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Serum from Asthmatic Mice Potentiates the Therapeutic Effects of Mesenchymal Stromal Cells in Experimental Allergic Asthma

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Key Words. Bone marrow stromal cells • Cell therapy • Lung • Animal models • Eosinophils • Macrophages

ABSTRACT

Asthma is a chronic inflammatory disease characterized by airway inflammation and remodeling, which can lead to progressive decline of lung function. Although mesenchymal stromal cells (MSCs) have shown beneficial immunomodulatory properties in preclinical models of allergic asthma, effects on airway remodeling have been limited. Mounting evidence suggests that prior exposure of MSCs to specific inflammatory stimuli or environments can enhance their immunomodulatory properties. Therefore, we investigated whether stimulating MSCs with bronchoalveolar lavage fluid (BALF) or serum from asthmatic mice could potentiate their therapeutic properties in experimental asthma. In a house dust mite (HDM) extract asthma model in mice, unstimulated, asthmatic BALF-stimulated, or asthmatic serum-stimulated MSCs were administered intratracheally 24 hours after the final HDM challenge. Lung mechanics and histology; BALF protein, cellularity, and biomarker levels; and lymph-node and bone marrow cellularity were assessed. Compared with unstimulated or BALF-stimulated MSCs, serum-stimulated MSCs further reduced BALF levels of interleukin (IL)-4, IL-13, and eotaxin, total and differential cellularity in BALF, bone marrow and lymph nodes, and collagen fiber content, while increasing BALF IL-10 levels and improving lung function. Serum stimulation led to higher MSC apoptosis, expression of various mediators (transforming growth factor-β, interferon-γ, IL-10, tumor necrosis factor-α-stimulated gene 6 protein, indoleamine 2,3-dioxygenase-1, and IL-1 receptor antagonist), and polarization of macrophages to M2 phenotype. In conclusion, asthmatic serum may be a novel strategy to potentiate therapeutic effects of MSCs in experimental asthma, leading to further reductions in both inflammation and remodeling than can be achieved with unstimulated MSCs.
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SIGNIFICANCE STATEMENT

Exposure of MSCs to serum from asthmatic mice, a biologically relevant sample, led to increased MSC apoptosis and expression of various anti-inflammatory and proresolution mediators. Administration of these cells to mice with allergic airway inflammation was associated with a further reduction of inflammation and remodeling, as well as improvement in lung function. These observations provide a platform for future clinical trials in patients with asthma.

INTRODUCTION

Asthma is a chronic inflammatory disease with a significant societal and economic burden. It is characterized by chronic immune and inflammatory responses that can promote several structural changes in the airways, all of which contribute to pathologic airflow obstruction and airway hyper-responsiveness [1]. Although most asthmatic patients can achieve disease control with the use of inhaled corticosteroids and long-acting β_2-adrenoceptor agonists, these approaches do not reverse established remodeling, and a subset of patients with severe asthma can be resistant to treatment [2–4]. Despite some progress with monoclonal antibody therapies, novel therapeutic strategies are required.

In recent years, studies have shown that mesenchymal stromal cells (MSCs) secrete a range of factors that induce immunomodulatory effects on various models of allergic

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airway inflammation [5–12]. MSCs can detect specific inflammatory microenvironments and tailor their anti-inflammatory responses accordingly [13]; however, they have proven less effective in reversing the remodeling process [6, 10, 11], which may be attributable to a lower expression of certain cytokines and growth factors needed to repair injured tissue [13].

Based on these observations, recent studies have tried to potentiate the therapeutic properties of MSCs by stimulating them before administration. Strategies include physical, chemical, or biological methods, such as mechanical stress and hypoxia, as well as exposure to biologically relevant fluids, including bronchoalveolar lavage fluid (BALF) and serum. A growing number of studies demonstrate that such practice can enhance the secretion of cytokines and growth factors, as well as alter expression of genes that regulate MSC survival [12–18]. Arguably, these reflect in vivo inflammatory conditions that can affect MSC survival and potentiate clinical effects.

In the present study, we investigated whether prior stimulation of bone marrow-derived MSCs with BALF or serum obtained from asthmatic mice could increase the therapeutic properties of these cells, thus enhancing their ability to mitigate not only inflammation but also the remodeling process in experimental allergic asthma. For this purpose, MSCs stimulated or not with BALF or serum from asthmatic mice were administrated in a murine model of house dust mite (HDM)-induced allergic asthma to comparatively evaluate their therapeutic effects on lung mechanics, BALF total and differential cellularity, collagen fiber content in airways and lung parenchyma, and expression of cytokines involved in the inflammatory process of asthma. Furthermore, we analyzed the in vitro effects of MSC prestimulation with BALF or serum obtained from either control or HDM-challenged mice on cellular viability (apoptosis), secretion of selected mediators, and stimulation of macrophage polarization.

Materials and Methods

This study was approved by the Ethics Committee of the Federal University of Rio de Janeiro Health Sciences Center (CEUA-020/17). All animals received humane care by trained veterinarians and veterinary staff in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences.

Animal Preparation and Experimental Allergic Asthma Protocol

One hundred and eight C57BL/6 mice (100 female and 8 male, weight 20–25 g, age 8–10 weeks) were used. As bronchoalveolar lavage may affect lung morphological analysis, 40 female mice were used to evaluate lung mechanics and histology (n = 8 per group), whereas 40 others were used to analyze total and differential cellularity in BALF, bone marrow, and lymph nodes, as well as protein levels of mediators in BALF (n = 8 per group). Mice were randomly assigned into two groups. In the HDM group, mice were challenged by intranasal instillation of 25 μg of protein (25 μl) presented in HDM extract (*Dermatophagoides pteronyssinus*; Greer Laboratories, Lenoir, NC), 3 days weekly for 3 consecutive weeks [11, 19, 20]. In the control group (CTRL), the vehicle (phosphate-buffered saline [PBS] 1×) was administered using the same protocol. Twenty-four hours after the last challenge, the HDM group was subsequently randomized into four subgroups to receive sterile saline solution (0.9% NaCl, 50 μl, SAL) or MSCs stimulated or not with BALF (MSC-BALF) or serum (MSC-serum) obtained from asthmatic mice (10^5 MSCs per mouse), intratracheally. Three days after MSC administration, all data were analyzed. In 10 other female mice, asthma was induced or not (5 animals per group) using the above-mentioned protocol, and BALF and serum were collected. Additionally, 10 female mice were used so as to collect alveolar macrophages from HDM mice. Twenty-four hours after the last challenge, BALF supernatant and serum were collected, pooled, and stored at −80°C for stimulation of MSCs in vitro.

BALF was obtained by three sequential gentle aspirations of 0.4 ml PBS 1× (final volume 1.2 ml) injected into the airways via a tracheal cannula. In order to obtain the serum, the collected blood was centrifuged to separate the blood cells from the plasma fraction. After centrifugation, the fibrin clot was picked up in the upper layer and squeezed to separate the serum. The collected murine serum was than centrifuged to remove any remaining red cells.

Extraction and Stimulation of MSCs

Eight male C57BL/6 mice were anesthetized with intravenous ketamine (25 mg/kg) and xylazine (2 mg/kg) and used as cell donors. Bone marrow cells were obtained from femurs and tibias. After isolation, bone marrow-derived MSCs were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA) containing 15 mM HEPES (Sigma–Aldrich, St. Louis, MO), 15% inactivated fetal bovine serum (FBS; Invitrogen, CA), 100 units/ml penicillin, and 100 mg/ml streptomycin (P/S) antibiotic solution (Gibco, Grand Island, NM) at 37°C in a 5% CO_2 humidified atmosphere. When adherent cells reached ∼80% confluence, they were passaged with 0.05% trypsin–EDTA solution (Gibco) and maintained in DMEM with 10% FBS and P/S.

In Vitro Analysis of MSCs Stimulated with BALF or Serum

MSCs were cultured in 12-well plates (10^5 cells/well) using DMEM supplemented with 10% FBS, P/S, and 2 mM L-glutamine (Invitrogen Life Technologies, Grand Isle, NY). MSCs were exposed or not to BALF or serum (10% vol/vol) from CTRL Or HDM-challenged mice for 24 hours. The concentration of 10% vol/vol was based on pilot studies. Briefly, MSCs were stimulated with a pool of BALF and serum obtained from five CTRL or five HDM mice using a concentration curve (0%, 0.25%, 0.5%, 0.75%, 1%, 10%, 20%, 30%, 40%, and 50%) in DMEM supplemented with 10% FBS, P/S, and 2 mM L-glutamine. Concentration of cytokines and growth factors produced by the cells before and after activation was measured; the 10% concentration was found to be more effective in modulating the MSC secretome.

After MSC exposure, the supernatant was removed, and cells washed with PBS 1×, harvested from culture plates using 2.5% trypsin–EDTA (Invitrogen Life Technologies), and pelleted by centrifugation (600g for 5 minutes). Cells were lysed for RNA extraction by the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Total RNA

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concentration was measured by spectrophotometry in a Nanodrop ND1000 system and first-strand cDNA was synthesized from total RNA using an M-MLV Reverse Transcriptase Kit (Invitrogen).

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) was performed and relative mRNA levels were measured with a SYBR Green detection system using ABI 7500 real-time PCR (Applied Biosystems, Foster City, CA). All samples were measured in triplicate. The relative level of each gene was calculated as the ratio of the study gene to the control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and given as the fold changes relative to unstimulated MSCs. Expression of mRNA for the following genes was analyzed: caspase-3, Bax (a proapoptotic Bcl-2 family protein), Bcl-2 (B-cell lymphoma 2), transforming growth factor (TGF)-β, interferon (IFN)-γ, interleukin (IL)-10, tumor necrosis factor (TNF)-α-stimulated gene 6 protein (TSG-6), indoleamine 2,3-dioxygenase-1 (IDO-1), and IL-1 receptor antagonist (IL-1RN). The sequence of each PCR primer can be found in Supporting Information Table S1.

Flow Cytometry

The effects of exposure to BALF and serum from CTRL and HDM-challenged mice on MSC viability (early and late apoptosis) were analyzed by flow cytometry using annexin-V and propidium iodide (PI) staining (BD Biosciences, San Jose, CA) in a FACSCalibur system (BD Biosciences), following the manufacturer's instructions. In addition, lymphocyte subpopulations from BALF, bone marrow, and mediastinal lymph nodes were characterized using flow cytometry after incubation with specific antibodies. Analyses were carried out in FlowJo software version 10.0.7 (Tree Star Inc., Ashland, OR).

In Vitro Analysis of Alveolar Macrophage Phenotype

Alveolar macrophages were obtained from the BALF of HDM-challenged mice [21, 22]. BALF was centrifuged at 300*g* for 10 minutes and the cellular pellet was washed with PBS, resuspended in red blood cell lysis buffer (8.3 g NH_4Cl, 1 g $KHCO_3$, 1.8 ml of 5% EDTA in 1 l distilled water) for 5 minutes at room temperature, and centrifuged again at 300*g* for 10 minutes. The pelleted cells were resuspended and cultured in a 12-well culture plate at 37°C with 5% CO_2 at a concentration of 10^5 cells per well in 1 ml RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS, 1 mM pyruvate, 1% nonessential amino acids, 14 mM glucose, 17.9 mM $NaHCO_3$, 10 mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After 2 hours of incubation, nonadherent cells were washed off with PBS, and the medium was refreshed. Alveolar macrophages were stimulated with conditioned media obtained from MSCs stimulated or not with BALF or serum of either CTRL or HDM-challenged mice for an additional 24 hours. Alveolar macrophages were then washed with PBS 1×, harvested from the culture plates, and pelleted by centrifugation (600*g* for 5 minutes). RT-qPCR was performed as described above. The relative level of each gene was calculated as the ratio of the study gene to the control gene (*GAPDH*) and given as the fold change relative to the macrophage group (macrophages cocultured with unstimulated MSC medium). Then, mRNA expression for the following genes was analyzed: inducible nitric oxide synthase (iNOS), TNF-α, and IL-β (M1 markers) and arginase-2, IL-10, and TGF-β (M2 markers). The sequence of each PCR primer is provided in Supporting Information Table S1.

Mechanical Parameters

Three days after MSC administration, the animals were sedated (diazepam 1 mg/kg intraperitoneally), anesthetized (thiopental sodium 20 mg/kg intraperitoneally), tracheotomized, paralyzed (vecuronium bromide, 0.005 mg/kg intravenously), and ventilated using a constant-flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) set to RR = 100 breaths/minute, tidal volume (V_T) = 0.2 ml, and fraction of inspired oxygen (FIO_2) = 0.21. The anterior chest wall was surgically removed and a positive end-expiratory pressure (PEEP) of 2 cmH_2O applied. Airflow and tracheal pressure (Ptr) were measured [23, 24], and lung mechanics were analyzed by the end-inflation occlusion method [24]. In an open chest preparation, Ptr reflects transpulmonary pressure (P_L). Briefly, after end-inspiratory occlusion, there is an initial rapid decline in P_L (ΔP1,L) from the preocclusion value down to an inflection point (Pi), followed by a slow pressure decay (ΔP2,L), until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (Pel). ΔP1,L selectively reflects the pressure used to overcome the airway resistance. ΔP2,L reproduces the pressure spent by stress relaxation, or the viscoelastic properties of the lung, together with a small contribution of *pendelluft*. Static lung elastance (Est,L) was determined by dividing Pel by V_T. Lung mechanics measurements were obtained 10 times in each animal [23, 25]. All data were analyzed using ANADAT software (RHT-InfoData, Inc., Montreal, Quebec, Canada).

Lung Histology

Soon after determination of lung mechanics, laparotomy was performed and heparin (1,000 IU) was injected into the vena cava. The trachea was clamped at end-expiration (PEEP = 2 cmH_2O), and the mice were euthanized by exsanguination following transection of the abdominal aorta and vena cava. The lungs were then removed and flash-frozen by immersion in liquid nitrogen. The left lung was fixed with Carnoy's solution and paraffin-embedded [6, 26].

Lung morphometry was analyzed in sections (4-μm thick) cut and stained with hematoxylin–eosin. Briefly, an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines of known length was coupled to a conventional light microscope (Olympus BX51, Olympus Latin America-Inc., Brazil). The number of mononuclear (MN) and polymorphonuclear (PMN) cells in each section was determined by the point-counting technique [27, 28] across 10 random, noncoincident fields of view per mouse.

Collagen fibers were quantified in the airways and alveolar septa of sections using Picrosirius-polarization staining method (ImagePro Plus 6.0 software) [6, 25].

Cell Recovery and Counts

BALF was obtained by three sequential gentle aspirations of 0.4 ml PBS 1× (final volume 1.2 ml) injected into the airways via a tracheal cannula. BALF was centrifuged at 250*g* for 10 minutes at 4°C and the cell pellets were resuspended in PBS 1×. Similarly, bone marrow was obtained by gentle lavage of the right femur with 1 ml of PBS 1×. The mediastinal lymph nodes (mLNs) were carefully extracted, placed into 1 ml of

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PBS 1×, and cell suspensions obtained after mechanical homogenization. Total leukocytes from BALF, bone marrow, and mLN were counted in a Neubauer chamber after dilution with Turk's solution (2% acetic acid). BALF and bone marrow cells were pelleted onto glass slides by cytocentrifugation and stained by the May–Grünwald–Giemsa method. Differential cell counts were performed as described elsewhere [11, 19].

Enzyme-Linked Immunosorbent Assay

Levels of IL-4, IL-10, IL-13, and eotaxin were quantified in BALF by Enzyme-Linked Immunosorbent Assay (PeproTech, Rocky Hill, NJ/Biolegend, San Diego, CA) as per manufacturer instructions. Total protease (Pierce Fluorescent Protease Assay Kit, Thermo Fisher, Grand Isle, NY, US) was also measured.

Statistical Analysis

Differences between groups were assessed using one-way ANOVA followed by Tukey's test. For nonparametric results, Kruskal–Wallis test followed by Dunn's test was used. Parametric data were expressed as mean ± SD, whereas nonparametric data were expressed as median (interquartile range). All tests were performed in Prism 6.07 (GraphPad Software Inc., La Jolla, CA). Statistical significance was established at $p < .05$.

Results

Stimulation of MSCs with Serum, Compared with BALF, Led to Increased MSC Apoptosis and Expression of Anti-Inflammatory Mediators

MSCs exposed to HDM-BALF or HDM-serum exhibited a higher percentage of apoptotic cells, as observed in the annexin-V/PI assay (Fig. 1A). Caspase-3 and Bax expressions were also higher in HDM-BALF and HDM-serum-stimulated MSCs compared with unstimulated MSCs or those stimulated with CTRL-BALF and CTRL-serum (Fig. 1B, 1C). Notably, apoptotic cell counts and expression of caspase-3 and Bax were significantly increased in HDM-serum-stimulated MSCs compared with those stimulated with HDM-BALF. MSCs stimulated with BALF or serum from either CTRL or HDM mice exhibited reductions in Bcl-2 expression compared with unstimulated cells, with even lower expression in HDM-BALF- and HDM-SERUM-stimulated MSCs (Fig. 1D). Furthermore, mRNA expressions of TGF-β1, IFN-γ, IL-10, TSG-6, IDO-1, and IL-1RN were higher in HDM-BALF- and HDM-SERUM-stimulated MSCs than in unstimulated MSCs or those stimulated with CTRL-BALF or CTRL-serum. The expression of such mediators were even higher in MSCs stimulated with HDM-SERUM than HDM-BALF (Fig. 1E–1J).

Stimulation of MSCs with Serum from HDM Mice Induced Further Macrophage Polarization to M2 Profile

iNOS and TNF-α expressions were higher in alveolar macrophages cultured with conditioned media obtained from MSCs stimulated with BALF from HDM mice, but not those cultured with media obtained from serum-stimulated MSCs (Fig. 2A, 2B). However, IL-1β expression was higher in macrophages cultured in the conditioned media obtained from MSCs stimulated with either BALF or serum compared with unstimulated macrophages (Fig. 2C). Additionally, macrophages cultured in conditioned media from MSCs stimulated with BALF and serum from HDM mice exhibited a higher expression of arginase-2 and TGF-β compared with the unstimulated macrophage group. The expressions of arginase-2 and TGF-β were more pronounced in macrophages cultured with conditioned media from MSCs stimulated with HDM-SERUM than HDM-BALF. Only macrophages cultured with conditioned media from MSCs stimulated with HDM-SERUM demonstrated a significant increase in IL-10 expression. Increased expression of arginase-2, IL-10, and TGF-β indeed suggests a polarization to the M2 profile (Fig. 2D–2F).

Since MSCs stimulated with BALF and serum from CTRL mice exhibited similar in vitro behavior compared with unstimulated MSCs, whereas exposure to BALF and serum from HDM mice induced more robust effects by enhancing the expression of anti-inflammatory mediators and polarizing macrophages to M2 profile in vitro, we chose to continue the in vivo experiments only with MSCs stimulated with BALF and serum.

Stimulation of MSCs with Serum Yielded Further Modulation of Mediators and Reduction of Lung Inflammation and Remodeling In Vivo

BALF protein levels of IL-4, IL-13, and eotaxin were higher in HDM-SAL than CTRL mice (Fig. 3A–3C). Only serum-stimulated MSCs were able to significantly reduce protein levels of such mediators to levels comparable to those of the CTRL group. Furthermore, only serum-stimulated MSCs increased BALF IL-10 levels in HDM mice (Fig. 3D).

PMN cell counts in lung tissue were higher in the HDM-SAL compared with the CTRL group (Fig. 4A). Both BALF-stimulated and serum-stimulated MSCs reduced PMN cell counts in HDM mice to levels comparable to those of the CTRL group. No differences were observed in the MN cell count among the groups. Total and differential cell counts were also increased in BALF of HDM-SAL mice compared with the CTRL group (Fig. 4B–4I). Regardless of stimulation, MSC administration reduced eosinophil and total leukocyte counts, with a greater reduction in the total leukocyte count in serum-stimulated MSC animals. In addition, BALF-stimulated or serum-stimulated MSCs reduced neutrophil counts in HDM mice, but only serum-stimulated MSCs were able to reduce the number of macrophages, total lymphocytes, $B220^+$, and $CD4^+$ cells to CTRL-comparable levels. MSC administration did not affect $CD4^+CD25^+FoxP3^+$ cell count, regardless of stimulation.

Collagen fiber content in the lung parenchyma and airways was significantly increased in the HDM-SAL mice compared with the CTRL group (Fig. 5). Regardless of stimulation, MSC administration reduced collagen fiber content in both lung parenchyma and airways; however, only serum-stimulated MSCs reduced collagen fiber content in the airways of HDM-challenged mice to CTRL-comparable levels.

Stimulation of MSCs with Serum Led to Further Improvement in Lung Mechanics In Vivo

HDM-SAL mice exhibited higher Est,L, ΔP1,L, and ΔP2,L compared with the CTRL group (Fig. 6A, 6B). Regardless of stimulation, MSC administration reduced the Est,L; however, serum-stimulated MSCs were more effective at reducing this parameter. Furthermore, only serum-stimulated MSCs reduced ΔP1,L and ΔP2,L in HDM-challenged mice to CTRL-comparable levels.

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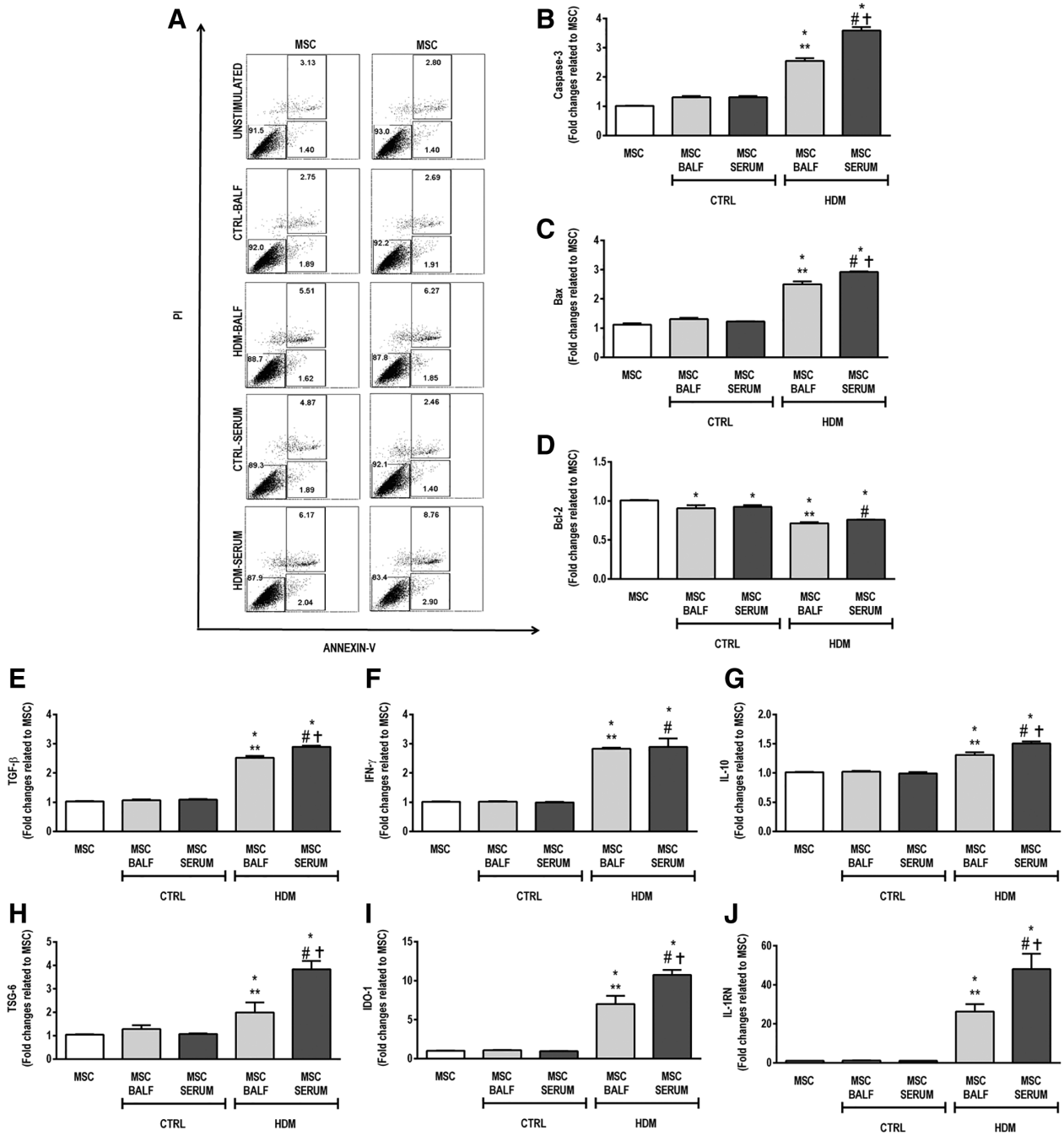

Figure 1. In vitro stimulation of mesenchymal stromal cells (MSCs) with serum yielded further cellular death and modified the MSC secretome. **(A):** Apoptosis was measured in MSCs by assessing the percentage of annexin-V$^+$/propidium iodide cells by flow cytometry. Representative plots for MSCs: unstimulated or stimulated with bronchoalveolar lavage fluid (BALF) or serum from control group (CTRL) and house dust mite (HDM)-challenged mice for 24 hours. Gene expression of caspase-3 **(B)**, Bax (a proapoptotic Bcl-2 family protein; **C)**, Bcl-2 (B-cell lymphoma 2; **D)**, transforming growth factor (TGF)-β1 **(E)**, interferon (IFN)-γ **(F)**, IL-10 **(G)**, tumor necrosis factor-α-stimulated gene 6 protein (TSG-6); **(H)**, indoleamine 2,3-dioxygenase-1 (IDO-1); **(I)**, and IL-1 receptor antagonist (IL-1RN); **(J)** was assessed by RT-PCR in cells after 24 hours of each specific stimulation. Data represent relative gene expression calculated as a ratio of average expression of the target gene compared with the reference gene (*GAPDH*) and expressed as fold change relative to unstimulated MSCs. Bars represent mean ± SD. *, Significantly different from MSC ($p < .05$). **, Significantly different from BALF-CTRL MSCs ($p < .05$). #, Significantly different from SERUM-CTRL MSCs ($p < .05$). †, Significantly different from BALF-HDM MSCs ($p < .05$).

Stimulation of MSCs with Serum Increased their Effectiveness in Reducing Bone Marrow and mLN Cellularity In Vivo

Total and differential cell counts were increased in the bone marrow of HDM-SAL mice compared with CTRL (Fig. 7A–7E). Regardless of stimulation, MSC administration reduced macrophage counts; however, only serum-stimulated MSCs were able to reduce total cell counts as well as neutrophils and eosinophils to CTRL-comparable levels. Total, CD4$^+$ T-cell and B-cell counts also increased in mLN of HDM-SAL mice

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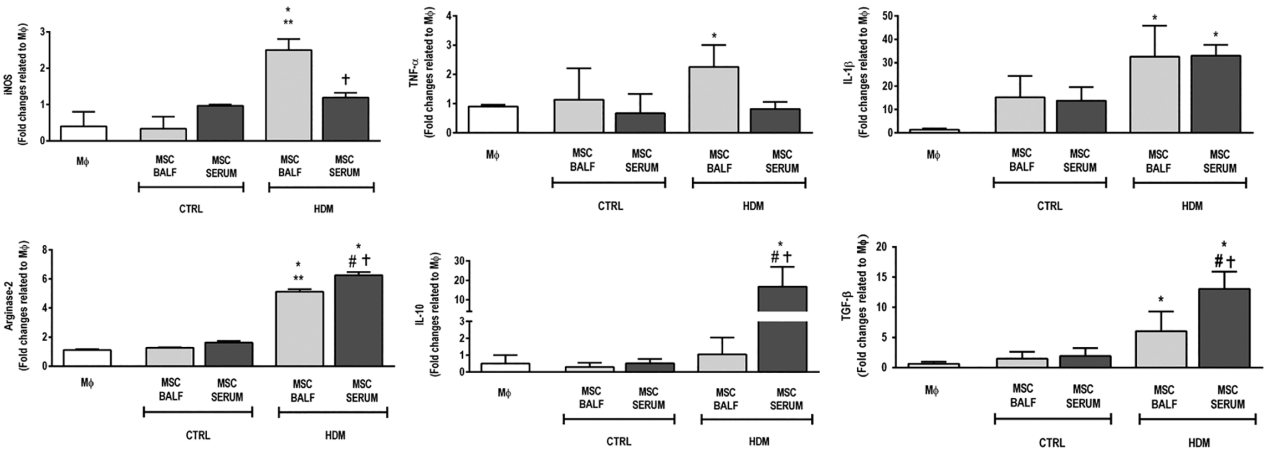

Figure 2. In vitro MSC stimulation with SERUM-HDM induced further macrophage polarization to the M2 profile. Alveolar macrophages (10^5 cells per well) from HDM mice were cultured with conditioned media obtained from MSCs (10^5 cells per well) either unstimulated or stimulated with BALF or SERUM from CTRL and HDM-challenged mice for 24 hours. Relative gene expression of **(A)** iNOS, **(B)** TNF-α, **(C)** IL-1β, **(D)** arginase-2, **(E)** IL-10, and **(F)** TGF-β was calculated as a ratio of the average gene expression levels compared with the reference gene (*GAPDH*) and expressed as fold changes relative to MΦ group (alveolar macrophages cultured with conditioned media from unstimulated MSCs). Bars are means + SD of four wells per condition. *, Significantly different from MΦ ($p < .05$). **, Significantly different from MSC-BALF-CTRL ($p < .05$). #, Significantly different from MSC-SERUM-CTRL ($p < .05$). †, Significantly different from MSC-BALF-HDM ($p < .05$). Abbreviations: MSC, mesenchymal stromal cell; iNOS, inducible nitric oxide synthase; IFN-α, interferon-α; IL, interleukin; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; HDM, house dust mite; BALF, bronchoalveolar lavage fluid.

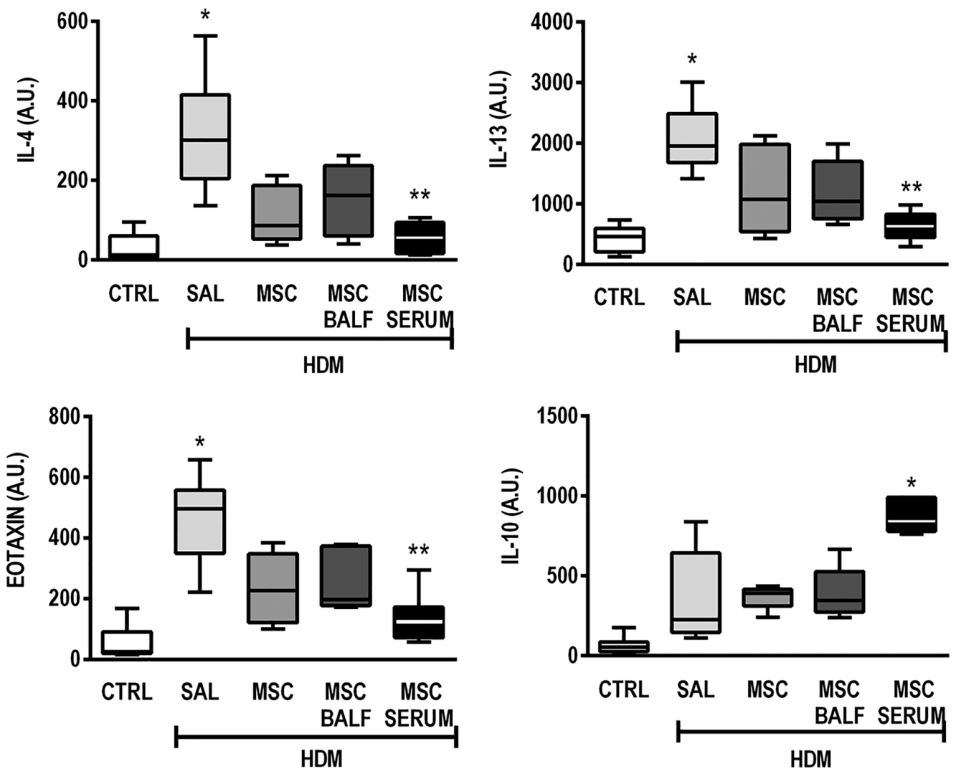

Figure 3. Stimulation of MSCs with serum yielded further modulation of mediators in the lung in vivo. Protein levels of IL-4, IL-13, eotaxin, and IL-10 in BALF. CTRL, phosphate-buffered saline-challenged mice; HDM, HDM-challenged mice; SAL, HDM mice treated with saline; MSC, HDM mice treated with unstimulated MSCs; HDM-MSC-BALF, HDM mice treated with MSCs stimulated with BALF from asthmatic mice; HDM-MSC-SERUM, HDM mice treated with MSCs stimulated with serum from asthmatic mice. Boxes show the interquartile range (P25–P75) range, whiskers denote the range (minimum–maximum), and horizontal lines represent the median of eight animals per group. *, Significantly different from CTRL ($p < .05$). **, Significantly different from HDM-SAL ($p < .05$). Abbreviations: MSCs, mesenchymal stromal cells; HDM, house dust mite; IL, interleukin; BALF, bronchoalveolar lavage fluid.

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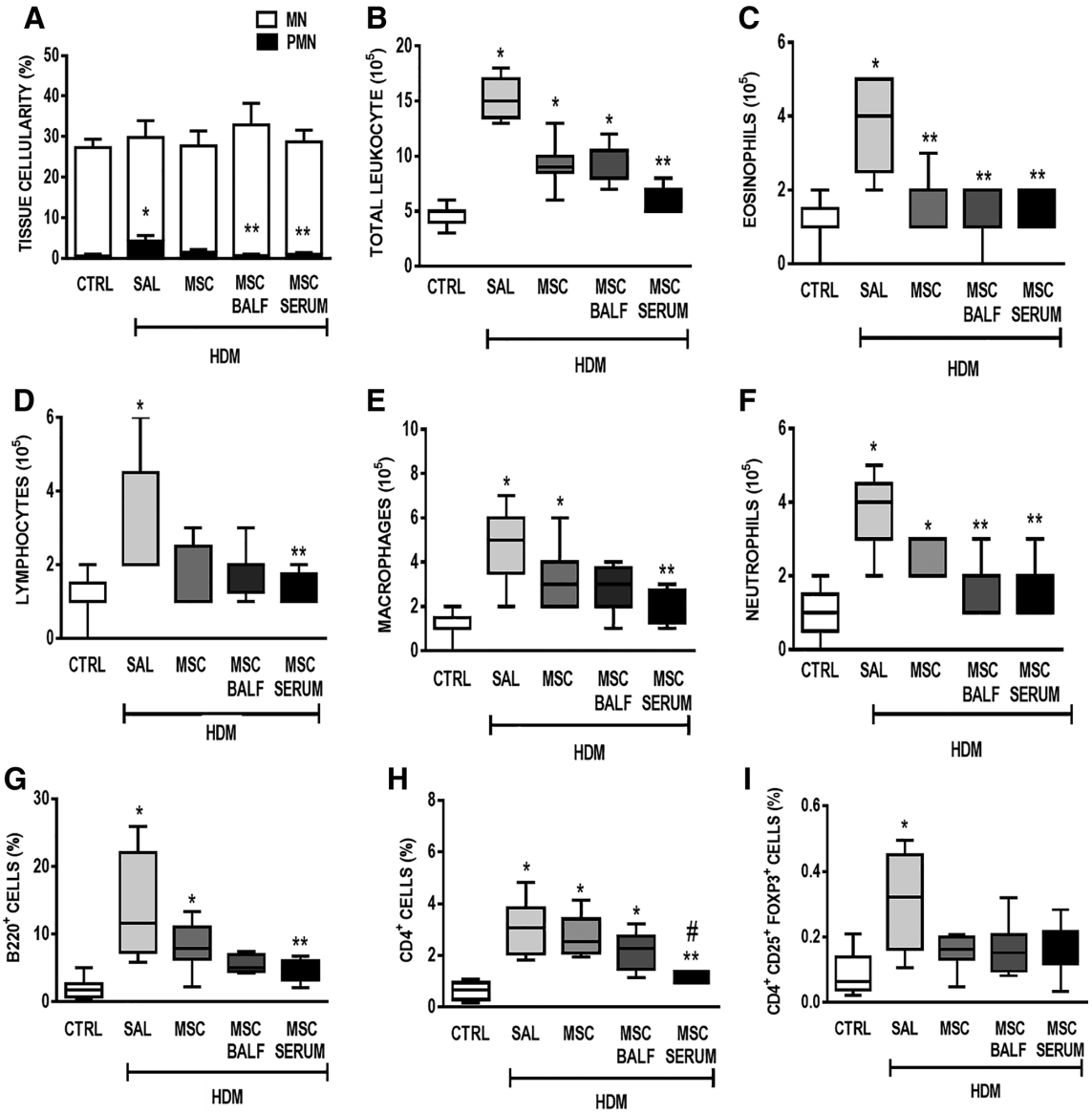

Figure 4. Ex vivo stimulation of MSCs with serum yielded further reduction of inflammation in the lungs in vivo. **(A):** Tissue cellularity: fractional area of mononuclear (MN, white bar) and polymorphonuclear (PMN, black bar) cells in lung tissue, **(B)** total leukocytes, **(C)** eosinophils, **(D)** lymphocytes, (**E**) macrophages, (**F**) neutrophils, **(G)** B220$^+$ cells, **(H)** CD4$^+$ cells, and **(I)** CD4$^+$CD25$^+$FoxP3$^+$ cells in BALF. CTRL, phosphate-buffered saline-challenged mice; HDM, HDM-challenged mice; SAL, HDM mice treated with saline; MSC, HDM mice treated with unstimulated MSCs; HDM-MSC-BALF, HDM mice treated with MSCs stimulated with BALF from asthmatic mice; HDM-MSC-SERUM, HDM mice treated with MSCs stimulated with serum from asthmatic mice. Data are presented as mean ± SD (A). Boxes show the interquartile (P25–P75) range, whiskers denote the range (minimum–maximum), and horizontal lines represent the median of eight animals per group (B–I). *, Significantly different from CTRL ($p < .05$). **, Significantly different from HDM-SAL ($p < .05$). #, Significantly different from HDM-MSC ($p < .05$). Abbreviations: BALF, bronchoalveolar lavage fluid; MSC, mesenchymal stromal cells; HDM, house dust mite.

compared with the CTRL group (Fig. 7F–7H). Only serum-stimulated MSCs were able to reduce total and B-cell counts in HDM-challenged mice to CTRL-comparable levels. MSC administration did not affect CD4$^+$ T-cell counts in HDM-challenged mice, regardless of stimulation.

Discussion

To the best of our knowledge, this is the first study in which MSCs were stimulated with BALF or serum from asthmatic mice prior to administration in an attempt to potentiate their ability to reduce both inflammation and remodeling in experimental HDM-induced allergic asthma. Compared with unstimulated or BALF-stimulated MSCs, serum-stimulated MSCs yielded greater therapeutic effects in HDM-challenged mice by further reducing levels of IL-4, IL-13, and eotaxin in BALF, as well as total and differential cell counts in BALF, bone marrow, and mLN and collagen fiber content in lungs, especially in the airways. Notably, compared with BALF-stimulated MSCs, cells stimulated with serum from HDM mice exhibited increased mRNA expressions of TGF-β1, IFN-γ, IL-10, TSG-6, IDO-1, and IL-1RN, and induced anti-inflammatory M2 macrophage polarization in vitro, Furthermore, serum from HDM mice also led to

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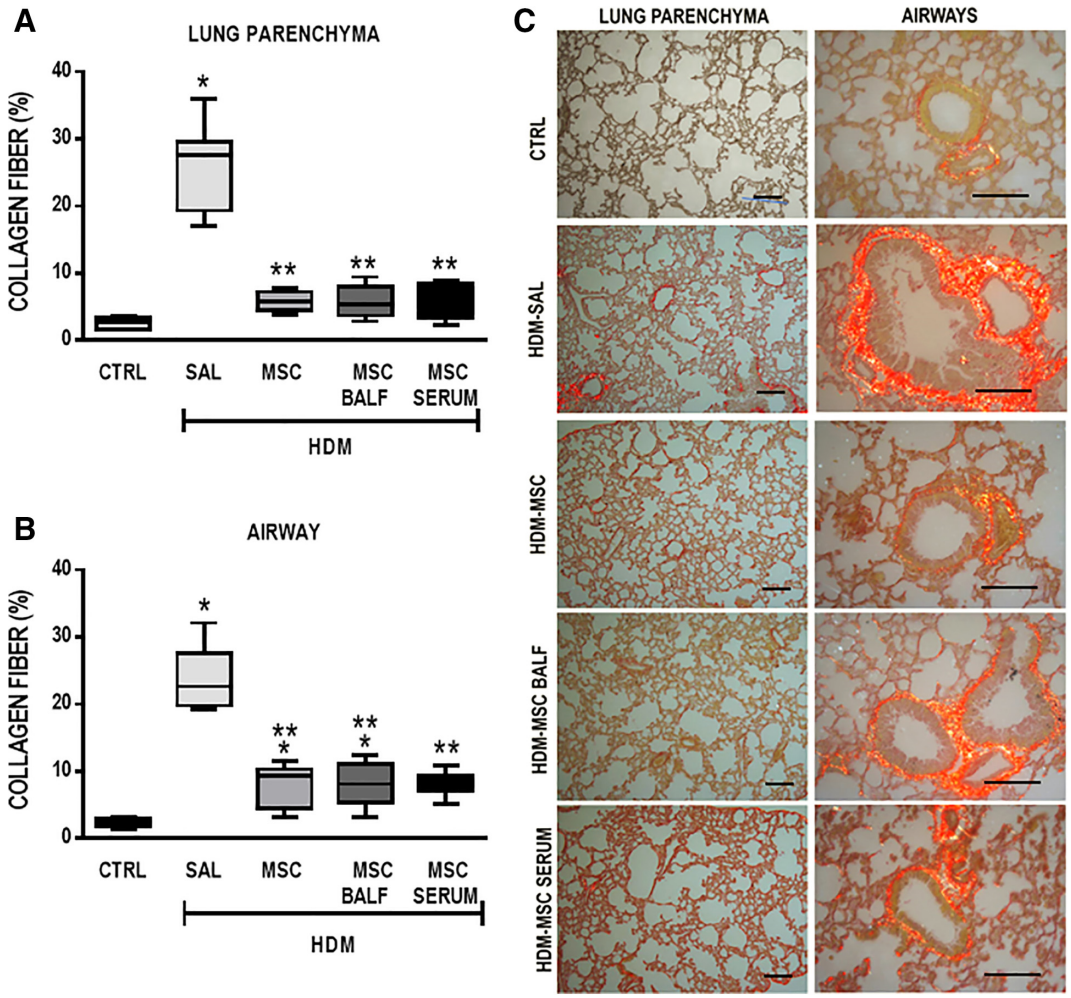

Figure 5. Ex vivo stimulation of MSCs with serum yielded further reduction of lung remodeling in vivo. Collagen fiber content in **(A)** lung parenchyma and **(B)** airway. Representative photomicrographs of lung parenchyma (original magnification: ×200) and airways (original magnification: ×400) stained by the Picrosirius-polarization method **(C)**. CTRL, phosphate-buffered saline-challenged mice; HDM, HDM-challenged mice; SAL, HDM mice treated with saline; MSC, HDM mice treated with unstimulated MSCs; HDM-MSC-BALF, HDM mice treated with MSCs stimulated with BALF from asthmatic mice; HDM-MSC-SERUM, HDM mice treated with MSCs stimulated with serum from asthmatic mice. Boxes show the interquartile (P25–P75) range, whiskers denote the range (minimum–maximum), and horizontal lines represent the median of eight animals per group. Scale bars: 100 µm. *, Significantly different from CTRL ($p < .05$). **, Significantly different from HDM-SAL ($p < .05$). Abbreviations: BALF, bronchoalveolar lavage fluid; MSC, mesenchymal stromal cells; HDM, house dust mite.

a higher percentage of apoptotic cells and expression of caspase-3 and Bax by MSCs compared with those unstimulated, stimulated with BALF from HDM mice, or stimulated with BALF or serum from CTRL animals.

Despite the widespread use of ovalbumin as an allergen to induce asthma in animal models, it is not a clinically relevant allergen, and requires peripheral sensitization and, in many cases, adjuvant administration to obtain a successful protocol [29]. Alternatively, HDM affects ∼85% of humans with asthma worldwide, and the HDM model resembles crucial features of chronic human asthma, such as local sensitization in the lungs and activation of complex inflammatory pathways, which involve the structural cells of the lung [30]. Another advantage of the model used herein is that only 3 weeks are needed to observe both the inflammation and remodeling characteristic of chronic asthma [11, 19, 20]. MSCs were extracted from bone marrow, since these cells exerted greater immunomodulatory responses compared with MSCs from other sources in previous studies by our group [6, 11]. Furthermore, MSCs were intratracheally administered 24 hours after the last challenge to mimic a clinically relevant situation.

Previous studies have demonstrated that MSC administration before sensitization or during challenge significantly reduced inflammatory-cell infiltration in the lungs, airway hyper-responsiveness, and mucus hypersecretion in models of ovalbumin-induced asthma [7, 9, 31, 32]. Prophylactic administration of MSCs also mitigated the inflammatory process in asthma models using clinically relevant allergens, including ragweed, *Aspergillus* hyphal extract, and HDM extract [5, 8, 33]. However, repair of injured tissue and reversal of the remodeling process, especially in the airways, were limited when MSCs were administered after lung function had been impaired and inflammation and remodeling had already been established [11, 19, 20]. One potential explanation is that the lung inflammatory environment after antigen challenge in sensitized mice somehow alters MSCs activity. More recent studies, conducted in other disease models, have tried to potentiate the immunomodulatory properties of MSCs by

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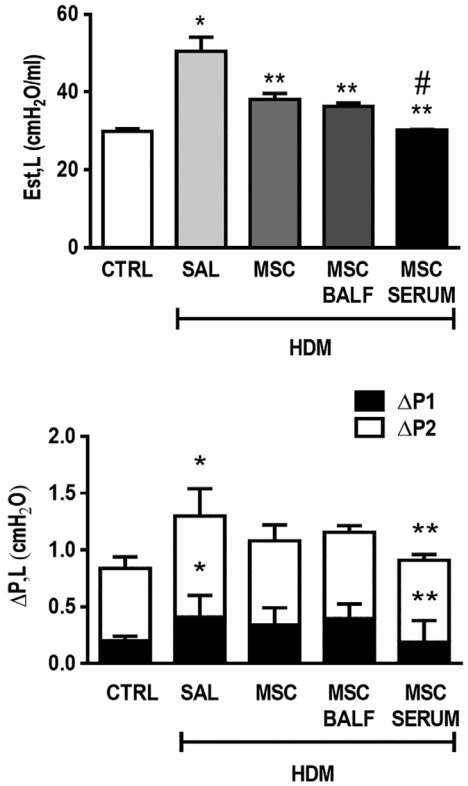

Figure 6. Ex vivo stimulation of MSCs with serum led to further improvement in lung mechanics in vivo. Static lung elastance (Est,L; upper panel), resistive (ΔP1,L), and viscoelastic (ΔP2,L) pressures (lower panel). CTRL, phosphate-buffered saline-challenged mice; HDM, HDM-challenged mice; SAL, HDM mice treated with saline; MSC, HDM mice treated with unstimulated MSCs; HDM-MSC-BALF, HDM mice treated with MSCs stimulated with BALF from asthmatic mice; HDM-MSC-SERUM, HDM mice treated with MSCs stimulated with serum from asthmatic mice. Bars represent mean ± SD of eight animals per group. *, Significantly different from CTRL ($p < .05$). **, Significantly different from HDM-SAL ($p < .05$). #, Significantly different from HDM-MSC ($p < .05$). Abbreviations: BALF, bronchoalveolar lavage fluid; MSC, mesenchymal stromal cells; HDM, house dust mite.

stimulating them prior to administration [15, 19, 34–36]. Several stimulation methods have been investigated, including incubation of MSCs in hypoxic and heat-shock environments or with small-molecule pharmacological agents, cytokines, growth factors, and biological fluids (BALF and serum) obtained from patients [15–19]. Both in vitro and in vivo studies have demonstrated that MSC survival is enhanced under hypoxic or serum-deprived conditions (34–36), whereas optimized sublethal stress conditions led to greater proliferation, chemotaxis, migration, engraftment, and differentiation of MSCs, potentiating the immunomodulatory effects of these cells [37]. In the present study, MSCs exposed to either BALF or serum from asthmatic mice demonstrated higher expression of caspase-3 and Bax, and apoptotic cell percentages, while increasing expression of several anti-inflammatory mediators (TGF-β1, IFN-γ, IL-10, TSG-6, IDO-1, and IL-1RN), with even greater effects induced by serum than BALF. In agreement with our in vitro findings, a recent study using a model of graft-versus-host disease observed that MSCs may be actively induced to undergo apoptosis by recipient cytotoxic cells. This process activates caspase-dependent IL-1 signaling, which strikingly enhances the immunomodulatory properties of MSCs, since the recipient phagocytes may engulf the MSCs under apoptosis [13, 38], leading to production of many mediators. In particular, increased expression of IDO-1 induces effective immunosuppression by interfering with amino acid metabolism in the inflammatory microenvironment [39, 40]. It is thus conceivable that BALF and serum from asthmatic mice would create an in vitro microenvironment similar to that in asthma, leading to alterations in MSC signaling pathways and, consequently, higher expression of anti-inflammatory mediators by MSCs, mainly after stimulation with serum (SERUM-HDM) compared with BALF (BALF-HDM). This may be explained by the higher protease levels in BALF compared with serum from HDM mice (7.2 [6.2–7.9] and 2.9 [1.8–4], respectively) in baseline conditions. In allergic asthma, the BALF almost invariably contains significant quantities of proteases [41]. MSCs present cell surface proteins, cell-adhesion molecules, signaling receptors, transporters, receptors, and enzymes [42, 43]. We hypothesized that the increased levels of proteases in the BALF may affect these MSC receptors and, consequently, MSC responses. Furthermore, if proteases result in damage to cell-adhesion molecules, contact of MSCs with other cells (e.g., macrophages) might be impaired, thus limiting their proinflammatory/anti-inflammatory activities and immunosuppressive effects as well.

Macrophages have also been implicated in the inflammatory process of allergic asthma. Classically, M1 macrophages are activated by Toll-like receptor ligands to secrete iNOS, IL-1β and TNF-α and mediate host defense, while alternatively activated M2 macrophages secrete anti-inflammatory mediators, including arginase-2, IL-10, and TGF-β, which stimulate proresolution responses and tissue repair [9, 19, 44]. In our study, conditioned media from MSCs stimulated with HDM-BALF, compared with HDM-SERUM, promoted the M1 alveolar macrophage phenotype, with increased iNOS and TNF-α expressions. In contrast, conditioned media from serum-stimulated MSCs induced macrophage polarization to the M2 phenotype, with increased expression of associated markers (arginase-2, IL-10 and TGF-β). In fact, increased BALF IL-10 levels in HDM-challenged mice after treatment with serum-stimulated MSCs can be correlated with both macrophage polarization to the M2 phenotype and MSC stimulation. Previous studies have shown that IL-10 mitigates inflammation by suppressing Th2 cytokine production [45, 46], in line with the anti-inflammatory effects of serum-stimulated MSCs in HDM-challenged mice.

Regardless of stimulation, MSCs administration inhibited eosinophilia, but only serum-stimulated MSCs significantly reduced levels of IL-4, IL-13, and eotaxin in BALF, which may suggest the involvement of other mediators in the process. In this line, MSCs may produce or induce lung-resident cells to produce IFN-γ, which then activates signaling in the airway epithelium to inhibit eosinophil recruitment [8, 47]. In addition, the reduction in neutrophil count in HDM-challenged mice after administration of BALF-stimulated or serum-stimulated MSCs may be correlated with increased TSG-6 expression in MSCs, as this mediator suppresses CXCL8-mediated chemotaxis of neutrophils [48, 49]. Although in vitro stimulation of MSCs with BALF or serum from asthmatic mice increased the expression of TGF-β1 and IL-10, the percentage of $CD4^+CD25^+FoxP3^+$ cells did not increase in BALF of HDM-challenged mice after MSC administration, regardless of stimulation. This finding stands in contrast to previous studies that reported upregulation of T-regulatory cells induced by MSCs [7, 50, 51]. One possible explanation for this conflicting result is the timing of

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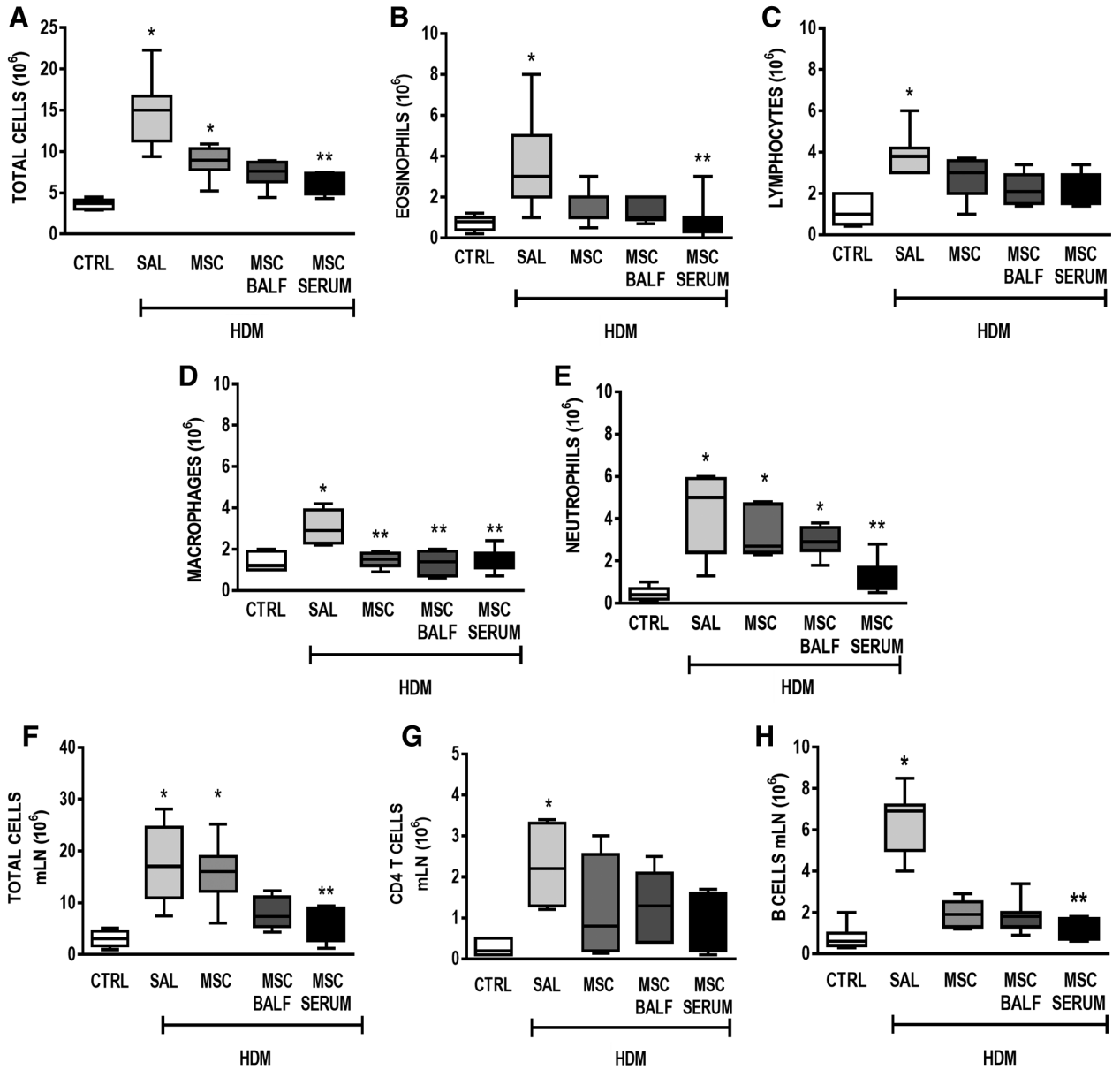

Figure 7. Ex vivo stimulation of MSCs with serum led to further reductions of cellularity in bone marrow and mLN in vivo. **(A):** Total leukocytes, **(B)** eosinophils, **(C)** lymphocytes, **(D)** macrophages, **(E)** neutrophils in bone marrow, **(F)** total leukocytes, **(G)** $CD4^+$ cells, and **(H)** B cells in mediastinal lymph nodes (mLN). CTRL, phosphate-buffered saline-challenged mice; HDM, HDM-challenged mice; SAL, HDM mice treated with saline; MSC, HDM mice treated with unstimulated MSCs; HDM-MSC-BALF, HDM mice treated with MSCs stimulated with BALF from asthmatic mice; HDM-MSC-SERUM, HDM mice treated with MSCs stimulated with serum from asthmatic mice. Boxes show the interquartile (P25–P75) range, whiskers denote the range (minimum–maximum), and horizontal lines represent the median of eight animals per group. *, Significantly different from CTRL ($p < .05$). **, Significantly different from HDM-SAL ($p < .05$). Abbreviations: BALF, bronchoalveolar lavage fluid; MSC, mesenchymal stromal cells; HDM, house dust mite.

treatment (prophylactic vs. therapeutic), which suggests that MSCs administered with therapeutic intent, as in our study, might not induce expansion of T-regulatory cells, since lymphocytes were already primed by the allergen.

Administration of serum-stimulated MSCs also induced a potent peripheral inflammatory modulation, yielding a decrease in immune cells in the bone marrow and mLN of HDM-challenged mice. As GFP-tagged MSCs were found neither in bone marrow nor in mLN in a previous study by our group using the same asthma model [11], cell engraftment would not be a feasible explanation for the mitigated inflammation in these tissues. It is possible that intratracheal administration of serum-stimulated MSCs boosted secretion of trophic factors that acted on the lungs in a paracrine manner and in bone marrow and mLN in an autocrine manner, as previously observed in other disease models and tissues [52, 53]. In addition, the reduction in mLN B-cell counts may have contributed to the resolution of lung inflammation after administration of serum-stimulated MSCs, as B cells promote secretion of allergen-specific immunoglobulins (mainly IgE) and are also involved in antigen presentation and T-cell proliferation, which intensify immune responses against the allergen [8, 54].

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Regardless of stimulation, MSCs administration reduced collagen fiber content in both lung parenchyma and airways; however, only serum-stimulated MSCs reduced collagen fiber content in the airways of HDM-challenged mice to CTRL-comparable levels. This fact may be attributable to a higher capacity of serum-stimulated MSCs to modulate secretion of mediators, including reduction in BALF IL-4 and IL-13 levels, since these mediators can induce fibroblast proliferation and extracellular matrix deposition [55, 56]. Taken together, the thickening of alveolar septa, increased inflammatory-cell count, and deposition of collagen fiber contributed to the impairment of lung function observed in HDM-challenged mice. Administration of serum-stimulated MSCs reduced inflammation and to a greater extent compared with unstimulated or BALF-stimulated MSCs, thereby improving lung function more efficiently.

This study has several limitations. First, we used a model of HDM-induced asthma based on the clinical relevance of this allergen, but MSC actions and effects may differ depending on the allergen as well as stage and severity of the disease. Second, a specific set of mediators was investigated, but a broader range of biomarkers could further elucidate the mechanisms of MSCs stimulation prior to administration. Third, MSCs stimulation with BALF or serum from CTRL mice was only analyzed in vitro, as BALF and serum from asthmatic mice were found to induce more robust therapeutic effects. Finally, analyses of the differences between BALF and serum effects on MSCs were focused on the levels of proteases. Further studies are needed to better understand how exposure to either serum or BALF alters the effects of MSCs in experimental asthma.

Conclusion

Exposure to either BALF or serum obtained from asthmatic mice potentiated expression and release of several mediators postulated to be involved in the anti-inflammatory actions of MSCs, while also inducing expression of apoptotic mediators. Asthmatic serum was more potent in exerting several of these effects than was asthmatic BALF. In parallel, MSCs exposed to asthmatic serum were found to subsequently have greater potency to ameliorate several but not all aspects of a clinically relevant model of allergic airway inflammation.

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Author Contributions

S.C.A.: conducted the experiments and study, contributed to data collection and analysis, interpreted the data, wrote and edited the manuscript; D.G.X., T.B.O., N.G.B., J.Z.K., and L.L.C.: conducted the experiments and contributed to data collection and analysis; M.L.P.: contributed to data collection, interpreted the data, wrote and edited the manuscript; P.C.O., M.M.M., and D.J.W.: interpreted the data, critically reviewed the manuscript for important intellectual content; P.R.M.R: contributed to the conception and design of the study, interpreted the data, and wrote, edited, and reviewed the manuscript; S.C.A., D.G.X., T.B.d.O., N.G.B., L.L.d.C., J.Z.K., P.C.O., M.L.-P., M.M.M., D.J.W., and P.R.M.R.: final version of the manuscript.

Disclosure of Potential Conflicts of Interest

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References

1 Global Strategy for Asthma Management and Prevention. Global Iniciative for Asthma; 2018. Available at https://ginasthma.org/2018-gina-report-global-strategy-for-asthma-management-and-prevention/. Accessed September 27, 2018.

2 Royce SG, Tang ML. The effects of current therapies on airway remodeling in asthma and new possibilities for treatment and prevention. Curr Mol Pharmacol 2009;2:169–181.

3 Barnes PJ. New therapies for asthma: Is there any progress? Trends Pharmacol Sci 2010;31:335–343.

4 Papierniak ES, Lowenthal DT, Harman E. Novel therapies in asthma: Leukotriene antagonists, biologic agents, and beyond. Am J Ther 2013;20:79–103.

5 Cruz FF, Borg ZD, Goodwin M et al. Systemic administration of human bone marrow-derived mesenchymal stromal cell extracellular vesicles ameliorates aspergillus hyphal extract-induced allergic airway inflammation in immunocompetent mice. Stem Cells Translational Medicine 2015;4:1302–1316.

6 Abreu SC, Antunes MA, Xisto DG et al. Bone marrow, adipose, and lung tissue-derived murine mesenchymal stromal cells release different mediators and differentially affect airway and lung parenchyma in experimental asthma. Stem Cells Translational Medicine 2017;6:1557–1567.

7 Nemeth K, Keane-Myers A, Brown JM et al. Bone marrow stromal cells use TGF-beta to suppress allergic responses in a mouse model of ragweed-induced asthma. Proc Natl Acad Sci USA 2010;107:5652–5657.

8 Goodwin M, Sueblinvong V, Eisenhauer P et al. Bone marrow-derived mesenchymal stromal cells inhibit Th2-mediated allergic airways inflammation in mice. Stem Cells 2011;29:1137–1148.

9 Mathias LJ, Khong SM, Spyroglou L et al. Alveolar macrophages are critical for the inhibition of allergic asthma by mesenchymal stromal cells. J Immunol 2013;191:5914–5924.

10 Marinas-Pardo L, Mirones I, Amor-Carro O et al. Mesenchymal stem cells regulate airway contractile tissue remodeling in murine experimental asthma. Allergy 2014;69:730–740.

11 Kitoko JZ, Nascimento AP, Abreu SC et al. Therapeutic administration of bone marrow-derived mesenchymal stromal cells reduces airway inflammation without upregulating Tregs in experimental asthma. Clin Exp Allergy 2018;48:205–216.

12 Malaquias MAS, Oyama LA, Jericó PC et al. Effect of mesenchymal stromal cells play a role the oxidant/antioxidant balance in a murine model of asthma. Allergol Immunopathol 2018;46:136–143.

13 Galleu A, Riffo-Vasquez Y, Trento C et al. Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. Sci Transl Med 2017;9(416).

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14 Cheng AS, Yau TM. Paracrine effects of cell transplantation: Strategies to augment the efficacy of cell therapies. Semin Thorac Cardiovasc Surg 2008;20:94–101.

15 Lan YW, Choo KB, Chen CM et al. Hypoxia-preconditioned mesenchymal stem cells attenuate bleomycin-induced pulmonary fibrosis. Stem Cell Res Ther 2015;6:97.

16 de Witte SF, Franquesa M, Baan CC et al. Toward development of imesenchymal stem cells for immunomodulatory therapy. Front Immunol 2015;6:648.

17 Feng Y, Luo S, Yang P et al. Electric pulse current stimulation increases electrophysiological properties of If current reconstructed in mHCN4-transfected canine mesenchymal stem cells. Exp Ther Med 2016;11:1323–1329.

18 Gharibi B, Cama G, Capurro M et al. Gene expression responses to mechanical stimulation of mesenchymal stem cells seeded on calcium phosphate cement. Tissue Eng Part A 2013;19:2426–2438.

19 Abreu SC, Lopes-Pacheco M, daSilva AL et al. Eicosapentaenoic acid enhances the effects of mesenchymal stromal cell therapy in experimental allergic asthma. Front Immunol 2018;9:1147.

20 Olsen PC, Kitoko JZ, Ferreira TP et al. Glucocorticoids decrease Treg cell numbers in lungs of allergic mice. Eur J Pharmacol 2015;747:52–58.

21 Zhang X, Gonçalves R, Mosser DM. The isolation and characterization of murine macrophages. Curr Protoc Immunol 2008; Chapter 14: Unit 14.1.

22 Ionescu L, Byrne RN, va Haaften T et al. Stem cell conditioned medium improves acute lung injury in mice: in vivo evidence for stem cell paracrine action. Am J Physiol Lung Cell Mol Physiol 2012;303:L967–L977.

23 Burburan SM, Xisto DG, Ferreira HC et al. Lung mechanics and histology during sevoflurane anesthesia in a model of chronic allergic asthma. Anesth Analg 2007;104:631–637.

24 Bates JH, Ludwig MS, Sly PD et al. Interrupter resistance elucidated by alveolar pressure measurement in open-chest normal dogs. J Appl Physiol 1988;65:408–414.

25 Antunes MA, Abreu SC, Cruz FF et al. Effects of different mesenchymal stromal cell sources and delivery routes in experimental emphysema. Respir Res 2014;15:118.

26 Rocco PR, Negri EM, Kurtz PM et al. Lung tissue mechanics and extracellular matrix remodeling in acute lung injury. Am J Respir Crit Care Med 2001;164:1067–1071.

27 Xisto DG, Farias LL, Ferreira HC et al. Lung parenchyma remodeling in a murine model of chronic allergic inflammation. Am J Respir Crit Care Med 2005;171:829–837.

28 da Silva AL, Magalhaes RF, Branco VC et al. The tyrosine kinase inhibitor dasatinib reduces lung inflammation and remodelling in experimental allergic asthma. Br J Pharmacol 2016;173:1236–1247.

29 Nials AT, Uddin S. Mouse models of allergic asthma: Acute and chronic allergen challenge. Dis Model Mech 2008;1:213–220.

30 Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. Trends Immunol 2011;32:402–411.

31 Kavanagh H, Mahon BP. Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells. Allergy 2011;66:523–531.

32 Duong KM, Arikkatt J, Ullah MA et al. Immunomodulation of airway epithelium cell activation by mesenchymal stromal cells ameliorates house dust mite-induced airway inflammation in mice. Am J Respir Cell Mol Biol 2015;53:615–624.

33 Lathrop MJ, Brooks EM, Bonenfant NR et al. Mesenchymal stromal cells mediate Aspergillus hyphal extract-induced allergic airway inflammation by inhibition of the Th19 signaling pathway. Stem Cells Translational Medicine 2014;3:194–205.

34 He A, Jiang Y, Gui C et al. The antiapoptotic effect of mesenchymal stem cell transplantation on ischemic myocardium is enhanced by anoxic preconditioning. Can J Cardiol 2009;25:353–358.

35 Hu X, Yu SP, Fraser JL et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg 2008; 135:799–808.

36 Oh JS, Ha Y, An SS et al. Hypoxia-preconditioned adipose tissue-derived mesenchymal stem cell increase the survival and gene expression of engineered neural stem cells in a spinal cord injury model. Neurosci Lett 2010;472:215–219.

37 Amiri F, Jahanian-Najafabadi A, Roudkenar MH. In vitro augmentation of mesenchymal stem cells viability in stressful microenvironments: in vitro augmentation of mesenchymal stem cells viability. Cell Stress Chaperones 2015;20:237–251.

38 Bartosh TJ, Ylöstalo JH, Bazhanov N et al. Dynamic compaction of human mesenchymal stem/precursor cells into spheres self-activates caspase-dependent IL1 signaling to enhance secretion of modulators of inflammation and immunity (PGE2, TSG6 and STC1). Stem Cells 2013;31:2443–2456.

39 Su J, Chen X, Huang Y et al. Phylogenetic distinction of iNOS and IDO function in mesenchymal stem cell-mediated immunosuppression in mammalian species. Cell Death Differ 2014;21:388–396.

40 Meisel R, Zibert A, Laryea M et al. Human bone marrow stromal cells inhibit allogeneic T-cell resposes by indoleamine 2, 3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619–4621.

41 Kim YH, Lee SH. TGF-beta/SMAD4 mediated UCP2 downregulation contributes to Aspergillus protease-induced inflammation in primary bronchial epithelial cells. Redox Biol 2018;18:104–113.

42 Shirjang S, Mansoori B, Solali S et al. Toll-like receptors as a key regulator of mesenchymal stem cell function: An up-to-date review. Cell Immunol 2017;315:1–10.

43 Zhou Y, Zhang T, Wang X et al. Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways. Cell Physiol Biochem 2015;36:631–641.

44 Song X, Xie S, Lu K et al. Mesenchymal stem cells alleviate experimental asthma by inducing polarization of alveolar macrophages. Inflammation 2015;38:485–492.

45 Vasandan AB, Jahnavi S, Shashank C et al. Human mesenchymal stem cells program macrophage plasticity by altering their metabolic status via a PGE2-dependent mechanism. Sci Rep 2016;6:38308.

46 Chung F. Anti-inflammatory cytokines in asthma and allergy: Interleukin-10, interleukin-12, interferon-gamma. Mediators Inflamm 2001; 10:51–59.

47 Mitchell C, Provost K, Niu N et al. IFN-γ acts on the airway epithelium to inhibit local and systemic pathology in allergic airway disease. J Immunol 2011;187:3815–3820.

48 Dyer DP, Thomson JM, Hermant A et al. TSG-6 inhibits neutrophil migration via direct interaction with the chemokine CXCL8. J Immunol 2014;192:2177–2185.

49 Dyer DP, Salanga CL, Johns SC et al. The anti-inflammatory protein TSG-6 regulates chemokine function by inhibiting chemokine/glycosaminoglycan interactions. J Biol Chem 2016;291:12627–12640.

50 Ge X, Bai C, Yang J et al. Effect of mesenchymal stem cells on inhibiting airway remodeling and airway inflammation in chronic asthma. J Cell Biochem 2013;114: 1595–1605.

51 Cho KS, Park MK, Kang SA et al. Adipose-derived stem cells ameliorate allergic airway inflammation by inducing regulatory T cells in a mouse model of asthma. Mediators Inflamm 2014;2014:436476.

52 Kyurkchiev D, Bochev I, Ivanova-Todorova E et al. Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells 2014;6:552–570.

53 Lunyak VV, Amaro-Ortiz A, Gaur M. Mesenchymal stem cells secretory responses: Senescence messaging secretome and immunomodulation perspective. Front Genet 2017;8:220.

54 Habener A, Behrendt AK, Skuljec J et al. B cell subsets are modulated during allergic airway inflammation but are not required for the development of respiratory tolerance in a murine model. Eur J Immunol 2017;47:552–562.

55 Lee CG, Link H, Baluk P et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. Nat Med 2004;10:1095–1103.

56 Bellini A, Marini MA, Bianchetti L et al. Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients. Mucosal Immunol 2012;5: 140–149.

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