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Therapeutic administration of bone marrow-derived mesenchymal stromal cells reduces airway inflammation without upregulating Tregs in experimental asthma

Running title: MSCs inhibit asthma without upregulating Tregs

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Abstract

Background Prophylactic administration of mesenchymal stromal cells (MSCs) derived from adipose (AD-MSC) and bone marrow tissue (BM-MSC) in ovalbumin-induced asthma hinders inflammation in a Treg-dependent manner. It is uncertain whether MSCs act through Tregs when inflammation is already established in asthma induced by a clinically relevant allergen.

Objective Evaluate the effect of therapeutic administration of MSCs on inflammation and Treg cells in house dust mite (HDM)-induced asthma.

Methods BM-MSCs and AD-MSCs were administered intratracheally to C57BL/6 mice 1 day after the last HDM challenge. Lung function, remodeling and parenchymal inflammation, were assayed 3 or 7 days after MSCs treatment, through invasive plethysmography and histology, respectively. Bronchoalveolar lavage fluid (BALF) and mediastinal lymph nodes (mLNs) were assessed for lymphocyte phenotyping by flow cytometry. MSCs were studied regarding their potential to induce Treg cells from primed and unprimed lymphocytes *in vitro*.

Results BM-MSCs, but not AD-MSCs, reduced lung influx of eosinophils and B cells and increased IL-10 levels in HDM-challenged mice. Neither BM-MSCs nor AD-MSCs reduced lung parenchymal inflammation, airway hyperresponsiveness or mucus hypersecretion. BM- and AD-MSCs did not upregulate Treg cell counts within the airways and mLNs, but BM-MSCs decreased the pro-inflammatory profile of alveolar macrophages. Co-culture of BM-MSCs and AD-MSCs with allergen-stimulated lymphocytes reduced Treg cell counts in a cell to cell contact independent manner, although co-culture of both MSCs with unprimed lymphocytes upregulated Treg cell counts.

Conclusions MSCs therapeutically administered exert anti-inflammatory effects in the airway of HDM-challenged mice, but do not ameliorate lung function or remodeling. Although MSC pre-treatment can increase Treg cell numbers, it is highly unlikely that the MSCs will induce Treg cell expansion when lymphocytes are allergenically primed in an established lung inflammation.

Key words. Asthma, airway inflammation, mesenchymal stromal cells, Treg.

Introduction

Asthma is a chronic inflammatory disease orchestrated by Th2 cells and associated with eosinophilia, airway hyperresponsiveness (AHR) and mucus hypersecretion [1]. Characteristically, Th2 cells release cytokines such as IL-4, IL-5 and IL-13, which contribute to airway inflammation in asthma [2]. Regulatory T (Treg) cells have been established as an important T-cell subset that mitigates the Th2 immune response, leading to resolution of inflammation in asthma [3]. Interestingly, Treg cell counts and their anti-inflammatory activity are impaired in asthmatic subjects as compared to healthy individuals, highlighting the importance of adequate Treg cell numbers to control allergic airway inflammation [4, 5].

Importantly, it has been shown that innate immune cells (e.g. dendritic cells and alveolar macrophages) also play an important role in attenuating lung inflammation in asthma, according to their phenotype [6, 7].

Glucocorticoids are the gold standard anti-inflammatory therapy for asthma. Nevertheless, they often fail to provide complete control in severe cases, and their prolonged systemic use at high doses leads to a broad range of adverse effects [8, 9]. The limitations of glucocorticoid therapy highlight the need to develop new strategies for asthma management. The immunomodulatory potential of non-hematopoietic multipotent precursor cells resident in connective tissue, namely mesenchymal stromal cells (MSCs), has encouraged studies of their application as an alternative to glucocorticoid therapy in asthma [10]. MSCs can be obtained from virtually all postnatal organs, being the MSCs derived from adipose tissue (AD-MSC) and bone marrow (BM-MSC) the most studied in experimental asthma [11-13]. The administration of MSCs has been performed before or immediately after sensitization with ovalbumin (OVA) in experimental asthma, hampering the Th2-biased immune response in a mechanism dependent on Treg cell expansion in the lung and in secondary lymphoid organs [12, 14, 15]. In addition, MSC administered prophylactic in OVA models reduces the capacity of lung dendritic cells to induce Th2 response, and shifts the alveolar macrophage phenotype toward an anti-inflammatory profile, thus mitigating lung alterations in asthma [16-18]. Nonetheless, prophylactic cell therapy in OVA models of asthma has some limitations. First, OVA is not clinically relevant to humans compared to allergens such as house dust mite (HDM) and grass pollen [19]. Second, prophylactic administration of MSCs in asthma is not a possible strategy in clinical application, as asthmatic subjects already present an established lung allergic inflammation.

Translational studies have shown that therapeutic administration of AD-MSCs in experimental asthma induced by HDM or Bermuda grass did not have anti-inflammatory

effects as prominent as those observed in OVA models; however, their effects on Treg cells were not assessed [20, 21]. Kapoor *et al.* showed that BM-MSCs co-cultured with peripheral-blood mononuclear cells (PBMCs) obtained from allergic asthmatic subjects fail to induce expansion of CD4⁺CD25⁺Foxp3⁺ T cells, suggesting that BM-MSCs do not upregulate Treg cell profile acquisition in lymphocytes harvested from an established inflammatory milieu [22]. MSCs can modulate It is still uncertain whether AD-MSC and BM-MSC therapy will have an anti-inflammatory effect by increasing Treg cell counts in established asthma induced by clinically relevant allergens. Therefore, we sought to test the effects of therapeutic administration of MSCs on lung changes, including airway inflammation, and mainly Treg cells in experimental asthma induced by HDM.

Methods

Mice

C57BL/6 or C57BL/6-Tg(GFP) mice (weight 20–25 g) were supplied with water and food *ad libitum*. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the Health Sciences Center of the Federal University of Rio de Janeiro (097/14; CEUA-UFRJ, Rio de Janeiro, Brazil). DO11.10 TCR transgenic mice were kindly donated by Dr. Marco Aurélio Martins, Oswaldo Cruz Foundation (FIOCRUZ).

MSC isolation, culture conditions, and characterization

MSCs were isolated from epididymal fat pads (AD-MSC) and from femurs and tibias (BM-MSC) of C57BL/6 mice and cultured as previously described [23]. AD-MSCs and BM-MSCs were harvested separately in passages 4–6 and then gradually cryopreserved at –80°C in a concentration of 1×10^6 cells in freezing solution containing DMEM (Life Technologies,

Grand Island, NY), fetal bovine serum (Life Technologies), and DMSO (Sigma-Aldrich, St. Louis, MO). Immediately prior to experimental use, cells were thawed and washed in 1X PBS [23, 24]. Cultured MSCs were phenotyped by flow cytometry and osteogenic differentiation was confirmed by Alizarin Red S staining, as previously described [23]. Adipogenic differentiation was confirmed by Sudan Black B staining [25]. Flow cytometry and differentiation assays confirmed the MSC phenotype for cultured AD-MSCs and BM-MSCs (Supplemental Fig. 1).

HDM-induced airway inflammation protocol

Mice were challenged by intranasal instillation with 25 µg of HDM protein (25 µL) (*Dermatophagoides pteronyssinus*; Greer Laboratories, Lenoir, NC), 3 days/week for 3 weeks [26]. Control mice were challenged with 25 µL of the HDM vehicle (1X PBS). One day after the last challenge, mice were euthanized and examined for the main features of an asthma-like phenotype in the HDM lung allergic model.

Administration of MSCs

Control and HDM-challenged mice were randomized to receive intratracheal instillation of saline (SAL, 0.9% NaCl, 50 µl), AD-MSC, or BM-MSC (1×10^5 cells), 1 day after the last challenge (Fig. 1A). Mice were culled 3 or 7 days after the MSC administration for evaluation of early and late effects (Fig. 1B). Control groups treated with AD-MSCs or BM-MSCs did not show relevant differences when compared to control group receiving only saline, therefore these data were omitted from the graphs.

Lung function assessment

AHR was measured in a FinePointe R/C Buxco platform (Buxco Electronics, Sharon, CT), as previously described [27]. Briefly, mice were tracheotomized, cannulated, and placed in dorsal recumbence on a heated bed in a plethysmography chamber. Airway resistance and dynamic compliance were determined in response to sequential inhalation of aerosolized PBS and 3, 9, 27, and 81 mg/ml of methacholine during each respiratory cycle. Dynamic compliance values were converted into lung elastance values.

Cell recovery and counts

BALF was obtained by gentle aspiration of 1X PBS injected into the airways of mice via a tracheal cannula. BALF was centrifuged at $250\times g$ for 10 minutes at 4°C. Cell pellets were resuspended in 1X PBS.

Mediastinal lymph nodes (mLNs) were carefully extracted, placed into 1 ml of 1X PBS, and cell suspensions obtained after homogenization.

Total leukocytes from BALF and lymph nodes were counted in a Neubauer chamber after dilution using Turk's solution (2% acetic acid). BALF cells were cytocentrifuged and stained by May-Grünwald-Giemsa. Differential cell counts were performed as previously described [27].

Lymphocyte co-cultures with MSCs

Mediastinal and inguinal lymph node cells were harvested from HDM-challenged C57BL/6 mice 1 day after the last challenge of the HDM-induced asthma protocol. Cells obtained from homogenization of each lymph node were plated separately at 10^6 and re-stimulated with HDM (0.05 to 0.1 $\mu\text{g}/\mu\text{L}$) in the presence of 10^5 AD-MSCs, BM-MSCs, or culture medium alone. Transwell chambers with a 4 μm pore membranes were used. MSCs were seeded on

the upper chamber and lymphocytes on the bottom at the same conditions mentioned before on direct co-culture. With 48 hours of incubation, lymphocytes were harvested and analyzed through flow cytometry.

Cells from mediastinal, inguinal, cervical, and axillar lymph nodes of DO11.10 TCR transgenic mice were harvested, pooled, and co-cultured at 10^6 with 10^5 of AD-MSCs, BM-MSCs, or culture medium alone. Co-cultures were prepared with or without ovalbumin (0.5 mg/mL; Sigma-Aldrich). After 48 hours, lymphocytes were harvested and analyzed through flow cytometry.

Flow cytometry assays

Cells obtained from BALF, lymph nodes, or lymphocytes from co-cultures were blocked with anti-CD16/32 and stained with monoclonal anti-mouse CD4 or B220 antibodies (eBiosciences; San Diego, CA) to assess T and B cell percentages, respectively, in the lymphocyte-gated population. Intracellular staining of CD4 T cells was performed using anti-IL-10-PE antibody (eBiosciences). Treg cells were characterized by staining for CD4, CD25, and Foxp3, according to the manufacturer's instructions (Mouse Treg Staining Kit, eBiosciences). Alveolar macrophages were characterized using anti-CD11c-PE, anti-CD11b-PEcy7 and anti-F4/80-APC antibodies ($CD11b^-CD11c^+F4/80^+$) (eBiosciences). Fluorescence activated cell sorting of alveolar macrophages retrieved from BALF were performed on MoFlo XDP (Beckman Coulter Inc., Miami, FL), and approximate purity of 85%. All data were acquired in a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo X 10.0.7 software (Tree Star Inc., Ashland, OR).

Histological analyses

The left lung of each animal was removed, fixed in Millonig's formalin, paraffin-embedded and sectioned into 5 μ m thick slices. Lung inflammation was evaluated by scoring perivascular and peribronchiolar inflammatory pockets in hematoxylin and eosin-stained sections on a scale of 0 to 5, increasing according to severity, as previously described [28]. Mucus-filled cells in the airways were revealed by periodic acid-Schiff staining and their presence scored as 0, 1, 2, 3, or 4. The values obtained from 6–10 airways were averaged and the resulting score considered as the final mucus score per mouse, as previously described [29]. All histological analyses were performed by a blinded, experienced investigator.

Cytokine detection assays

Cytokine levels were quantitated in lung-tissue homogenates. Briefly, lung tissues were isolated, homogenized in the presence of a lysis buffer solution, centrifuged ($600\times g$ for 5 minutes and $10,000\times g$ for 10 minutes), and the resulting supernatant was assayed. A sandwich ELISA for IL-10 was performed as per manufacturer instructions (BioLegend, San Diego, CA), as were assays for CCL11 and IL-4 (Peprotech, Rocky Hill, NJ) and TGF- β , IL-5 and CCL24 (R&D Systems, Minneapolis, MN).

RNA transcripts of IL-6, IL-10 and IL-1 β on sorted alveolar macrophages were measured by qRT-PCR as previously described [30]. RNA transcripts of IL-10 and TGF- β were evaluated in BM-MSC one day after stimulation *in vitro* using 10% BALF retrieved from HDM-challenged mice. The following primers were used: IL-10: [5'-TCC CTG GGT GAG AAG CTG-3' (forward) and 5'-GCT CCA CTG CCT TGC TCT-3' (reverse)]; TGF- β [5'-ATA CGC CTG AGT GGC TGT C-3' (forward) and 5'-GCC CTG TAT TCC GTC TCC T-3' (reverse)]; IL1- β [5'-GTT GAC GGA CCC CAA AAG-3' (forward) and 5'-GTG CTG CTG

CGA GAT TTG-3`]; IL-6: [5`-TCT CTG GGA AAT CGT GGA A-3` (forward) and 5`-TCT GCA AGT GCA TCA TCG T-3` (reverse)].

Statistical analysis

Data were presented as mean $\pm$ standard deviation or median (interquartile range) for a group of 3–10 animals. Statistical analysis was performed using a *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. Lung function data were analyzed using two-way ANOVA followed by Bonferroni correction. All tests were performed in GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA). *P*-values ≤ 0.05 were considered statistically significant.

Results

BM-MSCs and AD-MSCs did not reduce AHR, peribronchiolar inflammatory pockets, or airway mucus hypersecretion induced by HDM

Airway remodeling, AHR and increased leukocyte counts in the BALF were detectable in mice after 3 weeks of challenges using HDM (Supplemental Fig. 2). AD-MSCs or BM-MSCs were administered 1 day after the last HDM challenge and their effects were evaluated after 3 or 7 days. At 3 days, HDM-challenged mice exhibited significantly increased lung elastance and airway resistance, as compared to control mice, elicited by 27 and 81 mg/mL methacholine doses (Fig. 2). AD-MSCs were unable to reduce lung elastance and airway resistance, whereas BM-MSCs reduced lung elastance only when challenged with 81 mg/mL methacholine (Fig. 2). Seven days after cell therapy, HDM-challenged mice exhibited a

natural reduction in lung elastance and airway resistance; consequently, data were not significantly different across groups (Fig. 2).

No inflammatory pockets or mucus-filled cells were observed in the lungs of control mice. Conversely, both histological changes persisted in the lung of HDM-challenged mice at 3 days analysis (Fig. 3). Neither AD-MSCs nor BM-MSCs altered the presence of lung inflammatory pockets and mucus-filled cells at 3 and 7 days after cell therapy compared with HDM-challenged mice (Fig. 3).

BM-MSCs transiently reduced leukocyte counts in the airways without upregulating Treg cell counts from HDM-challenged mice

Three days after cell therapy, BM-MSCs had significantly reduced the number of airway leukocytes, including eosinophils, macrophages and neutrophils in the BALF of HDM-challenged mice (Fig. 4A). AD-MSCs only reduced macrophage and neutrophil counts but did not alter the number of eosinophils in BALF (Fig. 4A). Seven days after cell therapy, markers of inflammation in BALF had reduced naturally. This natural resolution of inflammation was not accelerated by AD-MSC or BM-MSC therapy (Fig. 4A). In addition, BM-MSCs reduced the numbers of B cells in the BALF of HDM-challenged mice, an effect that was appreciable only on 3-day analyses (Fig. 4B). AD-MSCs and BM-MSCs did not reduce CD4⁺ T cell counts significantly in the BALF of HDM-challenged mice at either time point of evaluation (Fig. 4B). Surprisingly, AD-MSCs and BM-MSCs slightly reduced the elevated numbers of BALF CD4⁺CD25⁺Foxp3⁺ T cells from HDM-challenged mice at 3-day analysis (Fig. 3B). Seven days after the cell therapy, lower numbers of CD4⁺CD25⁺Foxp3⁺ T cells were found in the BALF of HDM-challenged mice, but data from both MSC-treated groups did not reveal significant differences when compared to non-treated HDM-challenged mice (Fig. 4B).

Lymphocyte sub-populations from the lung-draining lymph nodes were phenotyped to elucidate the effect of intratracheal administration of MSCs. Elevated numbers of B cells present in mLNs of HDM-challenged mice were reduced only by BM-MSC at 3 and 7 days after cell therapy (Fig. 4C). Although BM-MSCs did reduce CD4⁺ T cell numbers in mLN, this effect was observed only 7 days after the cell therapy (Fig. 4C). Numbers of CD4⁺CD25⁺Foxp3⁺ T cells were increased by HDM challenge but were not altered significantly by either MSC therapy at any time point of analysis (Fig. 4C).

BM-MSCs, but not AD-MSCs, upregulated IL-10 levels in the lungs of HDM-challenged mice

To assess the effect of MSCs on lung cytokine levels, lung homogenates were analyzed. HDM-challenged mice increased CCL11 and CCL24 levels, particularly at day 3, but MSCs did not reduce them (Fig. 5A and B). At day 7, CCL11 levels did not differ significantly among groups, while CCL24 level is higher in HDM challenged mice compared to controls. IL-4 (Fig. 5C) and IL-5 (Fig. 5D) levels did not differ significantly among groups at any time point of analysis. On day 3 post therapy, TGF- β level was reduced in HDM-challenged mice compared to control, with no clear interference by MSC therapy (Fig. 5E). On day 7 post-therapy, TGF- β level was increased in all HDM-challenged groups, treated or not, compared to controls (Fig. 5E). BM-MSC therapy increased the low levels of IL-10 detected in the lung of HDM-challenged mice at day 3 (Fig. 5F). At day 7, HDM-challenged mice presented higher levels of IL-10 compared to control mice (Fig. 5F). Although HDM-challenged mice treated with AD-MSCs or BM-MSCs also presented high IL-10 levels in the lung, data did not differ from non-treated HDM-challenged mice (Fig. 5F).

BM-MSCs and AD-MSCs preferentially increase Treg cell percentages when lymphocytes are not allergenically primed

We next sought to confirm the effect of MSC therapy on T cells, especially Treg cells. One day after the last intranasal challenge with HDM, cells from mLN and inguinal lymph nodes were harvested and re-stimulated *in vitro* with HDM while co-cultured with AD-MSCs or BM-MSCs. Data obtained 48 hours after these co-cultures showed that, when mLN cells were co-cultured with BM-MSCs in the presence of HDM *in vitro*, the percentage of CD4⁺ T cells was reduced as compared to that found in cultures of solely HDM-re-stimulated mLN cells (Fig. 6A). AD-MSC co-culture with mLN cells re-stimulated *in vitro* with HDM did not change the percentage of CD4⁺ T cells when compared to cells re-stimulated in the absence of MSCs (Fig. 6A). However, neither AD-MSC nor BM-MSC co-cultures affected the percentage of mLN-obtained Treg cells in the presence of HDM re-stimulation (Fig. 6B). Interestingly, when inguinal lymph node cell in the presence of HDM were analyzed, we observed that the Treg cell percentage was increased by AD-MSC and BM-MSC compared to cultures of solely HDM-stimulated inguinal cells (Fig. 6C). To establish whether BM-MSC modulation of CD4⁺ T cells depends upon cell-to-cell contact we performed co-cultures of BM-MSC and lymphocytes at the same conditions before, but using Transwell chambers. BM-MSCs still reduces CD4⁺ T cells percentages when co-cultured with mLN derived lymphocytes in the presence of HDM, in a transwell chamber (Supplemental Fig. 3A). Interestingly, decreased percentage of mLN Treg cells induced *in vitro* by BM-MSCs was further exacerbated when they were not in direct contact (Supplemental Fig. 3B). BM-MSCs had similar effect on Treg cells from inguinal LN when cultured *in vitro* either in contact or separated by the Transwell membrane (Supplemental Fig. 3C). To confirm these findings, we tested a co-culture of MSC with lymph node cells obtained from DO11.10 TCR transgenic mice in the presence or absence of OVA. After 48 hours of co-culture, lymph node cells

stimulated with OVA and co-cultured with AD-MSCs or BM-MSCs presented decreased percentages of CD4⁺ T cells compared to cells stimulated solely with OVA (Fig. 6D). The presence of AD-MSCs or BM-MSCs decreased the elevated percentages of Treg cells observed in lymph node cells stimulated with OVA (Fig. 6E). When lymph node cells were not stimulated with OVA but were co-cultured with AD-MSCs or BM-MSCs, an increase in the percentage of Treg cells was observed (Fig. 6F).

BM-MSCs activated by HDM-induced lung inflammation produced more IL-10 and modulated alveolar macrophages

To elucidate how BM-MSCs modulate immune cells, we have cultured BM-MSCs for one day *in vitro* with BALF of HDM-challenged mice (retrieved 24h post-challenge). BM-MSCs activated by BALF of HDM-challenged mice expressed more IL-10 than BM-MSC not activated (Fig. 7A). Despite this, there is no substantial increase in TGF- β expression in BM-MSCs with BALF activation (Fig. 7A). BM-MSCs did not induce an increase in the percentage of lung CD4⁺ IL10 producing cells in HDM challenged mice (data not shown) and we did not observe an increase in IL-10 expression in lung CD4 T cells after BM-MSC therapy in HDM-challenged mice (Fig. 7B). BM-MSC therapy changed the pattern of cytokine expression on alveolar macrophages in the lungs of HDM challenged mice at 3 days post-therapy, as the expression of IL-10 tended to an increase (Fig. 7C), and the expression of pro-inflammatory cytokines IL-6 and IL-1 β decreased (Fig. 7D and 7E) after BM-MSC administration compared to HDM-challenged mice.

Discussion

The present study aimed to clarify whether therapeutic administration of MSCs in an established asthma model induced by HDM would have anti-inflammatory effects and act through the induction of Treg cells. Our results showed that BM-MSCs and AD-MSCs were incapable of upregulating Treg cell counts in the mLNs of HDM challenged mice, while reducing these counts in the BALF. Although single-dose MSCs treatment exerted an attenuation in the airway influx of inflammatory cells, there were no appreciable effects on lung parenchymal inflammation, mucus hypersecretion, or AHR, which may be related to the failure in Treg cell upregulation. These findings, corroborated by the BM-MSC modulation of alveolar macrophages, suggest that Treg-independent mechanisms may play an important role in blunting BALF inflammatory cell counts, mainly after BM-MSC therapy, but are not sufficient to abrogate lung structural changes.

Previous investigations have demonstrated an anti-inflammatory potential of AD-MSCs and BM-MSCs administered before sensitization or during challenge in OVA-induced asthma models [13-16, 31]. Overall, these models have shown that MSCs inhibit lung influx of eosinophils, AHR, and mucus hypersecretion. In addition, studies employing OVA-induced models of chronic asthma have shown that therapeutically administered MSCs also blunt inflammation, AHR, and most histological changes in the airways [31, 32]. Although OVA is widely used as an allergen in asthma models, it has some disadvantages, such as the need for peripheral sensitization and use of adjuvants for a successful protocol [19]. Other reports have alternatively employed HDM as allergens to induce asthma [26, 33], since it is the most clinically relevant allergen, affecting approximately 85% of asthmatic subjects worldwide [34]. In addition, HDM-induced asthma models resemble crucial features of clinical asthma, such as local sensitization in the lungs and activation of complex inflammatory pathways involving structural lung cells [34, 35]. In models using clinically

relevant allergens, prophylactic MSC administration has successfully mitigated allergic airway inflammation, as showed in Th17 related-*Aspergillus* hyphal extract induced asthma and in ragweed-induced asthma model [32, 36]. In addition, in cockroach induced asthma model, the increased TGF- β levels seem to mobilize more MSCs to the airways, demonstrating how allergen's particularities influence MSCs *in vivo* [37]. In HDM model, epithelial activation by proteases exacerbate lung inflammation, nevertheless, MSC administered prophylactically still prevents asthma by modulating epithelial cells [38, 39]. The obstacles for cell therapy seem to be highlighted when established asthma models are improved using relevant allergens. The HDM model used herein is well established before the treatment; accordingly, mice exhibited decreased lung function, inflammatory pockets in lung tissue, and mucus hypersecretion. The data obtained in our study corroborate those observed by Mariñas-Pardo *et al.* in their established HDM model, wherein therapeutic administration of AD-MSCs lead to little improvement in features of HDM-induced asthma 3 days after cell therapy [21]. It is important to note that the anti-inflammatory effect of the BM-MSCs observed early in the airways of HDM-challenged mice was transient, lasting less than 7 days after cell therapy. This can be partially explained by the poor engraftment of MSC-GFP on the lung of HDM-challenged mice, since only 1.08×10^2 AD-MSC-GFP and 0.92×10^2 BM-MSC-GFP cells could be found in BALF of HDM challenged mice 3 days post-challenge. Noteworthy, no MSC-GFP cells were found in the lungs, mLN, thymus or bone marrow of HDM-challenged mice in days 1 or 3 post HDM challenge (data not shown). To address this issue, additional doses of MSCs may be administered to help boost the anti-inflammatory potential of cell therapy in the lung. Our data also reveal that the effects of MSCs on lymphocytes in mLNs occurred later, as observed by the reduction of CD4⁺ T cell numbers in mLNs after BM-MSC therapy. In addition, MSC-GFPs were not found in mLN or thymus at any time (data not shown), excluding a direct effect of MSCs on lymphocytes in

mLN or even a central downregulation of lymphocyte numbers in the thymus, which could impact their migration into the lymph nodes. It is possible that MSCs induced an anti-inflammatory response in the airways, which may have had an indirect impact on inflammatory cell counts in the mLNs.

Treg cells have garnered considerable interest, as it has been demonstrated that CD4⁺CD25⁺Foxp3⁺ T cells are responsible for most of the immunomodulatory effects of MSCs in OVA models of asthma [14]. Studies have shown *in vivo* upregulation of Treg cell numbers and activity after prophylactic administration of MSCs in asthma models [12, 15, 40]. In addition, most *in vitro* studies have shown that Treg cells can be generated from PBMCs or purified lymphocytes when cultured with MSCs [41, 42]. By contrast, Zinocker and Vaage demonstrated, using conA-stimulated lymphocyte cultures, that MSCs reduced Treg counts, but the mechanisms involved in this phenomenon were not explored by the authors [43]. In our study, neither BM-MSCs nor AD-MSCs was able to upregulate Treg cell numbers. We performed *in vivo* studies with two different end points, in an attempt to better assess the resolution of inflammation in our model. Nevertheless, Treg cell numbers were not upregulated 7 days after cell therapy. Partially, these data are in line with those obtained by Lathrop *et al.* in their recurrent *Aspergillus* hyphal extract model, in which they observed negligible effects of cell therapy in the lung and no change in Treg cell counts in the spleen [36]. In addition, our group recently demonstrated that even in an OVA asthma model, therapeutic administration of MSCs or their extracellular vesicles leads to a decrease in Treg cell percentages in the BALF [44]. We observed here that allergenically primed lymphocytes polarized less to a Treg cell phenotype in the presence of MSC *in vitro*. On the other hand, unprimed lymphocytes efficiently polarize to the Treg phenotype in the presence of MSCs, even through a paracrine activity. Interestingly, the BM-MSC induced reduction of Treg cell percentage in HDM-primed lymphocyte co-cultures is more robust when cell-to-cell contact

is avoided, pointing to a paracrine effect of MSCs in downregulating Treg cell counts that can possibly be balanced by a cell-to-cell contact. These results strongly suggest that the immunomodulatory activity of MSCs, dependent on Treg cells, is influenced by the inflammatory status of the lung tissue at the time of the administration. The effects yielded by therapeutic administration of MSCs in the lung of HDM-challenged mice may be related to the status of lymphocyte differentiation and activation during antigen response, as we have seen that, when in contact with MSCs, primed lymphocytes are unlikely to acquire a Treg cell phenotype, as unprimed lymphocytes would. Most research models that apply cell therapy as a pre-treatment may be able to polarize Treg cells from unprimed T cells. Even in HDM models, when MSC were delivered before the lung antigen challenges, MSCs prevented AHR and inflammation [17]. However, this is very unlikely to occur in humans, as asthmatic subjects already have an established inflammatory response before treatment. Hence, a single dose of MSCs is highly unlikely to modulate Treg cells in subjects with allergic asthma if administered therapeutically. Moreover, asthmatic subjects already have low numbers of Treg cells compared to healthy subjects, and these Treg cells are functionally impaired, which could further hinder the Treg-mediated effects of MSCs [5, 45]. Our data corroborate the results obtained by Kapoor *et al.*, who found that PBMCs from asthmatic subjects sensitive to HDM, when co-cultured with MSC in the presence of HDM, did not upregulate Treg cell percentages, although IL-10 levels were increased [17]. Luz-Crawford *et al.* showed that MSCs only modulate Treg cells *in vitro* when co-cultured with lymphocytes in an early stage of differentiation, not when lymphocytes were already differentiated [46]. These results were confirmed *in vivo* in experimental autoimmune encephalomyelitis: early injection of MSCs after immunization leads to Treg upregulation, while MSCs injected later on fail to upregulate Treg cells [46]. It is important to state that the data presented here were not

consistent with any aggravating effect of MSCs on asthma features, although Treg cell numbers were decreased in BALF.

Kavanagh and Mahon observed that depletion of Treg cells ablates most of the anti-inflammatory effects of BM-MSCs in an asthma model, except reduction of eosinophil counts in BALF, suggesting that eosinophils are regulated independently of Treg cells [14]. We have also demonstrated that BM-MSC administration reduced eosinophil levels, despite a reduction in Treg cell counts in BALF. Although we did not explore the precise underlying mechanism, we can rule out a possible downregulation of chemoattraction mediated by eotaxin, as the levels of this chemokine produced in the lungs were not altered by BM-MSC administration. We also found that only BM-MSCs, but not AD-MSCs, upregulated levels of IL-10 in the lung, possibly produced by MSCs since BM-MSC stimulated with the BALF of allergenic challenged mice express more IL-10. Noteworthy, other immunosuppressive cell types, as alveolar macrophages, have been shown to readily respond to MSC therapy, no matter whether MSCs is delivered prophylactic or therapeutically [16, 30]. Altogether, the modulation of alveolar macrophage toward an anti-inflammatory profile and the excess of lung IL-10 may explain the anti-inflammatory effects on this HDM-asthma model induced by the BM-MSC therapy, since IL-10 is known to control eosinophilia [47]. Our study has limitations. We could not assess IL-10 levels in MSC-T cells culture supernatants because of the low levels of cytokines produced. Furthermore, regardless of Treg cell counts, we do not know how MSCs affect the suppressive function of these lymphocytes. These points will be elucidated in future studies.

The present study provides evidences that, although BM-MSCs have superior anti-inflammatory potential compared to AD-MSCs in lung HDM-challenged mice, both sources fail to induce increase in Treg cell counts. Furthermore, the timing of MSC administration seems to have a decisive impact on cell therapy efficacy, as unprimed lymphocytes can be

induced toward a regulatory profile, while primed lymphocytes are unlikely to undergo such a shift. The inability of MSCs to generate Treg cells in established experimental asthma could be related to the inefficiency of cell therapy in reverting airway mucus hypersecretion and improving lung function. As the majority of asthma models employed to study MSC therapy have many pitfalls, further research using more translational models is needed to provide reliable preclinical data. Importantly, our findings regarding the relevance of the timing of MSC administration could provide a basis for better prediction of the efficacy of MSC therapy according to existing inflammatory status in future clinical studies of asthma.

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Conflicts of interest

The authors have no potential conflicts of interest.

Figure legends

Figure 1. Study design. **(a)** Schematic flow chart. C57BL/6 mice were randomly divided into control and HDM experimental groups. HDM animals was allocated to subgroups according to treatment type: HDM-SAL, HDM-ADMSC, or HDM-BMMSC. **(b)** Timeline of study protocol. C57BL/6 mice were challenged by intranasal administration of HDM extract or PBS three times a week for 3 weeks. One day after the last challenge, mice were administered saline (control), AD-MSCs, or BM-MSCs intratracheally, and analyses were performed 3 or 7 days after cell therapy.

Figure 2. AD-MSCs and BM-MSCs did not improve lung function in HDM-challenged mice. Airway elastance and lung resistance values in mice subjected to bronchoprovocation with increasing doses of methacholine. All data were evaluated at day 3 or day 7 endpoints. Values represent mean $\pm$ SD of 4–8 animals. $*p < .05$ as compared with control-SAL group. $\S p < .05$ as compared with HDM-SAL group.

Figure 3. AD-MSCs and BM-MSCs did not mitigate lung inflammatory pockets or mucus hypersecretion in HDM-challenged mice. Histological score and representative photomicrographs of lung sections stained with hematoxylin and eosin-or periodic acid-Schiff stain (original magnification: $\times 400$). Scale bar indicates 100 μm . Black arrows denote mucus staining in the airways. All data were evaluated at day 3 or day 7 endpoints. Values represent mean $\pm$ SD of 4–8 animals. $*p < .05$ as compared with control-SAL group.

Figure 4. AD-MSCs and BM-MSCs transiently reduce inflammatory cell counts in BALF but do not upregulate Treg cells in HDM-challenged mice. **(a)** Total and differential leukocyte counts in BALF. **(b)** Absolute B-cell, CD4^+ T-cell, and $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ T-cell

counts in BALF. **(c)** Absolute B-cells, CD4⁺ T-cell, and CD4⁺CD25⁺Foxp3⁺ T-cell counts in mLNs. All data were evaluated at day 3 or day 7 endpoints. Values represent mean $\pm$ SD of 4–8 animals. * $p < .05$ as compared with control-SAL group. § $p < .05$ as compared with HDM-SAL group. + $p < .05$ as compared with HDM-ADMSC group.

Figure 5. BM-MSCs, but not AD-MSCs, increased levels of IL-10 in the lung of HDM-challenged mice. Levels of CCL11 **(a)**, CCL24 **(b)**, IL-4 **(c)**, IL-5 **(d)**, TGF- β **(e)** and IL-10 **(f)** in lung homogenates 3 or 7 days after cell therapy. All data were evaluated at the day 3 or day 7 endpoints. Values represent median (interquartile range) of 3–7 animals. * $p < .05$ as compared with control-SAL group. § $p < .05$ as compared with HDM-SAL group. + $p < .05$ as compared with HDM- ADMSC group.

Figure 6. AD-MSCs and BM-MSCs increased Treg cell percentages *in vitro* when lymphocytes were not antigenically stimulated. **(a)** Graphs and density plots represent percentage of CD4⁺ T cells derived from mLNs after stimulation using HDM in the presence or absence of MSCs. **(b)** Graphs and density plots represent percentage of CD4⁺Foxp3⁺ T cells derived from mLNs after stimulation using HDM in the presence or absence of MSCs. **(c)** Graphs and density plots represent percentage values of CD4⁺Foxp3⁺ T cells derived from inguinal lymph nodes after stimulation using HDM in the presence or absence of MSCs. **(d)** Graphs represent percentage of CD4⁺ T cells obtained from a pool of DO11.10 lymph node cells stimulated *in vitro* using OVA in the presence or absence of MSCs **(e)** Graphs represent percentage of CD4⁺Foxp3⁺ T cells obtained from a pool of DO11.10 lymph node cells stimulated *in vitro* using OVA in the presence or absence of MSCs. **(f)** Graphs represent percentages of CD4⁺Foxp3⁺ T cells from DO11.10 lymph node cells unstimulated in the presence or absence of MSCs. All data were evaluated by flow cytometry 48 hours after co-

culture of lymphocytes with or without MSCs. Values represent mean $\pm$ SD. Cells from inguinal lymph nodes were pooled. $^{\S}p < .05$ as compared with lymphocyte cultures stimulated with HDM or OVA alone.

Figure 7. BM-MSCs activated by the BALF of HDM-challenged mice express more IL-10 and leads to modulation of alveolar macrophages. **(a)** Fold change in expression of IL-10 and TGF- β from BM-MSCs stimulated *in vitro* with BALF of HDM-challenged mice. **(b)** IL-10 mean fluorescence intensity in CD4 T cells from lungs of mice challenged with saline or HDM and treated with either saline or BM-MSCs. Fold change in expression of IL-10 **(c)**, IL-6 **(d)** and IL-1 β **(e)** from alveolar macrophages of mice challenged with saline or HDM and treated with either saline or BM-MSCs. Values represent mean $\pm$ SD of 2-4 mice. $^{\S}p < .05$ as compared to-SAL mice or BM-MSCs cultures. $^{\S}p < .05$ as compared to HDM-SAL.

Supplemental figure legends

Supplemental Figure 1. Characterization of AD-MSCs and BM-MSCs. **(a)** Adherence of AD- MSC and BM-MSCs to plastic (original magnification: $\times 40$). **(b)** Osteogenic differentiation confirmed by Alizarin Red S staining of calcium in AD-MSCs and BM-MSCs cultures (original magnification: $\times 200$). **(c)** Adipogenic differentiation confirmed by Sudan Black B staining of lipid droplets (arrows) in AD-MSCs and BM-MSCs cultures (original magnification: $\times 200$). **(d)** AD-MSCs and BM-MSCs lack expression of CD45, CD11b, and CD34, but express CD29, Sca-1, and CD44 (grey histograms). Controls refer to unstained samples (black histograms).

Supplemental Figure 2. HDM challenge produces marked features of an asthma-like phenotype in C57BL/6 mice. **(a)** Total and differential leukocyte counts in BALF. **(b)** Different lymphocyte subsets in BALF and mLNs. **(c)** Levels of CCL11, IL-4, and IL-10 in lung homogenates. **(d)** Histological score and representative photomicrographs of lung sections stained with hematoxylin and eosin-or periodic acid-Schiff stain (original magnification: $\times 400$). Scale bar indicates 100 μm . Black arrows denote airway mucus. **(e)** Lung elastance and airway resistance values in mice subjected to bronchoprovocation with increasing doses of methacholine. All data were evaluated 24 hours after the last allergenic challenge. All values represent mean $\pm$ SD of 3–7 animals, except cytokine values, which are expressed as median (interquartile range). $^*p < .05$ as compared with control group.

Supplemental Figure 3. BM-MSCs modulate T cells also via paracrine effects *in vitro*. Percentage of **(a)** CD4^+ and **(b)** $\text{CD4}^+\text{Foxp3}^+$ T cells derived from mLNs after stimulation using HDM and in direct contact with BM-MSCs or indirect transwell. **(c)** Percentage values of $\text{CD4}^+\text{Foxp3}^+$ T cells derived from inguinal lymph nodes after stimulation using HDM, and in direct contact with BM-MSCs or indirect transwell culture conditions. All data were evaluated by flow cytometry 48 hours after co-culture of lymphocytes with or without BM-MSCs. Values represent mean $\pm$ SD of 2-3 animals. $^{\S}p < .05$ as compared with lymphocytes cultures stimulated with HDM alone. $^+p < .05$ as compared with lymphocytes cultures stimulated using HDM and in direct contact with BM-MSCs.













