

Mesenchymal Stromal Cells Mediate *Aspergillus* Hyphal Extract-Induced Allergic Airway Inflammation by Inhibition of the Th17 Signaling Pathway

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ABSTRACT

Systemic administration of mesenchymal stromal cells (MSCs) suppresses airway inflammation and methacholine-induced airway hyper-responsiveness (AHR) in mouse models of T helper cell (Th) type 2-mediated eosinophilic allergic airway inflammation (AAI); however, the efficacy of MSCs in mouse models of severe Th17-mediated neutrophilic AAI has not yet been demonstrated. We assessed MSC effects in a mouse model of mixed Th2/Th17 AAI produced by mucosal exposure to *Aspergillus fumigatus* hyphal extract (AHE). Following sensitization produced by oropharyngeal AHE administration, systemic (tail vein) administration of syngeneic MSCs on the first day of challenge significantly reduced acute AHR predominantly through reduction of Th17-mediated airway inflammation. In parallel experiments, MSCs also mitigated AHR when administered during recurrent challenge 10 weeks after initial sensitization and challenge through reduction in systemic Th17-mediated inflammation. Investigation into potential mechanistic actions of MSCs in this model demonstrated that although T regulatory cells were increased in all AHE-treated mice, MSC administration did not alter T regulatory cell numbers in either the acute or recurrent model. Differential induction of interleukin-17a secretion was observed in ex vivo restimulation of mediastinal lymph node mixed-cell cytokine analyses. Although the mechanisms by which MSCs act to decrease inflammation and AHR in this model are not yet fully elucidated, decrease in Th17-mediated airway inflammation appears to play a significant role. These results provide a basis for further investigations of MSC administration as a potential therapeutic approach for severe refractory neutrophilic asthma. *STEM CELLS TRANSLATIONAL MEDICINE* 2014;3:194–205

INTRODUCTION

Asthma is a clinically diverse disease characterized by airway obstruction, inflammation, and remodeling that affects approximately 300 million patients of all ages and ethnic groups globally [1, 2]. The majority of patients have persistent, mild to moderate asthma that can be relatively well controlled by the use of available medications, including inhaled bronchodilators and inhaled glucocorticoids. However, 5%–10% of patients suffer from severe disease that is poorly controlled clinically and resistant to corticosteroids and most other available treatments [1, 2]. These patients contribute disproportionately to morbidity, mortality, and health care expenditures in the asthmatic population [3–6]. This population of asthmatics tends to have interleukin-17 (IL-17)-mediated neutrophilic airway inflammation driven by antigen-specific Th17 CD4 T cells through mechanisms that are currently unclear

[7–10]. Although some antibody-based therapies may be helpful [11–13], new therapeutic options are needed.

Mesenchymal stromal cells (MSCs) are multipotent adult cells isolated from many source tissues, including bone marrow, adipose tissue, and umbilical cord blood, and are defined by expression of a panel of cell surface markers and the ability to differentiate into cartilage, fat, and bone tissues [14–18]. In addition, MSCs possess significant immunomodulatory functions, and adoptive transfer of non-human leukocyte antigen-matched allogeneic MSCs is feasible and appears to be safe. These properties have been demonstrated in a spectrum of animal models of inflammatory and immune-based diseases and have led to clinical trials in a wide variety of immune and inflammatory diseases [19–22] as well as recent clinical approval for therapeutic use in severe refractory pediatric graft-versus-host disease in Canada [23]. MSCs have also been demonstrated

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to have efficacy in mouse models of pulmonary diseases including acute lung injury, bronchopulmonary dysplasia, and chronic obstructive pulmonary diseases including asthma and emphysema [24, 25]. A recent clinical trial of allogeneic MSCs in patients with moderate-severe chronic obstructive pulmonary disease demonstrated safety and provides a firm basis for accelerating clinical investigations of MSCs in patients with lung diseases [26].

With respect to asthma, MSCs have been demonstrated to ameliorate experimentally induced T helper cell (Th) type 2-mediated eosinophilic allergic airway inflammation (AAI) in mice [27–38]. Immunogens investigated in models include sensitization and challenge with ovalbumin, ragweed pollen, dust mite antigen, and toluene diisocyanate, and comparable effects have been observed with syngeneic, allogeneic, or xenogeneic MSC administration. Postulated mechanisms of MSC actions include a shift from Th2 to Th1 antigen-specific CD4 T cells and an increase in T regulatory cells (T-regs) [31, 36, 39]. These results suggest that MSCs may be effective in clinical asthma. However, the effect of systemic MSC administration in preclinical models of Th-17-mediated neutrophilic severe AAI has not yet been assessed. Using a model of mixed Th2/Th17 AAI provoked by repeated airway mucosal exposure to *Aspergillus fumigatus* hyphal extract (AHE) [40, 41], we found that both Th2- and Th17-mediated inflammation is decreased following MSC administration in both acute and recurrent AHE exposures. A primary mechanism appears to be a decrease in Th17-mediated IL-17a production with no obvious effect on T-regs.

MATERIALS AND METHODS

Mice

C57Bl/6 mice (male, 8–12 weeks; Jackson Laboratories, Bar Harbor, ME, <http://www.jax.org>) were housed in microisolator cages and used in accordance with the University of Vermont's institutional animal care and use committee under all applicable Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

MSC Culture

Murine bone marrow-derived MSCs from C57Bl/6 mice at passage 5 were obtained from the Texas A&M University's stem cell core facility. These cells have previously been characterized extensively for cell surface marker expression and differentiation capacity [17, 42]. Cells were expanded in culture using Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY, <http://www.invitrogen.com>), 10% fetal bovine serum (HyClone, Logan, UT, <http://www.hyclone.com>), 10% horse serum (HyClone), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>), and 2 mM L-glutamine (Invitrogen) and used at passages 7–8. Cells were trypsinized for injection using 2.5% trypsin/ethylenediaminetetraacetic acid (Invitrogen), counted using a hemacytometer, and resuspended in 1× phosphate-buffered saline (PBS) to a final concentration of 1×10^6 cells per 200 μ l. An aliquot of the same PBS was made for injection of control mice. To inhibit soluble mediator secretion, MSCs were treated for 1 hour on ice with a freshly prepared 75 mM solution of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI; Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>)

in 0.9% sodium chloride [31]. Treated cells were washed three times in 1× PBS and prepared for injection as described.

Induction of AAI

AHE was generously provided by the Whittaker laboratory at the University of Vermont [40, 41]. AHE was produced by growing Af293 stock at 37°C for 5 days, after which the hyphal mat was washed with 1× PBS, homogenized by glass bead dissociation, and fixed in 1% paraformaldehyde before Bradford analysis of total protein content and concentration by endotoxin-free dialysis. Stocks of AHE at a concentration of 1.466 mg/ml in 1× PBS were stored at –80°C. For in vivo administration, AHE aliquots were thawed and vortexed immediately prior to use and diluted to a final concentration of 5 μ g of AHE in 40 μ l of sterile 1× PBS. Mice were anesthetized by isoflurane inhalation (2 units per 2 liters of oxygen per minute) until deep respiration was observed, and the AHE solution was administered by oropharyngeal inoculation. The mice were then allowed to recover from isoflurane and placed back in their cages. Mice were inoculated with 5 μ g of AHE on days 0 and 7 to initiate the immune response, and then challenged for 3 days on days 14, 15, and 16 with oropharyngeal inoculations using the same AHE dose (Fig. 1A) [40, 41]. AHE-inoculated mice were administered 1×10^6 MSCs in 200 μ l of 1× PBS (AHE-MSC) or 1× PBS control (AHE) systemically by tail vein injection on day 14. Some mice received MSCs treated with EDCI prior to injection to prevent release of soluble mediators, as described previously [31] (AHE-EDCI MSC). Naïve mice never exposed to AHE were used as the negative AAI control (Naïve), whereas naïve mice never exposed to AHE but injected with MSCs were examined to identify MSC effects (sham-MSC). Mice were euthanized on day 19, and inflammation and lung functions were measured as described below.

Mice undergoing long-term induction of AHE-induced AAI underwent the inoculation and challenge as described but with no administrations of MSCs. Following the initial inoculation, mice received a second round of AHE challenges on days 76, 77, and 78. MSCs or PBS control were administered on day 76, and the mice were euthanized on day 81 for assessments (Fig. 1A).

Respiratory Mechanics

Pulmonary function was analyzed using the forced oscillation technique (flexiVent; SCIREQ Scientific Respiratory Equipment, Tempe, AZ, <http://www.scireq-usa.com>), as described previously [31, 43]. The peak responses for airway resistance, overall tissue resistance, and elastance within the lung were determined in response to sequential inhalation of nebulized saline, 3.125 mg/ml, 12.5 mg/ml, and 25 mg/ml of methacholine (MCh) in saline.

Assessment of Airway Inflammation

Following examination of respiratory mechanics, mice were euthanized by lethal intraperitoneal injection of sodium pentobarbital. Bronchoalveolar lavage (BAL) fluid was generated by administering 1 ml of sterile 1× PBS to the airways through a tracheal cannula and rinsing the lungs three times prior to recovery. Final volume recovered was recorded and used to calculate total cell numbers within the BAL. Cells were pelleted by centrifugation at 5,000 rpm for 5 minutes at 4°C, and the supernatant was collected in separate tubes containing Protease Inhibitor Cocktail (Sigma-Aldrich) and stored at –80°C. The Bio-Plex Cytokine Assay System (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) was

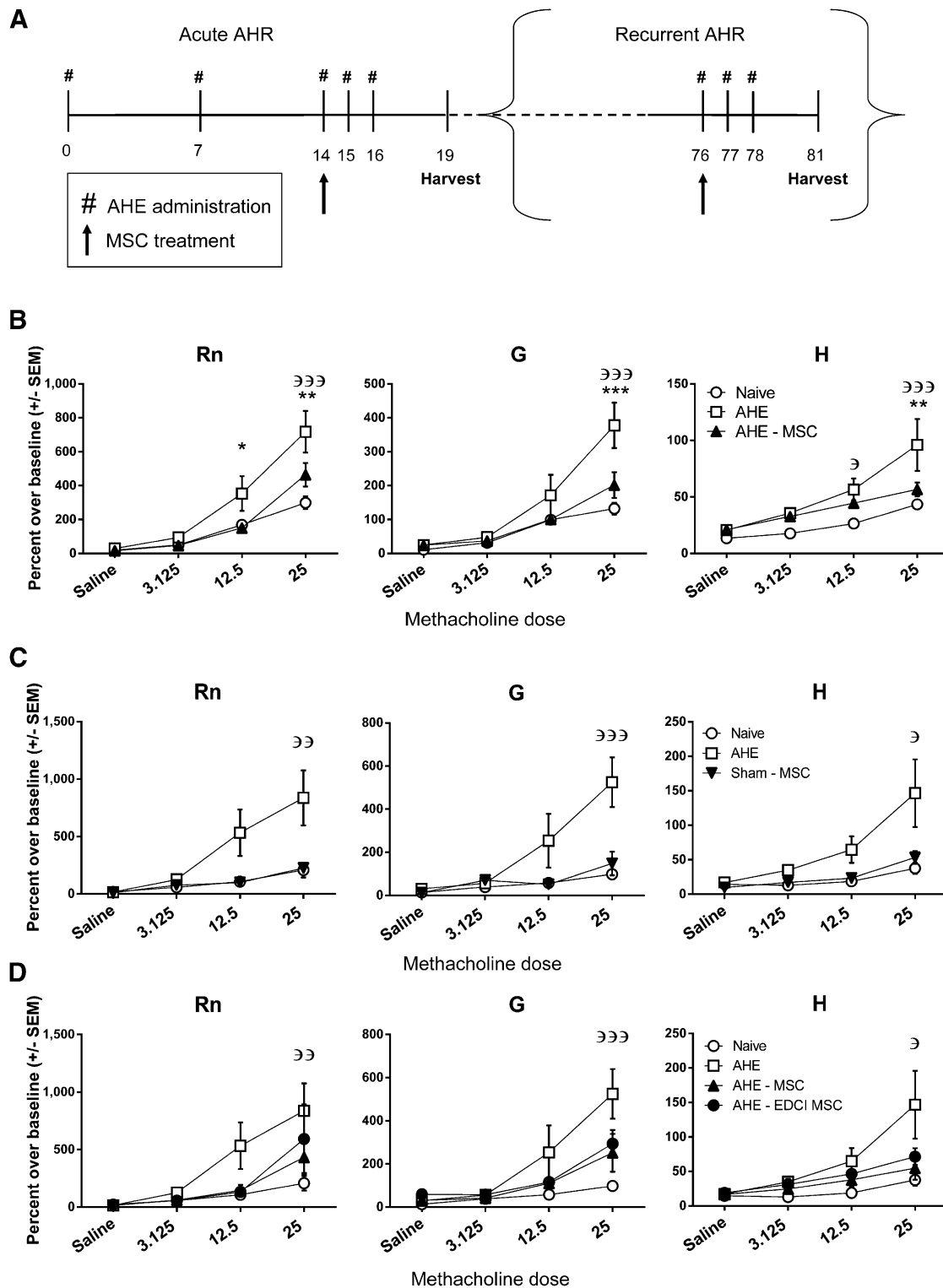


Figure 1. Systemic administration of MSCs significantly ameliorates the AHR induced by AHE. **(A):** Schematic of the acute and recurrent induction of AHR. **(B):** Airway resistance analysis of AHE with and without systemic MSCs (18 Naive, 23 AHE, 16 AHE-MSC). **(C):** Airway resistance analysis following systemic administration of MSCs to mice receiving control administrations of saline instead of AHE (sham-MSC). **(D):** EDCI-treated MSCs (AHE-EDCI MSC) systemically administered to AHE-sensitized mice. Data are presented as peak response normalized to the baseline and then expressed as percent increase over that baseline $\pm$ SEM. For **(C)** and **(D)**, sample sizes (7 Naive, 3 sham-MSC, 10 AHE, 6 AHE-MSC, 4 AHE-EDCI MSC) reflect direct comparison between experimental controls measured at the same time. $p \leq .05$ (single symbol), $p \leq .01$ (double symbols), $p \leq .001$ (triple symbols). $\supset$, AHE treatment was significantly increased over Naive. *, AHE-MSC was significantly reduced from AHE. Abbreviations: AHE, *Aspergillus fumigatus* hyphal extract; AHR, airway hyper-responsiveness; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; G, overall tissue resistance; H, elastance within the lung; MSC, mesenchymal stromal cells; Rn, airway resistance.

used to examine undiluted BAL fluid samples for soluble inflammatory cytokines using a mouse 23-plex panel. Concentrations were determined using the Bio-Plex Manager Software. The cell pellet was resuspended in 400 μ l of 1 $\times$ PBS, and 200 μ l was used to determine cell numbers with the ADVIA Hematology Analyzer (Siemens, Munich, Germany, <http://www.medical.siemens.com>). Cytospins were made using 5×10^4 cells centrifuged onto precleaned, pretreated glass slides (Corning Enterprises, Corning, NY, <http://www.corning.com>) at 800 rpm for 8 minutes, dried overnight, and stained using DiffQuick (Hema 3 Stain Set; Fisher Scientific International, Hampton, NH, <http://www.fisherscientific.com>). Cell populations were determined by blinded manual count of 200 cells performed by three separate individuals.

Following BAL, the trachea and heart/lung block were removed and the right lobes of the lung were removed and snap frozen on liquid nitrogen for mRNA analyses. The left lobe was then gravity fixed (20 cm H₂O) for 1 hour with 4% paraformaldehyde and 5- μ m paraffin sections subsequently stained with hematoxylin and eosin. Airway inflammation was scored on 10 airways per animal in a blinded fashion by three individuals, based on the presence and intensity of peribronchial cell infiltrates compared with known positive and negative controls; an established semiquantitative scoring system was used with a 0–3 range and 0.5-scale increments, as described previously [31, 44]. RNA was isolated from frozen lung tissue, and mRNA levels were assessed by TaqMan Gene Expression Assay (Life Technologies, Rockville, MD, <http://www.lifetech.com>), and analyses were normalized to GusB levels. Detailed protocols are presented in the supplemental online data.

Mediastinal Lymph Node Mixed Lymphocyte Assessments

The mediastinal lymph node (MLN) was isolated from each mouse and placed in T-cell media (RPMI, 5% fetal bovine serum, 1 $\times$ penicillin/streptomycin, 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 μ M β -mercaptoethanol). All MLNs per treatment group were pooled and pressed through a 40- μ m mesh filter into a single cell suspension. Cells were then washed twice in 1 $\times$ PBS and resuspended for counting. One million cells per time point (24-, 48-, and 72-hour stimulation) and unstimulated control cells were plated for each group (Naïve, sham-MSC, AHE, AHE-MSC, and AHE-EDCI MSC) in a 24-well dish in 500 μ l of T-cell media. Cells were stimulated with 1 μ g of AHE extract in the media for 24, 48, or 72 hours or left unstimulated for 72 hours. Total contents of each well were collected at the indicated time points, and cells were spun for 2 minutes at 10,000 rpm to pellet. Supernatants were removed to a new tube, and both supernatant and pellet were frozen at -20°C . Supernatants were assessed by Bio-Plex for soluble cytokines.

Assessment of T-Regulatory Cells

Detailed protocols are described in the supplemental online data.

Statistical Analyses

All data were graphed and analyzed using the GraphPad Prism 6 program (GraphPad Software, San Diego, CA, <http://www.graphpad.com>). Groups were compared for significant differences using either one-way or two-way ANOVA with a Fisher's

least significant difference post-test to compare all groups of columns or to perform direct analysis between two groups by Student's *t* test, using a Welch's correction for unequal variances, as appropriate [45]. Statistical significance was indicated as $p \leq .05$ (single symbol), $p \leq .01$ (double symbols), and $p \leq .001$ (triple symbols). Significance of MSC treatments compared with AHE controls is indicated by an asterisk, and significance between Naïve and AHE cell controls is indicated by a Δ symbol, whereas significance between Naïve and MSC treatments is indicated by the ∂ symbol. Any *p* values approaching significance noted in the figures are in reference to comparisons between AHE-MSC and AHE controls only.

RESULTS

Systemic MSC Administration on the First Day of Challenge Alleviates Acute AHE-Stimulated Airway Hyper-Responsiveness

AHE-induced AAI was examined in both acute and recurrent models (Fig. 1A). In the acute-exposure model, AHE-sensitized and challenged mice treated with control saline injections demonstrated a marked dose-dependent increase in each measure of airway hyper-responsiveness (AHR) with significant response compared with naïve mice observed by the 25 mg/ml MCh dose (Fig. 1B), as observed previously [40, 46]. AHE-sensitized and challenged mice receiving systemic syngeneic MSCs on the first day of challenge (AHE-MSC) demonstrated a trend toward reduction in airway resistance (Rn) and overall tissue resistance (G) by the 12.5 mg/ml MCh dose (Fig. 1B) that reached statistical significance by the 25 mg/ml MCh dose in airway resistance, overall tissue resistance, and elastance (H) within the lung (Fig. 1B). Sham-sensitized and challenged mice demonstrated no AHR in response to MSC administration (Fig. 1C). To investigate whether the effect of the MSCs was related to a secreted soluble mediator, in a parallel series of experiments MSCs were pretreated using EDCI to inhibit release of soluble mediators [31, 47] for 1 hour immediately prior to systemic injection to AHE-sensitized and challenged mice (AHE-EDCI MSC). A trend toward similar inhibition of AHE-stimulated increases in airway resistance, overall tissue resistance, and elastance within the lung was observed in AHE-EDCI MSC treatment compared with MSC-treated mice (Fig. 1D), but significance was not reached.

Systemic Administration of Syngeneic MSCs on the First Day of Challenge Alleviates Acute AHE-Stimulated Airway Inflammation

Mice inoculated with AHE showed a significant increase in the number of total cells in the BAL fluid compared with Naïve and sham-MSC mice (Fig. 2A). Treatment with either MSCs or EDCI-treated MSCs resulted in a significantly reduced number of cells within the population (Fig. 2A). As an indication of the mixed Th2/Th17 inflammatory response resulting from AHE sensitization and challenge, the BAL cell differentials showed a robust influx of both eosinophils and neutrophils. Treatment with either MSCs or EDCI-treated MSCs significantly decreased the population of neutrophils within the BAL (Fig. 2B). AHE-treated mice showed a corresponding robust increase in the inflammation score and infiltrating cells surrounding the airways of the lung compared with Naïve or sham-MSC mice (not indicated) that was significantly reduced by treatment with both MSCs and EDCI-treated

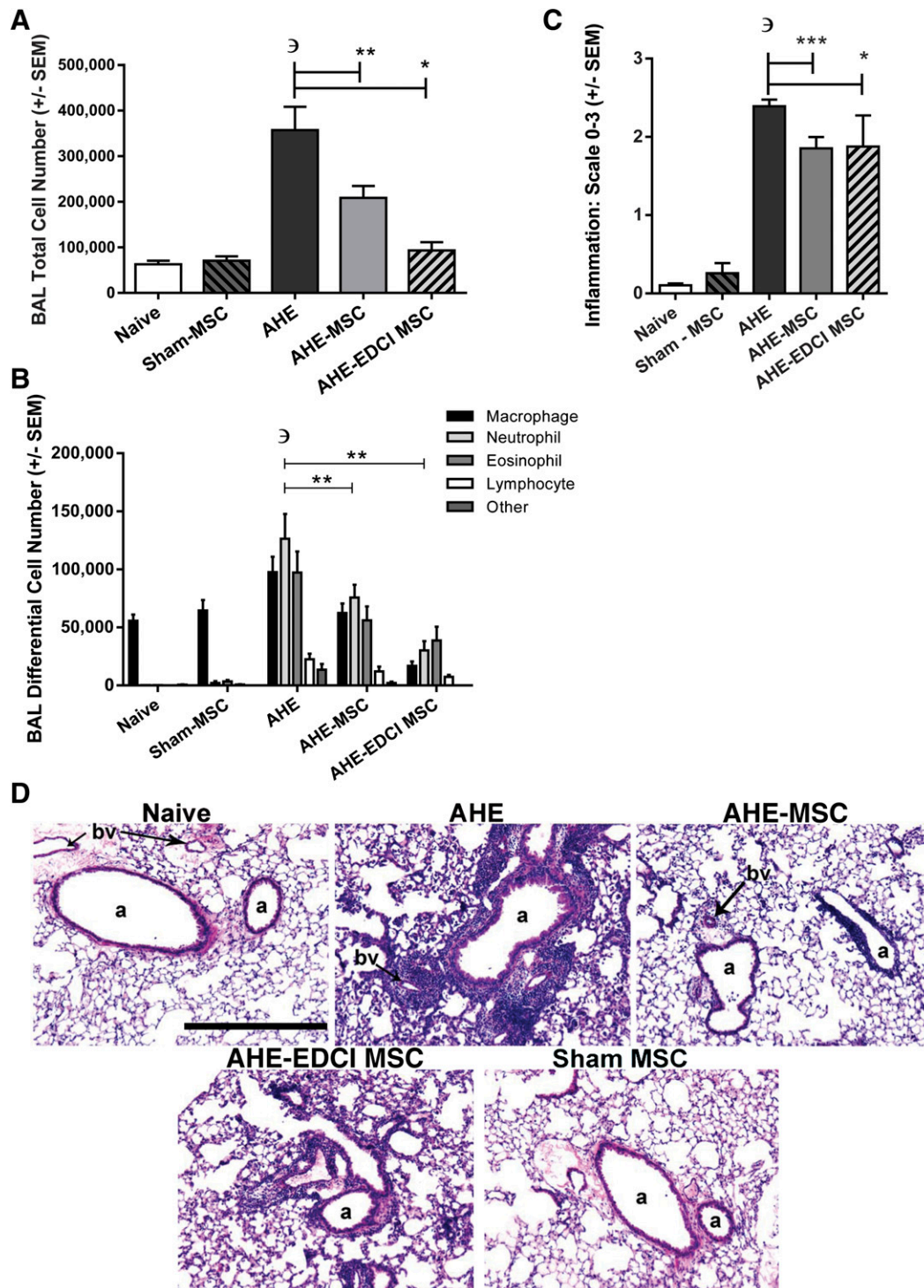


Figure 2. Systemic treatment with MSCs significantly inhibits the lung inflammation associated with acute AHE-induced AHR. **(A):** Total cell number within the BAL in AHE controls and mice administered AHE with either MSCs or EDCI-treated MSCs. **(B):** Differential cell population within the BAL, normalized to total cell numbers. Not indicated for clarity was a significant reduction in the total number of macrophages (at the $p = *$ level) when treated with EDCI-treated MSCs relative to AHE-treated mice. **(C):** Inflammation score of airways in AHE-MSC- and AHE-EDCI MSC-treated mice compared with AHE. **(D):** Representative images of hematoxylin and eosin-stained lung sections. Original magnification: $\times 10$. Scale bar = 500 μ m. Data are presented as means $\pm$ SEM. Sample sizes: 8 Naive, 3 sham-MSC, 23 AHE, 16 AHE-MSC, 4 AHE-EDCI MSC. Δ , AHE was significantly increased over Naive. *, AHE-MSC or AHE-EDCI MSC was significantly different from AHE. Abbreviations: a, airway; AHE, *Aspergillus fumigatus* hyphal extract; BAL, bronchoalveolar lavage; bv, blood vessel; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; MSC, mesenchymal stromal cells.

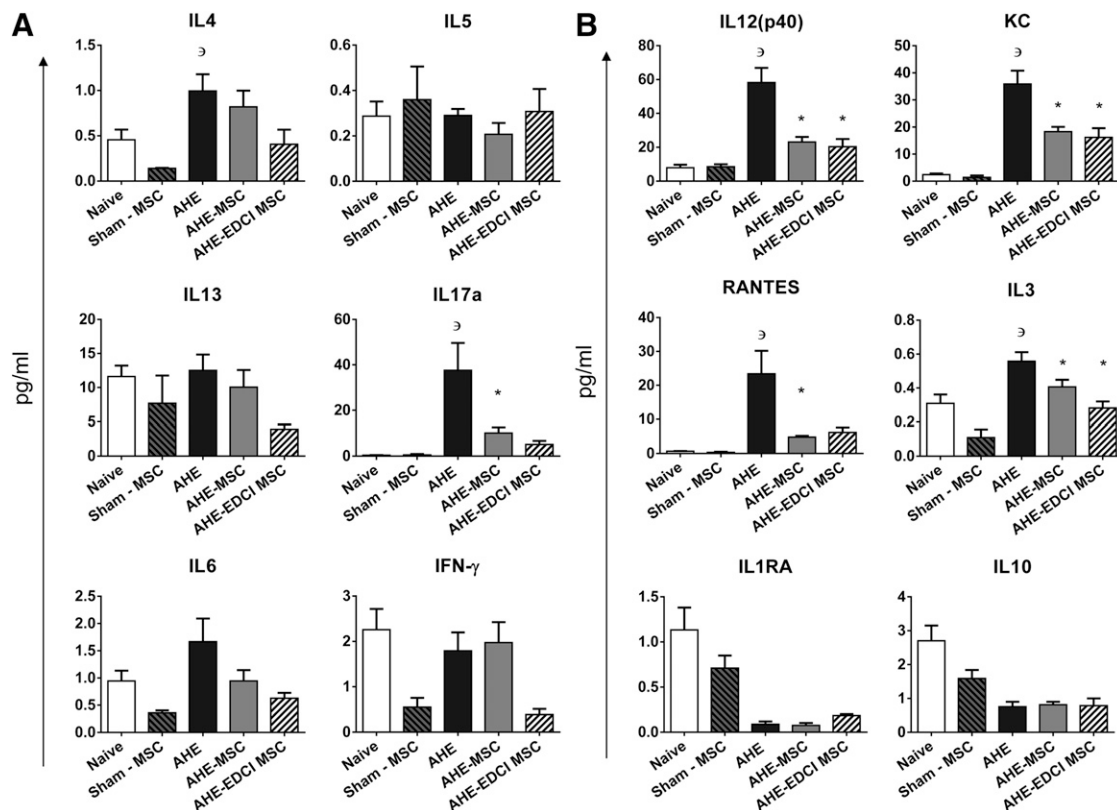


Figure 3. MSCs inhibit inflammatory soluble cytokines in AHE-induced airway hyper-responsiveness, indicating moderation of the T helper cell (Th) type 17 pathway. **(A):** Cytokines associated with Th2 (IL-4, IL-5, IL-13), Th17 (IL-6, IL-17a), and Th1 (IFN γ) inflammation. **(B):** Bio-Plex analysis of further Th17 inflammation-associated cytokines (IL-12(p40), KC), alternate inflammatory cytokines (RANTES, IL-3), and cytokines previously identified as secreted by MSCs in immunomodulation (IL-1RA, IL-10). All units are picograms per milliliter. Data are represented by the average $\pm$ SEM. $p \leq .05$ (single symbol). $\ominus$, AHE was significantly increased over Naive. *, AHE-MSC or AHE-EDCI MSC was significantly different from AHE. Sample sizes: 18 Naive, 3 sham-MSC, 23 AHE, 16 AHE-MSC, 4 AHE-EDCI MSC. Abbreviations: AHE, *Aspergillus fumigatus* hyphal extract; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; IFN, interferon; IL, interleukin; MSC, mesenchymal stromal cells; SEM, standard error of the mean.

MSCs (Fig. 2C, 2D). To assess whether the MSCs localized to lung and to gauge the degree of retention over a 24-hour period, labeled MSCs were injected into either Naive or AHE-treated mice. Flow cytometric analysis of whole-lung homogenates demonstrated that labeled MSCs can be found within the lung tissue of both Naive and AHE-treated mice 1 hour after administration. After 24 hours, some labeled MSCs remain detectable within AHE-treated lungs but none were detected in the Naive lungs (supplemental online Fig. 1).

MSC Inhibition of AHE-Induced AAI Is Associated With Decrease of Th2 and Th17 Soluble Mediators in BAL Fluid

Treatment with AHE results in a significant increase in the soluble mediators associated with a mixed Th2/Th17 response, namely, an increase in soluble IL-4, and IL-17a, with no changes in IL-5, IL-13, or interferon- γ (IFN γ) levels (Fig. 3). MSC administration resulted in a statistically significant decrease in IL-17a and a trend toward decrease in IL-4 and IL-6. No significant changes were observed in IL-5, IL-13, or IFN γ levels (Fig. 3A). In addition, AHE-stimulated mice exhibited increased levels of the inflammatory cytokines IL-3, IL-12(p40), KC, and RANTES, all of which were significantly reduced by treatment with MSCs and, with the exclusion of RANTES, were also significantly reduced by EDCI-treated MSCs (Fig. 3B). Neither IL-1RA nor IL-10, cytokines that have been

previously shown to be secreted by MSCs or by other inflammatory cells following exposure to MSCs, were increased in the BAL fluid of both sham-MSC treated and AHE-exposed mice [36, 48, 49]. The expression of CD4, IL-17f, IL-6, and IL-12 mRNA was examined in whole-lung RNA by quantitative polymerase chain reaction to determine whether the BAL cytokine levels were associated with changes in the resident lung lymphocytes. Gene expression was unchanged (supplemental online Fig. 2), suggesting that MSC effects in the lung in this model occur at least in part through inhibition of cytokine release and cell recruitment rather than direct effect on resident lung T-cell populations.

Systemic MSC Therapy in the Recurrence of AAI Is Effective in Reducing Lung Resistance but Not Markers of Inflammation

For effective clinical use of MSCs in severe asthma, patients will require treatment after the development of recurrent refractory asthma rather than at the initial onset of disease. Consequently, to determine whether administration of MSCs would be effective at the onset of a recurrent episode of AHE-induced AAI, mice received the initial day 0–16 stimulation with AHE but no MSC treatment and were then challenged for 3 days a second time beginning on day 76, with a single systemic administration of MSCs on day 76 (Fig. 1A). Similar to the acute AHR, recurrent

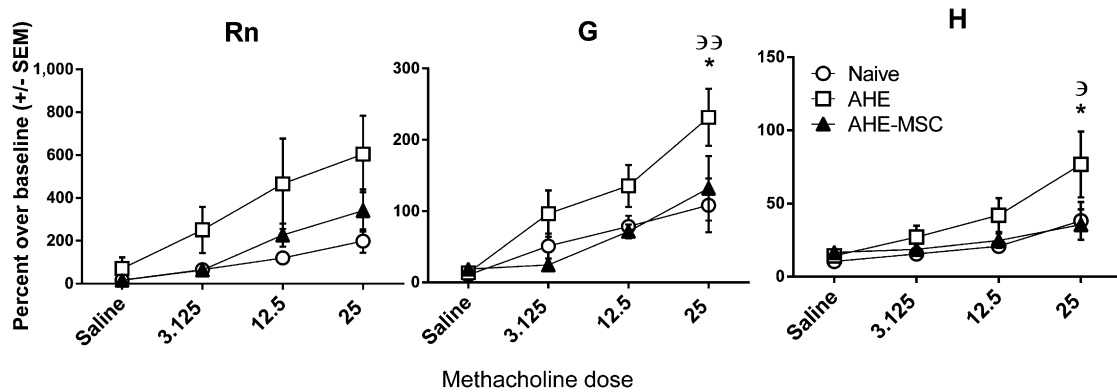


Figure 4. MSC administration significantly ameliorates airway hyper-responsiveness on recurrence of AHE-induced allergic airway disease. Stimulation with increasing doses of methacholine resulted in an increase in airway resistance, overall tissue resistance, and elastance (Rn, G, and H respectively) in AHE-treated mice over Naïve, whereas AHE-MSCs reduced the response, significantly where indicated. Data are presented as peak response normalized to the baseline and then expressed as percent increase over that baseline $\pm$ SEM. $\ominus$, AHE treatment was significantly increased over Naïve. *, AHE-MSC was significantly reduced from AHE. $p \leq .05$ (single symbol), $p \leq .01$ (double symbols). Sample sizes: seven Naïve, eight AHE, five AHE-MSC. Abbreviations: AHE, *Aspergillus fumigatus* hyphal extract; G, overall tissue resistance; H, elastance within the lung; MSC, mesenchymal stromal cells; Rn, airway resistance; SEM, standard error of the mean.

AHE administration generated an increased response to MCh in airway resistance, overall tissue resistance, and elastance within the lung in the control groups (AHE) and reached significance at the 25 mg/ml MCh dose in overall tissue resistance and elastance (Fig. 4). Treatment of recurrent AHR mice with systemic MSCs on day 76 reduced all measures of lung function nearly to naïve response levels (Fig. 4), with significant reductions occurring by the 25 mg/ml MCh dose in overall tissue resistance and elastance within the lung (Fig. 4). The decrease in airway resistance, although showing the same trend, did not reach significance (Fig. 4).

MSCs did not have the same effects of decreasing lung inflammation as those observed in the acute model. Total BAL cell number was not significantly reduced in the recurrent model (Fig. 5A). Furthermore, in contrast to the acute model, recurrent AHE exposure increased numbers of all cell types except macrophages. Paradoxically, MSC administration significantly further increased the numbers of neutrophils and lymphocytes, but not macrophages or eosinophils, compared with Naïve mice (not indicated) and significantly increased the neutrophil population relative to the AHE alone (Fig. 5B). Histological examination of lung tissue sections determined that in this repeated recurrent exposure, AHE resulted in less intense inflammation of the lungs and no further reduction in AHE-induced inflammation following MSC administration (Fig. 5C, 5D).

The pattern of elevated cytokines present in the BAL fluid following recurrent AHE exposure demonstrated similar trends to those seen in the acute model, with increases in levels of IL-4, IL-5, IL-6, and IL-17, confirming the development of the mixed Th2/Th17 inflammatory response to AHE in recurrent exposure. MSC administration resulted in a trend toward reduced levels of each of these cytokines; however, significance was seen only in IL-6 levels (Fig. 6A). No response was observed in IFN γ or IL-13 for either AHE- or AHE-MSC-exposed mice. As in the acute model, levels of IL-3, IL-12(p40), KC, and RANTES were significantly increased with recurrent AHE exposure, and MSC administration effected a nonsignificant trend toward reduction, particularly in IL-12(p40) and RANTES, following MSC administration (Fig. 6B). No significant levels of IL-1RA and IL-10 were observed in either sham-MSC-treated or AHE-exposed mice (Fig. 6B).

MSC Administration Does Not Significantly Alter the T-reg Population in AHE-Induced Acute or Recurrent AAI

Induction of T-reg proliferation has been proposed as a mechanism by which MSCs elicit immunomodulatory effects (discussed and reviewed in [36] and [50]). Treatment of mice with AHE induces a significant increase in the number of FoxP3 $^{+}$ /CD4 $^{+}$ T cells within the spleen in both acute models (supplemental online Fig. 3A) and recurrent models (supplemental online Fig. 3B). MSC administration (either native or EDCI treated) did not significantly alter the percentage of T cells within that population and did not alter the overall number of CD4 cells recovered (data not shown) or the percent of these cells that were CD25 $^{+}$ (data not shown). Analysis of FoxP3 mRNA expression levels by quantitative polymerase chain reaction of whole-lung RNA demonstrated comparable AHE-induced increase but no change following MSC administration in either acute or recurrent models (supplemental online Fig. 3C, 3D).

MSC Administration During Either Acute or Recurrent AHE-Induced AAI Alters Production of IL-17a by Ex Vivo Stimulated Mixed MLN Cells

Mixed lymphocytes isolated from MLNs of AHE-sensitized and acutely challenged mice exhibit increased IL-17a production at 48 hours following ex vivo stimulation with AHE. MSC administration did not affect the ability of these mixed lymphocytes to express IL-17a levels during ex vivo restimulation (Fig. 7A). Interestingly, these same increases were observed in mixed MLN lymphocytes obtained from AHE-EDCI MSC-treated mice. Because the MLN studies required pooling of samples from each experimental group, variance in measurements could not be determined for the different groups. In contrast, in the recurrent AHE-exposure model, pooled mixed MLN lymphocytes demonstrated a robust AHE-stimulated increase in IL-17a (>10-fold increase compared with acute model levels) when examined at 48 hours, whereas MSC administration resulted in a trend toward reduction in IL-17a (Fig. 7B). These results suggest that MSCs alter cell recruitment and secretion of IL-17a within the lungs following acute exposure but do not alter IL-17a secretion following

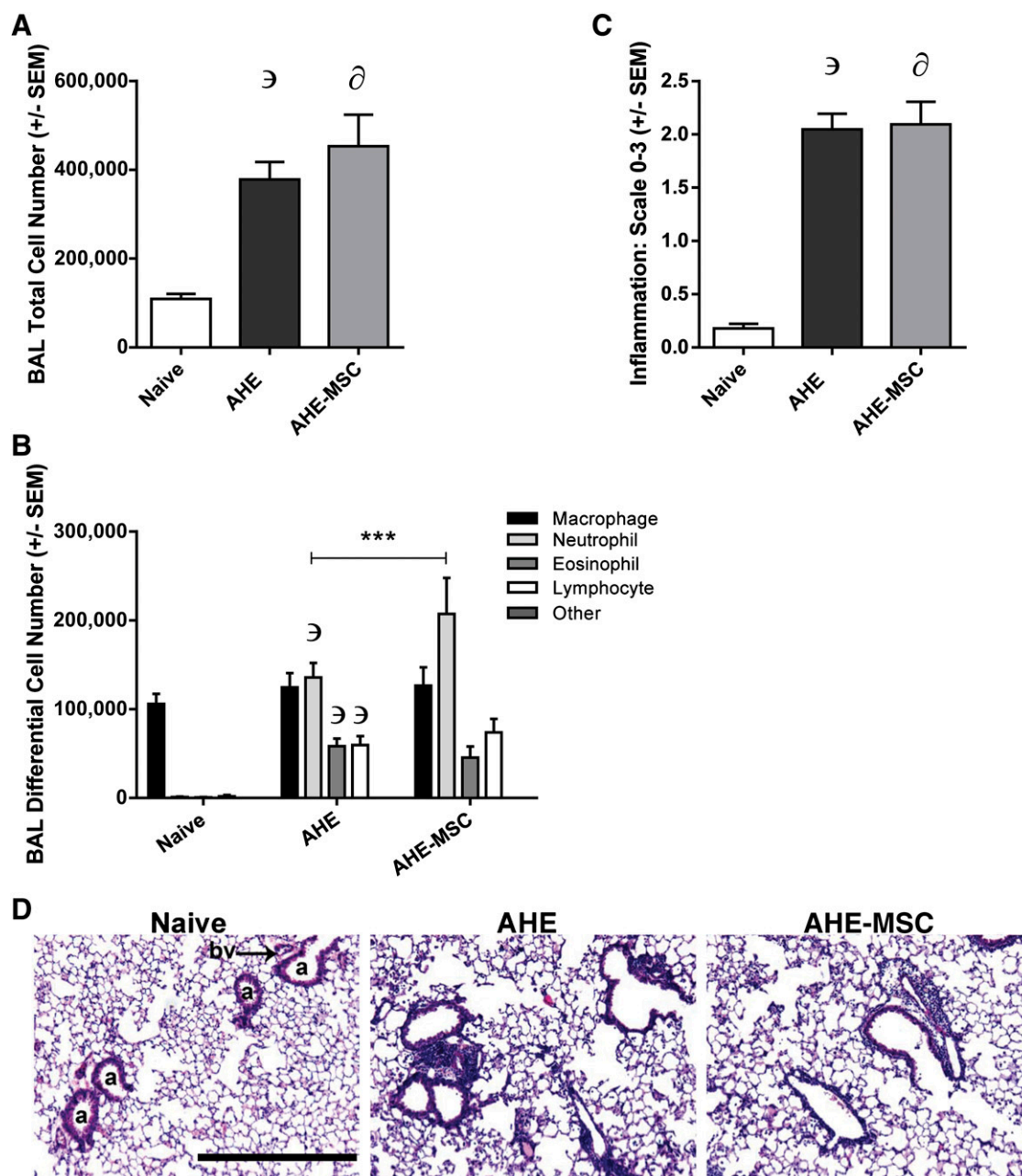


Figure 5. Recurrent airway hyper-responsiveness does not exhibit the same resolution of inflammation following MSC administration. **(A):** Total cell number within the BAL in a recurrent model of AHE, AHE controls and mice administered AHE and MSCs. **(B):** Differential cell population within the BAL, normalized to total cell numbers in the recurrence of AHR model. **(C):** Inflammation score of airways in AHE-MSC-treated mice compared with AHE in the recurrence of airway hyper-responsiveness model. **(D):** Representative images of hematoxylin and eosin-stained lung sections. Original magnification: $\times 10$. Scale bar = 500 μ m. Data are presented as means $\pm$ SEM. $p \leq .05$ (single symbol). $\ominus$, AHE was significantly increased over Naive. ∂ , AHE-MSCs were significantly greater than Naive. *, AHE-MSC was significantly different from AHE. Sample sizes: seven Naive, eight AHE, five AHE-MSC. Abbreviations: a, airway; AHE, *Aspergillus fumigatus* hyphal extract; BAL, bronchoalveolar lavage; bv, blood vessel; MSC, mesenchymal stromal cells.

restimulation, whereas in the recurrent model, MSCs appear to inhibit restimulation responses.

DISCUSSION

Asthma is a heterogeneous, immune-mediated lung disease in which a variety of immune cells play pathogenic roles. We and others have found that in mouse models of mild Th2-

eosinophilic mediated AAI, systemic administration of MSCs either during initial sensitization or during challenge significantly ameliorates both AHR and lung inflammation [27–38]. In the current study, we demonstrated that systemic MSC administration during challenge ameliorates AHR and lung inflammation in a more severe model of acute mixed Th2/Th17-mediated neutrophilic AAI induced by mucosal exposure to and challenge with adjuvant-free AHE. MSC administration was also found to reduce

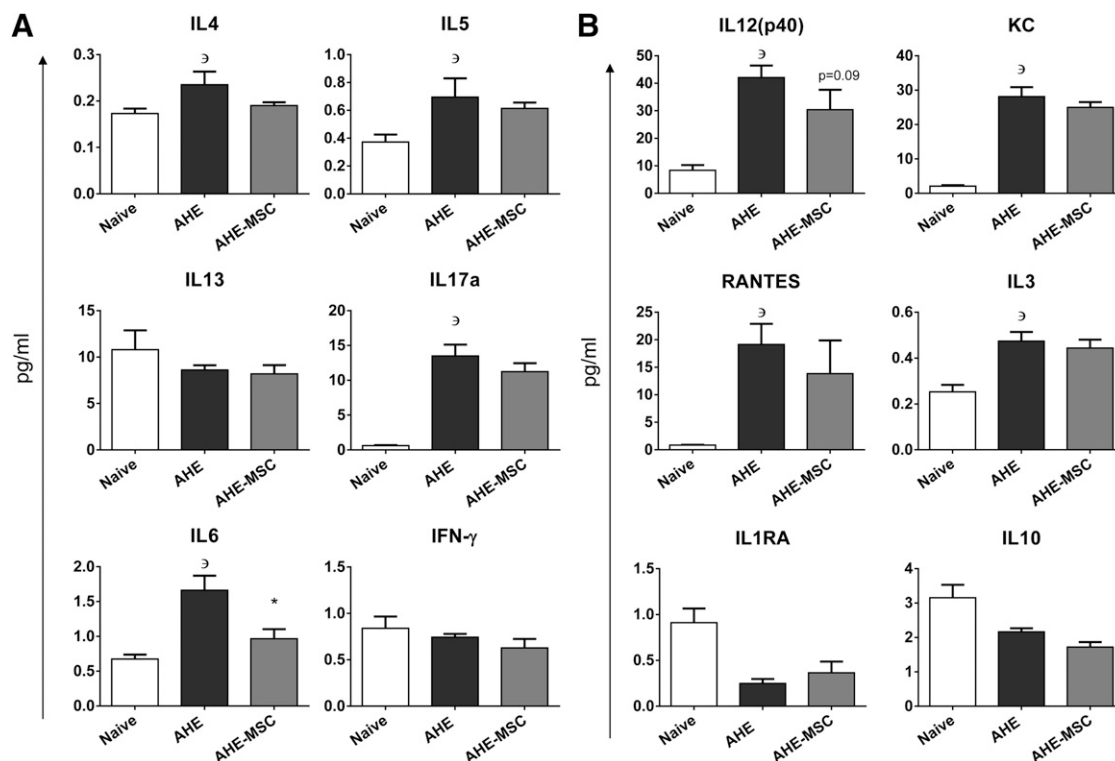


Figure 6. MSC administration in recurrent airway hyper-responsiveness inhibits the soluble cytokines associated with the T helper cell (Th) type 17 pathway. **(A):** Bronchoalveolar lavage supernatants from mice given recurrent AHE-induced AAI assessed by Bio-Plex for the presence of soluble cytokines associated with Th2 (IL-4, IL-5, IL-13), Th17 (IL-6, IL-17a), and Th1 (IFN γ) inflammation. **(B):** Bio-Plex analysis of recurrent model bronchoalveolar lavage supernatants of further Th17 inflammation associated cytokines (IL-12(p40), KC), alternate inflammatory cytokines (RANTES, IL-3), and cytokines previously identified as secreted by MSCs mediating immunomodulation (IL-1RA, IL-10). All units are picograms per milliliter. Data are represented by the average $\pm$ SEM. $p \leq .05$ (single symbol). $\exists$, AHE was significantly increased over Naive. *, AHE-MSC was significantly different from AHE. Sample sizes: seven Naive, eight AHE, five AHE-MSC. Abbreviations: AHE, *Aspergillus fumigatus* hyphal extract; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; IFN, interferon; IL, interleukin; MSC, mesenchymal stromal cells.

AHR but not the lung inflammation provoked by recurrent AHE administration. Importantly, administration of MSCs to sham-sensitized mice did not affect any of the inflammatory endpoints assessed. In addition, MSCs can be found localized within the lungs of AHE-treated mice longer than in the lungs of naïve controls. The MSCs appear to be working through release of soluble mediators and through cell contact; EDCI-treated MSCs were less able to resolve the AHR but produced similar inhibition of AHE-provoked lung inflammation. MSC administration differentially affected IL-17a secretion by ex vivo stimulated mixed MLN lymphocytes in the acute versus recurrent AHE-exposure models but did not affect levels of splenic or lung T-regs in either model.

In previous studies of mild acute Th2-mediated eosinophilic AAI in mice, the mechanism of MSC action has been suggested to be a balancing of the immune response, moderating the adjuvant induced Th2 inflammatory environment with either upregulation of T-regs [36] or by increasingly counterbalancing Th1 cytokines [31, 38, 51] both in BAL fluid and among those produced by antigen-specific T cells in ex vivo analyses. Increased BAL fluid levels of the anti-inflammatory cytokine IL-10 or increased IL-10 secretion by cultured splenocytes has also been observed [34]. In parallel, increased Th1- and decreased Th2-associated serum antigen-specific immunoglobulin levels were observed in MSC-treated animals [31, 38, 51]. Notably, allogeneic MSCs have comparable effects, as do syngeneic MSCs [31, 36], and control cell

types used, such as primary dermal fibroblasts, only partially mimicked the MSC effects, whereas an unrelated cell type such as bone marrow-derived macrophages did not [27, 31, 36]. In addition, xenogeneic MSC administration using rat bone marrow-derived MSCs decreased AHR and lung inflammation in an immunocompetent mouse model of acute AAI produced by toluene diisocyanate inhalation [35]. Comparably, xenogeneic administration of human MSCs in immunocompetent mice decreased lung inflammation in both acute and chronic models of ovalbumin-induced AAI, although effects on AHR were not assessed [27, 28].

The current studies expand the available information and demonstrate that systemic MSC administration can also ameliorate AHR and lung inflammation in a more severe model of acute mixed Th2/Th17 eosinophilic and neutrophilic AAI in mice. MSC administration significantly decreased BAL fluid IL-17a levels and the total number of neutrophils. Notably, in the syngeneic acute AHE-induced AAI model used in this study, no increase in BAL fluid of Th1 or Th2 cytokines was observed following MSC administration. Concurrent with decrease in BAL fluid neutrophils, cell recruitment-associated cytokines KC and RANTES were significantly reduced. Additional significant reductions are seen in IL-3, a mast cell attractant chemokine [52], and IL-12(p40), a key subunit of IL-23 that functions as an autocrine regulator of the Th17 phenotype [7, 53]; however, no change in IL-10 was observed. These data suggest that in an adjuvant-free model, MSCs may

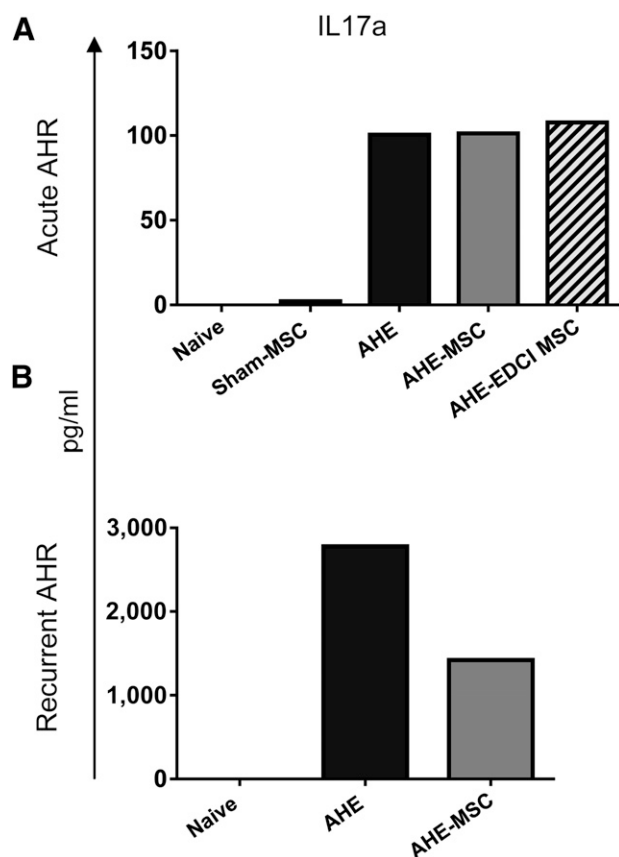


Figure 7. MSC administration alters IL-17a production in ex vivo restimulation of mediastinal lymphocytes from recurrent but not acute AHE exposure. **(A):** Assessment of supernatants from mixed mediastinal lymph node cell populations ex vivo restimulated for 48 hours with AHE antigen. The IL-17a levels were significantly increased in all AHE-treatment groups in the acute model. **(B):** IL-17a levels were significantly increased in the recurrent model, with MSC treatment leading toward a trend in reduced secretion. All units are picograms per milliliter. Data are derived from mediastinal lymph nodes pooled from mice within the same treatment group, and thus a single data point is indicated. Acute sample sizes: 18 Naïve, 3 sham-MSC, 23 AHE, 16 AHE-MSC, 4 AHE-EDCI MSC. Recurrent sample sizes: seven Naïve, eight AHE, five AHE-MSC. Abbreviations: AHE, *Aspergillus fumigatus* hyphal extract; AHR, airway hyper-responsiveness; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; IL, interleukin; MSC, mesenchymal stromal cells.

function by suppression of Th17 responses alone without T-reg induction or a counterbalancing increase in either Th1 or Th2 phenotype. This response is limited to the recruitment of cells and cytokine release because transcriptional events are not apparently altered. However, ex vivo mixed lymphocyte culture demonstrated that MSC administration in the acute AHE model did not alter restimulation-induced IL-17a secretion. In contrast, MSC administration decreased the markedly elevated levels of IL-17a found in ex vivo stimulated MLNs from the recurrent AHE model. The consistent amelioration of AHR by MSC administration in both models, with the variability in cytokine effects, suggests that MSCs will moderate inflammation in a situation-dependent mechanism. Acutely, MSCs appear to act locally on cell influx and cytokine secretion, whereas in a recurrent repeated exposure to antigen, MSCs primarily inhibit the restimulation response (AHE or MCh induced). Further study with timed responses and

isolated antigen-specific T cells, dendritic cells, and other components of the inflammation response will help elucidate these observations.

EDCI-treated MSCs had similar effects compared with MSCs in the acute AHE-exposure model, but this reached significance only in airway inflammation and BAL cytokine profiles, with more limited effect on AHR. In this mixed Th2/Th17 model, these results suggest a more complex mechanism of action of MSCs within the lung. These findings are consistent with possible mechanisms of MSC action in models of Th2-mediated eosinophilic AAI. In the eosinophilic models, pretreating the MSCs with EDCI to inhibit release of soluble mediators demonstrated clear differences compared with effect of naïve MSC administration, suggesting that release of soluble mediators, or perhaps release of microsomal particles, is a critical mechanism in that model [31, 34, 54, 55]. Importantly, systemic administration of conditioned media obtained from bone marrow-derived MSCs reproduced many of the effects produced by administration of intact functional MSCs in both acute and chronic ovalbumin-induced AAI [32]. In these studies, adiponectin released by the MSCs was a critical mediator of the effects of the conditioned media. Comparably, transforming growth factor- β release by the MSCs has been postulated as a critical soluble mediator of the MSC effects in acute ragweed pollen-induced AAI [36]. At present, the specific soluble mediators, possibly including microsomes, that play a role in MSC amelioration of AHE-induced AAI have not yet been clarified.

MSC-stimulated increase in T-regs has also been postulated as a mechanism for alleviating AAI and for other models of T-cell-mediated inflammation [32–34, 50, 56]. In the current studies, AHE exposure stimulated an increase in the T-reg population of both the spleen and lung; however, levels were not affected by MSC administration in either the acute or recurrent AHE models. This is consistent with the lack of MSC effects on IL-10 levels in BAL fluid or in conditioned media obtained from MLN mixed lymphocyte cultures but is contrary to reports that MSCs suppress Th17 differentiation of splenic T cells by secretion of soluble mediators including IL-10 [56–58].

An important aspect of the current study is evaluation of MSCs in a model of recurrent antigen exposure in previously sensitized mice. Limited available data suggest that MSCs or MSC-conditioned media can have effects in models of chronic AAI, but the mechanisms are unclear [27–32]. In the current studies, MSC administration during a recurrent challenge decreased AHR but not lung inflammation and paradoxically stimulated a significant increase in BAL fluid neutrophils. AHR and lung inflammation may not always occur synchronously and can be affected by different mechanistic pathways and by genetic background [52, 59–62]. Consequently, the dissociation of AHR from inflammation in recurrent MSC-treated, AHE-induced AAI may result from differential effects of MSCs on one or more of these pathways. The timing of AHR response in the recurrent model may also play a role in interpreting the current observation. Peak inflammatory responses to rechallenge, indicated by changes in BAL fluid cells or cytokine levels, may occur within an earlier time window than effects on T-regs, MLN cytokine expression, and/or AHR. More detailed study of the time course of MSC effects in the chronic and acute models is warranted in future studies.

The observed results suggest a mechanism of MSC action on the most prevalent cells associated with the inflammation as it occurs. Consistent suppression of AHR, decrease in BAL fluid cell number, and significant cytokine reduction following MSC

administration in acute but not recurrent AHR would suggest that MSCs may function to moderate the inflammation by eliciting a blockade of recruitment of inflammatory cells in the acute model, whereas in the recurrent model, cells associated with antigen-specific responses within the lymphatic system are the target of MSC suppression, with less MSC-mediated effect on inflammatory cells and cytokines within the lung itself. This important distinction examines the ability of MSCs to be used clinically because patients are not available to be treated with MSCs at the onset of sensitivity or initial asthma presentation during antigen challenge; instead, it is at the recurrence or during chronic persistence of asthma symptoms that patients look for treatment.

CONCLUSION

The current study extends previous observations of MSC effects in models of experimentally induced Th2-mediated eosinophilic AAI to demonstrate potent MSC effects in a mixed model of Th2/Th17 neutrophilic AAI. Furthermore, MSCs can have efficacy following recurrent exposure to AHE. Although the mechanisms of MSC actions remain unclear, these studies provide a firm initial basis for the potential clinical application of MSC-based cell therapy approaches to patients with severe neutrophilic steroid-resistant asthma.

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AUTHOR CONTRIBUTIONS

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

D.J.W. has a compensated consultancy.

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