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Alveolar Macrophages Are Critical for the Inhibition of Allergic Asthma by Mesenchymal Stromal Cells

Louisa J. Mathias,^{*,1} Sacha M. L. Khong,^{*,1} Lisa Spyroglou,^{*} Natalie L. Payne,[†] Christopher Siatskas,^{*} Alison N. Thorburn,[‡] Richard L. Boyd,^{*} and Tracy S. P. Heng^{*}

Multipotent mesenchymal stromal cells (MSCs) possess reparative and immunoregulatory properties, making them attractive candidates for cellular therapy. However, the majority of MSCs administered i.v. encounter a pulmonary impasse and soon disappear from the lungs, raising the question of how they induce such durable immunosuppressive effects. Using a mouse model of allergic asthma, we show that administration of MSCs isolated from human bone marrow, umbilical cord, or adipose tissue provoked a pronounced increase in alveolar macrophages and inhibited hallmark features of asthma, including airway hyperresponsiveness, eosinophilic accumulation, and Th2 cytokine production. Importantly, selective depletion of this macrophage compartment reversed the therapeutic benefit of MSC treatment on airway hyperresponsiveness. Our data demonstrate that human MSCs exert cross-species immunosuppressive activity, which is mediated by alveolar macrophages in allergic asthma. As alveolar macrophages are the predominant immune effector cells at the air–tissue interface in the lungs, this study provides a compelling mechanism for durable MSC effects in the absence of sustained engraftment. *The Journal of Immunology*, 2013, 191: 000–000.

Multipotent mesenchymal stromal cells (MSCs) are fibroblastic precursor cells, initially identified in the bone marrow, that have the stem cell–like ability to expand in culture and differentiate into osteoblasts, chondrocytes, and adipocytes (1, 2). Beyond their multilineage potential, MSCs came into prominence as a highly immunosuppressive cell population exhibiting extensive immunomodulatory properties that are independent of histocompatibility (3). As MSCs do not express MHC class II or costimulatory molecules, a single injection of MSCs does not appear to elicit a strong immune response (4, 5). In addition, clinical experience indicates that MSC administration is

safe and can promote hematopoietic engraftment and tissue repair (3). When delivered i.v., MSCs are cleared rapidly from the blood and are localized briefly in the lungs, where they secrete anti-inflammatory molecules that have local and distal effects (6, 7). Therefore, pulmonary conditions could benefit from the immunosuppressive and anti-inflammatory activity of MSCs in the lungs, as demonstrated in animal studies using bleomycin-induced pulmonary fibrosis (8–10).

Asthma is characterized by bronchial inflammation, hyperresponsive airways, and airflow obstruction caused by increased mucus secretion. Most asthma cases are classified as allergic asthma, in which the sensitized immune system reacts adversely to subsequent inhalation exposure to innocuous environmental triggers (11). Animal studies using model allergens have shown that administration of mouse bone marrow MSCs during airway challenge suppressed Th2-driven allergic responses by producing TGF- β and recruiting regulatory T cells (Tregs) to the lungs (12, 13). Other studies have indicated that mouse MSCs inhibit Th2-mediated allergic airway inflammation by promoting IFN- γ production (14, 15). Similarly, human bone marrow MSCs have been shown to suppress airway inflammation in mouse models of acute and chronic asthma (16, 17), but an improvement in lung function was not demonstrated. Although the use of human MSCs (hMSCs) is more clinically relevant, the mechanisms underlying their effects in asthma are not known, as species variation in MSC-mediated immunosuppression has been documented (18). The therapeutic effects of hMSCs have been attributed to paracrine mechanisms (7), but their limited engraftment suggests that these soluble factors would have to mediate long-term immunomodulatory effects. We therefore sought to determine the critical cellular responses that mediate the therapeutic effects of a variety of clinically applicable hMSC types.

Materials and Methods

Animals

Female 6- to 8-wk-old BALB/c mice were obtained from Monash Animal Services and the Walter and Eliza Hall Institute, and maintained under specific pathogen-free conditions at Monash University Animal Research Laboratories. All animal experimentation and procedures were conducted

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Abbreviations used in this article: AHR, airway hyperresponsiveness; AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; Cdyn, dynamic compliance; CFU-F, fibroblastic CFU; DLN, draining lymph node; eGFP, enhanced GFP; hADMSC, human adipose tissue MSC; hBMSC, human bone marrow MSC; hMSC, human MSC; hUCMSC, human umbilical cord MSC; IRES, internal ribosomal entry site; MSC, multipotent mesenchymal stromal cell; PAS, periodic acid–Schiff; RI, lung resistance; Treg, regulatory T cell.

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in accordance with institutional guidelines and approved by the Monash University Animal Ethics Committee (approval #SOBSA/MISCL/2007/51, MARP/2011/146).

OVA-induced allergic asthma model

Female BALB/c mice were sensitized with an i.p. injection of 50 μ g OVA (OVA, grade V; Sigma-Aldrich) with 2 mg aluminum hydroxide gel (alum; Sigma-Aldrich). Control mice received PBS with 2 mg alum. On days 8, 9, and 10, mice were challenged with 50 μ g OVA by intranasal administration under light anesthesia. To examine the effects of MSC administration upon OVA challenge, mice received 1×10^6 MSCs in 200 μ l sterile Dulbecco's PBS (Invitrogen) via tail vein injections on days 5, 6, and 7. To examine the effects of alveolar macrophage (AM) depletion, mice received 60 μ l clodronate-encapsulated liposomes (5 mg/ml clodronate in liposomal structures containing 18.8 mg/ml phosphatidylcholine and 4.2 mg/ml cholesterol in PBS; Encapsula NanoSciences) via intranasal administration under light anesthesia on days 3 and 5. Control mice received plain liposomes.

Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured by restrained invasive plethysmography 1 d after the last intranasal OVA challenge. Mice were anesthetized by i.p. injection of a mixture containing 5 mg/ml ketamine hydrochloride (Parnell, Australia) and 1 mg/ml xylazine hydrochloride (Ilium Xylazil-20, Troy Laboratories, Australia) at a dose of 75 mg/kg and 15 mg/kg body weight, respectively. A small incision was made to expose the trachea, and a cannula was inserted to connect to an inline nebulizer and ventilator. Mice were challenged with aerosolized PBS followed by increasing doses of methacholine (Sigma-Aldrich). Airway resistance and dynamic compliance (C_{dyn}) were determined by analysis of pressure and flow waveforms (Buxco).

Bronchoalveolar lavage and lung digestion

Mice were euthanized by pentobarbitone overdose 2 d after the last intranasal OVA challenge. Bronchoalveolar lavage fluid (BALF) was obtained by instilling three washes of 0.4 ml PBS with 0.1% BSA. BALF was centrifuged at $470 \times g_{\max}$ for 5 min, and cells were enumerated and labeled for flow cytometric analysis. For lung digestion, lungs were perfused with PBS and 0.6–0.8 ml 300 U/ml collagenase type I (Sigma-Aldrich), and 50 U/ml DNase I (Roche) in RPMI 1640 was injected into the trachea. Lungs were then removed, minced into small pieces, and digested at 37°C for 30–40 min. Lung pieces were disrupted with a syringe plunger, filtered through nylon mesh, and centrifuged. The cell pellet was resuspended in RBC lysis buffer, washed, enumerated, and labeled for flow cytometric analysis.

Lung histology

Mice were euthanized by pentobarbitone overdose and bled out by severing the aorta in the lower abdominal cavity. Lungs were perfused with PBS by injection into the right atrium of the heart, inflated with 10% neutral buffered formalin via the trachea, tied off, and fixed in formalin overnight. Lungs were then washed in 70% ethanol, embedded in paraffin, sectioned, and stained with H&E for detection of inflammatory cells and periodic acid-Schiff (PAS) for detection of mucin in goblet cells (mucus-secreting cells) by light microscopy.

Bone marrow MSC culture

Human bone marrow-derived MSCs were purchased from the Tulane Center for Gene Therapy (Tulane University, New Orleans, LA). Briefly, mononuclear cells in bone marrow aspirates were separated using density gradient centrifugation, and nonadherent cells were removed from culture after 18–24 h. Adherent cells were expanded by plating at 60 cells per cubic centimeter in α -MEM supplemented with 16.5% batch-tested FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μ g/ml streptomycin (MSC media) in a 37°C, 5% CO₂ humidified incubator. MSC media were changed every 2–3 d, and cells were harvested when 70–80% confluent. Passage 3 cells were used in all experiments, unless otherwise stated.

Umbilical cord MSC isolation

Umbilical cords from scheduled healthy caesarean sections (Southern Health ethics approval #2008000257; performed with informed patient consent) were collected in HBSS (Invitrogen) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) and kept at 4°C for no longer than 24 h before being processed. The cord was cut into 1-cm-long

pieces and washed thoroughly with cold PBS until clean of cord blood and blood clots. Pieces were finely minced and digested in 0.1% collagenase B (Roche) in PBS at 37°C on an orbital shaker at 170 rpm for 4 h. The suspension was then diluted 1:5 with PBS at room temperature and filtered through 100- μ m sterile nylon cell strainers and centrifuged at $840 \times g_{\max}$ for 10 min. Cells were plated at 4000–6000 cells per cubic centimeter in MSC media, and nonadherent cells and debris were removed after 24–48 h. Adherent cells were cultured as for bone marrow MSCs. Passage 3 cells were used in all experiments, unless otherwise stated.

Adipose MSC isolation

Subcutaneous adipose tissue was obtained from outpatient liposuction procedures as described (19), with some modifications. Briefly, human lipoaspirate was obtained from elective liposuction procedures (Monash human ethics approval #2007/1798; performed with informed patient consent). Lipoaspirate was filtered through a sterile stainless steel sieve, and the collected tissue was rinsed thoroughly with PBS to remove blood. The clean fat was then weighed and digested in 1.5 mg/ml collagenase type 1 (Sigma-Aldrich) at 37°C on an orbital shaker for 1.5 h. The vascular fraction of the digest supernatant was removed, filtered through a sterile 100- μ m nylon mesh, and centrifuged at $470 \times g_{\max}$ for 10 min. The cell pellet was then treated with RBC lysis buffer (STEMCELL Technologies) according to the manufacturer's instructions. Cells were washed and plated at 1.5×10^5 cells per cubic centimeter in MSC media, and nonadherent cells were removed after 24 h. Adherent cells were cultured as for bone marrow MSCs. Passage 3 cells were used in all experiments, unless otherwise stated.

Fibroblastic CFUs assay

Fibroblastic CFU (CFU-F) frequency was determined by plating, in triplicate, 100 cells in 10 ml MSC media in a 10-cm² dish at 37°C, 5% CO₂ for 14 d. MSC media were washed off, and cells were then stained with 3% (w/v) crystal violet in methanol for 5–10 min, and colonies 2 mm or larger were counted. The average of three plates is represented as percent CFU-F.

Adipogenic differentiation

Confluent cells were cultured in MSC medium supplemented with 0.5 μ M dexamethasone, 0.5 μ M isobutylmethylxanthine, and 50 μ M indomethacin for 21 d, with media changes every 3–4 d. Cells were then fixed in 10% neutral buffered formalin and stained with 0.3% Oil Red O for 20 min. Lipid droplets were assessed by bright-field microscopy.

Osteogenic differentiation

Confluent cells were cultured in MSC medium supplemented with 10 nM dexamethasone, 20 mM β -glycerophosphate, and 50 μ M L-ascorbic acid 2-phosphate for 21 d. Cells were then fixed in 10% neutral buffered formalin and stained with 1% Alizarin Red S for 20 min. Calcified structures were assessed by bright-field microscopy.

Lentiviral transduction of MSCs

The cDNA for firefly luciferase (fluc) was subcloned and blunt-end ligated upstream of the internal ribosomal entry site (IRES)–enhanced GFP (eGFP) cassette of the parental pWPI lentiviral plasmid (obtained from Professor Didier Trono, Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland), generating the transfer vector pWPI-fluc-IRES-eGFP. Viral stocks were generated by transfecting pWPI-fluc-IRES-eGFP together with pSPAX2 and pMD.2G, using FuGENE 6 (Roche) into 293T cells (ATCC CRL-11268). Cell culture supernatant was collected and passed through a 0.22- μ m filter, and the viral titer was calculated after flow cytometric determination of eGFP in transduced HeLa cells (ATCC CCL-2). Typically, $0.5\text{--}1 \times 10^7$ transducing units per milliliter of unconcentrated vector was generated in this manner. Passage 3 human bone marrow MSCs (hBMMSCs) were transduced twice at a multiplicity of infection of 50 over a 48-h period with unconcentrated lentiviral supernatant diluted 1:2 with culture medium in the presence of 8 μ g/ml protamine sulfate (Sigma-Aldrich). Transduction medium was replaced with fresh medium 16 h after each transduction.

Bioluminescence imaging

Mice were transplanted via the lateral tail vein with 1×10^6 transduced hBMMSCs on days 5, 6, and 7. To track gene-modified cells, mice were injected i.p. with 200 μ l D-luciferin (15 mg/ml in PBS, VivoGlo Luciferin; Promega) on days 7, 8, 10, 11, and 12. On these days, mice were anesthetized for 10 min with 2.5% isoflurane in oxygen prior to bioluminescence imaging, which was performed with an IVIS 200 system (Xenogen). The fluc luminescent signal intensity was analyzed using Living Image 3.2 Software (Xenogen).

Fixation and permeabilization of MSCs

MSCs were fixed in BD Cytofix/Cytoperm solution (BD Biosciences) at a cell concentration of 1×10^6 cells per 250 μ l at 4°C for 20 min, then washed twice and resuspended in sterile Dulbecco's PBS for i.v. injection.

Human MLR

PBMCs were isolated by density gradient centrifugation, and 1×10^5 cells from two different donors were mixed in 96-well round-bottom plates with irradiated (30 Gy; to prevent any unintentional proliferation) MSCs at 1:10 MSC/PBMC ratio at 37°C, 5% CO₂ for 6 d. Cells were pulsed with [³H]-thymidine during the last 18–24 h, then harvested using a Tomtec plate harvester (Tomtec) onto a glass fiber filter (PerkinElmer). MicroScint-O liquid scintillation mixture (PerkinElmer) was added to the dried filter, and [³H]-thymidine incorporation was measured on a TopCount NXT (PerkinElmer).

In vitro proliferation assays

For anti-CD3 activation of T cells, 96-well round-bottom plates were coated with 50 μ l per well of 10 μ g/ml purified NA/LE anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) (BD Biosciences) in triplicates, sealed with parafilm and incubated at 4°C overnight. Wells were then washed twice with sterile PBS to remove excess Abs. Next, 30 Gy-irradiated MSCs were added to triplicate wells containing 2.5×10^5 spleen cells from untreated mice at various MSC/spleen cell ratios for 3 d.

For OVA restimulation, 100 μ g/ml OVA was added to each well containing 2.5×10^5 bronchial and mediastinal lymph node cells from OVA-sensitized mice and 30 Gy-irradiated MSCs at 1:10 MSC/lymph node cell ratio. Cells were cultured for 4 d and pulsed with [³H]-thymidine during the last 18 h.

Flow cytometric analysis

All Abs were purchased from BD Biosciences, unless otherwise stated. MSCs were labeled with mAbs specific for human CD14 (clone 61D3), CD19 (clone H1B19), CD31 (clone WM59), CD34 (clone 581), CD44 (clone G44-26), CD73 (clone AD2), CD86 (clone 2331), CD90 (clone 5E10), CD105 (clone SN6), CD133/1 (clone AC133; Miltenyi Biotec), CD146 (clone P1H12), HLA-ABC (clone G46-2.6), HLA-DR (clone Tü39), HLA-G (clone 4H84), and platelet-derived growth factor receptor α (clone PRa29; R&D Systems). Mouse cells were labeled with mAbs specific for mouse CD3 (clone 145-2C11), CD4 (clone RM4-5; eBioscience), CD8 (clone 53-6.7), CD11b (clone M1/70; BioLegend), CD11c (clone HL3), CD25 (clone PC61), CD45R (B220, clone RA3-6B2), CD193 (clone TG14/CCR3; BioLegend), F4/80 (clone BM8; BioLegend); MHC class II (I-A/I-E, clone M5/114.15.2; BioLegend and Miltenyi Biotec).

After incubation with primary Abs at 4°C for 15 min, cells were washed with PBS supplemented with 2% BSA, 4 mM EDTA, and 0.01% NaN₃ and were fixed in 1% paraformaldehyde or BD Cytofix/Cytoperm solution (BD Biosciences) after surface labeling. Foxp3 labeling was performed according to the manufacturer's instructions (clone FJK-16a; eBioscience).

Data were collected on a FACSCalibur with CellQuest software, or FACSCanto II with FACSDiva software (BD Biosciences), and analyzed using Gategistic (Invivai, Australia) or FlowJo software. The percentage expression of each marker on MSCs was determined by the percentage of positive events, as determined by the isotype-matched negative control. The cellular composition in BALF was determined according to the method in Ref. 20, with modification, and confirmed by differential cell counts of cytopins. Granulocytes were recognized as non-autofluorescent highly granular (side scatter^{hi}) cells, and within this gate, eosinophils were defined as cells expressing the eotaxin receptor CCR3 and very low to undetectable expression of CD3, B220, and MHC class II. AMs were identified as large autofluorescent cells expressing low levels of CD11b and high levels of CD11c. T lymphocytes were identified as forward scatter^{lo}/side scatter^{lo} cells expressing CD3, and within this gate, Tregs were defined as cells expressing CD4 and Foxp3 compared with isotype control staining.

OVA-specific cytokine production

A total of 4×10^5 bronchial and mediastinal lymph node cells from OVA-sensitized mice were added to 96-well flat-bottom plates containing 100 μ g/ml OVA per well. Supernatant was collected after 4 d of culture and analyzed for various cytokines, using BD Cytometric Bead Array Flex Sets (BD Biosciences) according to the manufacturer's instructions. Data were collected on a FACSCanto II and analyzed using FCAP Array software (BD Biosciences). In vivo cytokine production was also measured by analyzing the amount of cytokines detected in the BALF (0.4 ml) and supernatant (0.5 ml) from homogenized lungs.

RNA isolation, cDNA synthesis, and quantitative PCR analysis

AMs were FACS purified from BALF and enzymatically digested lungs 2 d after the last intranasal OVA challenge, and subjected to RNA isolation using the RNAqueous-Micro Kit (Ambion), according to the manufacturer's instructions. cDNA was synthesized using SuperScript III First-Strand Synthesis System (Invitrogen) with oligo(dT)20 oligonucleotides (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using Platinum SYBR Green Supermix-UDG (Invitrogen) on a Corbett Rotor-Gene 3000 (Corbett Research, Australia). The PCR

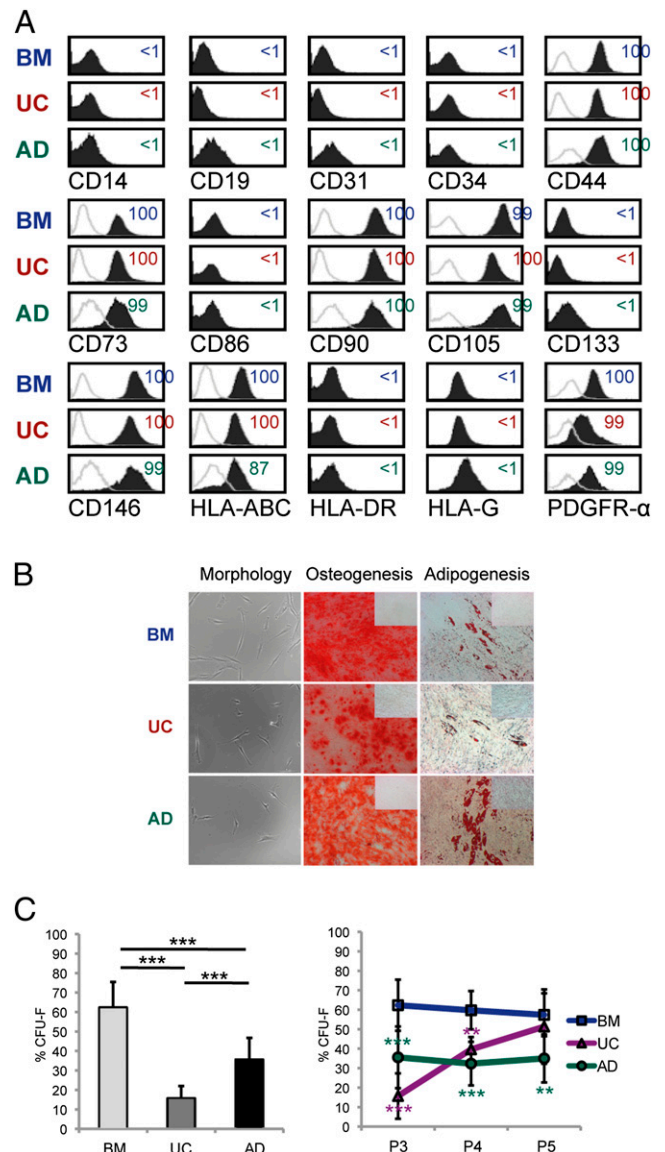


FIGURE 1. MSCs isolated from human bone marrow (BM), umbilical cord (UC) and adipose tissue (AD) exhibit similar characteristics. **(A)** Expression of cell surface markers (black histograms), compared with isotype controls (unshaded histograms), on passage 3 MSCs. Flow cytometric profiles are representative of three donors from independent experiments. **(B)** Spindle-like morphology of passage 3 MSCs (left vertical panels), osteogenic differentiation demonstrated by Alizarin Red S-stained calcium (middle vertical panels), and adipogenic differentiation demonstrated by Oil Red O-stained lipid droplets (right vertical panels). Controls are MSCs cultured in normal MSC medium (inset panels). **(C)** Average CFU-F frequency of passage 3 MSCs from three donors for each tissue source, and CFU-F frequency of MSCs between passage (P) 3, 4, and 5. Results show mean $\pm$ SD of two experiments performed in triplicate ($n = 6$). $**p \leq 0.01$, $***p \leq 0.001$, compared to every other group (one-way ANOVA, Tukey posttest) or compared to BM (one-way ANOVA, Bonferroni posttest).

cycling protocol used consisted of an initial hold for 2 min at 50°C (UDG incubation) to prevent the reamplification of carryover PCR products, followed by a hold for 2 min at 95°C for enzyme activation, followed by 45 cycles of 95°C for 15 s (denaturation) and 60°C for 60 s (amplification and extension). Finally, a melting curve analysis was performed from 60°C to 95°C in which the temperature was increased 0.5°C for 5 s per step. Relative transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method, where ΔC_t is the difference between the threshold cycle for the gene of interest and the threshold cycle for β -actin, and normalized to unsensitized (PBS) controls. Prevalidated primers for IL-10 (QT00106169) and TNF- α (QT00104006) were purchased from QIAGEN. All other primers were purchased from Geneworks (Australia), and their sequences are listed in Table I.

Statistics

Statistical analysis was performed with the unpaired two-tailed Student *t* test (for comparison between two groups), one-way ANOVA with the Tukey posttest (for comparison between three or more groups), or repeated-measures ANOVA with the Dunnett posttest (for AHR dose-response curves), using GraphPad Prism software. Data were represented as mean $\pm$ SEM unless otherwise stated. A $p \leq 0.05$ was considered significant.

Results

MSCs derived from human bone marrow, umbilical cord, and adipose tissue exert cross-species immunosuppressive activity

Bone marrow aspiration is a painful and invasive process that yields a low frequency of MSCs. As umbilical cord and adipose are tissues that are normally discarded, we have investigated these as readily available alternative sources of MSCs. To verify that the cells isolated were MSCs, we examined and compared their culture characteristics, cell surface marker phenotype, and differentiation capacity to conventional human bone marrow MSCs (hBMMSCs) according to the method of Ref. 21. MSCs isolated from digested human umbilical cord (hUCMSCs) and adipose tissue (hADMSCs) could be propagated over several passages without displaying karyotypic abnormalities and exhibited the surface marker phenotype (Fig. 1A) and fibroblastic morphology (Fig. 1B) of hBMMSCs. All three types of hMSCs demonstrated the capacity for differentiation into mesenchymal lineages in vitro (Fig. 1B) and the ability to form fibroblastic CFUs (Fig. 1C). We found that hBMMSCs and hADMSCs, compared with hUCMSCs, differentiated more readily into osteocytes and adipocytes. Similarly, hBMMSCs demonstrated greater colony-forming efficiency than did hADMSCs and hUCMSCs.

The immunosuppressive activity of MSCs has been well documented. When hBMMSCs, hUCMSCs, or hADMSCs were added to a two-way MLR, proliferation of allogeneic human PBMCs was inhibited in a dose-dependent manner (Fig. 2A). This property of MSCs was not restricted by the species barrier, as all three types of hMSCs were equally capable of inhibiting the proliferative response of anti-CD3-activated mouse splenocytes (Fig. 2B). Similarly, when splenocytes obtained from OVA-immunized mice were restimulated in vitro with OVA, Ag-specific proliferation was inhibited in the presence of hMSCs (Fig. 2C).

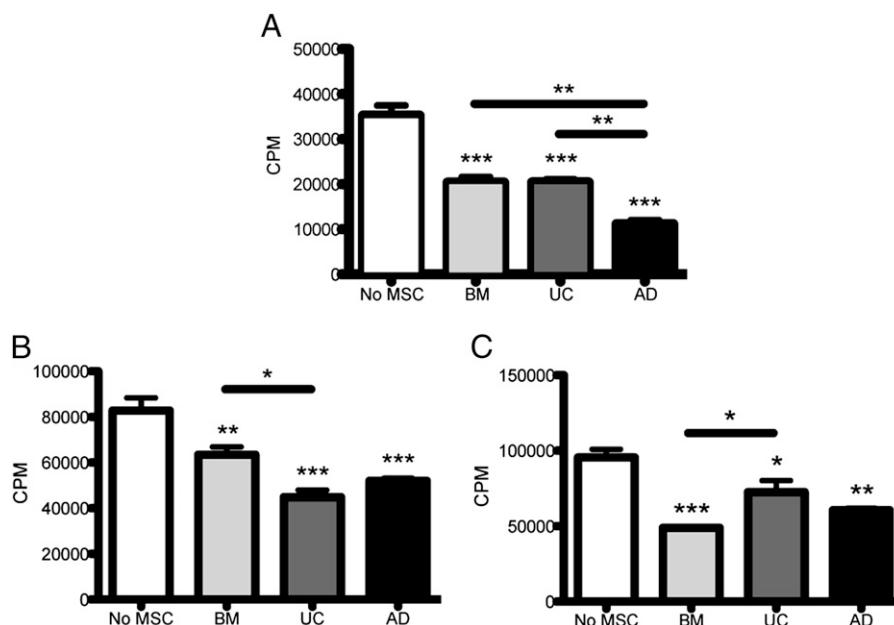
Hence, hMSCs derived from bone marrow, umbilical cord, or adipose tissue all exert potent cross-species immunosuppressive activity that affects lymphocyte proliferation in response to allogeneic stimulation, nonspecific TCR activation, as well as Ag-specific stimulation.

hMSC administration inhibits hallmark features of allergic asthma

As i.v. injected MSCs are retained for a short period in the lungs, where they exert anti-inflammatory effects (7), we used a well-established mouse model of allergic asthma to compare the effects of hUCMSCs and hADMSCs with those of conventional hBMMSCs (Fig. 3A) (22). In this model, mice sensitized to alum-adsorbed OVA, and exposed to repeated airway provocation with the same model allergen, developed AHR, as demonstrated by a dose-dependent increase in lung resistance (RI) and decrease in Cdyn in response to a cholinergic stimulus (methacholine) (Fig. 3B; OVA versus PBS). Administration of hMSCs just prior to acute allergen challenge significantly inhibited AHR, with hUCMSCs and hADMSCs being as effective as hBMMSCs (Fig. 3B). The inhibitory effects of hMSC administration were also evident histologically, as lung tissue sections from hMSC-treated mice showed a reduction in mucus-secreting goblet cells in the airway epithelium and inflammatory cells in airway tissue (Fig. 3C).

Eosinophilic accumulation in the pulmonary airways is another hallmark feature of OVA-induced allergic asthma. Analysis of BALF from OVA-sensitized mice showed that the influx of inflammatory cells was dominated by eosinophils, which constituted

FIGURE 2. hMSCs inhibit lymphocyte proliferation in response to allogeneic stimulation, nonspecific TCR activation, and Ag-specific stimulation across species. (A) Two-way MLR with human PBMCs from two different donors. (B) Anti-CD3-activated mouse splenocytes. (C) OVA-restimulated splenocytes from OVA-immunized mice. hMSCs were added at 1:10 MSC/PBMC or 1:10 MSC/splenocyte ratio. Proliferation was measured by [^{3} H]-thymidine incorporation, and results are expressed as average counts per minute (CPM) of triplicate samples. (A) is representative of two independent experiments, whereas (B) and (C) are average counts from three to five mice, expressed as mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, compared with No MSC (one-way ANOVA, Tukey posttest).



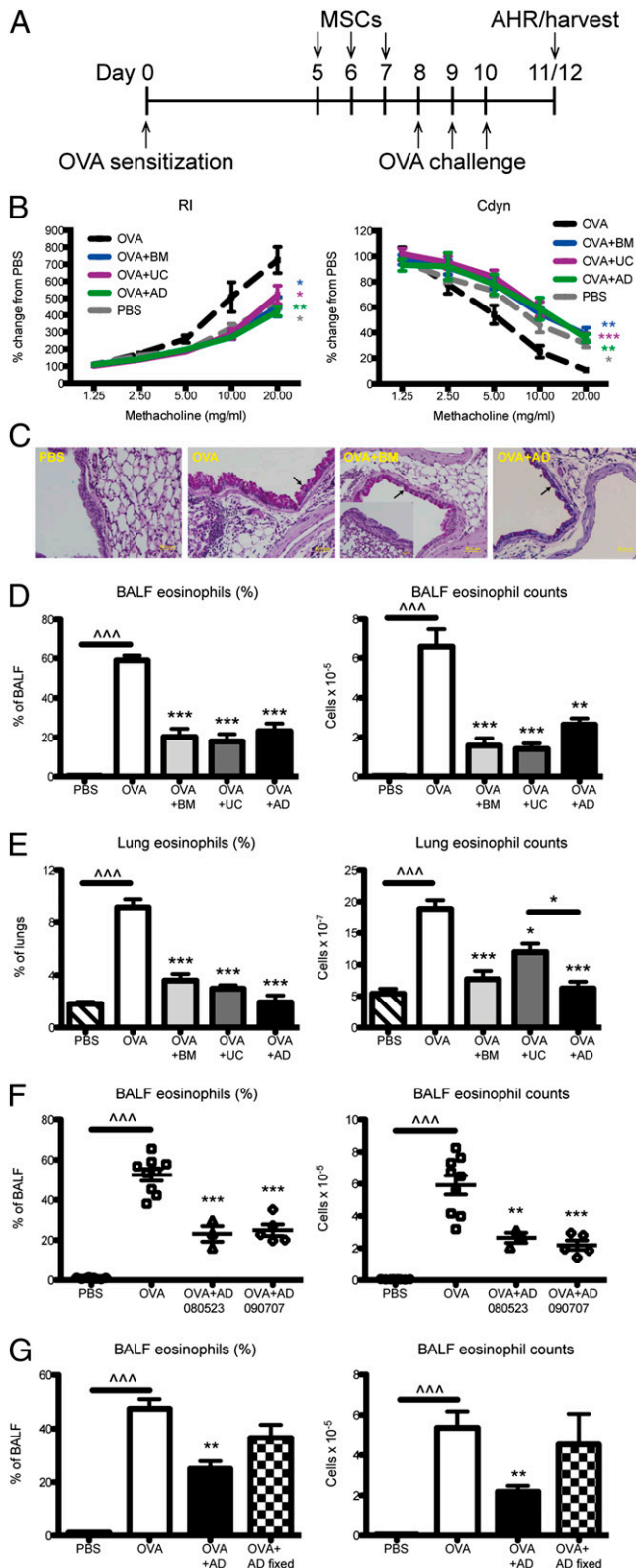


FIGURE 3. hMSC administration inhibits hallmark features of allergic asthma. **(A)** Experimental protocol to assess the effects of hMSC administration on OVA inhalation challenge. **(B)** RI and Cdyn in response to increasing doses of methacholine of unsensitized mice (PBS), OVA-sensitized mice (OVA), and OVA-sensitized mice treated with hMSCs (OVA + BM, OVA + UC, OVA + AD). Results were obtained from six to eight mice per group, expressed as mean $\pm$ SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, compared with OVA (repeated measures ANOVA with the Dunnett posttest for the entire dose-response curve). **(C)** Lung sections stained with H&E and PAS for detection of inflammatory cells and mucus-secreting

goblet cells (arrows). Some areas of lung sections from OVA-sensitized mice treated with hMSCs showed absence of mucin-positive cells (OVA + BM, inset). Histological images were representative of two independent experiments with five mice per group. Original magnification $\times 40$. **(D)** Percentage and cell count of BALF eosinophils and **(E)** lung eosinophils. **(F)** Percentage and cell count of BALF eosinophils from OVA-sensitized mice treated with hADMSCs obtained from different donors. **(G)** Percentage and cell count of BALF eosinophils from OVA-sensitized mice treated with unfixed or fixed/permeabilized hADMSCs (OVA + AD fixed). Results were obtained from three to five mice per group, expressed as mean $\pm$ SEM and representative of three independent experiments. $^{***}p \leq 0.001$ compared with PBS (unpaired two-tailed Student *t* test), $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, compared with OVA (one-way ANOVA, Tukey posttest).

~ 60% of total BALF cells (Fig. 3D). Increased eosinophils in lung digests were also evident in OVA-sensitized mice (Fig. 3E). In hMSC-treated mice, a 3-fold decrease in the proportion and number of eosinophils recovered in the BALF and lungs (Fig. 3D, 3E) was observed. These anti-inflammatory effects of hMSC administration were observed with MSCs obtained from different donors (Fig. 3F). In addition, a reduction in eosinophilic accumulation was not observed when nonviable paraformaldehyde-fixed hMSCs were injected (Fig. 3G), indicating that viable hMSCs are required for this inhibitory function.

To probe the mechanisms of hMSC-mediated inhibition of AHR and eosinophilia, we assayed key mediators of disease in this model. Th2 cells initiate and perpetuate disease by producing Th2-type cytokines that drive key pathophysiological features of allergic asthma (23). IL-4 is important for allergen sensitization and IgE production, IL-5 is crucial for eosinophil recruitment and survival, whereas IL-13 has a central role in AHR and tissue remodeling (24). Upon restimulation with OVA, T cells in the bronchial and mediastinal lymph nodes [draining lymph nodes (DLNs)] of OVA-sensitized mice produced high levels of IL-4, but only hADMSC-treated mice showed a decrease in IL-4 (Fig. 4A). IL-5 release from T cells in the DLNs of OVA-sensitized mice was also very high but was decreased in hMSC-treated mice (Fig. 4B). A decrease in IL-5 levels was also observed in the BALF and lungs of hADMSC-treated mice (Fig. 4C). Similarly, very high levels of IL-13 were produced in OVA-sensitized mice, whereas treatment with hMSCs inhibited this release (Fig. 4D).

IFN- γ production has been shown to inhibit Th2-mediated allergic airway inflammation in several models (23, 24). Studies have also shown an increase in CD4⁺ T cell production of IFN- γ in mice treated with mouse ADMSCs (15), and a failure for mouse BMSCs to inhibit allergic inflammation in IFN- γ receptor null mice (14). However, we did not detect a significant increase in OVA-specific IFN- γ secretion by T cells in the DLNs of hMSC-treated mice (Fig. 4E), indicating that this mechanism is not mediating the attenuation of allergic asthma following hMSC treatment.

Hence, treatment of OVA-sensitized mice with viable hMSCs suppressed the hallmark features of allergic asthma, including AHR, goblet cell hyperplasia, accumulation of BALF and lung eosinophils, and OVA-induced Th2 cytokine release.

Sustained engraftment of hMSCs in the lungs is not required for their inhibitory effects on allergic inflammation and AHR

To determine whether long-term engraftment was required for hMSCs to exert their inhibitory effects on allergic asthma, we used a noninvasive bioluminescence imaging technique to track the biodistribution of injected hMSCs in OVA-sensitized mice.

goblet cells (arrows). Some areas of lung sections from OVA-sensitized mice treated with hMSCs showed absence of mucin-positive cells (OVA + BM, inset). Histological images were representative of two independent experiments with five mice per group. Original magnification $\times 40$. **(D)** Percentage and cell count of BALF eosinophils and **(E)** lung eosinophils. **(F)** Percentage and cell count of BALF eosinophils from OVA-sensitized mice treated with hADMSCs obtained from different donors. **(G)** Percentage and cell count of BALF eosinophils from OVA-sensitized mice treated with unfixed or fixed/permeabilized hADMSCs (OVA + AD fixed). Results were obtained from three to five mice per group, expressed as mean $\pm$ SEM and representative of three independent experiments. $^{***}p \leq 0.001$ compared with PBS (unpaired two-tailed Student *t* test), $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, compared with OVA (one-way ANOVA, Tukey posttest).

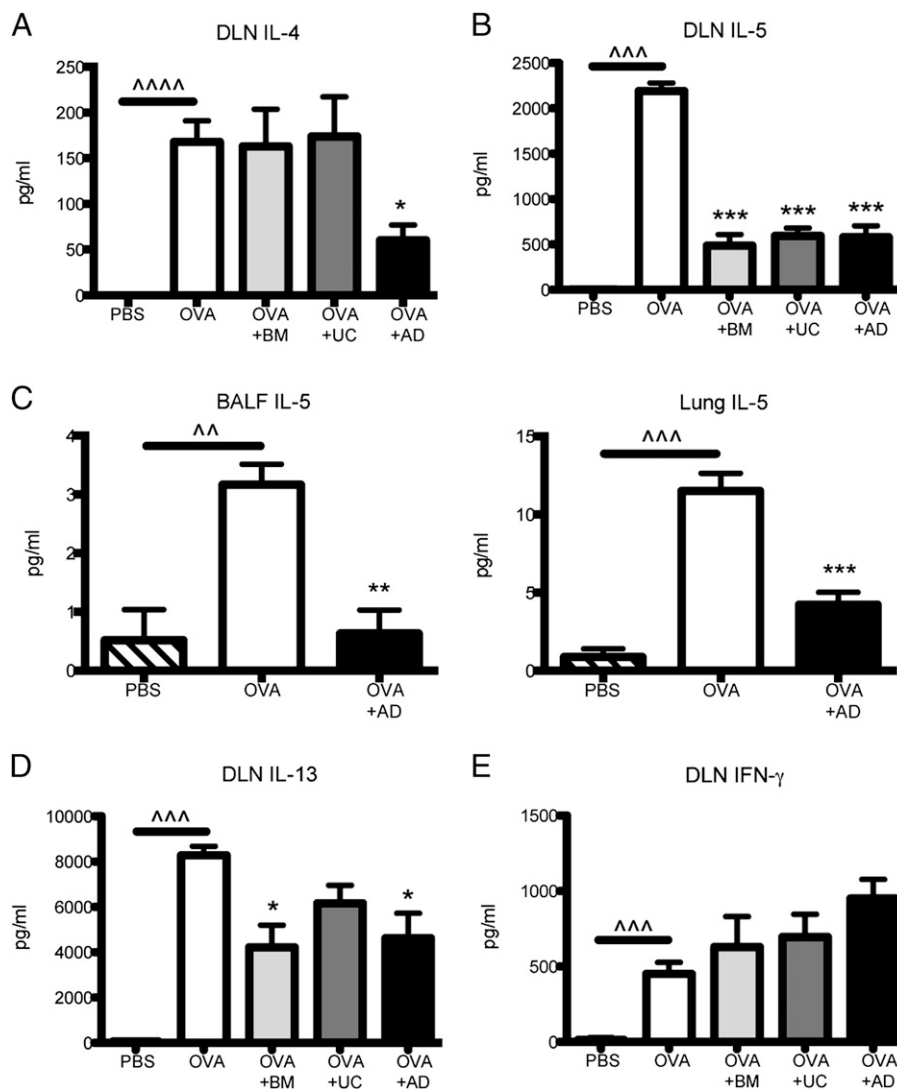


FIGURE 4. hMSC administration suppresses OVA-induced Th2 cytokines. **(A)** Administration of OVA-sensitized mice with hMSCs decreased IL-4 and **(B)** IL-5 release from bronchial and mediastinal lymph nodes (DLNs) following OVA restimulation. **(C)** IL-5 was also decreased in the BALF and lungs of hADMSC-treated mice. **(D)** A decrease in IL-13 release occurred, but no change in **(E)** IFN- γ levels in the DLNs following OVA restimulation. Results were obtained from three to five mice per group, expressed as mean $\pm$ SEM and representative of three independent experiments. $^{\wedge}p \leq 0.01$, $^{\wedge\wedge}p \leq 0.001$, compared with PBS (unpaired two-tailed Student *t* test). $^{\wedge\wedge\wedge}p < 0.0001$. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, compared with OVA [one-way ANOVA, Tukey posttest, or unpaired two-tailed Student *t* test for (C)].

hBMMSCs were transduced with a bicistronic lentiviral vector encoding firefly luciferase (fluc) and eGFP (Fig. 5A) and then administered via tail vein injection prior to intranasal OVA challenge (Fig. 5B). The highest bioluminescence fluc signals were detected in the lungs after the third consecutive injection (day 7) (Fig. 5C), confirming the localization of hMSCs in the lungs. Over the following 48 h, fluc signals diminished rapidly and could no longer be detected by day 10 (Fig. 5C). As bioluminescence imaging may not be sensitive enough to detect low numbers of hBMMSCs remaining, lungs from injected mice were digested and analyzed for GFP expression by flow cytometry. A very small population (0.5% of live cells) of GFP-expressing cells could be detected at day 10, but not at later time points (Fig. 5D), suggesting that substantial persistence of hMSCs in the lungs is not required for the inhibitory effects observed.

In vivo depletion of AMs abrogates effects of hMSC treatment on allergic inflammation and AHR

The short half-life of hMSCs in the lungs could indicate a role for AMs in clearing these cells. In unsensitized mice, the majority of cells recovered in the BALF were CD11b^{lo}CD11c⁺ AMs (25) (Fig. 6A). In OVA-sensitized mice, however, the proportion of AMs decreased to $\sim 20\%$ of total BALF cells (Fig. 6A). Interestingly, hMSC-treated mice exhibited an increase in AMs, both in proportion and in absolute number (Fig. 6B).

To determine whether the increase in AMs was important for the anti-inflammatory effects of hMSCs, we examined the impact of in vivo AM depletion with clodronate-encapsulated liposomes via the intranasal route. Upon phagocytosis and digestion of the liposomes, clodronate is released and accumulates intracellularly, killing macrophages in the alveolar spaces, but not in the lung parenchyma (Fig. 6C, 6D) (26). When OVA-sensitized mice were administered clodronate (Fig. 6E), depletion of AMs did not affect AHR in mice that did not receive hMSCs (Fig. 6F). We then measured AHR in clodronate-treated mice that received hADMSCs. Consistent with our previous data, hADMSC treatment attenuated AHR in OVA-sensitized mice (Fig. 6G, green line) and OVA-sensitized mice that received plain liposomes (Fig. 6G, blue line). However, hMSC treatment was ineffective in OVA-sensitized mice that received hMSCs and clodronate (Fig. 6G, red line), returning AHR to levels equivalent to those in OVA-sensitized mice that did not receive hMSCs (Fig. 6G, black dotted line).

The AHR results were supported by histological staining of lung sections, which showed a reduction in mucus-secreting goblet cells and inflammatory cells in the airways of hMSC-treated mice, but not in mice that received clodronate prior to hMSCs (Fig. 6H). Thus, in vivo depletion of AMs was sufficient to abrogate the beneficial effects of hMSCs on lung function in OVA-sensitized mice.

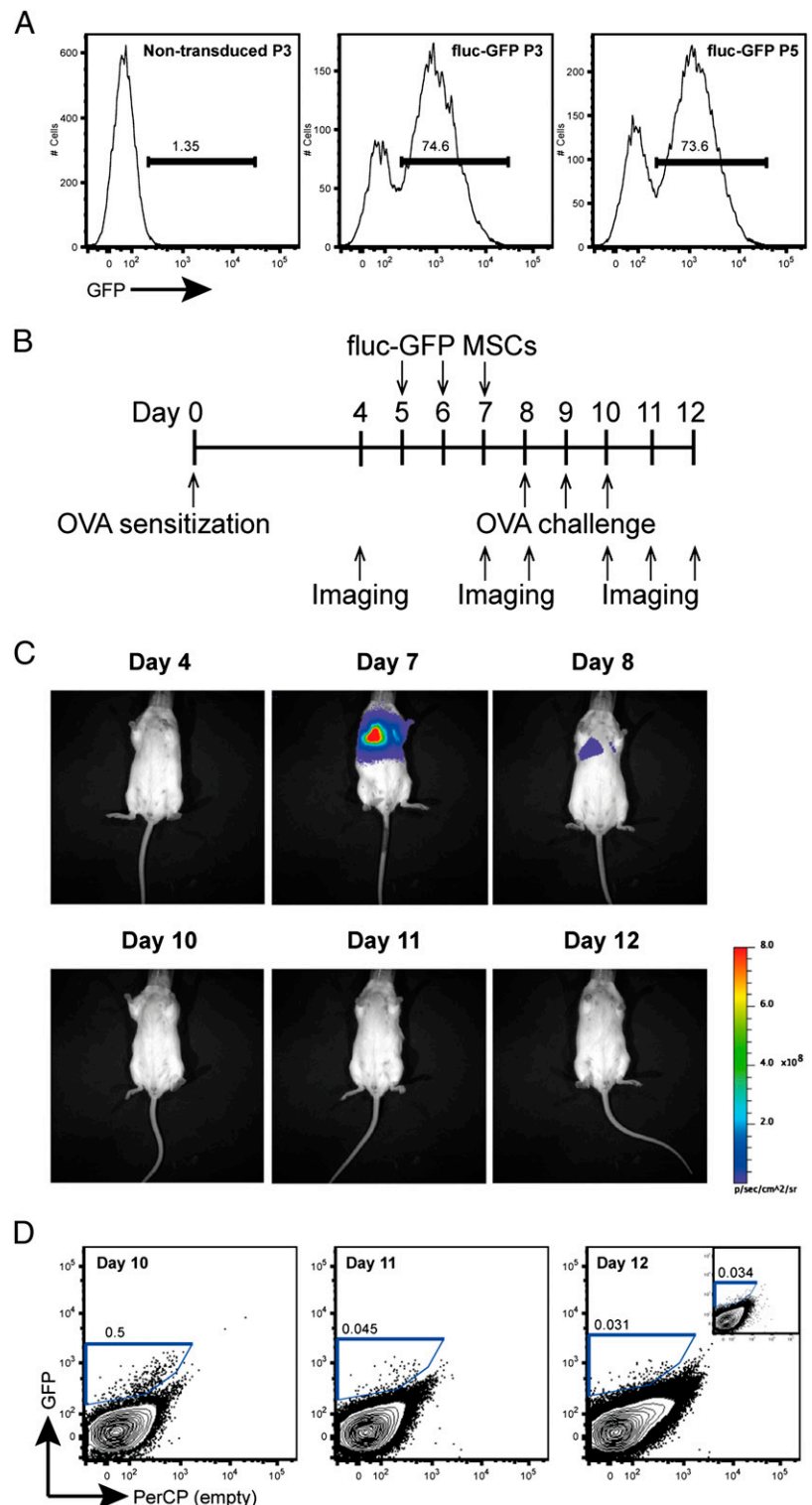


FIGURE 5. The i.v. administered hMSCs are cleared from the lungs within days. **(A)** Flow cytometric profiles of nontransduced hBMSCs and fluc-GFP-hBMSCs, showing stability of fluc-GFP expression following expansion of cells from passage (P) 3, when cells were transduced, to P5, when cells were injected for bioluminescence imaging. **(B)** Experimental protocol to track hMSCs following i.v. injection. **(C)** Bioluminescent images of OVA-sensitized mice at different time points before and after injection with fluc-GFP-hBMSCs. Injected cells could be detected at days 7 and 8, but not at day 10 and later. **(D)** Flow cytometric profiles of digested lungs from OVA-sensitized mice injected with fluc-GFP-hBMSCs. GFP-expressing cells could be detected at day 10, but not at later time points (*inset* shows background GFP levels of noninjected control).

Inhibition of allergic asthma by hMSC administration is dependent on AM-mediated induction of IL-10 production from other cell sources

TSG-6 is an anti-inflammatory protein found to be secreted by hMSCs activated in the lungs (7); it interacts with macrophages to decrease release of proinflammatory mediators (27). In a mouse model of acute lung injury, knockdown of TSG-6 expression in hMSCs by RNA interference abrogated the anti-inflammatory effects of hMSCs, whereas local administration of recombinant

TSG-6 reduced inflammation in the lungs (28). We therefore measured AHR in OVA-sensitized mice administered TSG-6 via the intranasal route instead of i.v. hMSCs. However, we found that TSG-6 could not fully replicate the beneficial effects of hMSCs in OVA-induced allergic asthma (data not shown).

Macrophages are highly heterogeneous cells that rapidly respond to local signals to change their effector function. Classically activated M1 macrophages are activated by IFN- γ and TLR ligands to mediate host defense, whereas alternatively activated M2

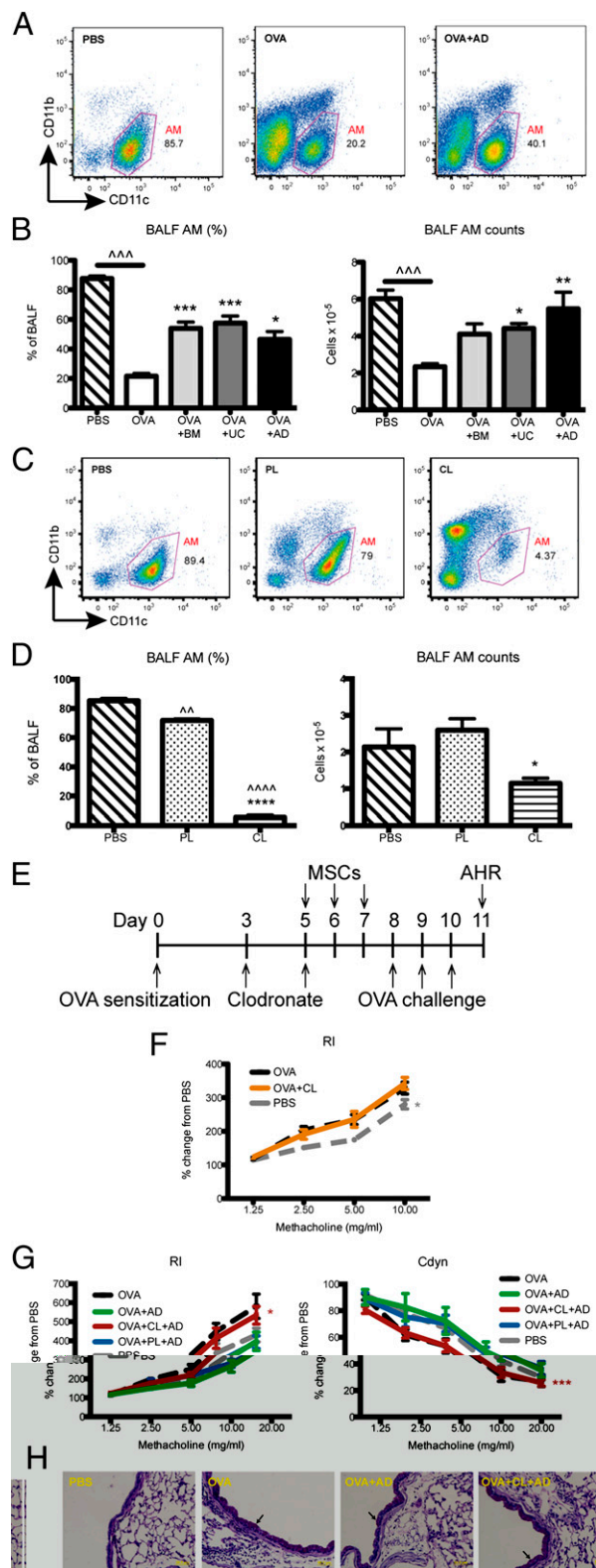


FIGURE 6. In vivo depletion of AMs abrogates effects of hMSC treatment on AHR. **(A)** Representative flow cytometric profiles of BALF cells from unsensitized (PBS) mice, OVA-sensitized mice (OVA), and OVA-sensitized mice treated with hADMSCs (OVA + AD). AMs were identified as CD11b^{lo}CD11c⁺ large autofluorescent cells with high forward and side scatter. **(B)** Proportion (left panel) and cell count (right panel) of AMs in BALF. Results were obtained from three to five mice per group, expressed as mean $\pm$ SEM and representative of three independent experiments. $^{***}p \leq 0.001$ compared with PBS (unpaired two-tailed Student *t* test). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, compared with OVA

macrophages are activated by IL-4 or IL-13 to promote anti-inflammatory function and tissue repair (29). Coculture studies have demonstrated that BMMSCs promote switching of monocytes/macrophages to an M2 phenotype with high IL-10 production (30–32). Therefore, to further define the interactions between hMSCs and macrophages in the lungs, CD11b^{lo}CD11c⁺ AMs were purified from hMSC-treated mice following intranasal OVA challenge for quantification of transcripts for various markers associated macrophage activation, polarization, and function (Table I).

M1 macrophages express proinflammatory cytokines, such as TNF- α (Fig. 7A), and inducible NO synthase (Fig. 7B), whereas M2 macrophages express arginase 1 (Fig. 7C), chitinase Chi3l3 (Fig. 7D), and mannose receptor Mrc1 (Fig. 7E). Although Arg1 and Chi3l3 expression appeared to be lower in lung macrophages from hADMSC-treated mice, these changes were not significant. Therefore, we found no evidence of M1/M2 switching in hADMSC-treated mice following OVA challenge, based on quantification of mRNA expression. We also did not find increased macrophage expression of TGF- β (Fig. 7F), which is involved in generating Tregs in the lungs to promote airway tolerance (33). Likewise, lung macrophages from hADMSC-treated mice appeared to express lower levels of IL-10, but this was not found to be a significant difference (Fig. 7G).

However, quantification of IL-10 protein in whole-lung homogenates showed an increase in OVA-sensitized mice treated with hADMSCs (Fig. 7H; OVA + AD) compared with mice that did not received hMSCs (Fig. 7H; OVA) and mice that were depleted of AMs prior to hMSC administration (Fig. 7H; OVA + CL + AD). IL-10 is a pleiotropic cytokine produced by many cell types, including CD4⁺ Foxp3⁺ Tregs (34). Tregs have been demonstrated to suppress established airway inflammation and AHR in animal models of allergic airway disease (35), by inducing secretion of anti-inflammatory factors such as IL-10 (36). We therefore analyzed the levels of Foxp3⁺ Tregs in digested lung tissues by flow cytometry. OVA-sensitized mice treated with hADMSCs had in-

(one-way ANOVA, Tukey posttest). **(C)** Representative flow cytometric profiles of BALF cells from mice that received PBS, plain liposomes (PL), or clodronate-encapsulated liposomes (CL) via the intranasal route 3 d and 1 d prior to analysis. **(D)** Proportion (left panel) and cell count (right panel) of CD11b^{lo}CD11c⁺ AMs in BALF after intranasal administration of PBS, PL, or CL. Results were obtained from three to four mice per group, expressed as mean $\pm$ SEM. $^{*}p \leq 0.01$, $^{***}p \leq 0.0001$ compared with PBS. $^{*}p \leq 0.05$, $^{***}p \leq 0.0001$ compared with PL (one-way ANOVA, Tukey posttest). **(E)** Experimental protocol to assess the effects of depleting OVA-sensitized mice of AMs on hMSC administration. **(F)** RI in response to increasing doses of methacholine of unsensitized mice (PBS), OVA-sensitized mice (OVA), and OVA-sensitized mice treated with clodronate (OVA + CL). **(G)** RI and Cdyn in response to increasing doses of methacholine of unsensitized mice (PBS), OVA-sensitized mice (OVA), and OVA-sensitized mice treated with hADMSCs (OVA + AD), OVA-sensitized mice treated with clodronate and hADMSCs (OVA + CL + AD), and OVA-sensitized mice treated with plain liposomes and hADMSCs (OVA + PL + AD). Results were obtained from 5 to 10 mice per group, expressed as mean $\pm$ SEM. $^{*}p \geq 0.05$, $^{***}p \leq 0.001$, compared with OVA (repeated-measures ANOVA with the Dunnett posttest for the entire dose-response curve). **(H)** Lung sections stained with H&E and PAS for detection of inflammatory cells and mucus-secreting goblet cells (arrows). OVA + AD mice had fewer inflammatory cells and mucus-producing cells than did OVA mice and OVA + CL + AD mice. Histological images were representative of two independent experiments with five mice per group. Original magnification $\times 40$.

Table I. Primer sequences for quantitative PCR analysis of transcripts from sorted AMs

Gene	Forward (5'–3')	Reverse (5'–3')
<i>Arg1</i>	ATTGGCTTGCGAGACGTAGA	AATCGGCCTTTTCTTCCTTCC
<i>Mrc1</i>	GAGTGATGGAACCCAGTGA	CCCAGTATCCATCCTTGCCCT
<i>Chi3l3</i>	GTTCCTGGTGAAGGAAATGCGTA	GTAGCAGCCTTGGAAATGTCTTT
<i>TGF-β</i>	CCGCAACAACGCCATCTA	CTCTGCACGGGACAG
<i>iNOS</i>	GTTCCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
<i>β-actin</i>	CTGGCCCGGACCTGACAGACTACC	ATCGGAACCGCTCGTTGCCAATAG

creased proportion and number of lung Tregs, compared with OVA-sensitized mice that did not receive hMSCs (Fig. 7I).

In summary, hMSC treatment inhibited the hallmark features of OVA-induced allergic asthma, an effect that was dependent on AM-mediated induction of IL-10 production from other cell types.

Discussion

In this study, we successfully isolated hMSCs from umbilical cord and adipose tissue as alternatives to conventional hBMMSCs. These hMSCs exhibited in vitro cross-species immunosuppressive capacity and, when administered i.v., localized to the lungs to inhibit cardinal features of OVA-induced allergic asthma in mice. Sustained engraftment of hMSCs in the lungs was not required for their inhibitory effects. In hMSC-treated mice, an increase in AMs was observed. Importantly, depletion of AMs with clodronate-encapsulated liposomes abolished the beneficial effects of hMSC treatment on AHR and lung disease, indicating a critical role for AMs in mediating the in vivo suppression of allergic asthma.

AMs are the predominant immune effector cells at the air–tissue interface in the lungs (37), continuously encountering inhaled substances and serving as important sentinels in the recognition of pathogens and apoptotic cells. AMs are kept in a quiescent state to avoid mounting inappropriate immune responses to harmless Ags while actively controlling pulmonary immune responses under steady-state conditions (26). Early studies have shown that in vivo depletion of AMs resulted in upregulation of Ag-presenting capacity of dendritic cells (38) and secondary Ab responses to passively inhaled Ags (39), supporting a suppressive function for AMs.

A role for AMs in suppressing Th2 responses has also been demonstrated in a mouse model of allergic asthma, whereby OVA-sensitized mice that were depleted of AMs developed increased eosinophilic inflammation and Th2 responses subsequent to airway challenge (40). A concern thus arising is that depletion of AMs might mask any real MSC-dependent effects in our clodronate studies. However, OVA-sensitized mice treated with clodronate exhibited the same pattern of AHR as those treated with plain liposomes; therefore, clodronate treatment by itself did not affect AHR in our experiments (Fig. 6F). Importantly, the macrophage effects are not just a consequence of their accumulation in the presence of foreign cell entities, as we did not observe the same beneficial effects when mice were treated with fixed/permeabilized hMSCs (Fig. 3G).

Macrophages are remarkably responsive to their cytokine milieu and/or TLR stimulation and become polarized to distinct functional subsets, broadly classified as M1 (classical) or M2 (alternative) (29). Whereas M1 macrophages produce high levels of proinflammatory cytokines and express strong antimicrobial activity, M2 macrophages produce anti-inflammatory cytokines, such as IL-10, and exhibit efficient phagocytic activity. In the Th2-driven lung environment during asthma, IL-4 and IL-13 can contribute to priming and skewing of M2 macrophages (41). Although M2

macrophages can inhibit the production of proinflammatory molecules and are traditionally described as immunosuppressive, their role in allergic asthma remains controversial, as some studies have demonstrated a role for M2 macrophages in promoting allergic inflammation (41, 42). Our data indicate that the interaction between MSCs and AMs in this setting is not be as straightforward as modulating M1/M2 polarization.

The beneficial effects of BMMSC treatment in mouse models of cecal ligation and puncture-induced sepsis (43) and acute myocardial infarction (44) have been demonstrated to involve IL-10-producing M2 macrophages. Similarly, intrapulmonary delivery of mouse BMMSCs in a mouse model of endotoxin-induced acute lung injury was shown to induce increased production of IL-10, which could have been MSC derived or host derived (45). IL-10 is a pleiotropic cytokine involved in the regulation of many effector functions associated with Th2 responses and allergic disease (46). An increase was observed in IL-10 secretion in the lungs of hMSC-treated mice, which was abolished when AMs were depleted prior to hMSC treatment. However, we did not detect upregulation of IL-10 expression in AMs from hMSC-treated mice following intranasal OVA challenge, suggesting that IL-10 is produced by AMs prior to OVA challenge or that AMs induce IL-10 production from other cell sources. The increase in Tregs in the lungs of hMSC-treated mice may not be the exclusive source of IL-10 production, as IL-10 is broadly expressed by cells of both innate and adaptive immune systems (34). It has also been demonstrated that Treg-mediated suppression of allergic airway inflammation, although dependent on IL-10, does not require IL-10 production by Tregs themselves (36).

An increase in IL-13 was observed in the lungs of mice with experimental acute lung injury after BMMSC administration, and it was suggested that the reduction in lung injury in MSC-treated mice was due to downregulation of proinflammatory Th1 cytokines and upregulation of anti-inflammatory Th2 cytokines (45). In contrast, in our model of Th2-mediated allergic asthma, the beneficial effects of hMSC treatment are associated with decreased IL-5 and IL-13 production and eosinophil infiltration. These findings support studies that suggest MSCs are able to sense their environment and restore the balance in disorders associated with a predominance of either Th1 or Th2 responses (47).

Although mouse MSCs have been shown to suppress allergic inflammation by inducing host IFN- γ production or Treg recruitment to the lungs, it is important to understand the mechanisms used by clinically relevant hMSCs. We show in this article that the cross-species effects of hMSCs in allergic asthma are dependent on AMs inducing the production of IL-10 from other cell sources. The finding that hMSCs activate the predominant immune effector cell type in the pulmonary system provides a compelling mechanism for their durable effects despite limited engraftment. Further elucidation of the molecular mechanisms governing the AM response to hMSCs in the context of allergic asthma will be important for understanding how MSCs modify the host response to therapeutic effect.

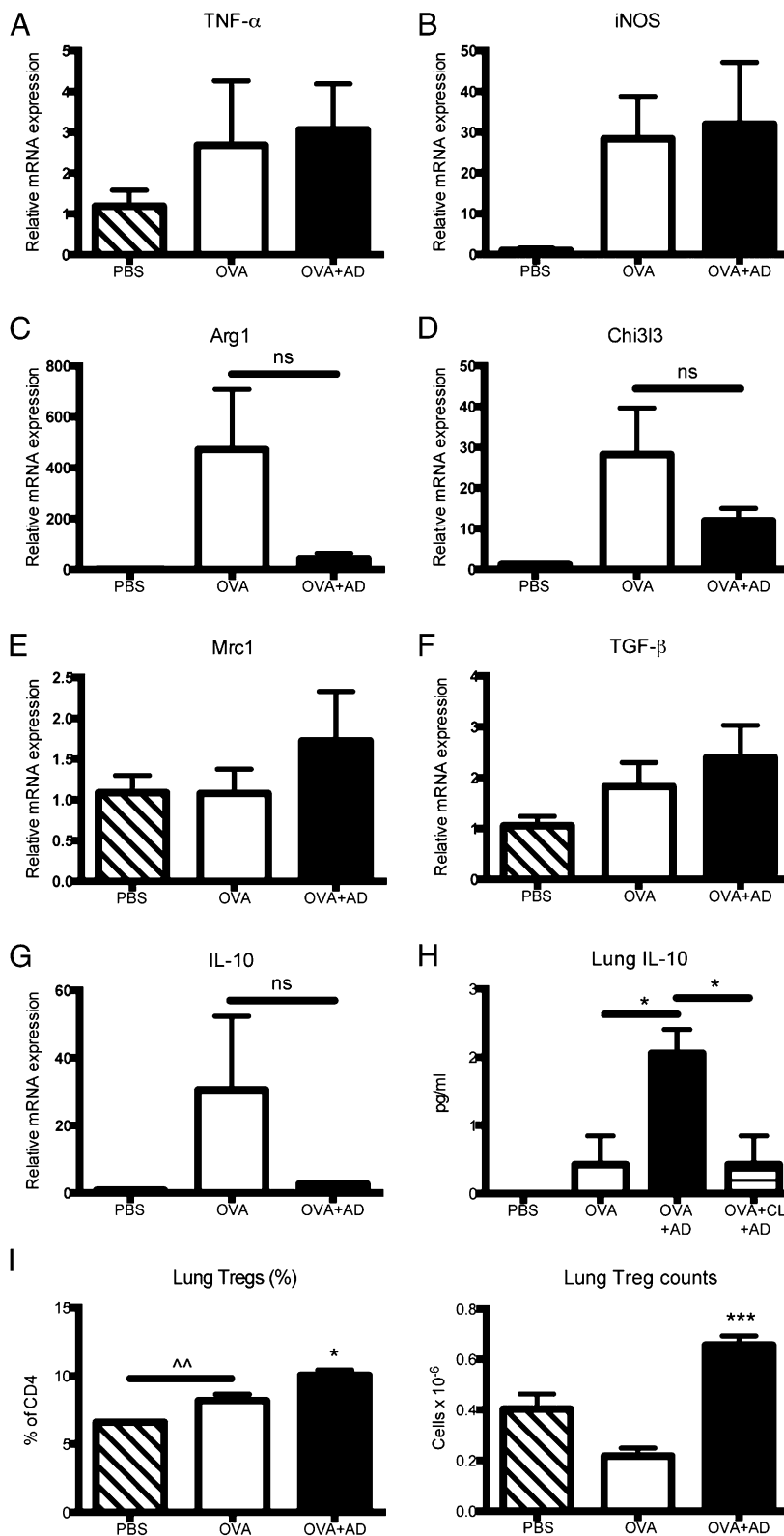


FIGURE 7. Transcription of genes associated with macrophage activation and polarization. CD11b^{lo} CD11c⁺ AMs were FACS purified from digested lungs of individual mice and analyzed for transcripts of (A) TNF- α , (B) inducible NO synthase (iNOS), (C) Arg1, (D) Chi3l3, (E) Mrc1, (F) TGF- β , and (G) IL-10. Relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method where ΔCt is the difference between the threshold cycle for the gene of interest and the threshold cycle for β -actin, and normalized to unsensitized (PBS) levels. Results were obtained from four to five mice per group, expressed as mean $\pm$ SEM. (H) OVA-sensitized mice treated with hADMSCs (OVA + AD) had increased IL-10 levels in lung tissue, compared with OVA-sensitized mice (OVA) and OVA-sensitized mice treated with clodronate and hADMSCs (OVA + CL + AD). Results were obtained from five mice per group, expressed as mean $\pm$ SEM. * $p \leq 0.05$ (one-way ANOVA, Tukey posttest). (I) OVA + AD mice had increased numbers of Tregs in lung tissue. Results were obtained from three to five mice per group, expressed as mean $\pm$ SEM. ^^ $p \leq 0.01$ compared with PBS. * $p \leq 0.05$, *** $p \leq 0.001$ compared with OVA (unpaired two-tailed Student t test).

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Disclosures

The authors have no financial conflicts of interest.

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