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CD11b+ and Sca-1+ Cells Exert the Main Beneficial Effects of Systemically Administered Bone Marrow-Derived Mononuclear Cells in a Murine Model of Mixed Th2/Th17 Allergic Airway Inflammation

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Key Words. Bone marrow-derived mononuclear cells • Mesenchymal stromal cells • Inflammation • Monocyte • Asthma • Mouse

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ABSTRACT

Systemic administration of bone marrow-derived mononuclear cells (BMDMCs) or bone marrow-derived mesenchymal stromal cells (MSCs) reduces inflammation and airway hyperresponsiveness (AHR) in a murine model of Th2-mediated eosinophilic allergic airway inflammation. However, since BMDMCs are a heterogeneous population that includes MSCs, it is unclear whether the MSCs alone are responsible for the BMDMC effects. To determine which BMDMC population(s) is responsible for ameliorating AHR and lung inflammation in a model of mixed Th2-eosinophilic and Th17-neutrophilic allergic airway inflammation, reminiscent of severe clinical asthma, BMDMCs obtained from normal C57Bl/6 mice were serially depleted of CD45, CD34, CD11b, CD3, CD19, CD31, or Sca-1 positive cells. The different resulting cell populations were then assessed for ability to reduce lung inflammation and AHR in mixed Th2/Th17 allergic airway inflammation induced by mucosal sensitization to and challenge with *Aspergillus* hyphal extract (AHE) in syngeneic C56Bl/6 mice. BMDMCs depleted of either CD11b-positive (CD11b+) or Sca-1-positive (Sca-1+) cells were unable to ameliorate AHR or lung inflammation in this model. Depletion of the other cell types did not diminish the ameliorating effects of BMDMC administration. In conclusion, in the current model of allergic inflammation, CD11b+ cells (monocytes, macrophages, dendritic cells) and Sca-1+ cells (MSCs) are responsible for the beneficial effects of BMDMCs. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:488–499*

SIGNIFICANCE

This study shows that bone marrow-derived mononuclear cells (BMDMCs) are as effective as bone marrow-derived mesenchymal stromal cells (MSCs) in ameliorating experimental asthma. It also demonstrates that not only MSCs present in the pool of BMDMCs are responsible for BMDMCs' beneficial effects but also monocytes, which are the most important cell population to trigger these effects. All of this is in the setting of a clinically relevant model of severe allergic airways inflammation and thus provides further support for potential clinical use of cell therapy using MSCs, BMDMCs, and also adult cells such as monocytes in patients with severe asthma.

INTRODUCTION

Cell-based therapies hold potential promise as a new treatment approach for asthma. This is potentially applicable to 5%–10% of patients with asthma who suffer from severe disease, which, in many cases, is caused by Th17-mediated neutrophilic airway inflammation [1–3]. This population is poorly clinically controlled and resistant to corticosteroids and most other available treatments [4–6]. Although there has been some potential promise with anti-immunoglobulin E-mediated therapies [7, 8] new therapeutic options are still urgently needed.

A growing body of literature demonstrates that systemic administration of syn-, *allo*- or, xenogeneic mesenchymal stromal cells (MSCs) isolated from bone marrow and other sources can mitigate airway hyperresponsiveness (AHR) and lung inflammation in preclinical (mouse) models of both Th2-mediated eosinophilic allergic airway inflammation [9–17] and in mixed Th2-eosinophilic/Th17-mediated neutrophilic allergic airway inflammation [18–20]. The ameliorating effects occur when the MSCs are administered either during initial antigen sensitization or at the onset of or during antigen challenge [9–20]. Some data also suggest that MSC

administration can also protect against chronic or delayed antigen challenges [9, 18]. Proposed mechanisms of MSC actions include upregulation of T-regulatory cells and also shift from Th2 to a counter-regulatory Th1 phenotype of antigen-specific CD4 cells [16–20]. As such, MSCs may provide a potential therapeutic approach for severe asthma.

However, use of MSCs poses some logistical difficulties, including isolation and expansion of the cells for subsequent administration as well as consideration of allogeneic MSC use [21–23]. An alternative approach uses a heterogeneous population of autologous bone marrow-derived mononuclear cells (BMDMCs). One potential advantage of this approach is that autologous BMDMCs can be harvested and reinfused systemically, avoiding the need for cell expansion or other alterations that might occur during cell culture. We recently demonstrated that systemic administration of syngeneic BMDMCs ameliorated AHR and lung inflammation in a model of Th2-mediated AHR in mice [24–26]. However, BMDMCs have not yet been evaluated in a more severe model of mixed Th2/Th17 allergic airway inflammation. Moreover, there is no information as yet available about the cell or cells in the heterogeneous BMDMC population responsible for the ameliorating effects. Notably, the BMDMC population does contain a small proportion of MSCs, but whether these alone are responsible is as yet unknown.

To these ends, we investigated whether systemic administration of syngeneic BMDMCs could ameliorate mixed Th2/Th17-mediated lung inflammation and AHR provoked by mucosal sensitization to and challenge with *Aspergillus* hyphal extract (AHE) [27, 28]. We further investigated which cell(s) conveyed the ameliorating effects of the BMDMCs, by sequentially depleting specific cell types (CD45-positive [$CD45^+$], $CD34^+$, $CD3^+$, $CD19^+$, $CD11b^+$, $CD31^+$, Sca-1^+) from the BMDMC fraction prior to administration.

Materials and Methods

Mice

C57Bl/6 mice (male, 8–12 weeks, $n = 72$, Jackson Laboratory, Bar Harbor, ME, https://www.jax.org) were housed in microisolator cages and used in accordance with the University of Vermont (UVM) Institutional Animal Care and Use Committee under all applicable Association for Assessment and Accreditation of Laboratory Animal Care International guidelines.

Mesenchymal Stromal Cell and Fibroblast Culture

Murine bone marrow-derived mesenchymal stromal cells (mMSCs) from C57Bl/6 mice were obtained from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine (Temple, TX, http://medicine.tamhsc.edu/irm/msc-distribution.html) [29]. These cells have previously been extensively characterized for cell surface marker expression and differentiation capacity [30, 31]. mMSCs were expanded in culture using Iscove's Modification of Dulbecco's Medium (GE Healthcare Life Sciences, Rockford, IL, https://promo.gelifesciences.com/gl/hyclone), 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences), 10% horse serum (GE Healthcare Life Sciences), 1% penicillin/streptomycin (Pen/Strep) (Thermo Fisher Scientific, Grand Island, NY, https://www.thermofisher.com), and 2 mM L-glutamine (Thermo Fisher Scientific), and used at passages 4–6. mMSCs were maintained in culture at confluence no greater than 70%. Normal, adult human lung fibroblasts (HLF) (catalog number CCL-199; ATCC, Manassas, VA, http://www.atcc.org) were expanded in culture with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com), 10% FBS, 1% Pen/Strep, and 2 mM L-glutamine and used at passage 6 or lower. We have previously demonstrated that HLFs can be successfully used as a control cell population in immunocompetent mouse models of allergic airway inflammation [16, 19, 20].

For use in experiments, the cells were harvested from tissue culture plates using 2.5% trypsin/EDTA (Thermo Fisher Scientific). Cell density and viability was determined using trypan blue staining and counted using a hemacytometer (Sigma-Aldrich). Cell pellets were then resuspended in 1× sterile phosphate-buffered saline (PBS) to a final concentration of 1×10^6 cells per 200 μl of PBS, immediately prior to injection [16, 19, 20].

Bone Marrow-Derived Mononuclear Cell Extraction, Characterization, and Depletions

Bone marrow cells from 20 adult male C57BL/6 mice were flushed from the femurs and tibias with Dulbecco's modified Eagle's medium (DMEM). After a homogeneous cell suspension was achieved, cells were centrifuged (400*g* for 10 minutes), resuspended in DMEM, and added to Ficoll-Hypaque (Histopaque 1083; Sigma-Aldrich). The mononuclear fraction (i.e., BMDMCs) was then isolated, centrifuged (3 times each at 400*g* for 10 minutes), resuspended in PBS, and cells were counted in a Neubauer chamber with trypan blue for evaluation of viability. A final concentration of 1×10^6 cells suspended in 200 μl of PBS was prepared immediately prior to systemic administration [24, 25, 32].

Parallel aliquots of the BMDMCs were used for immunophenotypic characterization of the component cell populations by flow cytometry using the following antibodies: anti-mouse CD45-fluorescein isothiocyanate (FITC) (leukocytes, 1:200 dilution, catalog number MCD4501; Thermo Fisher Scientific), CD34-APC (hematopoietic precursors, 1:20 dilution, catalog number 128611; Biolegend, San Diego, CA, http://www.biolegend.com), CD3-APC (T lymphocytes, 1:20 dilution, catalog number 100311; Biolegend), CD19-Alexa700 (B lymphocytes, 1:200 dilution, catalog number 115527; Biolegend), CD11b-Alexa647 (monocytes and macrophages, 1:200 dilution, catalog number 101220; Biolegend), CD31-APC (endothelial cells, 1:200 dilution, catalog number 551262; BD Pharmingen, San Jose, CA, http://www.bdbiosciences.com) and Sca-1-FITC (progenitor cells: hematopoietic progenitor cells and mesenchymal stromal cells, 1:200 dilution, catalog number 557405; BD Pharmingen) [24, 25, 32].

To identify which cell fraction participated in the ameliorating actions of the mixed BMDMCs, specific cell populations of the BMDMCs were labeled with the following biotinylated antibodies (all eBioscience, San Diego, CA, http://www.ebioscience.com; unless otherwise indicated): anti-mouse CD45 (1:200 dilution, catalog number 13-0451-82, anti-mouse CD34 (1:200 dilution, catalog number 119304), anti-mouse CD3 (1:200 dilution, catalog number 100303), anti-mouse CD19 (1:200 dilution, catalog number 115503), anti-mouse CD11b (1:200 dilution, catalog number 101204), anti-mouse CD31 (1:200 dilution, catalog

number 102503), and Sca-1 (1:200 dilution, catalog number 130-101-995; Miltenyl Biotec, Gladbach, Germany, http://www.miltenyibiotec.com). Following antibody labeling of any given cell population or populations, the cell suspensions were mixed with a Dynabead solution (Dynabeads Biotin Binder, catalog number 11047; Thermo Fisher Scientific) and the specific cell populations depleted with magnetic plates according to manufacturer's instructions [33, 34]. After depletion, cell viability was evaluated through trypan blue; an aliquot was used for immunophenotypic characterization by flow cytometry with the aforementioned antibodies to confirm cell depletion, and a final concentration of 1×10^6 postdepletion cells suspended in 200 μl of PBS was prepared immediately prior to systemic administration.

Induction of Allergic Airway Inflammation

The study design is shown in schematic form in Figure 1. *Aspergillus* hyphal extract (AHE) aliquots at a concentration of 1.466 mg/ml in 1× PBS, generously provided by the Whittaker laboratory at UVM and previously used by us, were thawed and vortexed immediately prior to use, and diluted to a final concentration of 5 μg of AHE in 40-μl of sterile 1× PBS [18–20]. Mice were anesthetized by isoflurane inhalation and received an oropharyngeal administration of PBS (naïve [N]) or AHE solution (A) on days 0 and 7 to initiate the immune response (sensitization). Then they were challenged for 3 successive days on days 14, 15, and 16, with oropharyngeal inoculations using the same AHE preparation (Fig. 1A) [18–20].

Systemic Administration of HLFs, MSCs, BMDMCs, and Depleted BMDMCs

On day 14, immediately after the AHE inoculation, mice received systemic (tail vein) injection of 1×10^6 cells in 200 μl of 1× PBS or 1× PBS control (P) (n = 15). As previously described, animals received either HLFs (n = 6), mMSCs (n = 6), BMDMCs (n = 13), or BMDMCs depleted of either CD45 (n = 6), CD34 (n = 6), CD3 (n = 6), CD19 (n = 6), CD11b (n = 9), CD31 (n = 6), or Sca-1 (n = 6) cells (Fig. 1B) [16, 19, 20, 24, 25, 35, 36].

Respiratory Mechanics

Pulmonary function was analyzed using the forced oscillation technique (flexiVent; SCIREQ Scientific Respiratory Equipment, Tempe, AZ, http://www.scireq.com) as previously described [18–20, 37, 38]. The peak responses for airway resistance (R_N), overall tissue resistance (G), and lung elastance (H) were determined in response to sequential inhalation of nebulized saline, followed by 3.125 mg/ml, 12.5 mg/ml, or 25 mg/ml methacholine (MCh).

Assessment of Airway Inflammation

Following evaluation of lung mechanics, mice were euthanized by lethal intraperitoneal injection of sodium pentobarbital (260 mg/kg). Bronchoalveolar lavage fluid (BALF) was collected by administering 1 ml of sterile 1× PBS to the airways through a tracheal cannula and rinsing the lungs 3 times before recovery. BALF was centrifuged at 2,460g for 5 minutes at 4°C and the supernatant was collected in separate tubes and stored at −80°C. The Bioplex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com) was used to examine undiluted BALF samples for soluble inflammatory cytokines, using a mouse 23-plex panel. Concentrations were determined using the Bio-Plex Manager Software (Bio-Plex MAGPIX multiplex reader; Bio-Rad Laboratories). The cell pellet was resuspended and an aliquot was used to determine total cell count, using the ADVIA Hematology Analyzer (Siemens Diagnostics, Johnson City, TN, http://usa.healthcare.siemens.com). Cytospins were performed using 3×10^4 cells centrifuged onto precleaned, pretreated glass slides (Corning, Corning, NY, https://www.corning.com) at 79g for 8 minutes, dried overnight, and stained using Diff-Quick (Hema 3 Stain Set; Thermo Fisher Scientific). Different cell populations were determined by blinded manual count of 200 cells performed by 3 individuals. Following BALF collection, the trachea and heart/lung block were removed. The right lung was then removed and flash frozen in liquid nitrogen. The left lobe was gravity fixed (20 cm H_2O) for 1 hour with 4% paraformaldehyde, and 5-μm paraffin sections subsequently were stained with hematoxylin and eosin. Airway inflammation (10 airways per animal, at least 6 animals in each group) was evaluated by 3 people in blinded fashion, based on the presence and intensity of peribronchial cell infiltrates compared with positive and negative controls, using an established semiquantitative scoring system, using a 0–3 range as previously described [18–20].

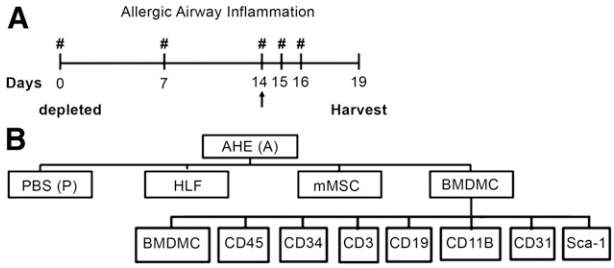

Figure 1. Study design. **(A):** Protocol of allergic airway inflammation induction. OP administration of PBS (naïve) or AHE solution on days 0 and 7 to initiate the immune response, then challenged for 3 days on days 14, 15, and16 with OP inoculations of AHE. Cell administration was performed on day 14; harvest was on day 19. **(B):** Experimental groups. Animals exposed to AHE solution **(A)** were randomly divided to receive treatment with the vehicle PBS, HLFs, mMSCs, BMDMCs, and BMDMCs depleted of CD45, CD34, CD11b, CD3, CD19, CD31, and Sca-1 positive cells. Abbreviations: #, oropharyngeal *Aspergillus* hyphal extract solution administered; ↑, human lung fibroblasts, mouse mesenchymal stem/stromal cells, bone marrow-derived mononuclear cells and depleted bone marrow-derived mononuclear cells administered; A, animals exposed to AHE solution; AHE, *Aspergillus* hyphal extract; BMDMC, bone marrow-derived mononuclear cell; HLF, human lung fibroblast; mMSC, mouse mesenchymal stem/stromal cell; OP, oropharyngeal; P, group receiving phosphate-buffered saline; PBS, phosphate-buffered saline.

Mediastinal Lymph Node Mixed Lymphocyte Assessments

Mediastinal lymph nodes (MLNs) were isolated by dissection from each mouse and placed in T-cell medium (Roswell Park Memorial Institute medium; 5% FBS; 1× Pen/Strep; 2 mM L-glutamine; 2,500 mg/ml glucose; 1 mg/ml folate in 2 g/l sodium bicarbonate; 1 mM sodium pyruvate; and 50 μM beta-mercaptoethanol). To ensure we would have enough cells for the assay, MLN cells from mice of the same experimental group (at least 6 animals in each group) were pooled and pressed through a 40-μm mesh filter into a single cell suspension. Cells were then washed twice in 1× PBS and resuspended for counting. One million cells were plated in duplicate for each group in a 24-well dish in 500 μl of T-cell medium. In half of the wells, cells were stimulated with 1 μg of AHE in

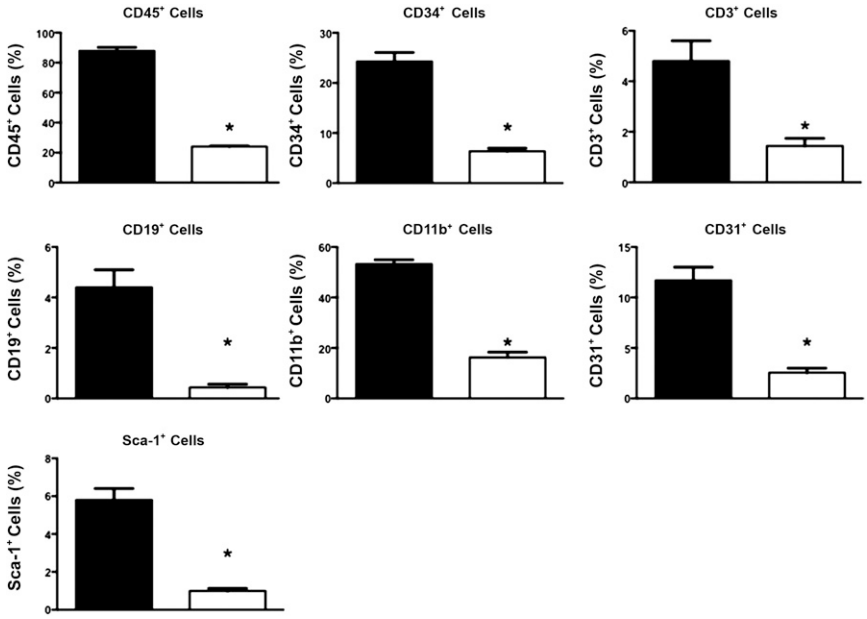

Figure 2. Bone-marrow mononuclear cell characterization before and after specific Dynabead depletions. Percentage of CD45-, CD34-, CD3-, CD19-, CD11b-, CD31-, and Sca-1-positive cells evaluated by flow cytometry analysis of bone-marrow derived mononuclear cells before and after magnetic depletions. Representative depletions are shown. Data are presented as mean + SD (n = 3). ∗, significance compared with BMDMC before depletion.

the medium for 48 hours; the other wells were left unstimulated for the same length of time. The total contents of each well were collected at the indicated time points and were centrifuged for 5 minutes at 2,460g to pellet cells and debris. Supernatants were moved to a new tube and frozen at −20°C. Contents of representative Th1, Th2, and Th17 soluble mediators (interleukin [IL]-4, IL-5, IL-17, and interferon [IFN]-γ) were assessed by enzyme-linked immunosorbent assay (Biolegend) [18–20].

Statistical Analysis

All data were graphed and analyzed using the GraphPad Prism version 6.0 statistical software package (GraphPad Software, La Jolla, CA, http://www.graphpad.com). The normality of the data and the homogeneity of variances were tested using the Kolmogorov-Smirnov test with Lilliefors correction and Levene median test, respectively. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. Nonparametric data were analyzed using ANOVA on ranks followed by Dunn's post hoc test [19, 20]). Parametric data were expressed as mean ± SD and nonparametric as median (interquartile range). Statistical significance was established at $p < .05$.

Results

Dynabead Depletions Significantly Reduced Specific Cells From the BMDMCs

Figure 1 depicts the experimental design. Flow cytometric analyses of representative naïve BMDMCs demonstrated 87.9% ± 2.3% $CD45^+$, 24.3% ± 1.8% $CD34^+$, 4.8% ± 0.8% $CD3^+$, 4.4% ± 0.7% $CD19^+$, 53.2% ± 1.7% $CD11b^+$, 11.7% ± 1.3% $CD31^+$, and 5.8% ± 0.6% Sca-1^+ cells (Fig. 2). After antibody labeling and magnetic bead depletions, there was a marked reduction of each specific cell type, as follows: 72.6% ± 1.6% $CD45^+$, 73.8% ± 1.2% $CD34^+$, 70.0% ± 0.5% $CD3^+$, 90.0% ± 0.6% $CD19^+$, 69.5% ± 0.4% $CD11b^+$, 78.1% ± 0.9% $CD31^+$, and 82.9% ± 0.5% Sca-1^+ (Fig. 2).

Systemic Administration of MSCs, Total BMDMCs, or CD34-, CD3-, CD19-, or CD31-Depleted BMDMCs Significantly Ameliorated Airway Hyperresponsiveness Induced by AHE Sensitization and Challenge

Sensitization and challenge with AHE and treatment with PBS (A-P group) resulted in a significant increase in R_N, tissue resistance (G), and lung elastance (H) compared with N (Fig. 3). Systemic administration of either mMSCs or BMDMCs significantly decreased each measure of MCh-induced AHR in doses 12.5 and 25 mg/ml of MCh, compared with the A-P group, whereas the administration of the HLF control cell population led to no effect (Fig. 3A). Administration of either CD34-, CD3-, CD19-, or of CD31-depleted BMDMCs also reduced AHR to control levels. In contrast, administration of CD45-, CD11b-, or Sca-1-depleted BMDMCs had minimal effects on any measure of AHR (Fig. 3B, 3C).

Systemic Administration of MSCs, Total BMDMCs, or CD34-, CD3-, CD19-, or CD31-Depleted BMDMCs Significantly Inhibited AHE-Provoked Lung Inflammation

AHE sensitization and challenge resulted in a significant increase in histologic inflammation and BALF inflammatory cell content compared with N (Figs. 4A, 4B, 5A, 5B). Administration of HLF did not yield any alterations in AHE-provoked lung inflammation. Systemic administration of either mMSCs, BMDMCs, or of CD34-, CD3-, CD19-, or CD31-depleted BMDMCs each significantly decreased both histologic inflammation (Fig. 4A, 4B) and BALF total and differential cell counts, compared with the A-P group (Fig. 5A, 5B). Administration of BMDMCs depleted of CD45+ cells did not

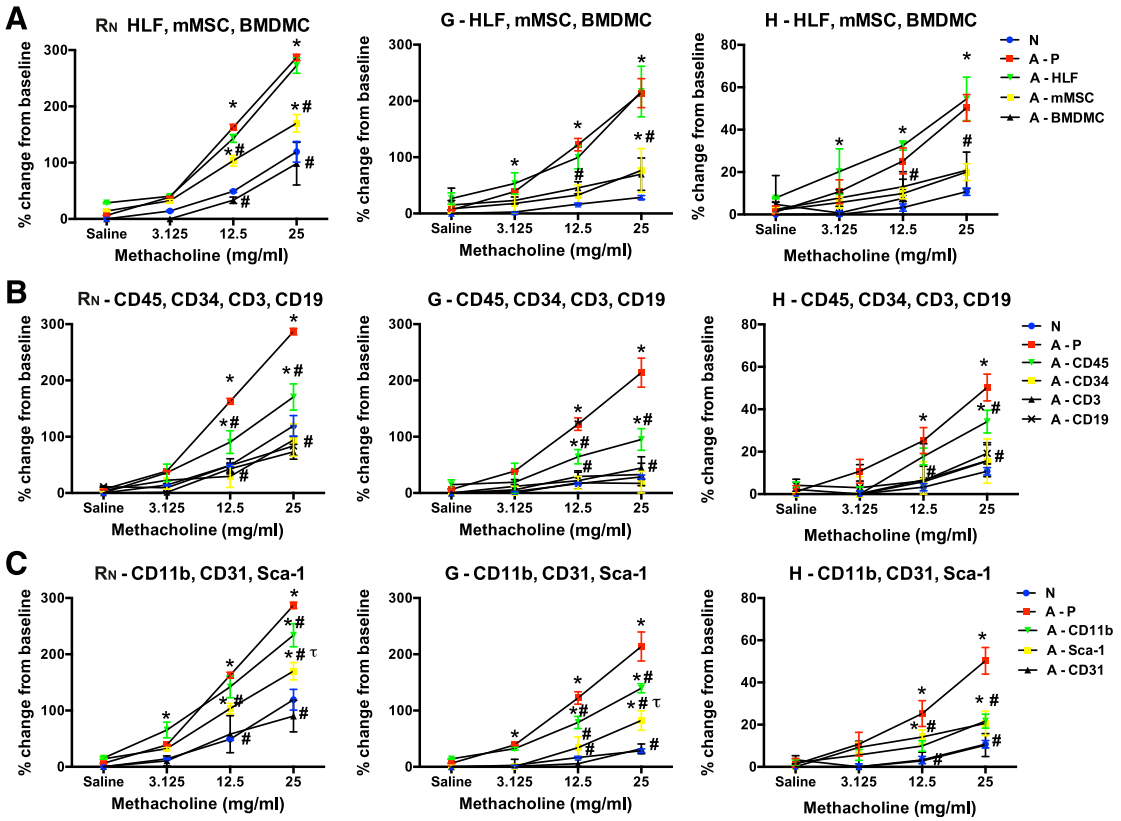

Figure 3. Systemic administration of mMSCs, BMDMCs, CD34-, CD3-, CD19-, and CD31-depleted BMDMCs, but not HLFs, significantly ameliorated the airway hyperresponsiveness induced by *Aspergillus* hyphal extract (AHE). Administration of CD11b-, Sca-1-, and CD45-depleted BMDMCs did not present the same beneficial effects. **(A):** Analysis of RN, G, and H according to methacholine dose in N and A-P, or A-HLF, A-mMSC, and A-BMDMCs (N, n = 17; A-P, n = 15; A-HLF, n = 6; A-mMSC, n = 6; A-BMDMC, n = 13). **(B):** Analysis of RN, G, and H in N, A-P, A-CD45, A-CD34, A-CD3, and A-CD19 (N, n = 17; A-P, n = 15; A-CD45, n = 6; A-CD34, n = 6; A-CD3, n = 6; and A-CD19, n = 6). **(C):** Analysis of RN, G, and H in N, A-P, A-CD11b, A-CD31, and A-Sca-1 (N, n = 17; A-P, n = 15; A-CD11b, n = 6; A-CD31, n = 6; and A-Sca-1, n = 6). Data are presented as peak response normalized to the baseline, and then expressed as percent increase over the baseline, ±SD. *, $p \leq .05$ compared with N; #, compared with A-P; **, compared with A-BMDMC; τ, compared with A-CD11b. Abbreviations: A, *Aspergillus* hyphal extract-exposed mice; A-BMDMC, *Aspergillus* hyphal extract-exposed mice treated with bone marrow-derived mononuclear cells; A-CD3 (or A-CD11b, A-CD19, A-CD31, A-CD34, A-CD45, A-Sca-1), *Aspergillus* hyphal extract-exposed mice treated with CD-3- (or CD11b-, CD19-, CD31-, CD34-, CD45-, and Sca-1-) depleted bone marrow-derived mononuclear cells; A-HLF, *Aspergillus* hyphal extract-exposed mice treated with human lung fibroblasts; A-mMSC, *Aspergillus* hyphal extract-exposed mice treated with mouse mesenchymal stem/stromal cells; A-P, *Aspergillus* hyphal extract-exposed mice treated with phosphate-buffered saline; BMDMC, bone marrow-derived mononuclear cell; G, overall tissue resistance; H, lung elastance; HLF, human lung fibroblast; mMSC, mouse mesenchymal stem/stromal cell; N, naïve; RN, airway resistance.

reduce AHE-provoked increases in histologic inflammation and had only minimal effects on BALF cellularity. Administration of CD11b-depleted BMDMCs did not reduce either AHE-provoked increases in histologic inflammation or BALF total and differential cellularity. Administration of Sca-1-depleted BMDMCs significantly decreased AHE-provoked increase histologic lung inflammation but had only minimal effects on BALF cellularity (Figs. 4A, 4B, 5A, 5B).

Systemic Administration of MSCs, Total BMDMCs, or of CD34-, CD3-, CD19-, or CD31-Depleted BMDMCs Significantly Inhibited AHE-Provoked Increase in BALF Th2 and Th17 Proinflammatory Cytokines

Animals that underwent the AHE oropharyngeal administration and received PBS (A-P group) had significantly higher BALF levels of IL-4, IL-5, IL-6, IL-12, IL-17, keratinocyte chemoattractant (KC), and RANTES (regulated on activation, normal T-cell expressed and secreted), and also, lower levels of IFN-γ and IL-10 in the BALF, compared with N. mMSCs and BMDMCs had comparable effects in significantly decreasing the AHE provoked increases in BALF IL-4, IL-17, and KC levels, trending toward a reduction of IL-5, IL-6, and RANTES levels. In parallel, both mMSCs and BMDMCs comparably significantly increased the AHE-provoked reduction in IFN-γ levels and produced a trend toward increase of IL-10 levels (Fig. 6A, 6B). Administration of CD34-, CD3-, CD19-, or CD31-depleted BMDMCs each resulted in similar effects. Administration of CD45-depleted BMDMCs did not significantly reduce IL-4 or IL-17 levels but trended toward reduced AHE-provoked increases of IL-5, IL-6, KC, and RANTES levels compared with the A-P group. The CD45-depleted BMDMCs promoted a significant increase in the BALF levels of IFN-γ and showed a trend toward increased IL-10 levels compared with the A-P group. CD11b-depleted BMDMC administration had no effects on the AHE-provoked increase in IL-4, IL-5, IL-17, IL-6, KC, or RANTES levels and also did not increase either IL-10 or IFN-γ levels. Administration of Sca-1-depleted

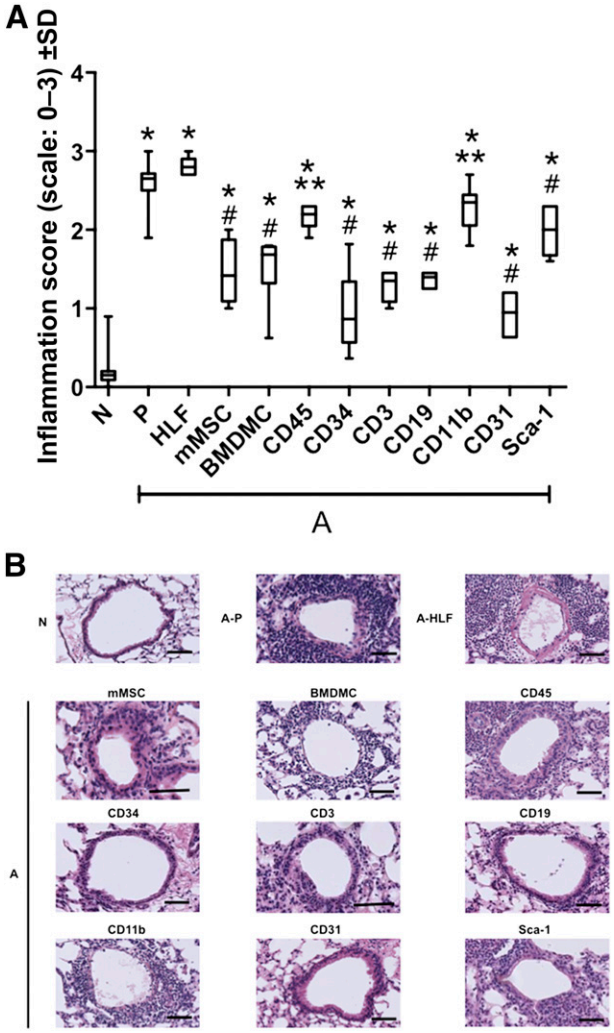

Figure 4. Systemic administration of mMSCs, BMDMCs, CD34-, CD3-, CD19-, CD31-, and Sca-1-depleted BMDMCs, but not HLFs, or CD11b-, and CD45-depleted BMDMCs, significantly reduced histologic lung inflammation provoked by AHE sensitization and challenge. **(A):** Inflammation score of the airway in N and A mice treated with HLF, mMSCs, BMDMCs, CD45-, CD34-, CD11b, CD3, CD19, CD31-, and Sca-1-depleted BMDMCs (N, n = 17; A-P, n = 15; A-HLF, n = 6; A-mMSC, n = 6; A-BMDMC, n = 13; A-CD45, n = 6; A-CD34, n = 6; A-CD11b, n = 9; A-CD3, n = 6; A-CD19, n = 6; A-CD31, n = 6; A-Sca-1, n = 6). Data are presented as median and interquartile ranges. **(B):** Representative photomicrographs stained with hematoxylin and eosin. Original magnification, ×10. Scale bar = 25 μm. ∗, $p \leq .05$ compared with N; #, compared with A-P; ∗∗, compared with A-BMDMC. Abbreviations: A, *Aspergillus* hyphal extract-exposed mice; A-CD3 (or A-CD11b-, A-CD19-, A-CD31-, A-CD34-, A-CD45-, A-Sca-1-), *Aspergillus* hyphal extract-exposed mice treated with CD-3- (or CD11b-, CD19-, CD31-, CD34-, CD45-, and Sca-1-) depleted bone marrow-derived mononuclear cells; A-HLF, *Aspergillus* hyphal extract-exposed mice treated with human lung fibroblasts; A-P, *Aspergillus* hyphal extract-exposed mice treated with phosphate-buffered saline; BMDMC, bone marrow-derived mononuclear cell; HLF, human lung fibroblast; mMSC, mouse mesenchymal stem/stromal cell; N, naïve; P, phosphate-buffered saline.

BMDMCs reduced the AHE-provoked increase in IL-4 levels, showed a trend toward a reduction of IL-5 and IL-6, but had no effects on levels of IL-17, KC, IL-10, RANTES, or IFN-γ levels. Administration of HLFs did not result in any alterations of AHE-provoked increase in inflammatory mediators.

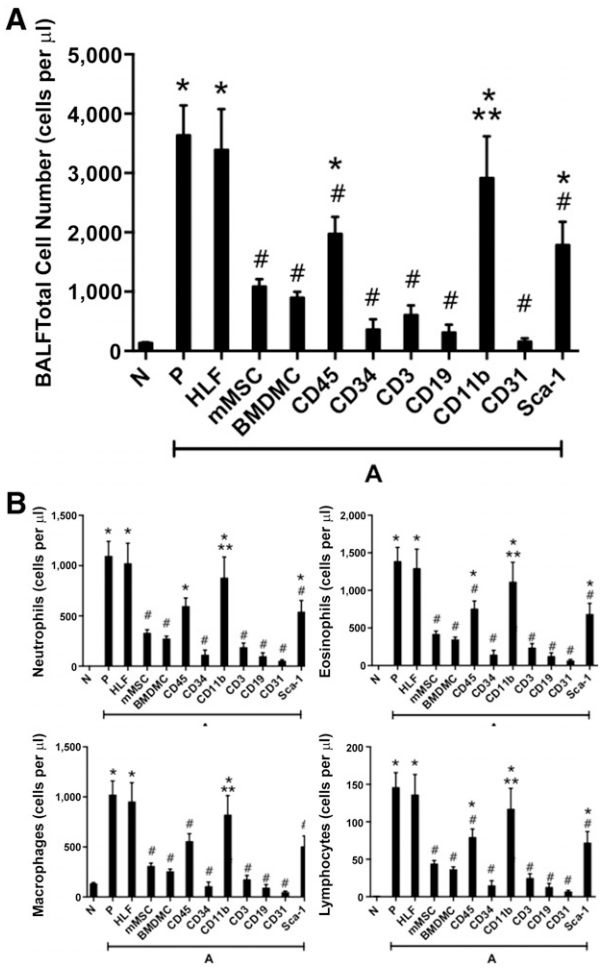

Figure 5. Systemic administration of mMSC, BMDMCs, CD34-, CD3-, CD19-, CD31-, CD45-, and Sca-1-depleted BMDMCs, but not HLFs and CD11b-depleted BMDMCs, significantly reduced the number of inflammatory cells in BALF provoked by *Aspergillus* hyphal extract sensitization and challenge. **(A):** Total cell number within the BALF in N and A mice treated with PBS, HLF, mMSCs, BMDMCs, CD45-, CD34-, CD11b, CD3, CD19, CD31-, and Sca-1-depleted BMDMCs. **(B):** Differential cell population within the BALF normalized to total cell numbers. Total number of neutrophils, eosinophils, macrophages, and lymphocytes (N, n = 17; A-P, n = 15; A-HLF, n = 6; A-mMSC, n = 6; A-BMDMC, n = 13; A-CD45, n = 6; A-CD34, n = 6; A-CD11b, n = 9; A-CD3, n = 6; A-CD19, n = 6; A-CD31, n = 6; A-Sca-1, n = 6). Data presented as mean ± SD. ∗, $p \leq .05$ compared with N; #, compared with A-P; ∗∗, compared with A-BMDMC. Abbreviations: A-CD3 (or A-CD11b-, A-CD19-, A-CD31-, A-CD34-, A-CD45-, A-Sca-1-), *Aspergillus* hyphal extract-exposed mice treated with CD-3- (or CD11b-, CD19-, CD31-, CD34-, CD45-, and Sca-1-) depleted bone marrow-derived mononuclear cells; BALF, bronchoalveolar lavage fluid; BMDMC, bone marrow-derived mononuclear cell; HLF, human lung fibroblast; mMSC, mouse mesenchymal stem/stromal cell; N, naïve; P, phosphate-buffered saline.

Systemic Administration of MSCs, Total BMDMCs, or CD34-, CD3-, CD19-, or CD31-Depleted BMDMCs Significantly Inhibited AHE-Provoked Antigen-Specific Release of Th2 and Th17 Mediators in Mixed Mediastinal Lymphocyte Cultures

AHE sensitization and challenge (A) resulted in a significant increase in IL-4, IL-5, and IL-17 release by mixed MLN cultures following ex vivo antigen stimulation (Fig. 7). This was most notable at 48 hours, particularly for the increase in IL-17 levels. No

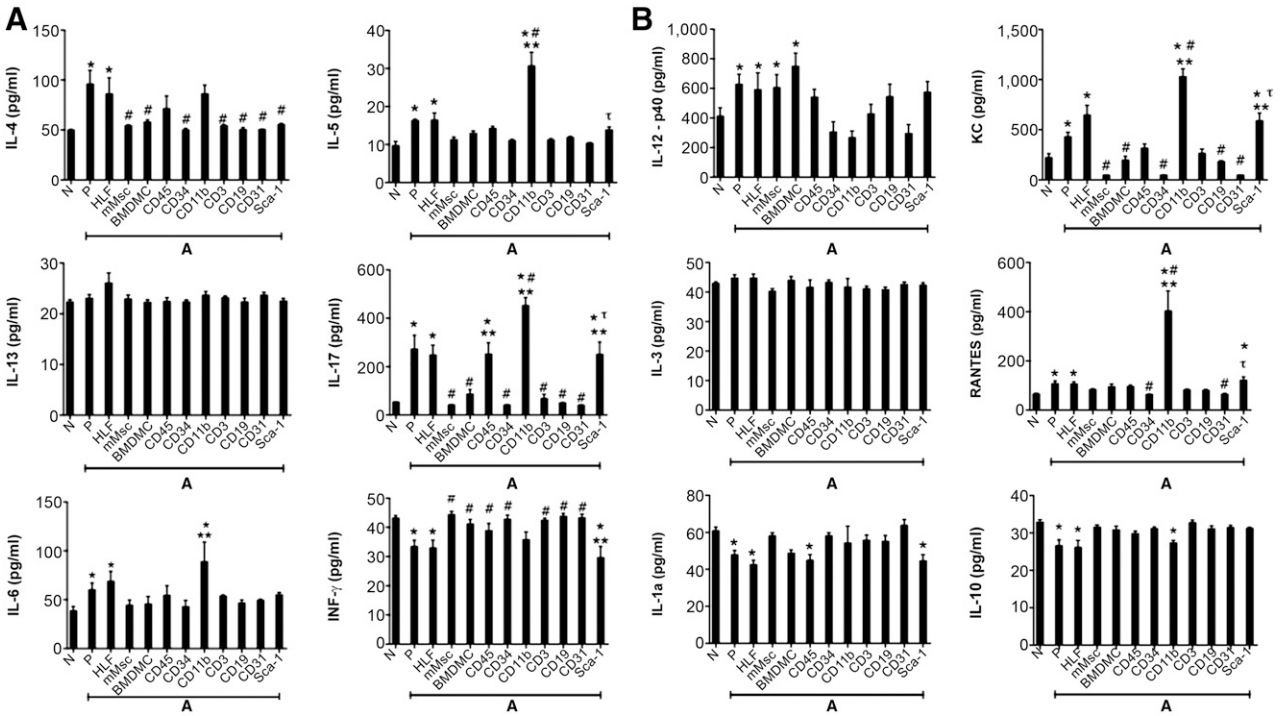

Figure 6. Systemic administration of mMSCs, BMDMCs, CD34-, CD3-, CD19-, and CD31-depleted BMDMCs, but not HLFs and CD11b- and Sca-1-depleted BMDMCs, significantly reduced the increased levels of proinflammatory soluble cytokines and chemokines in BALF, provoked by AHE sensitization and challenge. **(A):** Cytokines associated with Th2 (i.e., IL-4, IL-5, IL-13), Th17 (i.e., IL-6, IL-17a), and Th1 inflammation (i.e., IFN-γ). **(B):** Additional Th17 inflammation-associated cytokines (i.e., IL-12, KCs), alternate inflammatory cytokines (i.e., IL-3, RANTES), and cytokines previously identified as secreted by MSCs in immunomodulation (i.e., IL-1A, IL-10) (N, n = 17; A-P, n = 15; A-HLF, n = 6; A-mMSC, n = 6; A-BMDMC, n = 13; A-CD45, n = 6; A-CD34, n = 6; A-CD11b, n = 9; A-CD3, n = 6; A-CD19, n = 6; A-CD31, n = 6; A-Sca-1, n = 6). Data presented as mean $\pm$ SD. $*$, $p \leq .05$ compared with N; #, compared with A-P; $**$, compared with A-BMDMC; τ, compared with CD11b. Abbreviations: A-CD3 (or A-CD11b-, A-CD19-, A-CD31-, A-CD34-, A-CD45-, A-Sca-1-), *Aspergillus* hyphal extract-exposed mice treated with CD-3- (or CD11b-, CD19-, CD31-, CD34-, CD45-, or Sca-1-) depleted bone marrow-derived mononuclear cells; BMDMC, bone marrow-derived mononuclear cell; HLF, human lung fibroblast; IFN, interferon; IL, interleukin; KC, keratinocyte chemoattractant; mMSC, mouse mesenchymal stem/stromal cell; N, naïve; P, phosphate-buffered saline; RANTES, regulated on activation, normal T-cell expressed and secreted.

significant changes in levels of IFN-γ were observed. Systemic administration of either mMSCs or BMDMCs, but not HLFs, comparably resulted in significant reductions in release of IL-4, IL-5, and IL-17, and significant increase in IFN-γ release (Fig. 7). Administration of CD34-, CD-3-, CD-19-, or CD-31-depleted BMDMCs tended to reduce IL-4, IL-5, and IL-17 levels and promoted increase in IFN-γ levels. Administration of CD45-, CD11b-, or Sca-1-depleted BMDMCs tended not to reduce AHE-induced increases in IL-5 and IL-17 levels and also failed to promote increase in IFN-γ levels.

Discussion

Systemic administration of syngeneic BMDMCs during antigen challenge comparably reduced AHR and lung inflammation resulting from AHE-mediated allergic airway inflammation. This effect had not previously been demonstrated in this model with BMDMCs, to our knowledge, and is comparable to effects observed with systemic administration of syngeneic bone marrow-derived MSCs. Both cell types also comparably shifted phenotype of antigen-specific splenic CD4 cells from a Th2/Th17 toward a counter-regulatory Th1 phenotype. Using an antibody-mediated cell-depletion approach, removal of hematopoietic progenitors, endothelial cells, or of either T or B lymphocytes from the heterogeneous BMDMC populations had no effect, whereas removing either CD11b+ (monocyte/macrophages/dendritic cells) or Sca-1+ cells, likely the MSC fraction, of the BMDMCs each inhibited the ability of the BMDMCs to ameliorate either lung inflammation or airway hyperresponsiveness. These results demonstrate that for this model of lung injury, both MSCs and monocytes are the critical effector cells contained in the BMDMC population.

A large and steadily increasing number of studies have demonstrated beneficial effects of systemic or intratracheal administration of syngeneic, allogeneic, or xenogeneic MSCs derived from bone marrow, adipose tissue, placenta, and other sources in a wide spectrum of preclinical lung disease models [39–45]. With respect to asthma, MSC administration during either antigen sensitization or challenge mitigated both airway hyperresponsiveness and lung inflammation in a variety of models of allergic airway inflammation in mice [9–20]. Proposed mechanisms include MSC-stimulated increase in T-regulatory cells and also a shift from a Th2 or mixed Th2/Th17 phenotype to a counter-regulatory Th1 phenotype of antigen-specific CD4 cells [16, 18–20, 46]. How this occurs remains unclear, although recent data suggest that conditioned media—more specifically, extracellular vesicles released by the MSCs—can convey the same protective effects as the cells themselves [20].

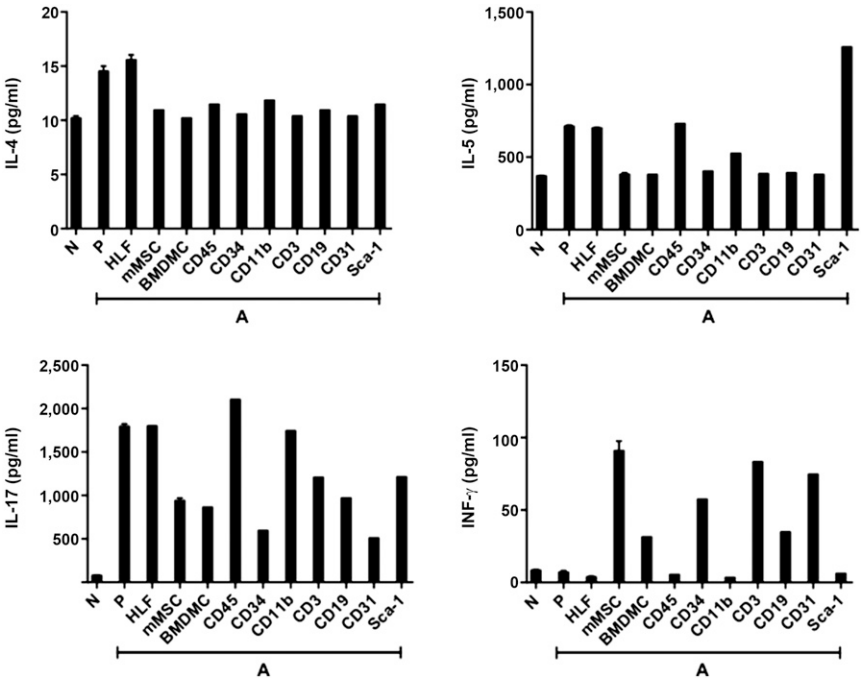

Figure 7. Systemic administration of mMSCs, BMDMCs, and postdepletion BMDMCs altered IL-4, IL-5, IL-17, and IFN-γ production in ex vivo restimulation of mediastinal lymphocytes. Assessment of IL-4, IL-5, IL-17, and IFN-γ levels in supernatants from mixed mediastinal lymph node cell populations ex vivo restimulated for 48 hours with *Aspergillus* hyphal extract antigen (N, $n = 17$; A-P, $n = 15$; A-HLF, $n = 6$; A-mMSC, $n = 6$; A-BMDMC, $n = 13$; A-CD45, $n = 6$; A-CD34, $n = 6$; A-CD11b, $n = 9$; A-CD3, $n = 6$; A-CD19, $n = 6$; A-CD31, $n = 6$; A-Sca-1, $n = 6$). Data presented as mean of duplicates (pooled data). Abbreviations: A-CD3 (or A-CD11b, A-CD19, A-CD31, A-CD34, A-CD45, A-Sca-1), *Aspergillus* hyphal extract-exposed mice treated with CD-3- (or CD11b, CD19, CD31, CD34, CD45, or Sca-1-) depleted bone marrow-derived mononuclear cells; BMDMC, bone marrow-derived mononuclear cell; HLF, human lung fibroblast; IFN, interferon; IL, interleukin; mMSC, mouse mesenchymal stem/stromal cell; N, naïve; P, group receiving phosphate-buffered saline.

Use of MSCs for cell-based therapies is based on several attributes, including their ability to support adequate tissue repair and suppress inflammation [47, 48]. However, the use of MSCs, particularly allogeneic MSCs, has some logistical drawbacks, including the need for culture expansion, potential alterations in cell properties that can occur in culture [21, 49, 50], and consideration of allogeneic MSC use [51]. An alternative approach is to use autologous BMDMCs, a heterogeneous pool of cells that can be administered safely and easily on the day of harvesting [52, 53]. Potential benefits include lack of need for culture expansion, no changes in cell properties that might occur in culture, and lower overall costs [54]. As such, the use of autologous BMDMCs may provide a viable alternative to MSCs. The premise of autologous BMDMC transplantation is based on the assumption that an affected individual will have BMDMCs that will be beneficial. The animal data suggest that in certain models, the BMDMCs may very well reflect underlying disease and, thus, arguably may be unsuitable for therapeutic use [55, 56]. In contrast, we have found that BMDMCs obtained from mice with allergic airway inflammation, albeit that produced by ovalbumin, and also from mice with lipopolysaccharide-induced acute lung injury are as potent as BMDMCs from normal syngeneic mice in reducing the specific disease inflammatory endpoints. Similar findings were observed in an animal model of acute lung injury [26, 57]. As such, there may be instances in which autologous BMDMCs obtained from individuals with a given lung disease may be appropriate [26, 57]. Furthermore, it is imperative to understand which cell populations in the BMDMCs might convey beneficial effects for use in any given disease. In preclinical models of lung diseases, BMDMCs have been shown to abrogate airway inflammation and remodeling and to promote lung repair in mouse models of acute lung injury, chronic obstructive pulmonary disorder, silicosis and asthma [24–26, 32, 36, 58]. With respect to asthma, we have previously compared BMDMCs with MSCs in a mild Th2-mediated eosinophilic allergic airway disease induced by ovalbumin sensitization and challenge [12]. In that study, to ascertain whether the outcomes of BMDMC administration resulted from the balance between cell types rather than strictly from MSCs present in the BMDMCs, similar numbers of MSCs were administered (4% MSCs in 2×10^6 BMDMCs, approximately 1×10^5 MSCs). That study demonstrated that BMDMC administration led to greater improvement in lung mechanics and a greater reduction in fractional area of alveolar collapse, collagen fiber content in the alveolar septa, and growth factor levels (transforming growth factor [TGF-β] and vascular endothelial growth factor) as compared with MSCs [12]. These results suggested that other cell types in the heterogeneous BMDMC mix might also be playing a role in ameliorating asthma.

The current study expands these initial observations to comparisons of BMDMC versus MSC administration in a more severe model of acute mixed Th2/Th17-mediated neutrophilic allergic airway inflammation induced by mucosal exposure to and challenge with adjuvant-free AHE. Notably, administration of either 10^6 BMDMCs or 10^6 MSCs yielded

similar beneficial effects on lung mechanics, airway inflammation, and behavior of antigen-specific splenic T cells, an effect not mimicked by a control fibroblast cell population. The reduction in AHE-induced increases in soluble Th2 (IL-4 and IL-5) and Th17 (IL-17) cytokines in BALF and in mixed lymphocyte cultures and the accompanying increase in IFN-γ levels after systemic BMDMC administration suggest a comparable shift of Th2/Th17 inflammatory response toward a counter-regulatory Th1 response, as observed with administration of MSCs alone. Reduction in AHE-stimulated increases in BALF levels of the neutrophil chemoattractants KC and RANTES suggests that BMDMCs and MSCs may also act in other ways to decrease neutrophil recruitment to the allergically inflamed lung. Additionally, BMDMC or MSC administration resulted in a decrease in IL-6 levels; IL-6 is an important regulator of effector CD4 T-cell fate, promoting IL-4 production during Th2 differentiation, inhibiting Th1 differentiation, and, together with TGF-β, promoting Th17-cell differentiation [59]. Furthermore, control levels of IL-10, an important anti-inflammatory cytokine, were found after treatment with BMDMCs or MSCs.

We next sought to identify the cell or cells in the BMDMCs that mediated the observed effects in this model. The heterogeneous BMDMC population does include a small percentage of Sca-1$^+$ CD45$^-$ cells that likely include MSCs. The approximate 5%–6% Sca-1$^+$ CD45$^-$ content of these cells is higher than those traditionally described in mouse bone marrow and so may also contain other Sca-1$^+$ cells. Nonetheless, it is reasonable to assume that this fraction does contain MSCs. Thus, either a small number of MSCs (i.e., the approximately 5×10^4 MSCs contained in the BMDMCs) was sufficient to mitigate the effect or another cell type in the BMDMC also played a role. Previous studies in other disease models have suggested that the functional effects of the BMDMCs result from a balance between different cell types, with potential beneficial involvement of all component cells [24–26, 32, 36, 52, 53, 57, 58]. BMDMCs include a variety of cells: progenitor cells (hematopoietic progenitor cells and mesenchymal stromal cells), leukocytes (B and T lymphocytes, and monocytes/macrophages), and endothelial cells. Using established cell surface markers for each of these cells, it was feasible to remove individual cell categories (i.e., total leukocytes) or individual cell types (i.e., T or B cells, monocytes, endothelial cells, or hematopoietic progenitor cells) with antibody depletion techniques. Regarding MSC surface markers, there is no one characteristic cell surface marker; rather, a set of suggested positive and negative markers has been described [60]. In the mouse, this includes Sca-1$^+$, CD45$^-$, and CD34$^-$ cells [60, 61]. As such, we used depletion of Sca-1 as a marker to deplete a cell population that would include MSCs, and compared this to BMDMC populations depleted of CD34 cells, which included hematopoietic progenitor cells [62, 63]. As HSCs differentiate into common progenitors, Sca-1 expression is downregulated [64]; thus, the number of Sca-1-positive cells is lower than CD34-positive cells.

These depletion studies demonstrated that bone marrow-derived CD11b$^+$ mononuclear cells were as potent, if not more so, than bone marrow-derived Sca-1+ mononuclear cells in conveying the beneficial effects of BMDMC administration in this model. CD11b-positive cells include a variety of cells, but, importantly, include dendritic cells or their precursors, and also monocytes/macrophages. Dendritic cells have been shown to activate T cells and trigger their differentiation into regulatory T cells following exposure to antigens. Once activated, regulatory T cells can engage in bystander suppression, whereby they suppress immune responses in an antigen-independent manner via cell-cell contact or by the secretion of inhibitory cytokines such as IL-10 and TGF-α [65]. Moreover, there are several mechanisms by which bone marrow-derived monocytes/macrophages could ameliorate allergic airway inflammation, including expression of anti-inflammatory cytokines and other mediators [66–71]. Although the inflammatory process in asthma is dominated by a Th2 inflammation, increasing evidence supports the parallel development and involvement of both M1 and M2 macrophages [65]. M1 macrophages, which are increased in bronchoalveolar lavage of patients with corticosteroid-resistant asthma compared with those with corticosteroid-sensitive asthma, are responsible for the recruitment of neutrophils, which are the major effector cells in severe phenotypes of asthma [65]. The cytokines IL-4 and IL-13 are abundantly present in the lungs of patients with asthma, and since these cytokines are M2 polarization inducers, it may not come as a surprise, therefore, that M2 macrophages have been associated with asthma [65]. However, previous studies did not conclusively prove that M2 macrophages play a causative role in the development of allergic airway inflammation, and Nieuwenhuizen et al. recently demonstrated that M2 macrophages are not necessary for allergic airway disease and may only be a consequence of the elevated Th2 response [72].

Notably, a growing body of literature demonstrates that MSCs induce macrophages to polarize into a M2 subtype that secretes several anti-inflammatory cytokines, such as IL-10 and TGF-β, and that also can induce CD4$^+$ T cells to adopt a CD25$^+$FoxP3$^+$ TGF-β-1$^+$ functional suppressor phenotype (T regulatory cells) [73, 74]. Thus, bone marrow-derived macrophages that are in constant contact with MSCs in that microenvironment can have anti-inflammatory properties and are reported to produce high levels of anti-inflammatory cytokines when compared with macrophages from other sources (e.g., spleen and peritoneum) [75]. Furthermore, M2 macrophages express receptors involved in phagocytosis and in the engulfment and digestion of dead cells, debris, and various extracellular matrix components that could promote tissue-damaging M1 macrophage responses [76, 77]. More interestingly, M2 macrophages produce factors that induce the apoptosis of myofibroblasts as well as matrix metalloproteinases and tissue inhibitors of metalloproteinases that control extracellular matrix turnover and play an important role in wound healing, angiogenesis, and fibrosis by helping to restore tissue homeostasis [78]. Thereby, administration of bone marrow-derived monocytes/macrophages can conceivably produce beneficial effects in allergic airway inflammation models because of their anti-inflammatory properties and their remodeling capacity [65, 66].

Although the immune magnetic-bead-depletion technique used in this study is a simple, feasible, and efficient method to deplete specific cells, no cell type was completely removed. Thus, it is conceivable that small numbers remaining for each cell type following depletion from the BMDMCs might have played a role in the AHE model. In future studies, bone

marrow-derived CD11b$^+$ and Sca-1$^+$ cells will be isolated and administered in the Th2/Th17-mediated allergic airway inflammation model. We will also further characterize the phenotype of the CD11b+ cells and compare effects with that of CD11b$^+$ cells extracted from other sources such as the blood, peritoneum, or the bronchoalveolar lavage fluid.

One other consideration is age of the mice being studied. Brandenberger et al. showed in 2014 that in BALB/c mice sensitized and challenged with house dust mites, the severity and character of allergic airway disease were age dependent, with a bias toward a Th17 immune response with aging. Thus, they concluded that elderly patients with asthma may be prone to develop severe allergic airway inflammation with a mixed Th2/Th17 immune response [79]. On the other hand, and corroborated by previous studies [16, 18–20], our group used younger immunocompetent C57Bl6 mice (8–12 weeks old) that have been shown to develop a severe neutrophilic-mediated allergic hyperresponsiveness and inflammation with mixed Th2/Th17 responses reflective of severe refractory asthma, induced by repeated mucosal exposure to AHE over a period of weeks. Thus, future studies will comparatively assess MSCs, BMMDCs, and BMMDC subpopulations in different age groups.

Conclusion

Systemic administration of either syngeneic BMDMCs or syngeneic MSCs were similarly effective in reducing airway inflammation and improving lung function in a mixed Th2/Th17 model of allergic airway inflammation in mice. However, depleting the heterogeneous BMDMC population of either monocytes/macrophages or of bone marrow-derived Sca-1+ cells did not produce the same improvement in lung mechanics and histology, suggesting that both cell types, and likely the interaction between these two cell types, play an important role in these processes. Future studies will clarify the respective contributions and interactions between these cell types in this model.

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Author Contributions

F.F.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Z.D.B., M.G., A.L.C., and D.E.W.: collection and/or assembly of data, final approval of manuscript; P.R.M.R.: conception and design, final approval of manuscript; D.J.W.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

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