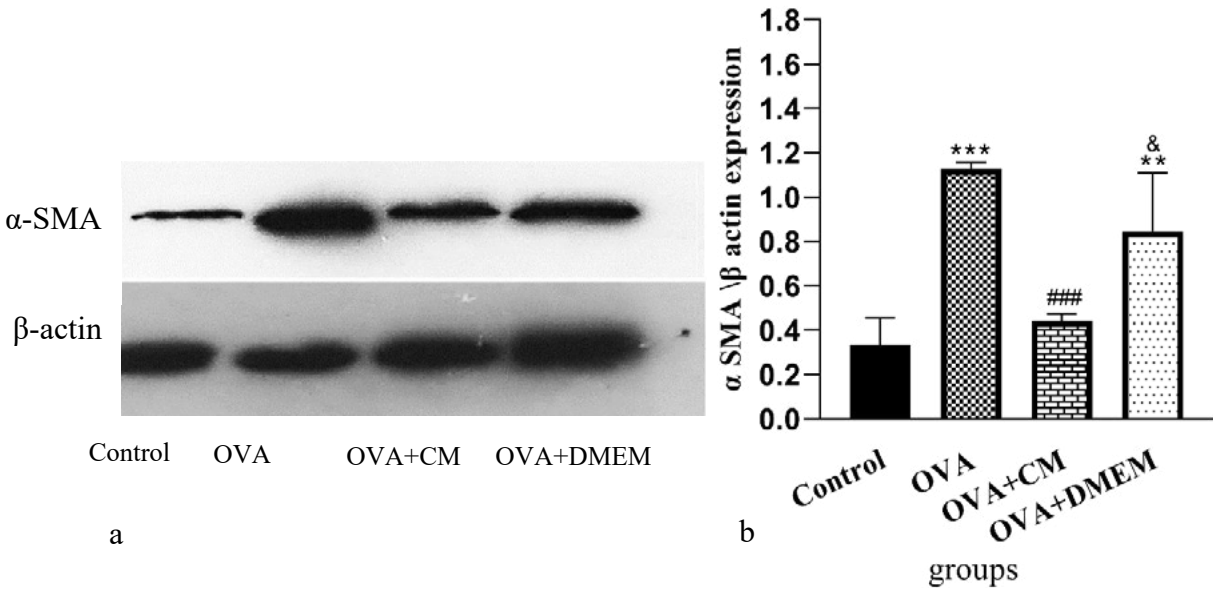

Fig 2. The effect of hAM-MSCs-CM on the α-SMA level in OVA-sensitized mice. (a) Western blots of the α-SMA. (b) Densitometric histograms of the western blot bands.
**P<0.01 and ***P<0.0001 vs. control group, ###P<0.0001 vs. OVA group and &P<0.05 vs. CM-treated one (OVA+CM). OVA: Ovalbumin; CM: conditioned medium.

the splenocytes were released by perfusion method. Splenocytes were then washed and approximately 10^6 cells were resuspended in 1 mL complete medium and cultured in a 24-well plate. The plate was incubated for 48 h in a CO2 incubator. Then, the contents of each well were mixed and the level of IL-10, IFN-γ, IL-4, and TGF-β (R&D Systems, Canada), as well as IgE (Abnova, Heidelberg, Germany) in the supernatant was analyzed by ELISA based on their instructor's manuals.

DATA ANALYSIS

Data were reported as mean ± SEM. P values less than 0.5 were defined as statistically significant. Statistical analysis of data was done by one-way ANOVA as well as Tukey post hoc tests on SPSS v.22 to determine the differences between groups.

RESULTS

The effect of hAM-MSCs-CM on α-SMA expression in lung tissue

Based on our western blot results, α-SMA was markedly increased in OVA and OVA+DMEM groups compared to controls (P<0.0001 and P=0.005, respectively). CM therapy could significantly underexpress α-SMA in the OVA+CM group compared to OVA and OVA+DMEM groups (P<0.0001 and P=0.013, respectively). However, there was no significant difference between the control and CM-treated group (P>0.999), as well as OVA and OVA+DMEM groups (P=0.176) (Figure 2).

The effect of hAM-MSCs-CM on IL-10, IFN-γ, IgE, IL-4, and TGF-β levels in splenocyte culture supernatant

In the OVA and OVA+DMEM groups, IL-10 and IFN-γ were significantly reduced compared to the control group (P<0.0001 for both groups and P=0.001, P=0.004, respectively). However,in animals treated with CM, IL-10 (P<0.0001 for both groups) and IFN-γ (P<0.0001, P=0.003, respectively) were significantly increased compared to untreated asthmatic animals and animals receiving DMEM. As expected, DMEM treatment did not significantly increase these cytokines compared to the OVA group (P>0.999) (Figures 3a and b).

Figures 3c, d, and e also show that IL-4 (P<0.0001, P=0.001, respectively), IgE (P<0.0001 for both groups), and TGF-β (P=0.001, P=0.002, respectively) in the OVA and OVA+DMEM groups increased significantly compared with the controls. The level of these cytokines after treatment with CM was significantly reduced compared with the OVA and OVA+DMEM groups (P<0.0001 for IL-4 and IgE and P<0.01 for TGF-β).

In addition, No significant difference was observed between the OVA and DMEM treatment groups (P>0.999), and the level of these factors remained high in the OVA+DMEM group.

DISCUSSION

A lot of people are suffering from asthma as a usual respiratory condition recognized by the chronic inflammatory responses of the innate and adaptive immune cells (4). Effective treatments are still needed for this disorder, as current therapies are not sufficient.

Stem cells have achieved some successful outcomes in the therapy of various human disorders but it has some disadvantages, too. To fill this practical gap in asthma treatment, we used hAM-MSCs-CM for the

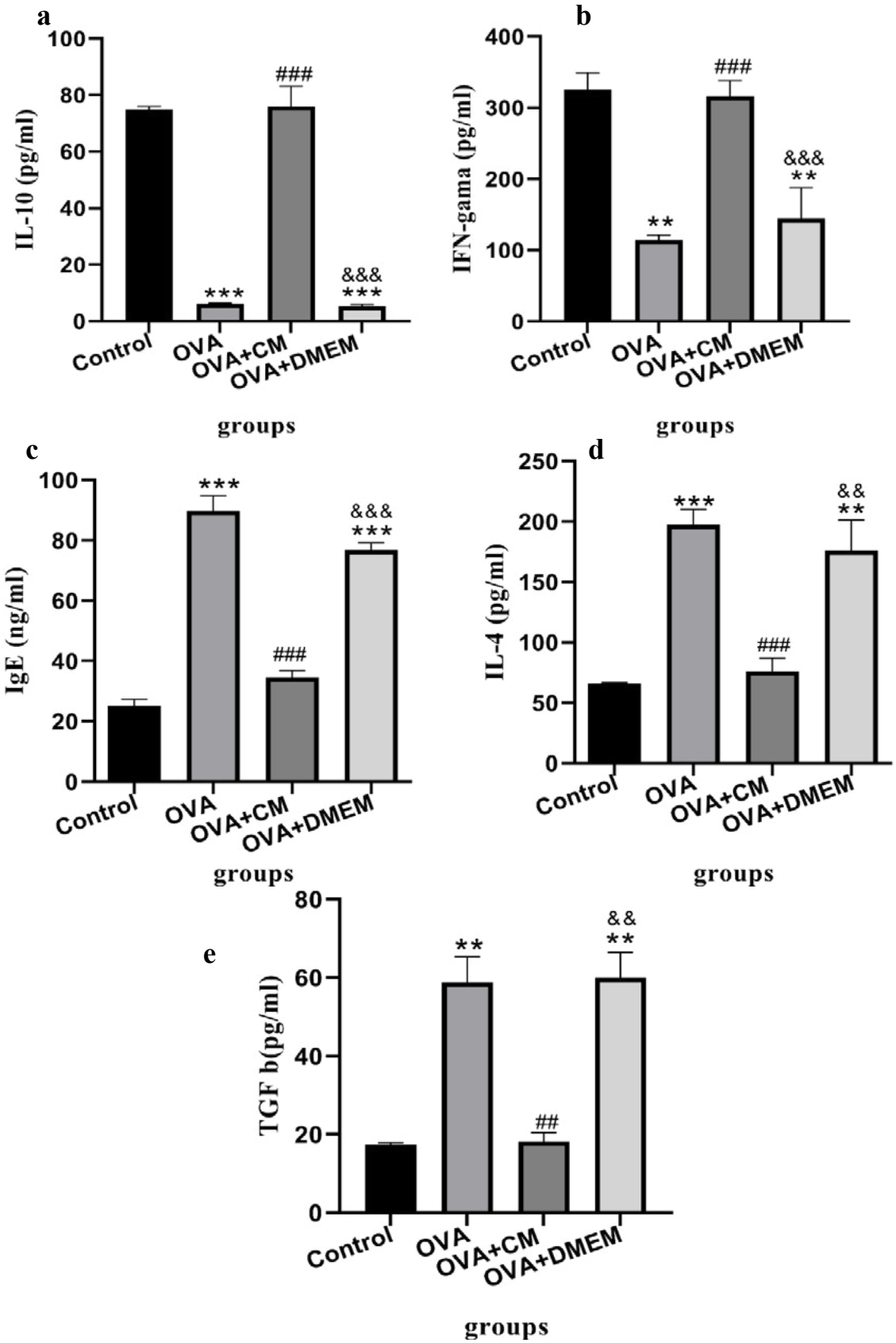

Fig 3. Effect of hAM-MSCs-CM on cytokines levels of spleen culture supernatant in the asthmatic animal model. a) IL-10, b) IFN-γ, c) IgE, d) IL-4, and e) TGF-β.
**P<0.01 and ***P<0.0001 vs. control group, ##P<0.01 and ###P<0.0001 vs. OVA group and &&P<0.01 and &&&P<0.0001 vs. group treated with CM (OVA+CM). OVA: Ovalbumin; CM: conditioned medium.

first time to decrease inflammation in the spleen and fibrosis in the lungs of asthmatic mouse models.

Bronchial wall remodeling and chronic inflammation occur at the same time with the differentiation of the fibroblasts to myofibroblasts. In recovering tissues, fibroblasts are characterized by the de novo contractile phenotype of α-SMA expression, which is associated with a lot of secretion of collagen and fibronectin as ECM components. This overactivity of myofibroblasts causes fibrosis and dysfunction (23, 24).

Our findings indicate that the use of CM improved asthma by decreasing the expression of α-SMA in the treated group compared to the untreated group. CM-treatment also increased IL-10 and IFN-γ while decreasing IgE, IL-4, and TGF-β in splenocytes. The current data confirms the data obtained in our previous study on asthmatic mice in which we analyzed these factors in the serum of animals and evaluated their lung tissue and reported that hAM-MSC-CM reduced inflammation, fibrosis, and oxidative stress (22). Our results were consistent with those of Koopmans et al., who found increased levels of IgE and Th2 cytokines, α-SMA expression, mucus production, and eosinophil infiltration in OVA-sensitized mice (8).

Studies show that α-SMA expression in asthmatic patients is higher compared with the controls. The most important finding in the differentiation of myofibroblasts from fibroblasts is α-SMA expression which its expression correlates with this differentiation. Furthermore, TGF-β plays an important role in promoting fibroblast differentiation to myofibroblasts (24). As Figure 1 shows, α-SMA was overexpressed in the OVA group compared to controls, which was significantly reduced with the CM treatment.

According to the study by Ramos-Barbón et al., there was a direct correlation between T cell proliferation and α-SMA expression in asthma airway tissue, and airway and sub-epithelial smooth muscle mass was increased in asthma (11). α-SMA can also produce TGF-β and collagen in fibrous tissues. Sun et al. showed that the amount of α-SMA in the lungs of bleomycin-induced fibrosis mice was increased compared with controls (25). Liu et al. showed that pulmonary fibrosis was associated with increased differentiation of fibroblasts to myofibroblasts, collagen secretion, and α-SMA expression. They also showed that underexpression of TGF-β could reduce the differentiation of fibroblasts to myofibroblasts and subsequently, fibrosis (26). In addition, α-SMA expression can be reduced by inactivating the TGF-β in pulmonary fibroblasts. Also, Hinz et al. reported that by TGF-β downregulation, myofibroblasts activity, and α-SMA mass are also reduced (27).

IL-10 is a pleiotropic cytokine secreted by divergent immune cells. For example, tolerogenic dendritic cells overexpress IL-10 to induce T-reg. Furthermore, for immune homeostasis, natural regulatory cell (nT-reg) secretes IL-10 in response to IL-2 (28). IL-10 can suppress allergic conditions like asthma. The administration of IL-10 with immune-regulating cells will lead to successful allergen immunotherapy (29). Based on studies, IL-10 is underexpressed in asthma pathogenesis. In severe persistent asthma, the IL-10-producing and releasing monocytes are decreased. Furthermore, IL-10 absence elevates eosinophilic inflammation of the airway and IL-5 overexpression (30).

In a study, Hou et al. overexpressed IL-10 in isolated bone marrow's mesenchymal stem cells (MSCs) and treated asthma mouse models with transgenic MSCs. They observed that eosinophilic inflammation and mucus secretion were more controlled in the transduced MSCs with a vector expressing IL-10 (MV-10) group compared to nontransduced MSCs (M) and transduced MSCs with expression vector (MV) groups. Based on this research, MSCs managed allergic asthma pathophysiology with their immune-modulatory characteristics, and IL-10 overexpression could testify to this effect (30).

Furthermore, our study found that reducing IgE in the CM group was a significant achievement . As IgE plays crucial role in allergic reactions, its reduction or neutralization can help manage the outcomes. The first approved humanized mAb which was administered to patients suffering from moderate to severe asthma was Omalizumab. It inhibited IgE function via binding to the Fc fragment of free IgE. So, IgE could not bind to the high-affinity FcεRI receptor on the surfaces of inflammatory immune cells like mast cells and basophils (31).

The spleen is involved in allergic diseases, as splenic immune responses play pivotal roles in their development. Based on studies, in inflammatory conditions such as allergies, mast cells grow in the spleen and produce IL-4, IL-6, and TNF-α in response to receptor-bound IgE. *In vitro*, stimulation of spleen cells in OVA-sensitized mice led to the development of mast cell populations in the spleen. Mast cells play a vital role in the Th2 induction, IL-4 overproduction, and Th1/Th2 imbalance. IFN-γ inhibits Th2 differentiation, so a decrease in IFN-γ intensifies the Th2 response and ultimately, causes allergic inflammation (12). Studies have shown that in OVA-sensitive mice, spleen cells secrete more IL-4, IL-5, IL-13, and IgE (32). Based on Gauvreau et al. research, IL-33 as an epithelial cell-derived mediator also can worsen asthma by inducing basophil-related IgE and IL-4 (33). The study by Li et al. showed that IL-4 was significantly upregulated in the spleen of the OVA group compared with controls (6). In the

study of Yun et al., the spleen cell culture supernatant IL-4 in the OVA group was more than in the controls, while IFN-γ was less than in the controls (5). Figure 2 shows our results in line with this content. IL-4, IgE, and TGF-β of splenic culture supernatant in the OVA group were higher than controls and IFN-γ and IL-10 were less than controls, and CM treatment reversed the results.

A decrease in IFN-γ and an increase in IL-4 can be induced by differentiating Th cells which are stimulated by Dendritic cells in the spleen as a secondary lymphoid organ and can be involved in many diseases, such as asthma, by affecting the immune response (34). Decreased number and functional defects of T regulatory (Treg) is a key cause of asthma. Evidence suggests that asthma is associated with Treg and its immune disorder (35). There is evidence for the role of Treg in maintaining immune tolerance in allergic diseases (16, 36). The study of Kianmehr et al. showed that in the presence of OVA, due to Th1/Th2 imbalance and decreased Treg function IL-4, TGF-β, and IL-17 were elevated in spleen cells of asthmatic animals and IFN-γ and Treg were decreased (37). Treg mediates the immune response by controlling the secretion of cytokines. Studies show that Treg levels in the OVA category are lower than that in controls. Jing's study also showed that Treg depletion in splenic lymphocytes can be effective in Th1/Th2 imbalance [5]. When naive CD4+ T cells express IFNγ they convert to the T helper type–1 (Th1) cells, while by expressing IL-4, they are converted to the Th2 cells (38). As in our study CM could increase IFNγ and decrease IL-4, we can conclude that the immune response was toward inducing Th1 and Asthma improvement. Cazzola et al. also introduced anti-TNF-α and Th1 cytokine-directed therapies as effective asthma treatment (39).

Previous studies have shown that MSC induces Treg in both mice and humans, both in vitro and in vivo. The elevated number of Treg and its activity are associated with MSC-regulated immunity. The study by Dai et al. shows that Treg/T lymphocytes in the spleen of asthmatic animals are significantly less than the healthy ones and MSC administration improved the percentage of Treg and reduced splenic cell infiltration [35]. According to the study by Huang et al., MSC and MSC-CM therapy share a common feature of significantly reducing macrophages in the liver and spleen. This way, MSCs are responsible for down-regulation proinflammatory macrophages, such asswitching proinflammatory macrophages (M1) to anti-inflammatory macrophages (M2) (40).

According to our results, CM can regulatethe expression of α-SMA protein and proinflammatory and antiinflammatory factors in asthmatic mice induced by OVA. Thus, our previous study and this one provide evidencethat hAM-MSCs-CM can improve asthma in mice.

Acknowledgments

This research was granted by the Iran University of Medical Sciences, Tehran, Iran. All animal studies were done under the Animal Care Committee of this university with the ethics code of no. IR.IUMS.FMD.REC.1398.050.

Ethical concerns

All animal studies were done under the Animal Care Committee of this university with the ethics code of no. IR.IUMS.FMD.REC.1398.050.

Conflicts of Interest

There is no conflict of interest.

Funding/support

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Data availability

All data is available.

Ethical statements

This investigation was approved by the Ethics Committee of the Iran University of medical science. All ethical principles are considered in this article.

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