Mesenchymal Stem Cells Attenuate Asthmatic Inflammation and Airway Remodeling by Modulating Macrophages/Monocytes in the IL-13-Overexpressing Mouse Model

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ABSTRACT

Mesenchymal stem cells (MSCs) are attractive alternatives to conventional anti-asthmatic drugs for severe asthma. Mechanisms underlying the anti-asthmatic effects of MSCs have not yet been elucidated. This study evaluated the anti-asthmatic effects of intravenously administered MSCs, focusing on macrophages and monocytes. Seven-week-old transgenic (Tg) mice with lung-specific overexpression of IL-13 were used to simulate chronic asthma. MSCs were intravenously administered four days before sampling. We examined changes in immune cell subpopulations, gene expression, and histological phenotypes. IL-13 Tg mice exhibited diverse features of chronic asthma, including severe type 2 inflammation, airway fibrosis, and mucus metaplasia. Intravenous administration of MSCs attenuated these asthmatic features just four days after a single treatment. MSC treatment significantly reduced SiglecF-CD11c-CD11b+ monocyte-derived macrophages (MoMs) and inhibited the polarization of MoMs into M2 macrophages, especially M2a and M2c. Furthermore, MSCs downregulated the excessive accumulation of Ly6c-Monocytes in the lungs. While an intravenous adoptive transfer of Ly6c-Monocytes promoted the infiltration of MoM and Th2 inflammation, that of MSC-exposed Ly6c-Monocytes did not. Ex vivo Ly6c-MoMs upregulated M2-related genes, which were reduced by MSC treatment. Molecules secreted by Ly6c-MoMs from IL-13 Tg mice lungs upregulated the expression of fibrosis-related genes in fibroblasts, which were also suppressed by MSC treatment. In conclusion, intravenously administered MSCs attenuate asthma phenotypes of chronic asthma by modulating macrophages. Identifying M2 macrophage subtypes revealed that exposure to MSCs transforms the phenotype and function of macrophages. We suggest that Ly6c-monocytes could be a therapeutic target for asthma management.

Keywords: Asthma; Mesenchymal stem cells; Macrophages; Monocytes; Interleukin-13
**INTRODUCTION**

Asthma is a common chronic inflammatory airway disease with symptoms such as coughing, shortness of breath, and wheezing. The incidence of asthma, which is a huge burden on public health, has been increasing worldwide in recent decades (1). Although asthma can be controlled with inhaled corticosteroids in most cases, certain subsets of severe asthma with progressive airway remodeling are unresponsive to conventional anti-asthmatic treatment, thereby necessitating the development of new therapeutics (2).

Mesenchymal stem cells (MSC) therapy can be a novel approach to the conventional treatment of asthma (3). MSCs are the heterogeneous populations of adult stem cells present in various tissues, such as bone marrow, umbilical cord, umbilical cord blood, and adipose tissue. Cell therapy using MSCs has shown promising efficacy in inflammatory diseases because MSCs are immunomodulatory and participate in tissue repair (4,5). MSCs regulate the immune system by suppressing the activation of dendritic cells (DCs) and T lymphocytes (including CD4+ or CD8+ T cells), and by inducing Tregs via cell-cell contact or secretion of anti-inflammatory molecules (6-8). However, the mechanism of immunomodulation by MSCs remains to be clarified.

Monocytes play a crucial role in asthma pathogenesis. Circulating blood monocytes are rapidly recruited to the airway and differentiate into macrophages during asthmatic inflammation; these macrophages are defined as monocyte-derived macrophages (MoMs) (9,10). They differentiate into M1 and M2 subsets with different phenotypic and functional characteristics (11). Along with Th2 cells, M2 macrophages are a major source of type 2 cytokines that aggravate asthmatic inflammation. Since macrophages are the most abundant immune cells in the airway and play a role in aggravating airway inflammation and remodeling, they can be a promising therapeutic target for anti-asthmatic treatment (12).

Systemic administration of MSCs in a murine acute asthma model effectively reduced type 2 cytokine production by Th2 cells and type 2 innate lymphoid cells (ILC2s) by regulating the differentiation of naïve CD4+ T cells and ILCs (8,13). However, the impact of MSCs on chronic type 2 inflammation and its related changes is not fully understood.

Therefore, we investigated the therapeutic effect of intravenously administered MSCs on macrophages/monocytes in a murine chronic asthma model, which overexpresses IL-13 in the lungs.

**MATERIALS AND METHODS**

**Preparation of human umbilical cord (hUC)-derived MSCs**

MSCs were collected and cultured as previously described (14). hUC tissues were obtained immediately after full-term births following cesarean section with informed consent. The hUCs (CTI-195; Cell2in, Seoul, Korea) were washed with PBS to remove blood vessels and amnion. Wharton’s jelly tissues within the hUC were isolated and minced. These explants were incubated with an enzyme mixture at 37°C for 3 h (Miltenyi Biotec, Bergisch Gladbach, Germany), filtered through a 100 μM cell strainer (BD Biosciences, Franklin Lakes, NJ, USA), and pelleted by low-speed centrifugation at 200 ×g for 10 min. The isolated WJ-MSCs were cultured in CellCor™ CD medium (Xcell Therapeutics, Seoul, Korea) supplemented with 2%
human platelet lysate (StemCell Technologies, Vancouver, BC, Canada) in a 37°C incubator. High-density MSCs were isolated from cultured MSCs using a fluorescent real-time thiol tracer (FreSHtracer, Cell2in) (15).

**IL-13 transgenic (Tg) mice and MSC injection**
Transgenic mice were provided by Professor Jack A. Elias (Department of Molecular Microbiology and Immunology, Brown University, RI, USA). Seven to eight weeks old littermate wild-type (WT) and IL-13 Tg C57BL/6 mice were intravenously injected with 1×10⁵ MSCs four days before sacrifice. Each group consisted of four mice. All results are representative of at least three independent experiments.

**Assessment of airway inflammation and histologic changes**
The mouse trachea was opened, and a tube was catheterized into the upper airway tract. Immune cells samples were obtained by administering 1 ml bronchoalveolar lavage fluid (BALF) twice into the open trachea. BALF sample pellets were cytocentrifuged and stained with Diff-Quik (Sysmex, Kobe, Japan). Approximately 300 inflammatory cells were counted using an optical microscope to assess the distribution of the immune cells. Histological analysis was performed with an optical microscope using H&E and periodic acid-Schiff staining (400× magnification) and Masson's trichrome staining (200× magnification). Semi-quantitative grading was based on the degree of inflammation, mucus secretion, and fibrosis (16-18).

**Collagen assay**
The amount of collagen in the mouse lung tissue was quantified using the Soluble Collagen Assay Kit (BioVision, Milpitas, CA, USA) according to the manufacturer’s protocol.

**Quantitative RT-PCR**
Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and chloroform. Reverse transcription was performed using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK), according to the manufacturer’s instructions. Gene expression was normalized using the housekeeping gene Hprt1, Ppia, or ACTB and relative gene expression was calculated using the ΔΔCt method. Primer sequences for each target gene are listed in Supplementary Table 1.

**Flow cytometry**
Chopped lung tissues were incubated in RPMI-1640 complete medium containing 10% type IV collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 90 min and filtered with a 0.45 μm sterile cell strainer. The cells in each tube (1×10⁶ cell density) were blocked with Fc receptor-blocking antibody (BD Biosciences) and then incubated with fluorochrome-labeled surface or cytokine antibodies (BioLegend, San Diego, CA, USA) at 4°C for 30 min. The antibodies used are listed in Supplementary Table 2. Flow cytometry was performed using a BD LSRFortessa™ X-20 (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (version 10.8) software (TreeStar, Woodburn, OR, USA).

**Ex vivo cell culture experiments**
Tregs were isolated from mouse spleen cells using a MojoSort™ Mouse CD4⁺CD25⁻ Regulatory T Cell Isolation Kit (BioLegend); ILCs were isolated from mouse lung cells using an EasySep™ Mouse Pan-ILC Enrichment Kit (StemCell Technologies).
For direct co-culture, $1 \times 10^4$ MSCs were added to each well of ILCs or Tregs and incubated for 1 to 2 days. For transwell co-culture, the 0.4 μm pore size Transwell Permeable Support (Costar, Kennebunk, ME, USA) was used with target cells plated at the bottom. Then, $1 \times 10^4$ MSCs were added to the upper chamber and cultured for 1 to 2 days (19).

**Co-culture of MSCs with bone marrow-derived monocyte from mouse**

Monocytes were isolated from the bone marrow of mice using a MojoSort™ Mouse Monocyte Isolation Kit (BioLegend). Isolated monocytes were cultured in RPMI medium with recombinant M-CSF protein (BioLegend) at 25 ng/ml for five days with or without $1 \times 10^4$ MSCs. Pre-conditioned bone marrow-derived macrophages were cultured with recombinant IL-13 protein (BioLegend) at 20 ng/ml for one day and harvested for analysis.

**Co-culture of MSCs with human PBMCs (hPBMCs)**

Blood samples from allergic asthma patients were collected and were isolated PBMCs using a Ficoll gradient (Cytiva, Marlborough, MA, USA). Isolated PBMCs were cultured in RPMI medium with recombinant human M-CSF (hM-CSF) protein (BioLegend) at 25 ng/ml for five days, and then, cultured with recombinant IL-13 protein (BioLegend) at 20 ng/ml for one day. Next, MSCs ($1 \times 10^4$ cells) were treated for one day and harvested for analysis.

For Th2 cell differentiation, a cell plate was coated with 1 μg/ml CD3 Monoclonal Antibodies (Thermo Fisher Scientific, Waltham, MA, USA) for one day. After coating, $1 \times 10^5$ PBMCs were seeded with 1 μg/ml CD28 Monoclonal Antibodies (Thermo Fisher Scientific) and stimulated with recombinant IL-2, IL-4, and IL-13 (10 ng/ml) for 3 days. Finally, following one day of MSC ($1 \times 10^4$ cells) exposure, cells were harvested for analysis.

**Adoptive transfer of Ly6c- monocytes**

Ly6c- monocytes were purified from bone marrow obtained from IL-13 Tg mice using MojoSort Mouse Ly6c separation Kit (BioLegend). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Ly6c- monocytes were co-cultured with or without MSCs using transwell for 24 h and harvested for adoptive transfer. The mice being transferred were stimulated by 10 μg house dust mite (HDM) extract for 3 days. A total of $5 \times 10^4$ Ly6c- monocytes or MSC pre-treated Ly6c- monocytes were adoptively transferred into HDM-stimulated mice via the tail vein. Control mice were treated with PBS. All the mice were sacrificed after 24 h.

**HDM-induced asthma model**

Female 6-week-old BALB/c mice were obtained from Orient Bio (Anyang, Korea). Mice were sensitized with intranasal injections of 10 μg HDM extract (Stallergenes Greer, London, UK) on day 0, and challenged by the intranasal injection of 10 μg HDM extract on day 7, 8, 9, 22, and 23. A total of $1 \times 10^7$ MSCs were administered intravenously on day 17. Each group consisted of four mice. All results are representative of at least three independent experiments.

**Airway hyperresponsiveness (AHR) measurement**

AHR was measured by Buxco® FinePointe Resistance and Compliance (Data Sciences International, New Brighton, MN, USA). The mice were anesthetized with sodium pentobarbital (50 mg/kg) and exposed to methacholine in concentrations of 10, 20, and 40 mg/ml for 3 min. Lung resistance ($R_L$) was measured and calculated by baseline values.
Statistical analysis
One-way ANOVA with Tukey's post-hoc test was used to compare four or more groups. The two groups were compared using the Mann–Whitney U test. Data are represented as mean ± SEM. Statistical significance was set at p<0.05. All statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, San Diego, CA, USA).

Ethical approval
All procedures involving human MSCs were approved by the Seoul National University Hospital Institutional Review Board (SNUH IRB No. C-1708-083-878). All procedures involving hPBMCs were approved by the Seoul National University Hospital Institutional Review Board (SNUH IRB No. 2006-142-1134). All subjects provided written informed consent. All experiments involving human subjects were performed in accordance with the principles of the Declaration of Helsinki. All animal experiments were performed in accordance with the guidelines of the Seoul National University Institutional Animal Care and Use Committee (IACUC No. SNU-20052541). Less than five mice were housed in each cage under standard conditions of temperature and humidity, according to the guidelines of the Biomedical Center for Animal Resource Development at Seoul National University in compliance with the ARRIVE guidelines and regulations.

RESULTS
Intravenous MSCs treatment attenuated chronic inflammation and remodeling in airways induced by IL-13 overexpression
To determine the anti-asthmatic effect of MSCs, 7–8 weeks old WT and IL-13 Tg littermate mice were intravenously treated with MSCs (1×10⁵ cells) and sacrificed four days after treatment (Fig. 1A). Compared to WT mice, IL-13 Tg mice showed a significant increase in inflammatory cells, including macrophages, neutrophils, eosinophils, and lymphocytes in the BALF. However, a significant decrease in the infiltration of total inflammatory cells, especially eosinophils and neutrophils, was observed in the BALF and lung tissue of intravenous MSC-treated IL-13 Tg mice (Fig. 1B and C). Similarly, histological analysis of lung tissue showed reduced histological quantification scores, improved goblet cell hyperplasia, and suppressed collagen deposition around the bronchi and vessels in the MSC-treated IL-13 Tg mice, compared to that in the IL-13 Tg mice (Fig. 1D, E, and F). Additionally, the expression of Muc5ac (a gene related to mucus secretion) was increased in IL-13 Tg mice compared to those in WT mice but was reduced by MSC treatment (Fig. 1G). The reduced soluble collagen content was also confirmed after MSC treatment in IL-13 Tg mice (Fig. 1H).

Collectively, the intravenous administration of MSCs alleviated the asthmatic characteristics, including infiltration of immune cells in the lung, airway fibrosis, and collagen deposition, which were observed in the IL-13 Tg mice.

Intravenous MSC treatment alleviated Th2 inflammation and promoted Tregs in the lung
To determine whether intravenously administered MSCs altered the CD4⁺ T cell population in the chronic asthma model, T cell subpopulations were analyzed based on cytokine and transcription factor expression using flow cytometry analysis (Supplementary Fig. 1). The percentages of IL-5⁺, IL-13⁺, and IL-17⁺ CD4⁺ T cells were higher in IL-13 Tg mice than in WT mice. MSC-treated IL-13 Tg mice showed diminished percentages of IL-5⁺, IL-13⁺, or IFN-γ⁺ CD4⁺ T cells compared to that in IL-13 Tg mice. An increase in IL-17⁺ CD4⁺ T cells was
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Figure 1. Intravenous MSCs administration attenuated the chronic IL-13 overexpression-associated changes, including Th2 inflammation. (A) MSCs (1×10^5 cells) were intravenously administered to 7–8 weeks old littermate WT and IL-13 Tg mice four days before sacrifice. (B) The number of inflammatory cells including macrophages, neutrophils, eosinophils, and lymphocytes in BAL fluid. (C) The number of eosinophils and neutrophils in the lungs by flow cytometry analysis. (D) Lung histology and semi-quantitative grading of lung inflammation score (H&E staining, 400× magnification). (E) Lung histology and semi-quantitative grading of mucus score (PAS staining, 200× magnification). (F) Semi-quantitative grading of fibrosis score (MT staining, 100× magnification). (G) The relative gene expression of Muc5ac in the lung. (H) The quantification of soluble collagen in the lung tissue by Sircol collagen assay.

PAS, periodic acid schiff; MT, masson’s trichrome; Muc5ac, mucin Sac.

*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
observed in MSC-treated WT mice (Fig. 2A). Compared to WT mice, IL-13 Tg mice showed an increased number of Foxp3+ CD4+ Treg cells. Both WT and IL-13 Tg mice showed enhanced Tregs population, especially the number of IL-10-secreting Tregs increased with MSC treatment in the lungs (Fig. 2B).
To determine the effect of MSCs on Treg proliferation, Tregs isolated from mice spleens were co-cultured with MSCs directly/indirectly for two days. Similar to the in vivo results, the Treg numbers increased in ex vivo cultures of IL-13 Tg mice spleen. Furthermore, this increase in Treg numbers was only in indirect MSC co-cultures, whereas direct MSC co-cultures showed no change (Fig. 2C).

In summary, intravenous MSCs administration in IL-13 Tg mice downregulated T-cell activation, especially that of the Th2 cells, while upregulating Treg proliferation via soluble factors.

Intravenous MSC treatment decreased the ILC2 population in the lung
To determine the effect of MSCs on the ILC population, we gated the ILC subpopulations based on cytokine expression (Supplementary Fig. 1). The percentages of IL-5+ or IL-13+ ILC2 and IFN-γ+ ILC1 were higher in IL-13 Tg mice than in WT mice. MSC-treated IL-13 Tg mice showed diminished percentages of IL-5+ or IL-13+ ILC2 compared to that in IL-13 Tg mice. The increase in IFN-γ+ ILC1 percentage caused by IL-13 overexpression was not affected by MSC treatment. In contrast, IL-13 or MSC-treatment did not alter the IL-17A+ ILC3s percentage (Fig. 3A).

Further, we assessed the MSCs-ILCs crosstalk using ILCs isolated from the mouse lung. ILCs and MSCs were co-cultured ex vivo, directly/indirectly, for one day. Compared to ex vivo cultures from WT mice, those from IL-13 Tg mice showed a significant increase in IL-5+, IL-13+, ST2+, and GATA3+ ILC2 populations. These increments were reduced in direct and indirect ILCs-MSCs co-cultures (Fig. 3B).

In summary, MSC treatment inhibited the upregulation of the ILC2 population in vivo (intravenous application) and ex vivo co-cultures.

Intravenous MSC treatment altered the spectrum of lung macrophage populations
Next, we evaluated how intravenously administered MSCs regulated a subset of lung macrophages that express CD11c and CD11b (Fig. 4A). Macrophage subpopulations were gated according to the strategy depicted in Supplementary Fig. 2. IL-13 overexpression increased the total number of macrophages. This increase was reduced by MSC treatment (Fig. 4B). The effect of MSC on resident and transient AM populations was analyzed based on the origin of alveolar macrophages (AMs). In IL-13 overexpressing lungs, SiglecFCD11c+CD11b- resident AMs were depleted, and SiglecFCD11c+CD11b+ transient AMs were increased. These changes remained unaffected with or without MSC treatment. In contrast, SiglecFCD11c+CD11b- macrophages and SiglecFCD11c+CD11b+ MoMs were increased in IL-13 Tg mice, but MSC treatment restored the SiglecFCD11c+CD11b+ MoMs number to control levels (Fig. 4C).

We further assessed SiglecFCD11c+CD11b- MoMs subtypes. While the MSCs did not affect the increased CD86+ M1 macrophages in IL-13 Tg mice, the increased number of CD206+ M2 macrophages in IL-13 Tg mice was effectively decreased by MSC treatment. Among M2 subsets, IL-13 Tg mice showed increased percentages of CD206+CD86+MHCI+ M2a macrophages, CD206+CD86+MHCI+ M2c macrophages, and CD206+CD86+MHCI+ M2b macrophages. Intravenously administered MSCs significantly reduced the percentages of CD206+CD86+MHCI+ M2c macrophages, while further increases in CD206+CD86+MHCI+ M2b macrophages were observed in IL-13 Tg mice. Although the decreases in the percentage...
of CD206+CD86+MHCII+ M2a macrophages in IL-13 Tg mice were not statically significant, the number of CD206+CD86+MHCII+ M2a macrophages showed significant reduction by MSC treatment (Fig. 4D).

To determine the differential effect of MSCs on macrophage subsets, we isolated CD11b- and CD11b+ macrophages, mainly representing AM and MoM populations, respectively, from the lung and co-cultured them with/without MSCs for one day. The mRNA levels of Cd163, Tgfb1,
**Figure 4.** Intravenous MSCs administration altered lung macrophage populations. (A) The FACS gating strategy of measuring macrophages subpopulation within total macrophages. (B) The number of total macrophages in the lung. (C) The percentages of SiglecF+CD11c+CD11b− among MoMs in the lung. The number of CD206+M2 among total macrophages. (D) The number of CD206+M2c and CD206+M2b among SiglecF+CD11c+CD11b− macrophages among total macrophages. (E) Isolated CD11b+AMs and CD11b+MoMs from the lung were co-cultured with MSCs (1×10⁶ cells) or PBS directly/indirectly for one day and the gene expression of M2 phenotype-related Ccl2, Tgfb1, and Ym1 were analyzed by qRT-PCR analysis. p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
and Ym1 were highly upregulated in IL-13 Tg mice compared to those in WT mice. Furthermore, among macrophages derived from IL-13 Tg mice, the expression of C1163 and Tgfβ1 was more prominent in the MoMs than in the AM. MSC treatment suppressed the increase in mRNA levels of C1163, Tgfβ1, and Ym1 in the Ly6c MoMs in IL-13 Tg mice (Fig. 4E). Finally, the gene expression of these markers was not detected in MSCs (Supplementary Fig. 3A).

In summary, in IL-13 Tg mice, intravenously administered MSCs reduced the infiltration of MoMs and suppressed polarization toward M2a and M2c.

**Effect of intravenous MSCs on non-classical Ly6c+ monocytes in the lung**

To assess the crosstalk between MSCs and monocytes, we analyzed monocyte subpopulations according to the gating strategy shown in Supplementary Fig. 4. The percentages of total monocytes, especially Ly6c- and Ly6c+ monocytes, were significantly higher in IL-13 Tg mice than in WT mice. MSC administration reduced the total monocyte and Ly6c- monocyte populations in IL-13 Tg mice, although there was no significant difference in the Ly6c+ monocyte population between IL-13 Tg mice and MSC-treated IL-13 Tg mice (Fig. 5A). The Ly6c+/Ly6c- ratio was significantly lower in MSC-treated IL-13 Tg mice than in IL-13 Tg mice (Fig. 5B). The direct effect of MSCs on monocyte differentiation was assessed using Ly6c- or Ly6c+ monocytes isolated from mouse bone marrow. Ly6c- or Ly6c+ monocytes were co-cultured, *ex vivo*, with MSCs for five days along with M-CSF stimulation and harvested two days later with/without IL-13 stimulation. The mRNA expression levels of integrin alpha M (Itgam) were assessed to identify the effect of MSCs on macrophage subsets. After MSC treatment, the gene expression of Itgam-producing CD11b was downregulated in both Ly6c- and Ly6c+ MoMs (Fig. 5C).

Genes related to M2 phenotypes were predominantly expressed in Ly6c- monocytes compared to Ly6c+ monocytes in IL-13 Tg mice. In Ly6c- monocytes, MSC treatment suppressed the IL-13 overexpression mediated upregulation of mRNA levels of Tgfβ1, Mrcl, Ym1, and histocompatibility 2, class II antigen A, beta (H2-Ab) which encodes major histocompatibility complex class II antigen. Excepting Mrcl expression, there were no differences in gene expression of Ly6c- monocytes after IL-13 and MSC treatment (Fig. 5C). Modulation of gene expression in both Ly6c- and Ly6c+ MoMs was similar to that in macrophages isolated from IL-13 Tg mice (Supplementary Fig. 5). In addition, the gene expression of these markers was not confirmed in MSCs (Supplementary Fig. 3B).

The effect of soluble factors secreted by Ly6c MoMs on fibroblasts was assessed to confirm if the microenvironment induced by Ly6c MoMs alone (without MSCs) affects fibrosis. Lung fibroblasts were cultured for three days on TGF-β stimulation in the medium obtained from Ly6c- monocyte cultures, co-cultured with or without MSCs during M-CSF stimulation. The lung fibroblasts grown in culture medium derived from Ly6c- monocytes of IL-13 Tg mice showed upregulation of mRNA levels of Fgf1 and Fnnl compared with those grown in media derived from Ly6c- monocytes of WT mice. Meanwhile, the medium containing soluble factors released from Ly6c+ MoMs and MSCs co-culture did not upregulate the expression of fibrosis-related genes (Fig. 5D).

In summary, MSC treatment suppressed the lung infiltration of monocytes, especially of Ly6c- monocytes, in IL-13 Tg mice. It also suppressed M2-related gene expression in macrophages derived from Ly6c- monocytes in *ex vivo* cultures. Furthermore, the secretory molecules of MSCs altered the fibrosis-related gene profiles of fibroblasts induced by Ly6c- monocytes.
MSC-exposed Ly6c⁺ monocytes suppress the infiltration of monocyte into the lung and Th2 inflammation

To confirm the direct effects of Ly6c⁺ monocytes in asthma, we performed intravenous adoptive transfer of Ly6c⁺ monocytes. Ly6c⁺ monocytes were co-cultured with or without...
MSCs using transwell for 24 h. WT mice were intravenously administered by HDM extract for 3 days to induce monocyte homing into inflammatory sites (Fig. 6A). A total of $5 \times 10^4$ Ly6c+ monocytes or MSC pre-treated Ly6c+ monocytes were adoptively transferred into HDM-stimulated mice via the tail vein. The Ly6c+ monogroup (i.e. Ly6c+ monocyte transfer group) showed a significant increase in total cell population, especially the macrophage population, in BALF compared to those of WT mice. However, they are reduced in the Ly6c+ monoMSC group (i.e. MSC pre-treated Ly6c+ monocyte transfer group) (Fig. 6B). Correspondingly, histological analysis of lung tissue showed reduced histological quantification scores, improved goblet cell hyperplasia, and suppressed collagen deposition around bronchi and vessels in the MSC-treated HDM group compared to that in the Ly6c+ monoMSC group (Fig. 6C). The percentages of IL-5+ and IL-13+ CD4+ T cells were higher in Ly6c+ monogroup than in WT mice, yet these increases were diminished in Ly6c+ monoMSC group. Compared to Ly6c+ mono group, Ly6c+ monoMSC group showed an increased the percentages of Tregs, especially IL-10-secreting Tregs (Fig. 6D). Next, we evaluated a subset of lung macrophages. The percentages of SiglecF CD11c+CD11b+ MoMs were increased in the Ly6c+ monogroup, but MSC pre-treated Ly6c+ monocyte transfer restored the percentages of MoMs to control levels. In contrast, the percentages of SiglecFCD11c+CD11b+ resident AMs were unaffected with or without Ly6c+ monocyte treatment. The percentages of M1 and M2 subsets in MoMs were increased in Ly6c+ monoMSC group compared to the Ly6c+ monocytes transfer group. Among M2 subsets, in the Ly6c+ monoMSC group, the percentages of CD206+CD86+MHCIIm2b were highly increased compared to the Ly6c+ monocytes transfer group, whereas CD206+CD86 MHCIim2a were slightly increased and CD206+CD86 MHCIim2c showed no changes. Also, dividing the MoMs according to Ly6c expressions, while the percentages of Ly6c+ MoMs were decreased in Ly6c+ monoMSC group compared to the Ly6c+ monogroup, that of Ly6c+ MoMs were increased in the Ly6c+ monoMSC group, compared other groups (Fig. 6E).

In summary, asthmatic characteristics, including infiltration of immune cells into the lungs, airway fibrosis, and collagen deposition, were exacerbated by intravenous adoptive transfer of Ly6c+ monocytes. Also, IL-5+ and IL-13+ Th2-mediated inflammation and SiglecF CD11c

Figure 6. MSC-exposed Ly6c+ monocytes suppress the infiltration of monocyte into the lung and Th2 inflammation. (A) MSC-exposed Ly6c+ monocytes were adoptively transferred into HDM-stimulated mice via the tail vein. (B) The number of inflammatory cells including macrophages, neutrophils, eosinophils, and lymphocytes in BAL fluid. (C) Lung histology and semi-quantitative grading of lung inflammation score (H&E staining, 400× magnification); Lung histology and semi-quantitative grading of mucus score (PAS staining, 200× magnification); Semi-quantitative grading of fibrosis score (MT staining, 200× magnification). (D) The percentages of IL-5+CD4+ T cells and IL-13+CD4+ T cells in the lung and their dot plot. The percentages of Foxp3+CD25+Tregs among CD4+ T cells and IL-10+ Tregs in the lung and their dot plot. (E) The percentages of SiglecFCD11cCD11b+ MoMs and SiglecFCD11cCD11b+ AMs among total macrophages. The percentages of CD86+M1 and CD206+M2 among MoMs in the lung. The percentages of CD206+CD86+MHCIIm2b MoMs, CD206+CD86 MHCIim2a MoMs, and CD206+CD86 MHCIim2c MoMs among total macrophages in the lung. The percentages of Ly6c+ non-classical macrophage and Ly6c+ classical macrophage among total macrophages in the lung, PAS, periodic acid schiff; MT, masson's trichrome.

*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

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CD11b+ MoM populations were increased. However, these increases were not observed in the Ly6c mono group. Compared to the Ly6c mono group, the Ly6c mono group displayed reduced infiltration of Ly6c MoM to the lung and increased CD206 CD86 MHCII M2b and Treg populations.

Figure 6. (Continued) MSC-exposed Ly6c monocytes suppress the infiltration of monocyte into the lung and Th2 inflammation. (A) MSC-exposed Ly6c monocytes were adoptively transferred into HDM-stimulated mice via the tail vein. (B) The number of inflammatory cells including macrophages, neutrophils, eosinophils, and lymphocytes in BAL fluid. (C) Lung histology and semi-quantitative grading of mucus score (H&E staining, 400× magnification); Semi-quantitative grading of fibrosis score (MT staining, 200× magnification). (D) The percentages of IL-5 CIA-D4+ T cells and IL-13 CIA-D4+ T cells in the lung and their dot plot. The percentages of Foxp3 CIA-CD25+ Tregs among CIA-D4+ T cells and IL-10 Tregs in the lung and their dot plot. (E) The percentages of SiglecF CIA-CD1+CD11b+ MoMs and SiglecF CIA-CD11b- AMs among total macrophages. The percentages of CD86+ M1 and CD206+ M2 among MoMs in the lung. The percentages of CD206+CD86 MHCII+ M2b MoMs, CD206+CD86 MHCII- M2a MoMs, and CD206+CD86 MHCII- M2c MoMs among total macrophages in the lung. The percentages of Ly6c non-classical macrophage and Ly6c classical macrophage among total macrophages in the lung. PAS, periodic acid Schiff; MT, Masson's trichrome.
Co-culture with MSCs suppressed the differentiation of M2 macrophages and Th2 cells of hPBMCs

To test if the effects of MSC treatment can be extended to human samples, we co-cultured hPBMCs isolated from allergic asthma patients with MSCs. hPBMCs were cultured with recombinant hM-CSF for five days and recombinant IL-13 for one day to induce M2 macrophages. Then, MSCs (1×10^4 cells) were treated for one day and harvested for analysis (Fig. 7A). IL-13 stimulated hPBMC-derived macrophages were highly expressed M2-related genes, including MRC1, HLA-DR, and TGFB1, but these increases were reduced by direct
MSC co-culture. There are no changes in the indirect co-culture of MSCs compared to IL-13 stimulated hPBMC-derived macrophages (Fig. 7B).

To determine whether MSCs can suppress the Th2 differentiation of hPBMC, we differentiated hPBMCs into Th2 cells and co-cultured them with MSCs. Using an anti-CD3 coated plate, $1 \times 10^5$ hPBMC were seeded with CD28 monoclonal antibodies and recombinant IL-2, IL-4, and IL-13 for 3 days. MSCs ($1 \times 10^4$ cells) were administered for one day, and cells were harvested for analysis (Fig. 7C). Typical Th2-secreted cytokines, including IL4, IL5, and IL13, were increased in Th2 cytokine stimulated hPBMCs. These increases were reduced by direct and indirect MSC co-culture (Fig. 7D).

To summarize, MSCs suppressed M2 polarization and Th2 differentiation in stimulated hPBMCs.

**DISCUSSION**

This study showed the anti-inflammatory effect and the reversal of remodeling by intravenous MSCs by modulating monocytes/macrophages in constitutional IL-13 overexpression mice which have diverse features of chronic asthma.

Asthma is a heterogeneous disorder characterized by diverse endotypes. T2-high asthma is mediated by Th2 cytokines such as IL-4, IL-5, and IL-13. IL-13 is a key central regulator of IgE synthesis, eosinophil infiltration, goblet cell hyperplasia, mucus hypersecretion, airway hyper-responsiveness, and fibrosis (20). Indeed, IL-13 Tg mice exhibit striking chronic asthma-like tissue and physiological features, including eosinophilic inflammation, mucus metaplasia, airway fibrosis, and airway hyper-responsiveness (21). Using this IL-13 Tg mouse model, we confirmed that MSC administration ameliorated the pathologic features of chronic asthma by inhibiting macrophage activation.

Macrophages are the most abundant innate immune cells in the lungs which play important role in the maintenance of homeostasis in both normal physiological condition and pathologic inflammatory conditions. AMs are the primary phagocytes of innate immune system in the lung by clearing infectious, toxic, or allergic particles in the airway and can be subdivided into resident AMs and MoMs. In the steady state, resident AMs are hyporesponsive to inhaled particles and dust with restricted plasticity. These tissue resident AMs are sentinels that maintain immune balance. In contrast, newly recruited MoM move to the alveoli following insults and develop their own features with more plasticity and their functions are mainly dependent on the lung microenvironment (22). MoMs can differentiate into M1 and M2 subtypes depending on their microenvironment (23). In the current study, we found that intravenous MSCs administration downregulated the number of MoM cells in WT compared to IL-13 Tg mice. M2 macrophages affect immune regulation and tissue remodeling by resolving inflammations (23). However, under asthmatic lung inflammation, they cause excessive type 2 inflammation and airway remodeling during allergic lung inflammation by secreting type 2 and anti-inflammatory cytokines such as IL-4, IL-13, and TGF-β (24). M2 macrophages have been categorized into three subtypes, M2a, M2c, and M2b, based on their phenotypic and functional features. M2a cells contribute to Th2 inflammation and eosinophil infiltration by secreting high levels of IL-13 and chemokines. M2c is involved in tissue remodeling and fibrosis by expressing anti-inflammatory cytokines such as IL-10 and TGF-β (11).
MSC treatment effectively reduces airway remodeling and type 2 inflammation in asthma models, but its impact on the regulation of macrophage polarization was not actively investigated. MSCs interact with macrophages and induce phenotypic and functionally changes toward attenuating asthma. Phagocytosed MSCs altered the transcriptional profile of macrophages, which induced recruitment of Treg cells and downregulated the expression of M2-related genes in murine lung fibrosis model (25). Exosomes derived from MSCs attenuated inflammation by suppressing M2 polarization in a murine asthma model (26). In this study, although we did not separate exosome from MSC, transwell co-culture experiment showed that secretory molecules of MSCs might exert regulatory effects. We clearly proved the effect of MSCs on downregulating M2 markers such as Ym1, Cd163, and Tgfb1. Ym1 is expressed on M2a macrophages and is involved in inflammatory response (23). Cd163 and Tgfb1 are major mediators of pulmonary fibrosis expressed on M2c macrophages (27,28). Inhibiting M2 macrophage in the lungs leads to anti-fibrotic effects (27). Therefore, the mitigation of M2 macrophages from the MoM population, especially M2a and M2c, could be a therapeutic target for asthma by attenuating type 2 inflammation and airway remodeling. MSC-exosomes downregulate pro-fibrotic M2 macrophages, which leads to the attenuation of fibrosis and pulmonary airway remodeling (29). In addition, MSCs upregulate the expression of MMP13, which is a major interstitial collagenase that cleaves fibrous collagen in M2 macrophages (30). In the current study, MSCs significantly alleviated the CD206+ M2 macrophages. Particularly, intravenous MSCs lowered the increased M2a and M2c in IL-13 Tg mice. In the ex vivo lung macrophage experiment, MSCs directly downregulated the gene expression related to M2 phenotypes in MoMs. Among M2 subtypes, M2b macrophages are regulatory macrophages that secrete high levels of IL-10 and promote the recruitment of Tregs, which elicit a Th2 response. Similarly, our results showed increased M2b in IL-13 Tg mice following MSC treatment, which may be related to decreased Th2 response and increased Treg. Meanwhile, there are contradictory reports that MSCs may upregulate M2 macrophage activation (31,32). However, since M2 subtypes plays different roles in inflammation, fibrosis, and immunomodulation (33), those studies had limitations in that they did not confirm changes in the M2 subset. In contrast, we determined the effect of MSCs on regulating the MoM subtypes, M2a, M2c, and M2b macrophages.

Monocytes sense the microenvironment, recruit quickly to the inflammatory site, and differentiate into several subtypes of macrophages (34). They are identified as distinct subtypes based on the expression of surface markers in both humans and mice. In mice, these populations correspond to Ly6c- classical monocytes and Ly6c+ nonclassical monocytes (35). Ly6c+ monocytes regulate tissue repair by secreting anti-inflammatory cytokines. However, excessive Ly6c+ monocyte recruitment in chronic inflammatory diseases is likely to be involved in persistent inflammation and pathologic fibrosis (36-38). Although the effects of MSCs on macrophages have been studied on asthma, the effect of MSCs on monocytes requires more attention. MSCs preconditioned with lung monocytes and macrophages have an immune tolerance ability to suppress T-cell inflammation (39). The contribution of Ly6c+ monocytes to asthma pathogenesis has not been clearly defined. Here, MSC treatment suppressed the significant increase in total monocytes, especially the Ly6c+ monocyte population, usually seen in IL-13 Tg mice. Ly6c+ monocytes accumulate in inflammatory tissues and are more likely to differentiate into M2 macrophages, exacerbating type 2 inflammation and fibrosis in asthma (34). Correspondingly, we showed that intravenous MSC administration inhibits M2 differentiation of Ly6c+ monocytes in ex vivo cultures.
Interestingly, in the experiment on the effects of the intravenous adoptive transfer of Ly6c- monocytes, Ly6c- monocytes induced inflammatory cells infiltration and airway remodeling, as well as increased the MoM population and Th2 inflammation in the lung. On the other hand, when Ly6c- monocyte were indirectly exposed to MSCs, MoM populations in the lung were reduced, most likely due to their loss of homing abilities. Moreover, a decrease in Th2 inflammation and an increase in Treg and M2b were observed; we are planning to perform an additional study to elucidate whether Ly6c- monocytes are differentiated into M2b because M2b is known to interact with Treg. We proved that while Ly6c- monocytes play key roles in asthma exacerbation, MSC exposure can confer anti-inflammatory properties to circulatory Ly6c- monocyte. These results suggest that MSCs can inhibit the infiltration of circulating Ly6c- monocytes into the lungs and their subsequent polarization. Furthermore, we annotated a function for Ly6c- monocytes by confirming the crosstalk between Ly6c- monocytes and fibroblasts. Ly6c- MoMs-secreted molecules promoted the upregulation of gene expression related to fibrosis in fibroblasts, while the Ly6c- MoMs co-cultured with MSCs, had a reduced ability to induce fibrosis.

Although the monocyte-macrophage system has not been defined as a therapeutic approach in asthma, our study suggests that the monocyte-macrophage can be a therapeutic target against asthma. Intravenously administered MSCs can effectively relieve asthmatic airway remodeling via modulating circulating Ly6c- monocytes and their polarization.

Two different routes have been frequently used for MSC administration in murine asthma models – 1) systemic administration by intravenous injection (via the tail vein) targeting circulating monocytes and 2) direct intratracheal administration targeting AMs. The differential effect of two different routes was validated by selective depletion of macrophage by liposomal clodronate using murine allergic asthma model. Depletion of AMs by intratracheal clodronate injection resulted in aggravation of asthma while depletion of monocytes by intravenous clodronate injection attenuated allergic inflammation (40). The intravenous administration of MSCs is also confirmed to be equally effective in cases of pulmonary inflammatory diseases (41). In our previous study, intratracheal administration of MSCs to IL-13 Tg mice resulted in the reduction of the CD11c+CD11b+ macrophage population which might be a transient form of MoMs evolving to AMs (42). On the other hand, intravenous administration of MSCs downregulated the CD11c+CD11b+ macrophage population representing MoMs. Therefore, we suggest that target macrophage populations may be dependent on MSC administration routes. Our results show that the intravenous injection of MSCs can effectively modulate inflammatory MoMs, by successfully suppressing their M2 differentiation mainly in Ly6c- monocyte populations without affecting AMs.

In this study, monocytes exposed to MSCs were shown to acquire the ability to regulate macrophages and T cells. However, we also confirmed the immunoregulatory properties of MSCs as previous studies had shown. MSCs suppress the antigen-presenting function of DCs by inhibiting DC maturation and migration, resulting in the decreased activation of effector T cells (43). The cDC2 population, which induces Th2 differentiation, was also decreased in MSC-treated IL-13 Tg mice (Supplementary Fig. 6).

Consistent with previous reports presenting the amelioration of type 2 inflammation mediated by Th2 and ILC2, which secretes IL-4, IL-5, and IL-13 by MSCs (8,44), we observed that the infiltration of Th2 and ILC2s in lung tissue was suppressed by intravenous MSC treatment in IL-13 Tg mice, whereas Th17 was unexpectedly increased in MSC-treated WT
mice. Although we did not delve deep into Th17 properties, some researchers have reported that the co-culture of hPBMCs with MSCs increases the proliferation of Th17 cells and downregulates the expression of Th17 related gene profiles, such as the hallmark gene RORγt (45). In addition, intravenous MSC treatment in mice increased Th17 cells and transformed them into Treg phenotype (46). In particular, we confirmed that indirect communication with molecules secreted by MSCs was more effective in reducing ILC2 differentiation and enhancing Tregs proliferation than direct cell-cell communication. MSC-small extracellular vesicles reduce ILC2s in ex vivo cultures of hPBMCs from patients with allergic rhinitis (47). Meanwhile, the direct co-culture between ILC2 and MSCs derived from human tissue did not affect ILC2 features (48). miRNA-146a-5p suppresses ILC2 activation and attenuates allergic inflammation (47). Furthermore, MSC-derived soluble factors inhibit T cell activation by supporting Treg expansion and function. Tregs suppress airway inflammation by releasing anti-inflammatory cytokines including IL-40 and TGF-β (44). We showed that mRNA levels of the indoleamine 2,3-dioxygenase (IDO) and Prostaglandin E2 Synthase (PTGES2) which induce Treg from naïve CD4+ T cells are highly expressed in MSCs (Supplementary Fig. 7). IDO suppresses inflammation and airway hyperresponsiveness, and PTGES2 is an enzyme to produce the PGE2 which has an immunomodulatory function by regulating the production of leukotriene B4 in AM, eosinophil infiltration and smooth muscle constriction (44,49-51). Our study clearly showed the reduction of type2 inflammation and increase of IL-10+ Tregs in IL-13 Tg mice by MSC treatment.

Also, although IL-13 can mimic asthmatic phenotypes closely, its overexpression alone does not fully explain the pathogenesis of asthma. Thus, we replicated this study using HDM-induced allergic asthma models involving various cytokines (released by diverse immune cells) and their complicated interactions. When MSC was single administrated, a significant therapeutic effect in the HDM group was observed, and asthma phenotypes were attenuated compared to those of IL-13 Tg mice (Supplementary Fig. 8). Since the IL-13 Tg mouse used in this study represents a chronic asthma model characterized by severe airway remodeling and type 2 inflammation, it seems necessary to determine the appropriate dose and number of MSC administrations according to the severity of asthma.

This study has limitations. Firstly, although we demonstrated that MSCs alleviate the asthmatic phenotype by regulating Ly6c+ monocyte recruitment and suppressing M2 polarization, but in detailed mechanism was not revealed in this study. Therefore, the exact anti-asthmatic mechanism of MSCs need to be investigated especially focused on the monocyte-macrophage system. Secondly, since indirect culture with MSCs was more effective in regulating ILCs and Tregs than cell-cell contact in our study, we inferred that soluble factors secreted by MSCs play an important role. Therefore, further research should investigate the factors that mediate the anti-asthmatic effect of MSCs. Finally, we carried out the experiment in a xenogeneic model to prove the therapeutic effects of human MSCs preclinically. It was shown in previous studies that allogeneic MSCs are not only effective in attenuating allergic inflammation, but also low in immunogenicity due to the low expression of MHC-1, and non-expression of MHC-II or costimulatory factors (52,53). We demonstrated the anti-asthmatic effects of human MSCs on human cells. Co-culture with human MSC suppressed the differentiation of M2 macrophages and Th2 cells of human PBMCs isolated from allergic asthma patients. Therefore, MSCs may have anti-asthmatic effects on humans, and further studies are needed to prove this.
In conclusion, we demonstrate that the intravenous administration of MSCs attenuated asthmatic phenotypes in a chronic asthma model by modulating innate immune cells, such as ILC2s, M2a, and M2c macrophages, especially those differentiating from Ly6c- monocytes. By identifying M2 macrophage subtypes, we elucidate that exposure to MSCs transforms the phenotype and function of macrophages – thus, monocytes could be a therapeutic target for asthma treatment.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Primer sequences used in qPCR amplification

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Supplementary Table 2
Flow cytometry antibody list

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Supplementary Figure 1
Gating strategy for T cells and ILCs.

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Supplementary Figure 2
Gating strategy for macrophages

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Supplementary Figure 3
Gene expressions of MSC by RT-qPCR using mouse primer

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Supplementary Figure 4
Gating strategy for monocytes.

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Supplementary Figure 5
Effect of MSCs on Ly6c⁺ or Ly6c⁻ monocyte from IL-13 Tg mice

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Supplementary Figure 6
Intravenous MSCs administration decreased the cDC2 population

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Supplementary Figure 7
The gene expressions of IDO and PTGES2 in MSC

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Supplementary Figure 8
Intravenous MSCs treatment attenuated asthmatic phenotypes of the HDM-induced asthma model

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