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J Immunol published online 19 January 2018
http://www.jimmunol.org/content/early/2018/01/19/jimmunol.1700562

Supplementary Material http://www.jimmunol.org/content/suppl/2018/01/19/jimmunol.1700562.DCSupplemental

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Published January 19, 2018, doi:10.4049/jimmunol.1700562

Mesenchymal Stem Cells Recruit CCR2$^+$ Monocytes To Suppress Allergic Airway Inflammation

Katsuyuki Takeda,*,[1] Tracy L. Webb,†,[1] Fangkun Ning,* Yoshiki Shiraishi,* Daniel P. Regan,† Lyndah Chow,† Mia J. Smith,† Shigeru Ashino,* Amanda M. Guth,† Sophie Hopkins,† Erwin W. Gelfand,*,[2] and Steven Dow†,[2]

Mesenchymal stem cells (MSC) exert immune modulatory properties and previous studies demonstrated suppressive effects of MSC treatment in animal models of allergic airway inflammation. However, the underlying mechanisms have not been fully elucidated. We studied the role of MSC in immune activation and subsequent recruitment of monocytes in suppressing airway hyperresponsiveness and airway inflammation using a mouse model of allergic airway inflammation. MSC administration prior to or after allergen challenge inhibited the development of airway inflammation in allergen-sensitized mice. This was accompanied by an influx of CCR2-positive monocytes, which were localized around injected MSC in the lungs. Notably, IL-10–producing monocytes and/or macrophages were also increased in the lungs. Systemic administration of liposomal clodronate or a CCR2 antagonist significantly prevented the suppressive effects of MSC. Activation of MSC by IFN-γ leading to the upregulation of CCL2 expression was essential for the suppressive effects, as administration of wild-type MSC into IFN-γ−deficient recipients, or IFN-γ receptor–deficient or CCL2-deficient MSC into wild-type mice failed to suppress airway inflammation. These results suggest that MSC activation by IFN-γ, followed by increased expression of CCL2 and recruitment of monocytes to the lungs, is essential for suppression by MSC in allergen-induced airway hyperresponsiveness and airway inflammation. ***The Journal of Immunology*****, 2018, 200: 000–000.**

Asthma is the most common chronic respiratory disease and the prevalence is increasing in most areas of the world (1). Many severe asthma patients are resistant to available treatments regardless of significant advances in care (2). This has prompted the search for newer and more creative ways to manage allergic airway disease and asthma, including treatment with mesenchymal stem cells (MSC).

MSC are known to exert potent immune modulatory properties, both in vitro and in vivo (3–6). The in vivo effects of these immune modulatory properties of MSC have been demonstrated in a number of different disease models, including inflammatory bowel disease, arthritis, allergic encephalitis, and respiratory diseases including asthma (4, 5, 7, 8). In addition, MSC have also been observed clinically to suppress immune responses in patients with severe graft-versus-host disease and in patients with inflammatory bowel disease (9, 10). Thus, immunomodulation with MSC has been viewed as a potential alternative or complement to conventional immunosuppressive therapy.

A number of mechanisms have been advanced to explain the immunomodulatory activities of MSC. These include production of immunoregulatory cytokines such as TGF-β or IL-10, release of biochemical mediators (e.g., PGE_2), and release of extracellular vesicles that contain microRNA or mRNA (3–6, 11). Activities of MSC on immune cells such as induction of regulatory T cells (Tregs) and monocytes and/or macrophages have also been described (4, 6).

Based on these findings, numerous studies following MSC treatment in rodent models of asthma have been reported (7, 12–16). Recently, we demonstrated the ameliorative effects of MSC treatment in a large animal (feline) model of asthma (17, 18). When MSC were injected i.v., most of the injected cells initially lodged in lung capillaries via filtration effects (6). The interaction of administered MSC with lung monocytes and/or macrophages as regulators of inflammatory responses in lung disease models, including asthma, has been previously investigated (19–23).

In the current study, we used an experimental model of allergen-induced airway inflammation to investigate changes in monocyte and macrophage populations following interaction with systemically administered MSC. We determined the mechanism underlying activation of MSC to initiate monocyte recruitment through chemokine (C–C motif) legend 2 (CCL2), which is a potent monocyte chemoattractant and shown to be secreted by MSC (5, 11). These studies revealed previously unreported pathways of interaction between MSC activation by IFN-γ, MSC production of CCL2, and recruitment of IL-10–expressing monocytes and/or macrophages as negative regulators of airway inflammation.

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Received for publication April 28, 2017. Accepted for publication December 3, 2017.

The work was supported by National Institutes of Health Grant K08AI071724 (to T.L.W.) and funding from the Shipley Foundation for the studies at Colorado State University and by National Institutes of Health Grants HL-36577 and AI-77609 (to E.W.G.) for studies at National Jewish Health.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; MSC, mesenchymal stem cell; PAS, periodic acid–Schiff; Treg, regulatory T cell; WT, wild-type.

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Materials and Methods

Animals

Adult C57BL/6 (wild-type [WT]) mice (female, aged 8–12 wk) were purchased from Jackson Laboratory (Bar Harbor, ME) and were housed in microisolator cages in the animal facilities at National Jewish Health and Colorado State University. Mice on the C57BL/6 background lacking a functional IFN-γ gene (IFN-$\gamma^{-/-}$) or IFN-γ receptor gene (IFN-$\gamma R^{-/-}$) were purchased from Jackson Laboratory as were mice lacking the CCL2 gene ($CCL2^{-/-}$). CCR2-GFP reporter mice (on the C57BL/6 background) were provided by Dr. E. Pamer (Memorial Sloan Kettering, NY). Foxp3-GFP reporter mice (C57BL/6 background) were provided by Dr. A. Rudensky (Washington University, St. Louis, MO).

Animal model of asthma protocol

All animal protocols used in these studies were approved by the Institutional Animal Care and Use Committees at National Jewish Health and Colorado State University. Airway inflammation was induced in mice using OVA as previously described (24). Briefly, mice were immunized twice, 14 d apart via i.p. injection with 20 μg OVA with 1.0 mg of alum (AlumImuject: Pierce, Rockford, IL) as an adjuvant. Fourteen days later, mice were challenged with 20-min inhalation of OVA (1% in saline) for three consecutive days by ultrasonic nebulizer (model NE-U07; Omron Healthcare, Vernon Hills, IL). Forty-eight hours after the last OVA nebulization, the mice were anesthetized and airway hyperresponsiveness (AHR) following methacholine challenge was determined in anesthetized, tracheostomized, mechanically ventilated mice. The mice were then euthanized and bronchoalveolar lavage fluid (BALF) with 1 ml of HBSS and lung tissues for histology were collected.

Generation of adipose tissue–derived MSC and treatment protocol

Adipose tissue–derived MSC were generated from abdominal adipose tissues. Adipose tissue–derived MSC were chosen for this study as we previously demonstrated that these cells were easier to propagate in vitro and shared a similar phenotype with bone marrow–derived MSC (25). Adipose tissue–derived MSC were generated from abdominal adipose tissues collected from WT or $CCL2^{-/-}$ mice. To prepare MSC cultures, adipose tissues were minced under sterile conditions and incubated in a 1 mg/ml solution of collagenase (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min to generate single cell suspensions in PBS. The cell suspension was then centrifuged at 1050 × *g* to pellet the stromal vascular fraction, rinsed twice, and resuspended in MSC culture medium. Culture medium consisted of low glucose DMEM (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with essential and nonessential amino acids (Invitrogen/Life Technologies) and 10% heat-inactivated FBS (Cell Generation, Fort

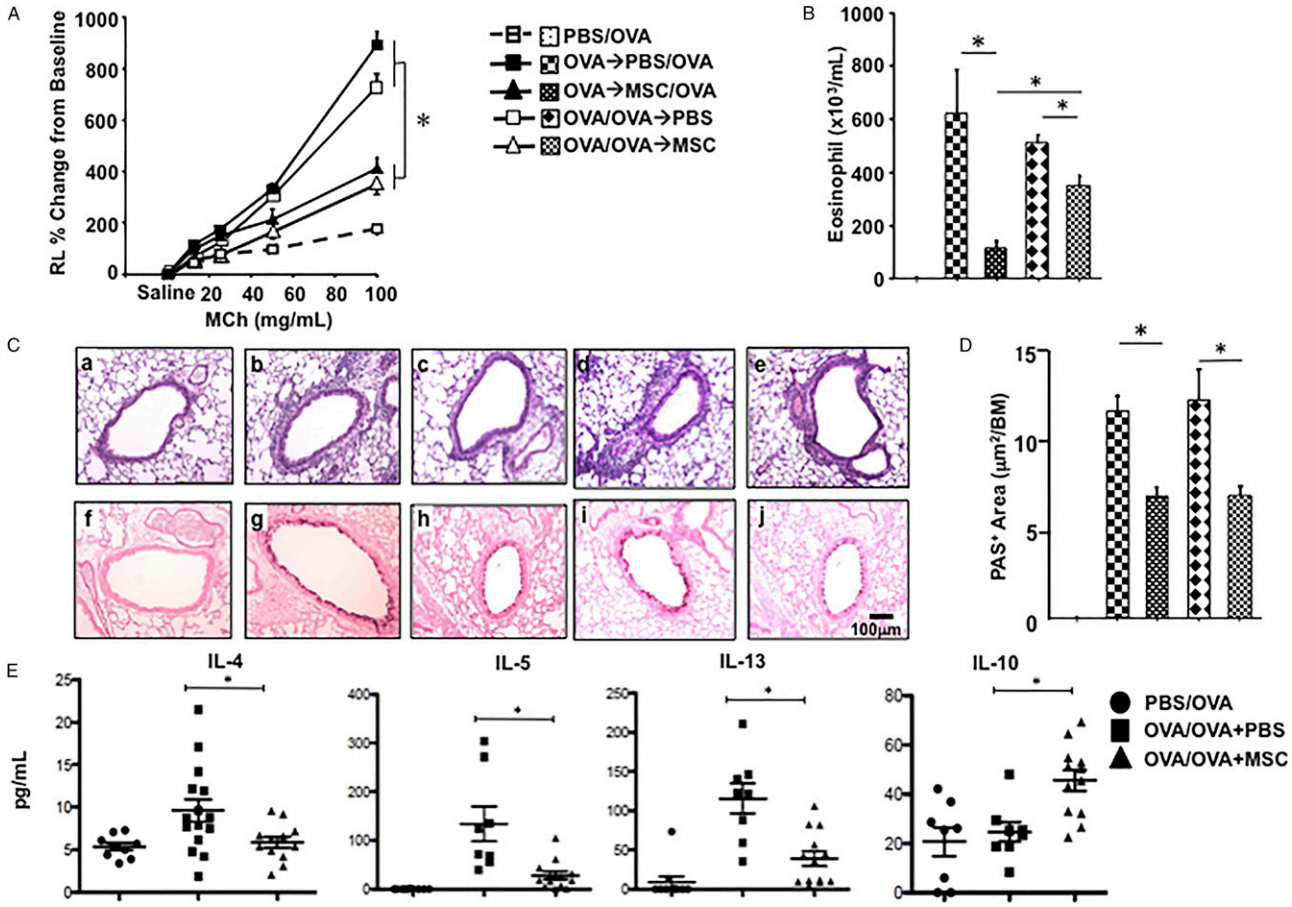

FIGURE 1. Effects of MSC injection on allergen-induced AHR and inflammation. C57BL/6 mice (n = 8 per group) were sensitized to OVA and then exposed to aerosol challenge with OVA, as described in *Materials and Methods*. (**A**) Effects of a single i.v. injection of 2×10^5 adipose tissue–derived MSCs immediately prior to first aerosolized-OVA challenge on AHR were assessed in allergen-sensitized mice (OVA→MSC/OVA) and compared with AHR in OVA-sensitized mice injected with PBS (OVA→PBS/OVA) and AHR in sham-sensitized mice (PBS/OVA). In some groups of mice, MSC or PBS were injected following allergen challenge in sensitized mice (OVA/OVA→MSC and OVA/OVA→PBS, respectively). Injection of MSC prior to or following allergen challenge significantly reduced AHR in OVA-sensitized and challenged mice. $*p < 0.05$ versus OVA/OVA+PBS. (**B**) Effects of MSC injection on numbers of eosinophils in BALFs were assessed. Mice injected with MSC had significantly fewer eosinophils in their airways than PBS-injected mice. $*p < 0.05$. (**C**) Representative photomicrographs of cellular responses in the lung tissue sections of sham-sensitized but OVA-challenged mice (**a** and **f**), OVA-sensitized and challenged mice receiving vehicle (**b** and **g**) or MSC injection (**c** and **h**) prior to OVA challenge, and vehicle (**d** and **i**) or MSC injection (**e** and **j**) following the last OVA challenge were assessed using routine histology; (a–e) H&E staining; (f–j) PAS staining. (**D**) PAS^+ areas were quantified and analyzed. PAS^+ areas were significantly decreased in mice that received MSC (n = 5 per group). $*p < 0.05$. (**E**) Effects of MSC injection on bronchoalveolar lavage concentrations of IL-4, IL-5, IL-13, and IL-10 were assessed. Concentrations of IL-4, IL-5, and IL-13 were significantly decreased in MSC-treated mice compared with untreated sensitized mice. Conversely, concentrations of IL-10 were significantly increased. $*p < 0.05$.

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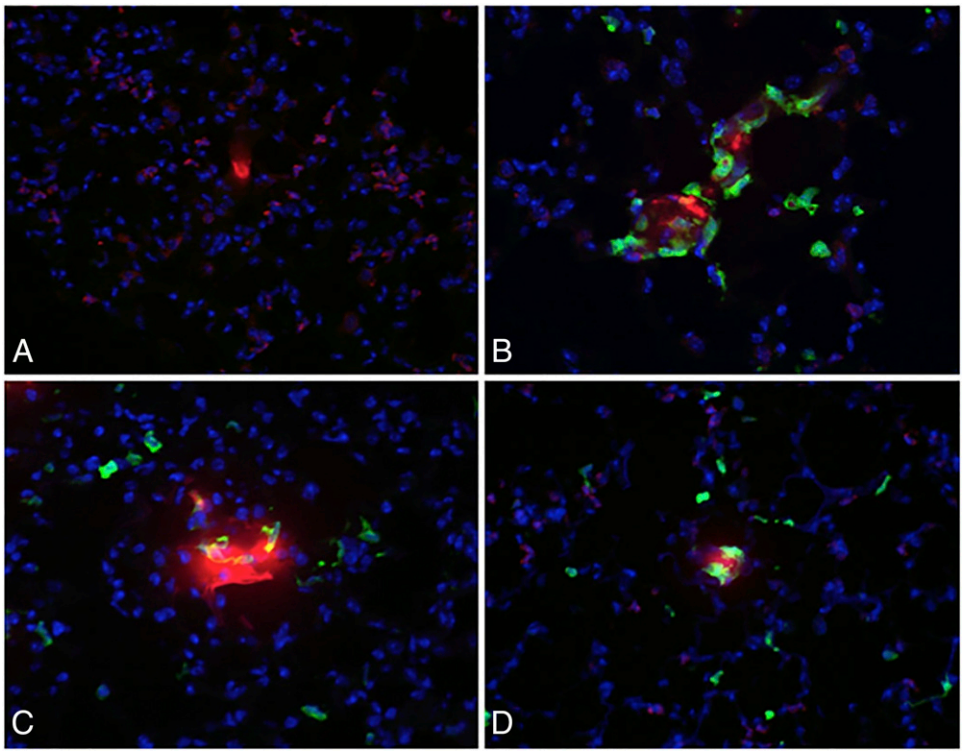

FIGURE 2. Pulmonary monocyte response to MSC administration. CCR2-GFP reporter mice (n = 3 per group) were administered 2×10^5 MSC labeled with CellTracker Orange by the i.v. route, and pulmonary $CCR2^+$ monocyte responses were assessed at 0 (**A**), 8 (**B**), 24 (**C**), and 72 h (**D**) of MSC injection using confocal microscopy. CCR2-GFP^+ monocytes were found to cluster around injected CellTracker Orange positive MSC, particularly at early time points after MSC injection.

Collins, CO), plus penicillin and streptomycin solution (Invitrogen/Life Technologies). Cells in medium were allowed to adhere in tissue culture flasks (BD Falcon; BD Biosciences, Bedford, MA) for 72 h, after which the nonadherent cells were removed, and fresh medium was added. When cells reached 70–80% confluence, they were passaged using trypsin-EDTA solution (Invitrogen/Life Technologies). All cells for in vivo experiments were used at passage 2–3. The cell surface phenotype of MSC was determined by flow cytometry and the ability of the MSC to undergo trilineage differentiation was assessed as described previously (17). MSC (2×10^5) were administered i.v. 24 h prior to the first OVA challenge or 2 h after the last OVA challenge in sensitized mice.

Histological studies

After BALF was collected, lungs were inflated with 1 ml 10% formalin through the trachea and fixed in formalin by immersion. Blocks of lung tissue were cut from around the main bronchus and embedded in paraffin; two to three tissue sections (4 μm) per mouse were then affixed to microscope slides and deparaffinized. The slides were then stained with H&E or periodic acid–Schiff (PAS). The pictures of slides were taken with a microscope (BX40; Olympus America, Melville, NY) equipped with a digital camera (Q-color 3; Olympus America). Goblet cell metaplasia was quantified from PAS+ areas along the airway epithelium using National Institutes of Health ImageJ software (version 1.38), available on the Internet at http://rsb.info.nih.gov/ij/download.html. Four different fields per slide in five samples from each group of mice were examined in a blinded manner.

Biochemicals

OVA was purchased from Thermo Fisher Scientific (Hampton, NH). Liposomal clodronate was administered by i.v. injection 24 h before and after MSC administration. Macrophage depletion activity was assessed using in vitro assays prior to in vivo experiments. To track injected MSC in the lung tissue, MSC were labeled with CellTracker Orange CMTMR dye (Thermo Fisher Scientific). The small molecule CCR2 antagonist RS102895 was purchased from Tocris Bioscience (Bristol, U.K.) and administered (5 mg/kg of body weight) by i.p. injection 24 h prior to the first OVA challenge and 2 h prior to each OVA challenge.

Tissue processing and flow cytometry

Lung tissue was collected, placed in tissue culture medium on ice, and then minced using scissors. Tissue was then digested in a 1% solution of collagenase (Sigma-Aldrich) in MEM medium with 2% FBS and DNase for 30 min at 37°C, erythrocytes lysed with ammonium-chloride-potassium solution, and single cell suspensions were filtered through cell strainers and washed twice with FACS buffer (PBS plus 2% FBS and 0.05% sodium azide). Cells were incubated with Abs in FACS buffer in 96-well round-bottom plates for 30 min at room temperature, then washed and incubated with streptavidin conjugates when necessary.

Intracellular cytokine staining was performed to identify IL-10–producing monocytes and macrophages. For these experiments, OVA-sensitized and challenged mice received MSC treatment after the last OVA challenge. Lung tissues were removed 7 h after MSC treatment. Lung cells were isolated as described above and the cells were cultured with 10 μg/ml LPS and brefeldin A followed by surface staining (19). Cells were then fixed and permeabilized prior to intracellular IL-10 staining with anti-mouse IL-10 (allophycocyanin, clone JES5-16E3) or rat IgG2b K allophycocyanin as an isotype control following surface staining with anti-CD11b (FITC, clone M1/70) and anti-F4/80(PE-Cyanine5, clone BM8). All of the Abs were purchased from eBioscience (San Diego, CA). Directly labeled Abs for flow cytometry and/or immunofluorescence staining were purchased from either eBioscience or Becton-Dickinson (Bedford, MA) except as noted, and included the following clones: mouse Ly6G (clone 1A8), mouse B220 (clone RA3-6B2), mouse CD20 (clone eBioL31), mouse CCR2 (clone 475301, R&D Systems, Minneapolis, MN), mouse CD4 (clone RM4-5), mouse CD8 (clone 53.6.7), and mouse NK1.1 (clone PK136). Abs used for characterization of mouse MSC included mouse CD44 (clone 1M7), mouse CD73 (clone TY/11.8), mouse CD34 (clone RAM34), mouse CD90 (clone HI551), mouse CD105 (clone 8A1), and mouse CD45 (clone 30-F11).

Flow cytometry was carried out using a Beckman Coulter Cyan ADP flow cytometer running Summit software (Fort Collins, CO) or LSRFortessa (Becton-Dickinson). Analysis was performed using FlowJo software (Ashland, OR). Cell counts were performed with an automated cell counter (Nexcelom Bioscience, Lawrence, MA). Confocal microscopy was performed using an Olympus IX83 instrument with CellSens software.

Immunohistochemistry

Lung tissues were snap frozen in OCT solution (VWR Scientific, Radnor, PA), and then crysosectioned to 4-μm thickness onto Superfrost slides (VWR Scientific). Tissues were fixed in acetone, nonspecific peroxidase activity blocked, then incubated with primary Abs diluted in PBS with 2% normal serum overnight at 4°C, rinsed, and incubated with biotinylated secondary Ab, followed by incubation with streptavidin-HRP (Vector Laboratories, Velencia, CA), then incubated with amino-ethyl carbazole substrate, followed by hematoxylin counterstain. Sections were sealed using cryomount (PolyScientific, Bay Shore, NY) and examined using a Zeiss microscope.

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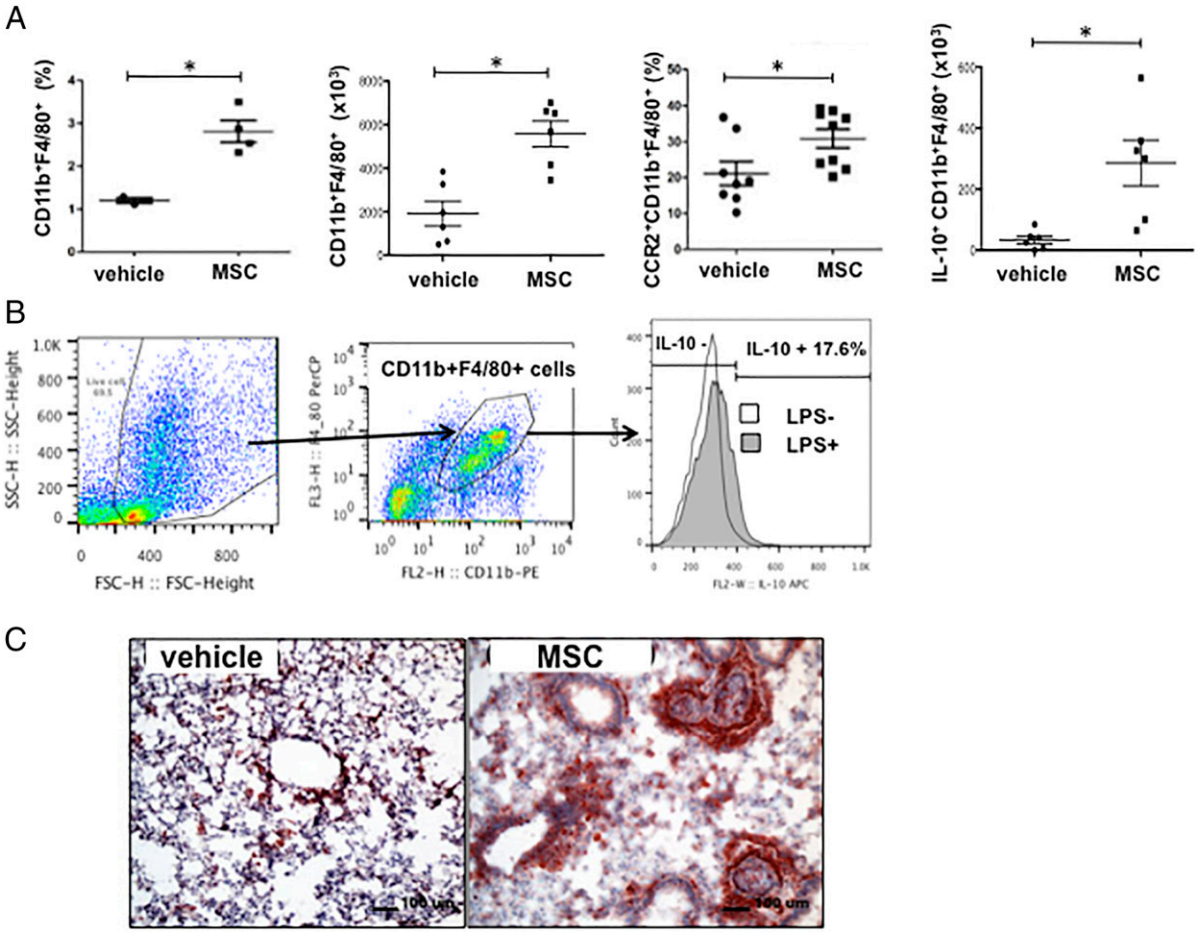

FIGURE 3. Monocyte and/or macrophage responses following MSC administration in the lungs of allergen-sensitized and challenged mice. C57BL/6 mice were sensitized and challenged with OVA, then injected i.v. with 2×10^5 MSC. (**A**) Seven hours after MSC injection, lung cells from MSC-injected (MSC) or PBS-injected (vehicle) mice were isolated, then immunostained for identification of $CD11b^+F4/80^+$ (per total lung mononuclear cells or numbers of cells per lung), $CCR2^+CD11b^+F4/80^+$ (per total $CD11b^+F4/80^+$ cells), or $IL\text{-}10^+CD11b^+F4/80^+$ (per lung) monocytes and macrophages by flow cytometry ($n = 6$ per group). $^*p < 0.05$. (**B**) Gating strategy to detect IL-10–positive monocyte/macrophage in the lung tissue and representative flow cytometry data. IL-10 levels in $CD11b^+F4/80$ cells were compared following with or without LPS stimulation as describe in *Materials and Methods*. (**C**) Lung tissues from PBS-injected (left panel) and MSC-injected (right panel) mice were immunostained for identification of $CD68^+$ monocytes and macrophages. Lungs from MSC-injected mice had prominent accumulations of $CD68^+$ cells (red stain) around airways compared with PBS-injected mice. Similar results were observed in two additional control and treated mice, processed similarly.

For immunofluorescence evaluation of MSC and monocyte trafficking to lungs, immediately following dissection lung tissues were immersion fixed in 1% paraformaldehyde-lysine-periodate fixative (1% paraformaldehyde in 0.2 M lysine-HCl, 0.1 M anhydrous dibasic sodium phosphate, with 0.21% sodium periodate) for 24 h at 4°C. Following fixation, lungs were placed in a 30% w/v sucrose solution for 24 h at 4°C, prior to embedding and freezing in Tissue-Tek OCT compound. Embedded tissues were sectioned at 5 μm for immunostaining. Tissue sections were immunostained with a rabbit Ab against GFP (Life Technologies, Carlsbad, CA) to enhance GFP signal intensity, followed by incubation with a donkey anti-mouse IgG Ab, conjugated to AF647 (Jackson ImmunoResearch, West Grove, PA), and then pseudo-colored green after image analysis.

Cytokine assays

Cytokine concentrations in BALF and in supernatants from MSC cultures were assayed using commercial ELISA kits and performed according to the manufacturers' directions. ELISA kits for IL-4, IL-5, IL-10, IL-13, and IFN-γ were purchased from eBioscience and the kit for CCL2 was purchased from R&D Systems (Minneapolis, MN). Recombinant murine IFN-γ was obtained from PeproTech (Rocky Hill, NJ).

Statistical analyses

Statistical comparisons between the data sets with two treatment groups were performed using nonparametric tests (Mann–Whitney *U* test). Comparisons between three or more groups were performed using ANOVA, followed by Tukey multiple means post hoc test. Analyses were performed using Prism5 software (GraphPad, La Jolla, CA). For all analyses, statistical significance was determined for $p < 0.05$. All results were expressed as the mean ± SEM from at least two independent experiments.

Results

MSC suppress airway inflammation following i.v. injection

MSC were administered by i.v. injection prior to allergen challenge or after completion of the 3 d of inhaled allergen challenge in allergen-sensitized mice. We observed that i.v. administration of MSC either prior to or following allergen challenge significantly suppressed development of AHR and airway eosinophilia in allergen-sensitized and challenged C57BL/6 (WT) mice, compared with sensitized and challenged mice not injected with MSC (Fig. 1A, 1B). Reduction of eosinophil numbers in BALF was more prominent in mice that received MSC prior to allergen challenge compared with mice that received MSC following allergen challenge. However, following both treatments the suppressive effects on AHR, airway inflammation, and goblet cell metaplasia were comparable (Fig. 1A, 1C, 1D). In addition, the administration of MSC prior to allergen challenge reduced BALF levels of IL-4, IL-5, and IL-13 in sensitized mice (Fig. 1E). Further,

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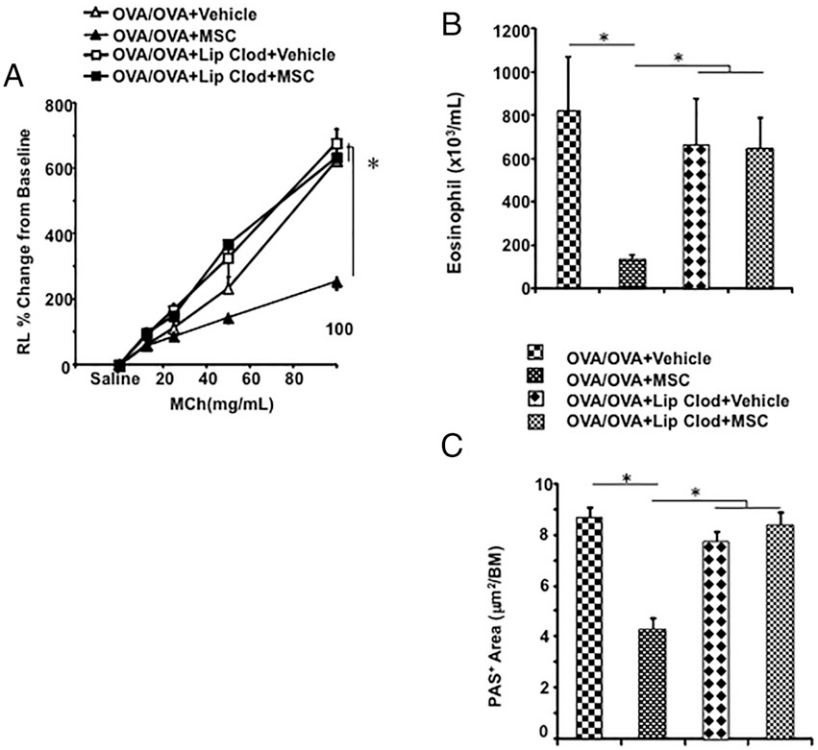

FIGURE 4. Effects of monocyte depletion and migration blockade on allergen-induced AHR and airway inflammation. OVA-sensitized and challenged mice ($n = 8$ per group) received a single i.v. injection of MSC. Monocytes were concurrently depleted in one group of mice by i.v. administration of liposomal clodronate (OVA/OVA+MSC+Lip Clod) or vehicle alone (OVA/OVA+MSC+Vehicle). Liposomal clodronate was injected i.v. 24 h prior to and following MSC injection. The effects of liposomal clodronate administration on MSC-induced suppression of (**A**) AHR, (**B**) eosinophil numbers in BALF, and (**C**) goblet cell metaplasia along the airways were assessed. $*p < 0.05$.

IL-10 levels in BALF were increased following MSC administration compared with vehicle-injected mice (Fig. 1E).

Impact of MSC administration on recruitment of monocytes to the lungs

Monocytes and/or macrophages play a critical role in MSC-induced anti-inflammatory activities (19). CCR2 is mainly expressed on monocytes and has been recognized as a key molecule in the recruitment of monocytes to inflammatory sites as well as differentiation into macrophages (26). We determined in this experimental model whether MSC could recruit monocytes to the lungs through activation of CCR2. To address this question, experiments were performed using labeled MSC to assess cell localization in the lungs following i.v. injection and to determine the impact of MSC injection on monocyte recruitment to the lungs using the CCR2-GFP reporter mice.

Labeled MSC were widely demonstrated throughout the lung parenchyma following i.v. administration (Supplemental Fig. 1A) and remained abundant in lung tissue for at least 72 h after injection; cells were still detected in the lungs up to 7 d after injection (data not shown). In addition, we observed that $CCR2^+$ monocytes were rapidly (within 8 h of MSC injection) recruited to the lungs following MSC injection and remained in the lungs for at least 7 d after MSC injection (Supplemental Fig. 1B). Notably, extensive clustering of $CCR2^+$ monocytes around injected MSC was observed 24 h after MSC administration in the lung tissue (Fig. 2). The association of $CCR2^+$ monocytes with injected MSC persisted through later time points as well (72 h, 7 d), although the clustering was not as tight as at 24 h. Thus, these studies revealed a rapid and persistent influx of monocytes into the lungs in response to injection of MSC.

Flow cytometry was also performed to assess the effects of MSC injection on monocyte and macrophage populations in the lungs. We observed a significant increase in $CD11b^+F4/80^+$ monocytes and/or macrophages in lung tissues at 7 h after MSC treatment (Fig. 3A). In addition, the numbers of $CCR2^+$ monocytes, which may include macrophages, were increased following MSC treatment. Further, IL-10–producing monocytes and/or macrophages were significantly increased in lung tissue following MSC injection compared with vehicle injection. Immunohistochemical analyses of MSC-injected lung tissues also revealed an increase in the numbers of monocytes and/or macrophages stained with anti-CD68 (a marker available for these cells in immunohistochemistry) in MSC-injected animals. Many of the cells were oriented around small airways (Fig. 3B). There was also a significant increase in the numbers of $CD4^+Foxp3^+$ Tregs in the lungs of MSC-injected mice (Supplemental Fig. 2), consistent with observations from previous reports (9). In contrast, MSC injection did not produce a significant change in the numbers of $CD4^+$ or $CD8^+$ T cells, neutrophils ($CD11b^+Ly6G^+$), B cells, or NK cells in the lungs at any time points examined (data not shown).

Monocyte depletion eliminates MSC-induced suppression of airway inflammation

To determine whether monocytes were recruited to the airways in response to MSC administration and mediated the suppressive effects of MSC on airway inflammation, liposome clodronate was systemically administered to deplete circulating monocytes in allergen-sensitized and challenged WT mice. As shown in Fig. 4, treatment with liposome clodronate abrogated the anti-inflammatory activities of MSC, preventing MSC-induced reductions in AHR and suppression of airway eosinophilia and goblet cell metaplasia along the airways. Liposome clodronate treatment did not affect the viability of MSC (Supplemental Fig. 3).

Role of the CCL2-CCR2 axis in MSC-induced anti-inflammatory effects

To further explore the mechanisms of monocytes recruited in MSC-mediated suppression of inflammation, the CCR2 antagonist (RS102895) was administered to mice injected with MSC. In allergen-sensitized and challenged WT mice treated with RS102895, the effects of MSC administration on AHR and airway inflammation were almost completely eliminated (Fig. 5A–C).

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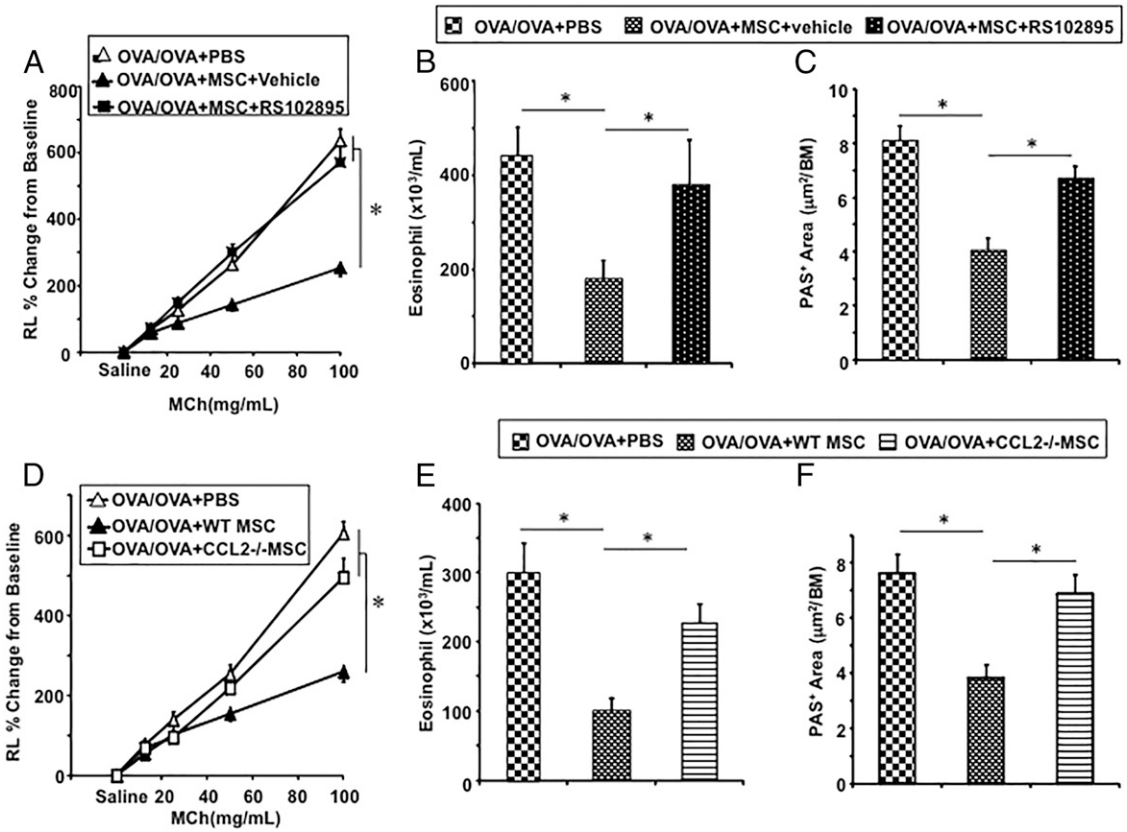

FIGURE 5. The role of CCL2-CCR2 axis for MSC-induced suppression of allergen-induced AHR and airway inflammation. OVA-sensitized and challenged mice (n = 8 per group) received MSC treated with CCR2 antagonist RS102895 (OVA/OVA+MSC+RS102895) or vehicle alone (OVA/OVA+MSC+Vehicle). (**A**) AHR, (**B**) eosinophil numbers in BALF, and (**C**) goblet cell metaplasia along the airways were assessed. *$p < 0.05$. To identify the source of CCL2, MSC were derived from $CCL2^{-/-}$ mice ($CCL2^{-/-}$ MSC) and the effects of administration into WT mice (OVA/OVA+$CCL2^{-/-}$ MSC) on (**D**) AHR, (**E**) eosinophil numbers in BALF, and (**F**) goblet cell metaplasia along the airways were compared with recipients of WT MSC (OVA/OVA+WT MSC) (n = 8 per group). *$p < 0.05$.

As CCL2 is a potent ligand for CCR2, studies were performed to determine whether CCL2 production by injected MSC was necessary to suppress AHR and airway inflammation. Allergen-sensitized WT recipient mice were injected with $CCL2^{-/-}$ or WT MSC prior to allergen challenge, and the effects on AHR and airway inflammation were assessed. Injection of $CCL2^{-/-}$ MSC had little effect on suppressing AHR, airway eosinophilia, or goblet cell metaplasia compared with the effects of injecting WT MSC (Fig. 5D–F). In addition, the numbers of monocytes and/or macrophages in the lungs decreased in mice treated with $CCL2^{-/-}$ MSC (data not shown).

IFN-γ is required for MSC production of CCL2 and in vivo MSC activation

IFN-γ has been reported previously to activate MSC production of several key immune regulatory enzymes and cytokines (27, 28). Indeed, we found that when MSC were treated in vitro with IFN-γ, they produced large amounts of CCL2 (Fig. 6A). We also demonstrated that IFN-γ levels were increased in the airways following allergen sensitization and challenge compared with challenge alone (Fig. 6B). These results suggested that IFN-γ played an important role in the regulation of MSC responses with respect to airway inflammation in vivo. To address this question, MSC generated from IFN-$\gamma R^{-/-}$ mice were injected into WT mice and their effects on airway responsiveness and inflammation were compared with those of injected WT MSC. Although WT MSC significantly suppressed AHR and airway inflammation, IFN-$\gamma R^{-/-}$ MSC exerted relatively little effect on AHR, airway eosinophilia, or goblet cell metaplasia in allergen-sensitized and challenged WT recipient mice (Fig. 6C–E).

The suppressive activities of MSC in the development of AHR and airway inflammation were also assessed in recipient mice unable to produce IFN-γ. WT MSC were administered to IFN-$\gamma^{-/-}$ recipient mice and the suppressive activities of MSC on AHR and airway inflammation were compared with vehicle recipients. As shown in Fig. 6F, WT MSC exerted little protective effect on AHR, as well as on airway eosinophilia or goblet cell metaplasia (data not shown), when administered to sensitized and challenged IFN-$\gamma^{-/-}$ recipient mice.

Both IFN-γ and CCL2 are required for MSC-dependent recruitment of IL-10–producing monocytes to the lungs

To determine the mechanisms underlying the impaired suppressive activities of administered IFN-$\gamma R^{-/-}$ MSC and $CCL2^{-/-}$ MSC, the numbers of IL-10–producing monocytes and macrophages in the lungs of allergen-sensitized and challenged mice following WT, IFN-$\gamma R^{-/-}$, or $CCL2^{-/-}$ MSC injection were monitored by flow cytometry. As shown in Fig. 7, IFN-$\gamma R^{-/-}$ or $CCL2^{-/-}$ MSC failed to recruit IL-10–producing monocytes and macrophages into the lung. Based on these findings, mechanisms of suppressive effects of MSC on allergen-induced AHR and airway inflammation were proposed as in Fig. 8.

Discussion

Our studies demonstrated that MSC administration following allergen sensitization but prior to allergen challenge inhibited the full complement of lung allergic responses, including development of

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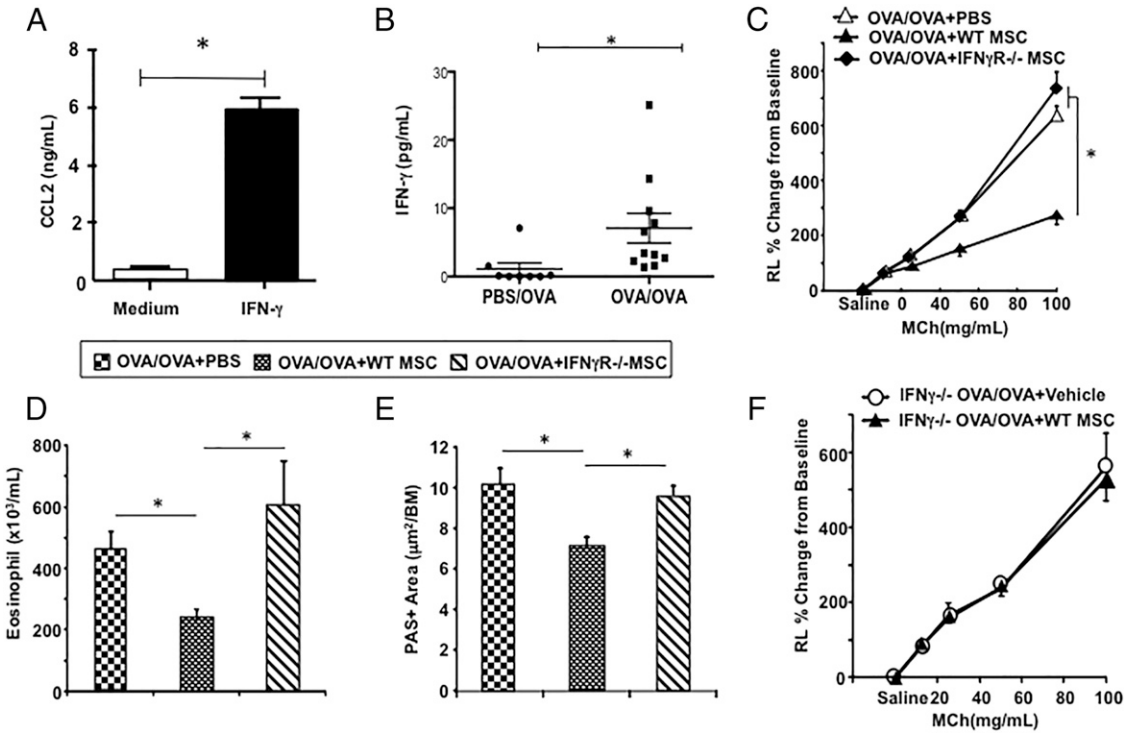

FIGURE 6. The role for IFN-γ in MSC-induced suppression of allergen-induced AHR and airway inflammation. (**A**) Cultured MSC were incubated with medium alone, or medium with 10 ng/ml recombinant mouse IFN-γ, and production levels of CCL2 in culture supernatants were determined by specific ELISA (three replicates with two repeats). $*p < 0.05$. (**B**) Bronchoalveolar lavage levels of IFN-γ from mice that received sham sensitization and OVA challenge (PBS/OVA) or OVA sensitization and challenge (OVA/OVA) were measured by high-sensitivity ELISA kit (n = 10). $*p < 0.05$. Allergen-sensitized WT mice received WT MSC (OVA/OVA+WT MSC) or MSC derived from mice lacking a functional IFN-γ receptor (OVA/OVA+IFN-γR$^{-/-}$ MSC) following sensitization but prior to challenge with OVA. The changes of (**C**) AHR and (**D**) eosinophil numbers in BALF were assessed following MSC treatments (n = 8 per group). $*p < 0.05$. (**E**) Goblet cell metaplasia along the airways were assessed in WT mice that received IFN-γ receptor–deficient MSC (OVA/OVA+IFN-γR$^{-/-}$ MSC) or WT MSC (OVA/OVA+WT MSC) following sensitization but prior to challenge with OVA (n = 5 per group). $*p < 0.05$. In mice lacking a functional IFN-γ gene (IFN-γ$^{-/-}$ mice), the effects of treatment with WT MSC (IFN-γ$^{-/-}$OVA/OVA+MSC) or vehicle (IFN-γ$^{-/-}$ OVA/OVA+Vehicle) on (**F**) AHR was analyzed following OVA sensitization and challenge (n = 8 per group).

AHR, increases in airway eosinophils and Th2-type cytokine levels in BALF, inflammatory cell infiltration, and goblet cell metaplasia in the airways. When MSC were administered after allergen challenge in sensitized mice, development of AHR and goblet cell metaplasia were inhibited. IL-10 levels in BALF were increased following MSC treatment prior to allergen challenge in sensitized mice. In the lungs of OVA-sensitized and challenged mice, Tregs as well as monocytes and/or macrophages were increased following MSC treatment. Among these cells, the numbers of IL-10–producing monocytes and/or macrophages were increased. Depletion of monocytes with systemic administration of liposomal clodronate or blockade of monocyte migration with a CCR2 antagonist abrogated the suppressive effects of the MSC. These findings supported the notion that monocytes played a critical role in the effector arm of MSC-suppressing lung allergic responses. The results from studies using CCL2-deficient MSC revealed that the CCL2-CCR2 axis played a crucial role in the suppressive activity of monocytes induced by administration of MSC. IFN-γ was shown to increase CCL2 release from MSC in vitro. In addition, in experiments with IFN-γ receptor–deficient MSC administered to WT mice combined with administration of WT MSC to IFN-γ–deficient mice, IFN-γ was shown to be an essential initiator of the suppressive effects induced by MSC in allergen-induced airway inflammation in vivo.

Previous studies of MSC administration in animal models of asthma proposed several immune mechanisms for suppression of airway inflammation. Studies with MSC in experimental models of asthma identified suppressive effects of MSC treatment, including increased levels of TGF-β and Th1-type cytokines, transfer of mitochondria to airway epithelial cells, increased numbers of Tregs, and decreased secretion of alarmins from epithelial cells (16, 19–31).

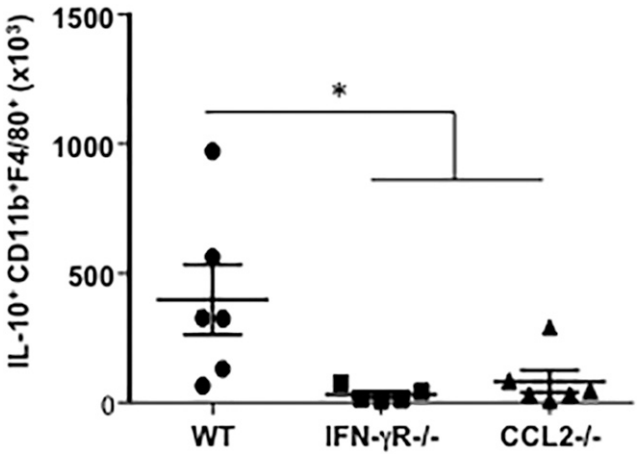

FIGURE 7. Monocyte and/or macrophage responses following WT, IFN-γR$^{-/-}$, or CCL2$^{-/-}$ MSC administration in the lungs of allergen-sensitized and challenged mice. C57BL/6 mice were sensitized and challenged with OVA, then injected i.v. with 2×10^5 of WT, IFN-γR$^{-/-}$, or CCL2$^{-/-}$ MSC. Seven hours after MSC injection, lung cells from MSC-injected mice were isolated, then immunostained for identification of IL-10$^+$ CD11b$^+$F4/80$^+$ monocytes and macrophages (per lung) by flow cytometry (n = 6 per group). $*p < 0.05$.

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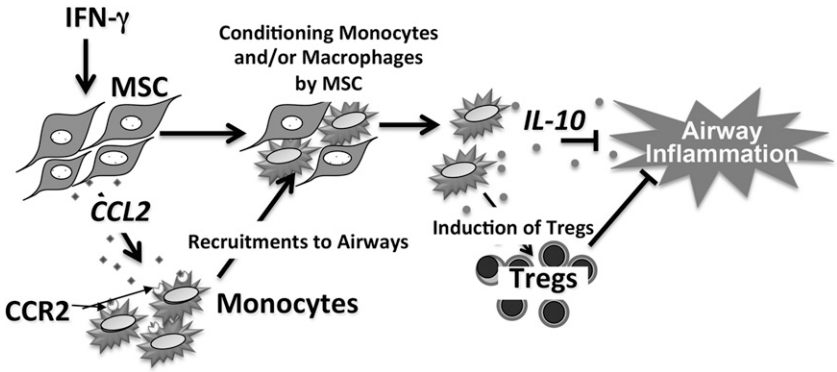

FIGURE 8. Proposed model for IFN-γ activation, CCL2 production, and monocyte recruitment in MSC-mediated suppression of AHR and airway inflammation. IFN-γ–activated MSC secrete CCL2 and recruit CCR2$^+$ monocytes and macrophages into the inflammatory site. Recruited monocytes and macrophages are triggered to produce IL-10 and consequently resolve inflammation.

In this study, increases in the numbers of Tregs in lung tissue were also demonstrated following MSC treatment. The potent roles of macrophages in MSC-induced anti-inflammatory activities have also been shown by MSC-induced reprogramming of monocytes and/or macrophages to produce IL-10 in a mouse model of sepsis (19). Mathias et al. (20) reported that alveolar macrophages play a role in human MSC-induced inhibition of the development of asthma in mice. Treatment with human bone marrow–derived MSC was shown to inhibit development of experimental asthma in mice through induction of M2-type macrophages and TGF-β secretion (21). In addition, MSC administration reduced allergen-induced airway inflammation and AHR by induction of TGF-β or IL-10–producing macrophages following phagocytosis of administered MSC (22). A number of in vitro studies similarly demonstrated that MSC were capable of conditioning macrophages to produce IL-10 (20, 32). In the current study, we also demonstrated that MSC treatment increased the numbers of monocytes and/or macrophages in the lungs of allergen-sensitized and challenged mice and that the cells were potent IL-10 producers. Furthermore, the recruited monocytes expressed CCR2, and the suppressive effects of MSC on allergen-induced airway inflammation and AHR were abrogated by treatment with a CCR2 antagonist or by administration of CCL2-deficient MSC. These results suggested that the recruitment of monocytes through activation of the CCL2-CCR2 axis in response to MSC administration was another critical mechanism underlying the immunomodulatory properties of MSC. Further support was gained in the studies with liposome clodronate, which also resulted in impaired MSC-suppressive effects as this treatment likely depleted circulating monocytes rather than resident alveolar macrophages (33).

The results of these investigations do not rule out an important role for Tregs in suppressing AHR and airway inflammation. As monocyte depletion or inhibition of CCL2-CCR2 signaling significantly abolished the suppressive effects of MSC treatment, Treg expansion could be a secondary event following expansion of IL-10–producing monocytes and/or macrophages. Recent studies implicated the distinct roles of subsets of macrophages such as M1 or M2 in asthma (23, 34), and some studies reported that MSC treatment in allergen-induced airway inflammation resulted in induction of M2 macrophages (21, 22). Based on current notions (35), it appears that IL-10–producing monocytes and M2 macrophages are important effector cells in MSC-induced suppression of allergen-induced airway inflammation.

We also demonstrated an essential role for IFN-γ in the MSC-mediated suppression of allergen-induced airway inflammation and AHR. Although asthma is thought to be a Th2 cytokine–associated disease, upregulation of IFN-γ has been reported in experimental asthma as well as human asthma (36–39). IFN-γ has been shown to activate MSC to secrete hepatocyte growth factor, TGF-β, or IDO to inhibit T cell proliferation (27, 28, 40). In this study, in vitro treatment with IFN-γ induced MSC release of CCL2, which was a critical chemoattractant for monocytes to the lung. Transfer of IFN-γ receptor–deficient MSC into WT mice or administration of WT MSC into IFN-γ–deficient mice failed to demonstrate the suppressive effects on lung allergic responses. As described previously (41), we confirmed that the levels of IFN-γ were increased in the airways of allergen-sensitized and challenged mice. Taken together, these findings implicate an important role for IFN-γ as a potent initiator of the immune-suppressive effects of MSC in allergen-induced airway inflammation and AHR.

Based on our findings, a new model to explain MSC-mediated suppression of airway inflammation and AHR is proposed (Fig. 8), in which MSC activated by IFN-γ secrete CCL2 and recruit monocytes to the lungs to amplify the overall immunomodulatory potential of MSC. MSC administration, not only prior to allergen challenge but also after completion of allergen challenge, demonstrated significant suppressive effects on AHR and airway inflammation. These findings may be relevant to the use of MSC in the treatment of asthma. For example, it may be possible to augment the effects of MSC treatment by preactivating the MSC with IFN-γ prior to administration as in clinical trials in allotransplantation or Crohn's disease (42, 43). Alternatively, it may also be possible to engineer MSC to overexpress chemokines such as CCL2 or CCL5 to augment recruitment of immune-suppressive monocytes to the lungs. Our data also provide in vivo evidence that injection of MSC can exert a significant dampening effect on airway inflammation even after airway inflammation is established, further supporting the potential for MSC therapy in lung inflammatory diseases.

In conclusion, these studies indicate that MSC activated in vivo by IFN-γ suppress airway inflammation via CCL2 production and recruitment of anti-inflammatory monocytes and macrophages produces IL-10 in the lungs.

Acknowledgments

We acknowledge the assistance of Dr. Scott Hafeman of Colorado State University for preparation of liposomal clodronate.

Disclosures

The authors have no financial conflicts of interest.

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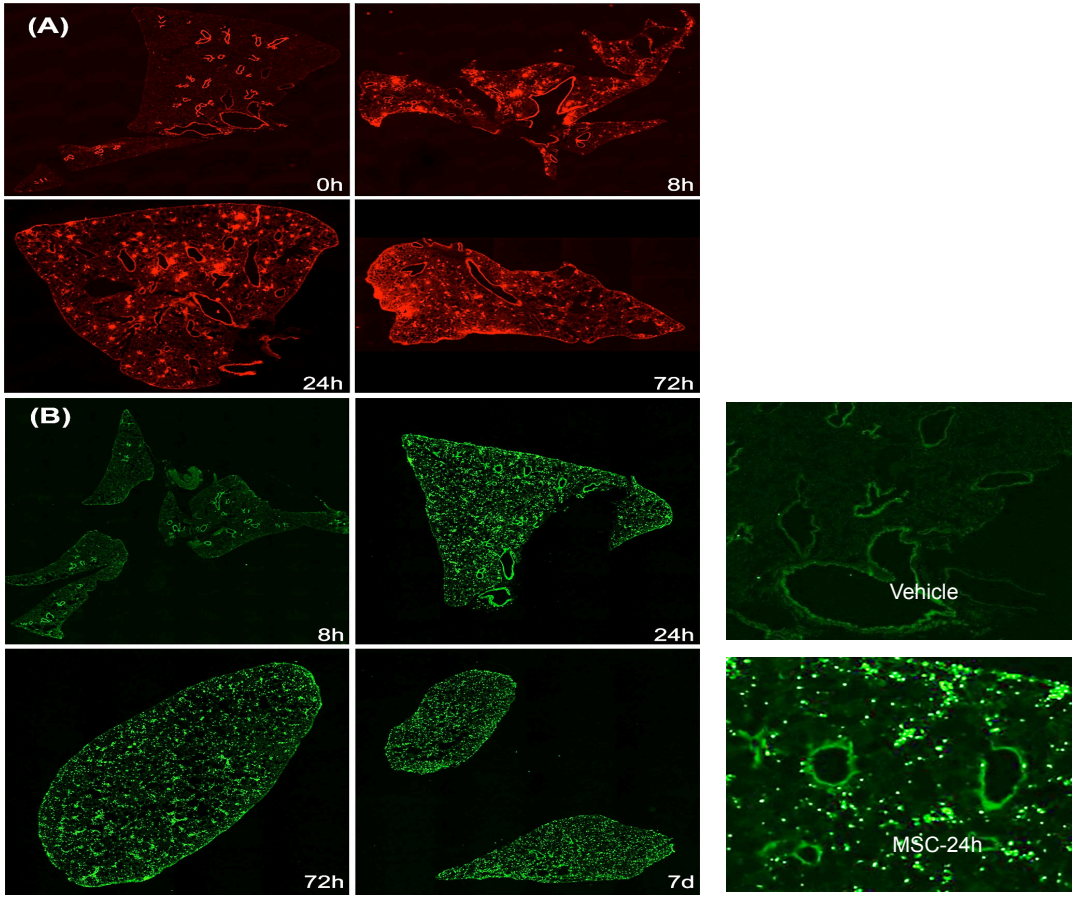
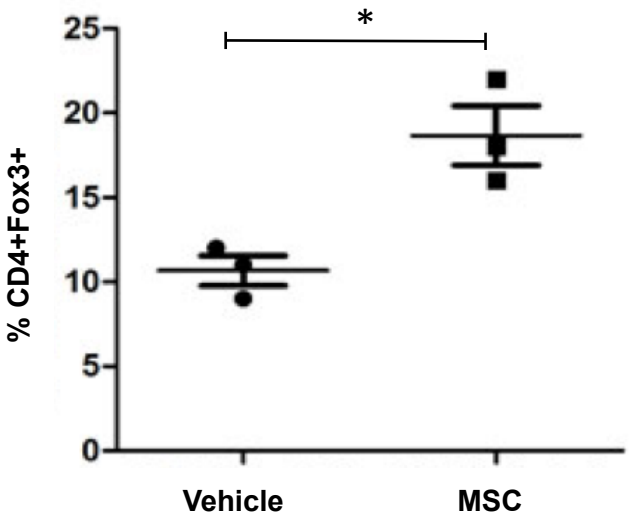
Supplementary Figure 1



Supplementary Figure 1. Localization of Injected MSC and CCR2+ Monocytes in the Recipients' Lung. CCR2-GFP reporter mice (n=3 per group) were administered $2x10^5$ MSC labeled with Cell Tracker Orange by the i.v. route and pulmonary $CCR2^+$ monocyte responses were assessed at various time points after injection using confocal microscopy. In (**A**), numerous Cell Tracker Orange positive MSC were detected in lung tissues throughout the observation period. Panels: 0, 8, 24, and 72 hours after MSC injection. Following MSC injection (**B**), large numbers of $CCR2\text{-}GFP^+$ cells appeared in the lungs within 8 hours of injection, and persisted throughout the 7 days observation period. Panels: 8, 24, and 72 hours, and 7 days after MSC injection. Right 2 panels represented 24 hours after vehicle or MSC injection in larger magnifications.

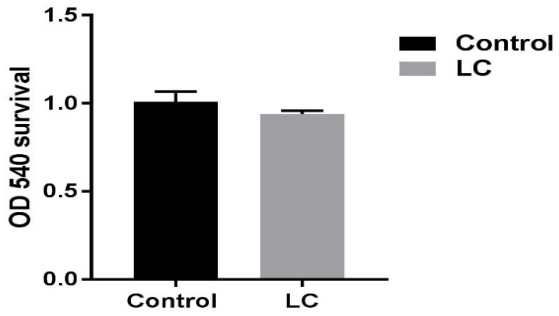
Supplementary Figure 2



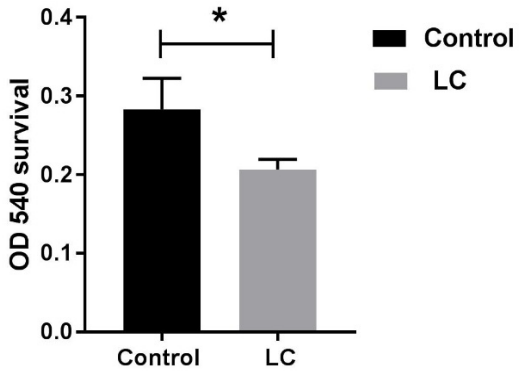
Supplementary Figure 2. Effects of MSC Administration on Treg Populations in Lungs of Allergen Sensitized and Challenged Mice. WT mice were sensitized and challenged with OVA. Prior to the first OVA challenge, mice received PBS alone (Vehicle) or MSC (MSC) by i.v. injection as described in Methods. Twenty-four hours after the last OVA challenge, lung cells were analyzed for $GFP^{+}CD4^{+}$ cells per total lung CD4+ cells (n=4 per group) (*$p < 0.05$).

Supplementary Figure 3

(A)



(B)



Supplementary Figure 3. Effects of Liposome Clodronate (LC) on MSC (A) and Macrophages (B). MSC and primary bone marrow derived macrophages were seeded at equal density (5 X 10^4 cells/well) in quadruplicate wells of 96-well flat bottom plates. Cells were incubated with 5% v/v LC for 24h, using a protocol based on a previous report (S1), then cell viability was determined using MTT assay (n=4)(*<0.05). S1. Hafeman S, C. London, R. Elmslie, S. Dow. 2010. Evaluation of liposomal clodronate for treatment of malignant histiocytosis in dogs. *Cancer Immunol Immunother*. 59: 441-452.