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Mesenchymal Stem Cells Exert their Anti-Asthmatic Effects Through Macrophage Modulation in a Murine Chronic Asthma Model

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Mesenchymal Stem Cells Exert their Anti-Asthmatic Effects Through Macrophage Modulation in a Murine Chronic Asthma Model

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ABSTRACT

Asthma is a chronic airway inflammatory disease characterized by shortness of breath, airway hyperresponsiveness, and excessive mucus production. Despite the numerous attempts to reveal the pathogenesis of asthma, the current studies are limited to correlating asthma only with changes in T_H2 cells and T_H2 cytokines. Therefore, the interrelation between asthma and other cell types need to be elaborated to unveil the complete mechanisms in the pathogenesis of asthma.

Stem cell therapy using mesenchymal stem cell (MSC)s has recently emerged as a potential therapeutic approach for diseases with no known cure, such as severe asthma. MSCs can proliferate and differentiate into various cell types to repair damaged tissues or suppress inflammation, and their therapeutic effects can be further enhanced through priming. However, though numerous studies have successfully demonstrated the anti-asthmatic effects of MSCs in vivo models, none has been successfully introduced to human. Therefore, MSCs-based cell therapies with better efficiency are needed to be established for human application.

This study aimed to evaluate the therapeutic effects of intratracheally administered primed MSCs, and to explain their action mechanisms with lung macrophages in addition to T_H2 cells. First, the anti-asthmatic abilities of

umbilical cord (UC)-derived MSCs primed with Liproxstatin-1, a ferroptosis inhibitor, were examined using a murine chronic asthma model. Then, the macrophage modulation in asthmatic conditions were investigated in attempt to explain the mechanism of the observed anti-asthmatic effects of the MSCs. Moreover, the study sought to evaluate the potential of macrophages as a new therapeutic target for asthma.

Genetically modified transgenic (TG) mice constitutively overexpressing interleukin (IL)-13 in lung were used to represent chronic persistent asthma for the study. IL-13 TG mice showed excessive eosinophilic infiltration in the airways, mucus metaplasia, and airway remodeling. No change in T_H2 or innate lymphoid cell populations were noted, but prominent differences in macrophage populations were observed; *$CD11b^{int}F4/80^{high}$ macrophages were greatly diminished while $CD11b^{high}F4/80^{int}$ macrophages increased*. Among the *$CD11b^{high}$ $F4/80^{int}$* macrophages recruited to the lung interstitium in response to the inflammatory cues, IL-13 TG mice showed an upregulation of alternatively activated (M2) macrophages. The M2 macrophages of IL-13 TG mice showed high expression levels of Ly6C, a conceivable pro-inflammatory marker. Additionally, total $CD11c^+CD11b^+$ macrophages, that are also known to be pro-inflammatory, were also increased in numbers in IL-13 mice. Among the $CD11c^+CD11b^+$ macrophages, upregulation of Ly6C-expressing populations was noted in the IL-13 TG mice.

Upon intratracheal administration of Liproxstatin-1-primed human UC-MSCs (hUC-MSCs), a significant reduction in the total number of inflammatory cells, eosinophils, neutrophils, and lymphocytes in bronchoalveolar lavage fluid was observed. In the lung tissues, a decrease in inflammatory cell recruitments around the airways, mucus production, and collagen deposition were seen. In addition, a reduction in the total number of *CD11b^{high}F4/80^{int}* macrophages and Ly6C^{+}M2 macrophages, as well as CD11c^{+}CD11b^{+} macrophages were observed. Among the CD11c^{+}CD11b^{+} macrophages, the reduction in CD11c^{+}CD11b^{+} Ly6C^{+} populations were conspicuous.

In contrast to the CD11c^{+}CD11b^{+} macrophages of healthy WT mice that showed high expressions of SiglecF, those of the IL-13 TG mice showed low expressions. In the IL-13 mice that received the hUC-MSC, both SiglecF^{-} and siglecF^{+} CD11b^{+}CD11c^{+} macrophages were discovered. Moreover, both CD11c^{+}CD11b^{+} classically activated (M1) and alternatively activated (M2) macrophages were decreased in the IL-TG mice treated with MSCs compared to those that did not receive the treatment.

This study confirmed that intratracheally administered Liproxstatin-1-primed hUC-MSCs have effective anti-asthmatic abilities. In addition, the study revealed that the observed anti-asthmatic effects of the MSCs were exerted

through modulation of macrophage phenotypes and therefore validated the potential of macrophages as a new therapeutic target molecule for asthma.

Keywords: asthma, mesenchymal stem cells, cell therapy, interleukin-13, macrophage

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Introduction

Asthma, characterized by airway hyperresponsiveness, shortness of breath, and excessive mucus production, is a chronic airway inflammatory disease that has affected over 300 million people around the world (1) . The prevalence rate of asthma is continuously increasing and it is expected to reach 400 million patients within the next 5 years. Advanced researches in drug development and drug delivery have led to improvements in asthma treatment for the past 50 years, however, currently available drugs show limited efficacy, sustained mortality, and considerable adverse effects (2).

Recent attempts to develop more efficacious treatment for asthma has incorporated mesenchymal stem (Stromal) cell (MSC)s, the multipotent stroma cells that have extensive abilities to differentiate into variety of cell types. Their reparative immunoregulatory properties (3) make them an attractive therapeutic agent, and many studies are being conducted to develop MSC-based cell therapies for asthma and other chronic diseases (4-7). The anti-inflammatory and anti-asthmatic effects of the MSCs have been successfully demonstrated through numerous studies with various origins, dosages, and frequencies of MSCs, but the early studies were limited to injecting the MSCs intravenously (8-12). Although a significant amount of intravenously injected stem cells are to known to be first localized in the lungs

(13), there exists a disadvantage in delivery efficiency, as a great number of cells are lost before reaching the lung (14). To complement this limitation, an intratracheal administration method was introduced, targeting to improve the delivery efficacy and augment the therapeutic effects of the MSCs. Another method attempted to enhance the therapeutic efficacy of the MSCs is priming, which refers to exposing the MSCs to diverse molecules prior to administration. Although cytokines and growth factors are most frequently used priming substances (15), there also exist studies with other compounds, such as ascorbic acid 2-glucoside and liproxstatin-1 (16). Liproxstatin-1, commonly known as a lipid metabolic substance that inhibits ferroptosis (17), is known to mitigate tissue injuries (18), to suppress tumor growth (19), and to ameliorate neurodegeneration (20). When used as a priming factor for stem cells, the resulting human pluripotent stem cell-derived cardiomyocytes showed a significant enhancement in beating rates and amplitudes (21).

Various allergens including house dust mites (HDM), pollen, and tobacco smokes are known to trigger asthma, but the underlying mechanisms leading to its pathology are still unclear. Although recent studies have revealed the pathologic relevance to other innate and adaptive immune cells including T helper type 17 (T_H17) cells (22, 23) and type 2 innate lymphoid cell (ILC2)s (24-26), asthma has long been considered as a T helper type 2 (T_H2)-dominant disease (27-29). Interleukin (IL)-13, one of the most predominant T_H2

cytokines, is known to play pivotal roles in exhibiting asthmatic phenotypes, including, but not limited to, eosinophilia in the respiratory organs, mucus hyperproduction, and airway remodeling (30, 31). Consistent with the findings in animal models, asthma patients showed elevated levels of IL-13 in the blood and the sputum (32-34). As a significant amount of IL-13 is secreted by T_H2 cells, changes in the number of T_H2 cells are therefore prevalently considered as a measure of the disease severity. As proven by clinical cases, however, a reduced IL-13 secretion from T_H2 cells does not always lead to an alleviation of asthma. Here, I hypothesized that there exists another cell population that induces T_H2-like responses and make a significant contribution to the pathogenesis of the asthma. Proven that asthma patients show an elevation in the number of alternatively-activated (M2) macrophages (32, 35) that secrete IL-13, I sought to investigate how macrophage modulation is affected under asthmatic conditions and examine their potential as another possible therapeutic target.

Macrophages are the most abundant immune cells in the lung under homeostatic conditions (36), and play an important role in the pathogenesis of asthma (37). When macrophages perceive foreign substances in the airway, they release pro-inflammatory cytokines which result in the activation of naive T cells. In homeostatic conditions, macrophages reside in alveolar spaces and interstitium. Upon asthmatic stimulations, however, monocyte-

derived macrophages are rapidly recruited to the lung interstitum from the blood streams. Some of the recruited macrophages stay in the interstitum and differentiate into interstitial macrophages, while some move into the alveolar spaces to assist alveolar macrophages with clearing the allergens.

Though there are several perspectives on distinguishing the macrophages, they are most commonly categorized into two distinct populations depending on their polarization status. Based on the perceived stimuli, macrophages polarize towards either M1 or M2 subsets and this status can easily be reversed upon environmental changes (38). Classically activated (M1) macrophages are characterized by the expression of CD86 and nitric oxide synthase 2, whereas alternatively activated (M2) macrophages characteristically express CD206 (MRC1), Ym1, and Arg1 (39, 40). In terms of their functions, M1 macrophages are commonly understood as pro-inflammatory cells that participate in proactive clearing of the invading substances, while M2 macrophages are believed to play regulatory roles by assisting with restoration of lost cells and intracellular matrices (41).

Macrophages can also be divided into two different groups based on their origins. Yolk-sac derived resident macrophages are known to express intermediate levels of CD11b and high levels of F4/80, whereas monocyte-derived infiltrated macrophages express high levels of CD11b and intermediate levels of F4/80 (42). Another method of grouping macrophages

is based on their expression of surface makers and the residing locations, which discriminate $CD11c^{-}F4/80^{+}$ interstitial macrophages from $CD11c^{+}F4/80^{+}$ alveolar macrophages. Alveolar macrophages are often further characterized by their high auto-fluorescence and simultaneous expression of CD86 and CD206 (43-45).

As MSCs are known to exert cross-species immunosuppressive activities (46), the study aimed to evaluate the anti-asthmatic effects of the human umbilical cord derived MSC (hUC-MSC)s primed with Liproxstatin-1, using a murine chronic asthma model. The murine model used for this study are genetically modified transgenic (TG) mice that constitutively overexpress IL-13 in lung. They not only represent the airway inflammation seen in the chronic asthma patients, but also portray severe airway remodeling that results in shortness of breath. Additionally, I sought to further evaluate the changes within the macrophage populations and their characteristics in asthmatic conditions to explain the yet undiscovered pathogenesis of asthma, while evaluating the potential of macrophages as an additional therapeutic target.

Materials and Methods

Human peripheral blood mononuclear cell (PBMC) isolation

Heparinized blood samples from allergic rhinitis patients were kindly donated for research purposes only. Samples were diluted to 2x volume with PBS buffer (pH 7.2) and 2mM EDTA. 35mL of diluted samples were layered with 15 mL of Ficoll-Paque (GE Healthcare, Chicago, IL) and centrifuged at 400xg for 30 minutes at 25˚C without brake. The PBMC layer was then isolated and resuspended in RPMI1640 (Biowest, Riverside, MO) with 2.5% FBS (Biowest), 2.5% human AB serum (Sigma, St. Louis, MO), and antibiotics (penicillin-streptomycin, Sigma). The experiment with human samples followed the guidelines of the Institutional Review Board of Seoul National University (IRB No. 2006-142-1134 and 1708-083-878).

Liproxstatin-1-primed human umbilical cord-derived MSCs

All hUC-MSCs used in this study were cultured, prepared, and primed from Professor In-kyu Kim's laboratory (College of Biomedicine, Seoul National University, Seoul, Korea). MSCs were primed with Liproxstatin-1 (Sigma) at a 10:1 ratio 24 hours prior to injection.

Co-culture with human PBMCs

$1x10^5$ PBMCs and 10ng/mL of HDM extracts (Dermatophagoides pteronyssinus; Stallergenes Greer, Cambridge, MA) were seeded in a 96 well

plate coated with 0.5μg/mL of CD3 and 1μg/mL of CD28 for 24 hours at 4˚C. After another 24-hour incubation at 37˚C, Liproxstatin-1-primed hUC-MSCs were added at 1:1 ratio. Activated PBMCs were shortly co-cultured with MSCs for 48 hours at 37˚C and harvested for quantitative real-time polymerase chain reaction (RT-qPCR).

Co-culture with macrophages

Alveolar macrophage cell line (ATCC® CRL-2456™, Manassas, VA) was purchased and $1x10^5$ cells were seeded in a 96 well plate. After 6 hours, CRL-2456™ were treated either with PBS or 20 ng/mL of recombinant IL-13 (Biolegend, San Diego, CA). Cells were harvested 24 hours after treatment.

IL-13 TG mice

The transgenic mice were generated and kindly donated by Professor Jack A. Elias (Department of Molecular Microbiology and Immunology, Brown University, RI, USA). They are on a C57BL/6 background and use the Clara cell 10-kDa protein (CC10) promoter to target transgene. The constructs and the method used for the generation of cc10-IL-13 TG (IL-13 TG) mice are previously published (47, 48). The IL-13 TG mice were inbred and only heterozygotes were used for the experiment. Less than Five mice were housed in each cage under standard conditions of temperature and humidity, according to the guidelines of Biomedical Center for Animal Resource Development at the Seoul National University. All animal experiments

followed the guidelines of Seoul National University Institutional Animal Care and Use Committee (IACUC No. SNU-200525-1-1).

MSC injection and preparation of the animal model

Seven-week-old WT and IL-13 TG C57BL/6 mice were anesthetized with isoflurane and were intratracheally injected either with PBS or Liproxstatin-1-primed hUC-MSCs, resulting in four experimental groups; *healthy control group (WT/PBS), MSC-treated healthy group (WT/MSC), disease control group (IL-13 TG/PBS), and the MSC-treated disease group (IL-13 TG/MSC)* The mice were sacrificed four days after the MSC treatment and the bronchoalveolar lavage (BAL) fluid and lung samples were collected for analysis.

Collection of BAL fluid

WT or IL-13 TG C57BL/6 mice were injected intraperitoneally with a mixture of 90 mg/kg ketamin and 10 mg/kg xylazine before sacrifice. The trachea was exposed and the lungs were lavaged twice with 1 mL of phosphate buffered saline (PBS) through an incision made in the upper trachea. The collected BAL fluid was centrifuged at 3,000 rpm at 4˚C for 15 minutes. The supernatants were stored at -80˚C for further analysis and the pellets were used for total and differential cell counting.

Total and differential cell counting

The pellets obtained from BAL fluid samples were resuspended in 100μL of PBS; 10μL were mixed with 90μL of trypan blue for total cell counting and another 20μL were diluted with 60μL (WT) or 120μL (IL-13 TG) of PBS for cytocentrifugation (Shandon CytoSpin III, Shandon, UK). Cytocentrifugated slides were then stained with Diff. quick stain (Sysmex Co., Kobe, Japan) for one minute for each solution. At least 300 cells from random locations for each slide were manually counted under microscope.

Histological analysis

The PBS-perfused lung tissues from each mouse were fixed in 4% paraformaldehyde at 4˚C for 24 hours. The fixed tissues were then embedded in paraffin, cut into 3μm sections, and stained either with hematoxylin/eosin (H&E), periodic acid Schiff (PAS), or Masson's trichrome (MT). Staining processes were performed by experts at the pathology core facility at the Seoul National University Hospital Biomedical Research Institute. For MT staining, 3-point semi-quantitative grading was performed to assess the severity of collagen deposition: 0, none or nearly none; 1, a thin layer of fibrotic deposition; 2, a thicker layer; 3, very thick layers of fibrotic deposition are detectable around the vessels and the alveoli.

Collagen assay

Collagen assay allows for the quantification of the soluble collagen contents. Left lobe of PBS-perfused lung tissues from each mouse was removed and

the initial weight of sample was measured. The tissue was homogenized in an acid-pepsin mixture (0.1 mg pepsin/1mL 0.5M acetic acid) at 4˚C overnight to solubilize acid-pepsin soluble extracellular matrix. A Sircol™ Soluble Collagen Assay kit (Biocolor, Antrim, UK) and subsequent steps followed the protocols provided by the manufacturer without any modification.

RT-PCR

Total RNA was isolated with Trizol (Life Technologies, Carlsbad, CA) and chloroform and reverse transcription was performed with SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK, Cat. No. BIO-65053) according to the manufacturer's instructions. RT-qPCR was performed using SensiFAST™ SYBR® No-ROX Kit (Bioline, Cat. No. BIO-94020) also according to the manufacturer's instructions. Each gene was normalized to the expression level of the housekeeping gene GAPDH and the relative gene expressions were calculated using -ΔΔCt method. The primers used in the study are listed in table 1 and table 2.

Flow cytometry

Minced lung tissues were incubated in 5 mL of RPMI1640 with 10% Type IV collagenase (Worthington Biochemical Corporation, Lakewood, NJ) at 37˚C for 90 minutes and sorted through sterile cell strainer for single cell preparation. Cells were blocked with Fc receptor binding inhibitor antibody at room temperature for 5 minutes, and then incubated with fluorochrome-

labeled antibodies directed against cell surface markers for 30 minutes at 4˚C. All antibodies were purchased from Biolegend. The antibodies used are as follows; anti-CX3CR1 (SA011F11), anti-CD86 (GL-1), anti-CD206 (C068C2), anti-F4/80 (MB8), anti-I-A/I-E (M5/114.15.2), anti-siglecF (S17007L), anti-Ly6C (HK1.4), anti-CD45 (A20), anti-CD11b (M1/70), anti-CD11c (N418). Flow cytometry was performed with the BD LSRFortessa X-20 and analyzed by using FlowJo (version 10.6) software.

Statistical analysis

All statistical and graphic data were presented as means ± SEM using GraphPad Prism 7 (San Diego, CA, USA), unless otherwise specified. A p value less than 0.05 was considered statistically significant.

Table 1. A list of RT-qPCR primers used for human PBMCs

Gene	Forward	Reverse
Human		
IL-5	5′-AGC TGC CTA CGT GTA TGC CA-3′	5′-GCA GTG CCA AGG TCT CTT TCA-3′
IL-13	5′-TGA GGA GCT GGT CAA CAT CA-3′	5′-CAG GTT GAT GCT CCA TAC CAT-3′
IL-17	5′-GGA CTG TGA TGG TCA ACC TGA-3′	5′-TCA TGT GGT AGT CCA CGT TCC-3′
FOXP3	5′-TCA TCC GCT GGG CCA TCC TG-3′	5′-GTG GAA ACC TCA CTT CTT GGT C-3′
GAPDH	5′-TGT AGA CCA TGT AGT TGA GGT CA-3′	5′-AGG TCG GTG TGA ACG GAT TTG-3′

The Abbreviation used are: *IL, interleukin; FOXP3, Forkhead box P3*

Table 2. A list of RT-qPCR primers used for murine samples

Gene	Forward	Reverse
Mouse		
Mrc1	5′-CAA GGA AGG TTG GCA TTT GT-3′	5′-CCT TTC AGT CCT TTG CAA GT-3′
Ym1	5′-GGG CAT ACC TTT ATC CTG AG-3′	5′-CCA CTG AAG TCA TCC ATG TC-3′
Arg1	5′-CTC CAA GCC AAA GTC CTT AGA G-3′	5′-AGG AGC TGT CAT TAG GGA CAT C-3′
Fgf1	5′-GGG AGA TCA CAA CCT TCG CA-3′	5′-CTG TCC CTT GTC CCA TCC AC-3′
Fgf2	5′-GAA ACA CTC TTC TGT AAC ACA CTT-3′	5′-GTC AAA CTA CAA CTC CAA GCA G-3′
Fn1	5′-CAC GGG AGC CTC GAA GAG-3′	5′-ACA ACC GGG CTT GCT TTG-3′
Tgf-β	5′-AGG AGA CGG AAT ACA GGG CT-3′	5′-CCA CGT AGT AGA CGA TGG GC-3′
Mmp-9	5′-AAC CTC CAA CCT CAC GGA CA-3′	5′-TCA TCG ATC ATG TCT CGC GG-3′
Mmp-12	5′-TCA GTC CCT CTA TGG AGC CC-3′	5′-CAC AGA TGC AGA GAA GCC CA-3′
Muc5ac	5′-GGA ACT GTG GGG ACA GCT CTT-3′	5′-GTC ACA TTC CTC AGC GAG GTC-3′
Gapdh	5′-GGA GTC AAC GGA TTT GGT CGT A-3′	5′-CAA CAA TAT CCA CTT TAC CAG AGT TA-3′

The Abbreviation used are: *MRC, mannose receptor C-type; Arg, Arginase; FGF, fibrosis growth factor; TGF, transforming growth factor; MMP, matrix metallopeptidase*

Results

Liproxstatin-1-primed MSCs exerted greater anti-asthmatic effects than naïve MSCs did

To verify the enhanced anti-asthmatic properties of hUC-MSCs that are primed with Liproxstatin-1, each of the naïve and Liproxstatin-1-primed hUC-MSCs were co-cultured with human PBMCs. MSCs primed with Liproxstatin-1 showed augmented oxidative stress-resistance capacity and colony formation (Data not shown), indicating that their stemness was improved by priming. When co-cultured with HDM-activated PBMCs from a donor who has allergic rhinitis, the Liproxstatin-1-primed hUC-MSCs reduced mRNA levels of IL-5, IL-13, and IL-17 more effectively than the naïve MSCs did (**Figure 1a-c**), while yielding a bigger increment of $Foxp3^+$ regulatory T (Treg) cells (**Figure 1d**).

When co-cultured with M2 murine alveolar macrophages (ATCC® CRL-2456™) that were stimulated with recombinant IL-13 to mimic asthmatic conditions, Liproxstatin-1-primed hUC-MSCs showed a greater reduction in the gene expressions of M2 macrophage-related genes, such as *Mrc1*, *Ym1*, and *Arg1* (**Figure 2a-c**). In addition, Liproxstatin-1-primed hUC-MSCs showed a significantly greater ability in reducing the relative mRNA expressions of airway-remodeling-related genes, including fibroblast growth

factor (FGF), transforming growth factor beta (TGF-β), and matrix metallopeptidase (MMP)-12 (**Figure 2d-f**). Together, Liproxstatin-1-primed MSCs were proved to exhibit enhanced overall anti-asthmatic capacities than the naïve MSCs, and that their therapeutic effects were retained in xenogeneic recipients.

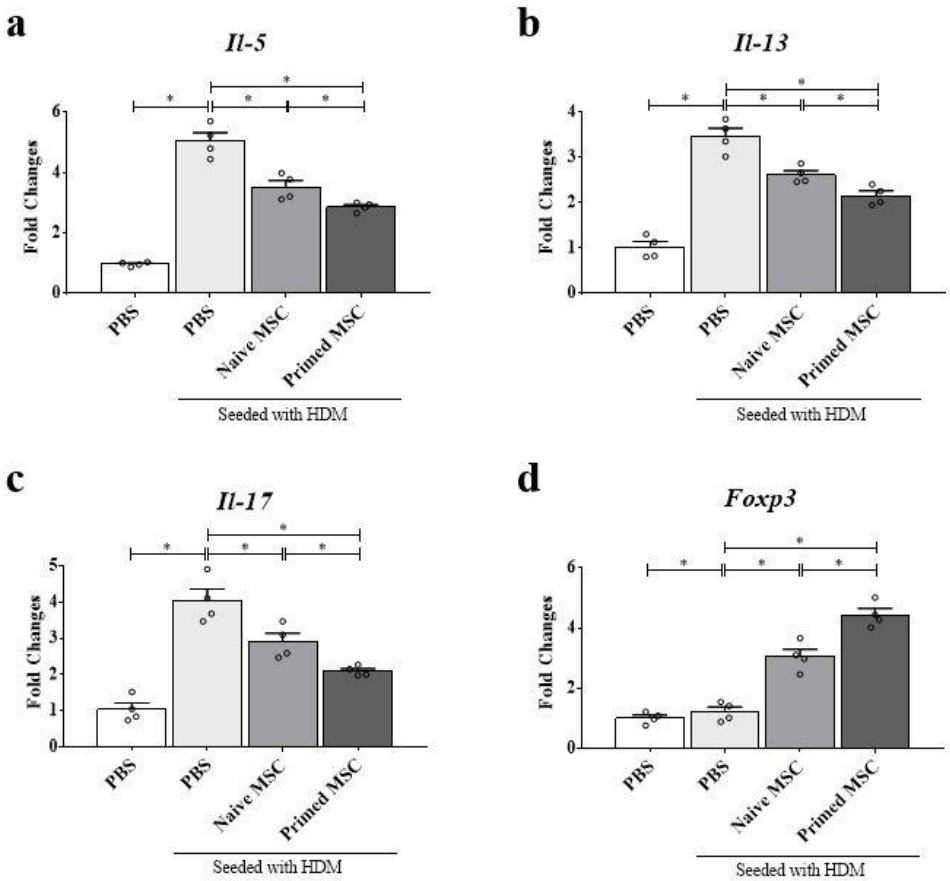

Figure 1. Effects of naïve and Liproxstatin-1-primed hUC-MSCs on human PBMCs.

(a-d) represents mRNA expressions relative to *Gapdh*. T_H2 cells (a-b), T_H17 cells (c), and regulatory T cells (d) were evaluated. Errors represent SEM and Mann-Whitney t-tests were used (*$p < 0.05$, $n = 4$). All results are representative of at least three independent experiments.

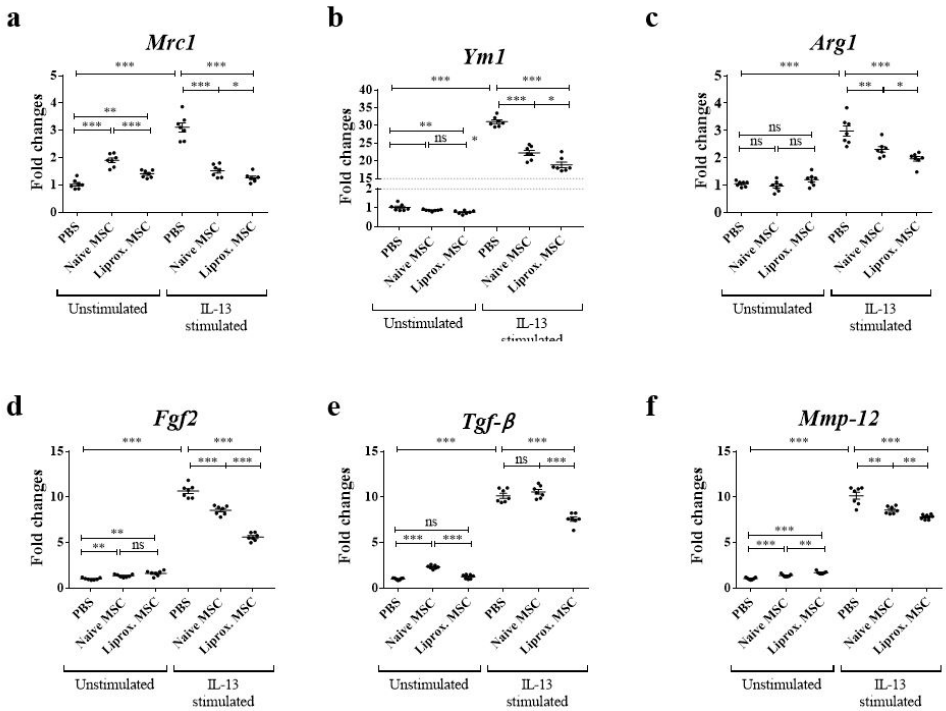

Figure 2. Effects of Naïve or Liproxstatin-1-primed hUC-MSCs on M2-related markers.

(a-f) represents mRNA expressions relative to *Gapdh*. M2 macrophage-related markers (a-c) and airway remodeling-related markers (d-f) were evaluated. Errors represent SEM and Mann-Whitney t-tests were used (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, $n = 6$). *ns* stands for not significantly different. All results are representative of at least three independent experiments.

Liproxstatin-1-primed hUC-MSCs reduced inflammatory cell infiltrations and mucus production in the airway of murine chronic asthma model

The anti-asthmatic abilities of the Liproxstatin-1-primed hUC-MSCs were further assessed using a murine model of chronic asthma that was genetically modified to constitutively overexpress IL-13 in the lung. In accordance to the hyperproduction of IL-13 in the lung, IL-13 TG mice showed elevated eosinophil counts in the airways, goblet cell hyperplasia, enlarged and multi-nucleated macrophages, and exacerbated subepithelial airway fibrosis.

Compared to the healthy control group (WT/PBS), the disease control group (IL-13 TG/PBS) showed a significant increase in total inflammatory cell counts with a rise in the numbers of macrophages, neutrophils, and eosinophils in the BAL fluid (**Figure 3a-c**). No significant difference in the numbers of total cells, macrophages, neutrophils, eosinophils, and lymphocytes were observed between the two WT mice groups, suggesting that Liproxstatin-1-primed hUC-MSCs themselves did not cause inflammatory responses in non-disease conditions, and that therefore, there was no observed xenogeneic effects to consider for the experiment. Between the two disease groups, the MSC-treated IL-13 mice showed a significant decrease in the numbers of all analyzed cell types, especially in eosinophils.

In accordance with the BAL fluid differential count data, histological analysis with H&E staining displayed decreased inflammatory cell recruitments around the vessels and the airways in the MSC-treated disease group, compared to the disease control group (**Figure 4a**). Quasi-absence of goblet cell hyperplasia (**Figure 4b**) and the reduced gene expression of *Muc5ac*, a gene linked to mucus secretion, in the MSC-treated IL-13 TG mice (**Figure 4c**) also indicated that Liproxstatin-1-primed hUC-MSCs exert anti-inflammatory effects in the murine model of chronic asthma.

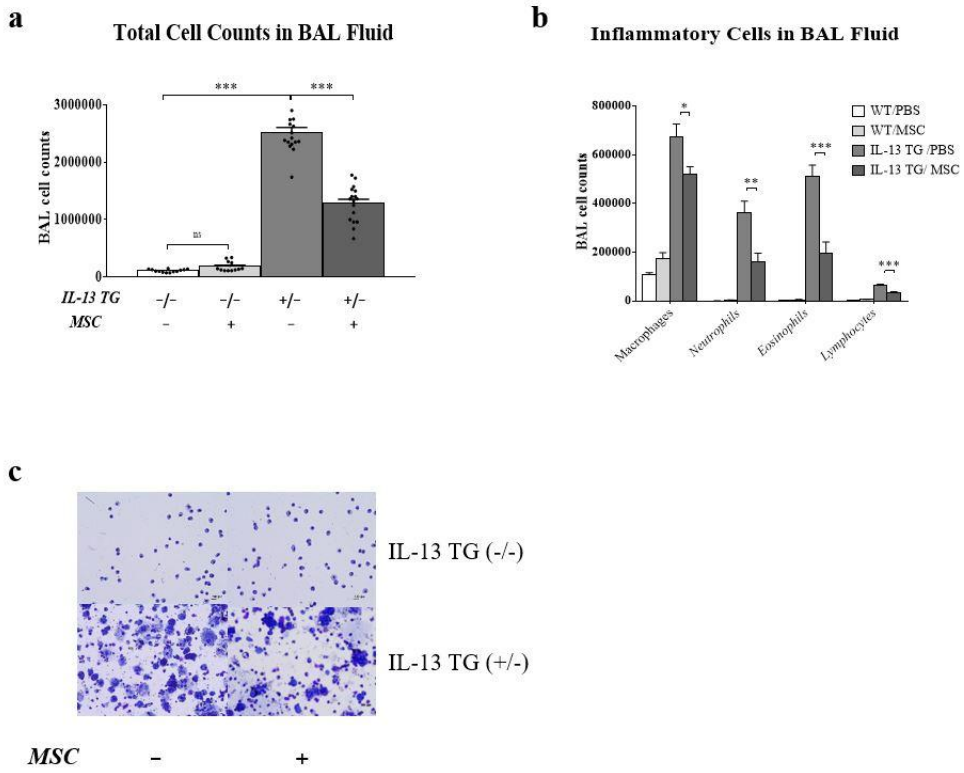

Figure 3. Liproxstatin-1-primed hUC-MSCs alleviated inflammation in the BAL fluid.

(a) shows the result of total cell counts and (b) shows differential counts of macrophages, neutrophils, eosinophils, and lymphocytes in BAL fluid. (c) shows microscopic images of Diff. Quick-stained slides of cytospined BAL fluid (x40). Samples from IL-13 TG groups were diluted with doubled amount of PBS compared with the samples from the wild type groups. Each bar represents the SEM of a minimum of thirteen animals, based on Mann-Whitney t-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. All results are

representative of at least three independent experiments.

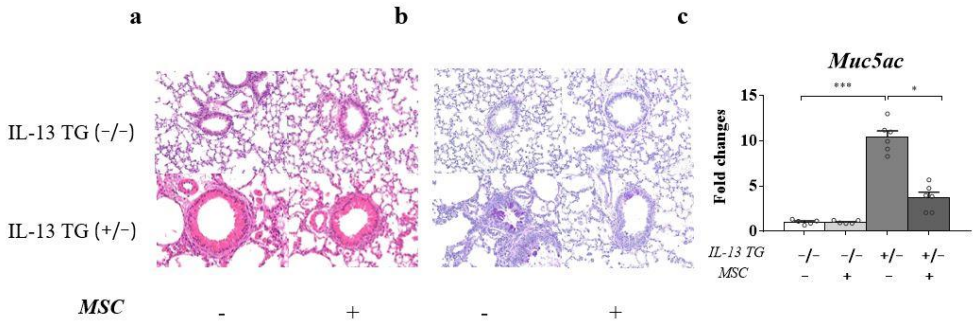

Figure 4. Liproxstatin-1-primed hUC-MSCs alleviated inflammatory cell recruitments and mucus production in the respiratory organs.

(a-b) shows histological comparison between the groups at a power of 20. H&E (a) and PAS (b) stains were used. (c) represents relative gene expressions of *Muc5ac to the housekeeping Gapdh.* (*$p < 0.01$, ***$p < 0.001$, $n > 5$).

Liproxstatin-1-primed hUC-MSCs suppressed airway remodeling in chronic asthma model

MT stained slides revealed excessive collagen deposition in the airway of the disease control group. In the MSC-treated disease group, on the other hand, a significant reduction of the collagen fibers was observed (**Figure 5a-b**). Sircol assay confirmed the histological data, that the amount of soluble collagen in the MSC-treated disease group showed approximately a 30% reduction compared to the disease control group (**Figure 5c**). The mRNA levels of fibrosis-related markers, Fgf1 and Fibronectin (Fn)1, and emphysema-related markers, *Mmp-9* and *Mmp-12*, were also compared among the groups. For all four markers, the MSC-treated IL-13 TG mice showed remarkable downregulations compared to those of the disease control group (**Figure 5d-g**), confirming that Liproxstatin-1-primed hUC-MSCs not only mitigated inflammation, but also abated airway remodeling at genetic levels.

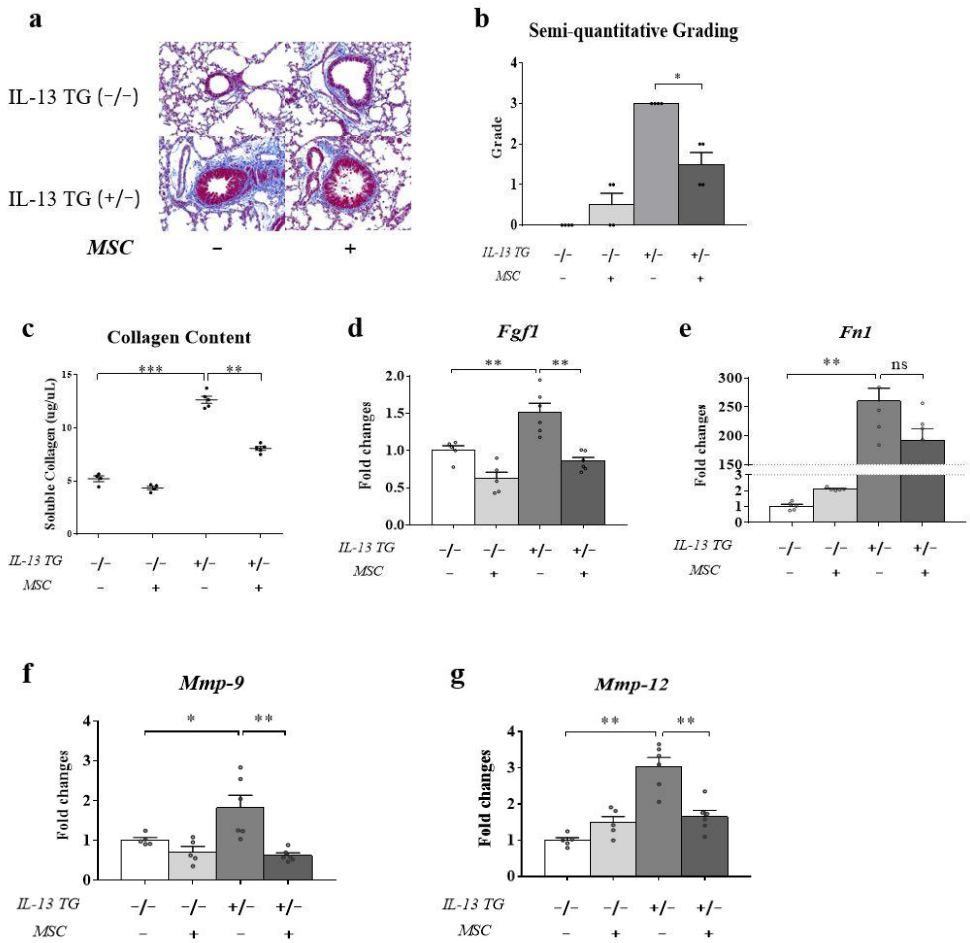

Figure 5. Anti-fibrotic ability of Liproxstatin-1-primed hUC-MSCs in the lung.

(a) shows Masson's Trichrome-stained slides of each group at low power (x20) and semi-quantitative grading was used to quantify the results (b). Then Sircol collagen assay was performed to quantify the soluble collagen contents in the homogenates of the lungs (c). (d-g) represents RT-qPCR results of fibrosis-related *Fgf1*(d) and *Fn1*(e), and airway remodeling-related *Mmp-9*

(f) and *mmp-12 (g)*. (d-g) represents fold changes relative to *Gapdh*. Errors bars reflect SEM and Mann-Whitney t-test was used (*$p < 0.05$, $n > 5$). *FGF, fibrosis growth factor; Fn, fibronectin; MMP, matrix metallopeptidase*.

Liproxstatin-1-primed hUC-MSCs led to alterations in the lung macrophage populations

The IL-13 TG mice showed no change in the numbers of T cells and ILCs (**Figure 6a-d**), indicating that the observed anti-asthmatic and anti-fibrotic effects of Liproxstatin-1-primed hUC-MSCs were not manifested through T cells or ILCs as is commonly acknowledged in allergen asthma models. The most evident differences between the WT and the IL-13 TG mice were shown within the macrophage populations. The disease control group showed a diminished count of $CD11b^{int}F4/80^{high}$ macrophages and a nearly doubled number of $CD11b^{high}F4/80^{int}$ macrophages compared to those of the wild type mice (**Figure 7a-c**). Upon the administration of Liproxstatin-1-primed hUC-MSCs, a partial recovery of the dissipated $CD11b^{int}F4/80^{high}$ macrophage population was observed (**Figure 7a-c**) in the IL-13 TG mice, indicating that Liproxstatin-1-primed hUC-MSCs caused alterations in the macrophage populations in the lung.

To further characterize the $CD11b^{int}F4/80^{high}$ macrophages and the $CD11b^{high}F4/80^{int}$ macrophages, their expression levels of Ly6C and CX_3CR1 were analyzed. A portion of $CD11b^{high}F4/80^{int}$ macrophages were found to be expressing high levels of Ly6C and the rest were expressing low levels, and their CX_3CR1 expression levels were low regardless of their Ly6C expressions. On the contrary, all $CD11b^{int}F4/80^{high}$ macrophages expressed

low levels of Ly6C and high levels of CX_3CR1 (**Figure 7d**). To investigate notable differences between the number of $CD11b^{high}F4/80^{int}$ macrophages that express high levels of Ly6C, the mean fluorescence intensity (MFI) of Ly6C were compared among groups. It was found that the MFI of Ly6C was extremely elevated in the disease control group, and Liproxstatin-1-primed hUC-MSC injection resulted in a significant downregulation (**Figure 7e**). The MFI of CX_3CR1 of $CD11b^{int}F4/80^{high}$ macrophages showed no significant difference between the groups (**Figure 7f**). Along with the elevated MFI, the percentages of Ly6C+ macrophages among $CD11b^{high}F4/80^{int}$ macrophages were upregulated in the IL-13 TG mice, and showed a significant reduction upon the intratracheal administration of hUC-MSCs (**Figure 7g**).

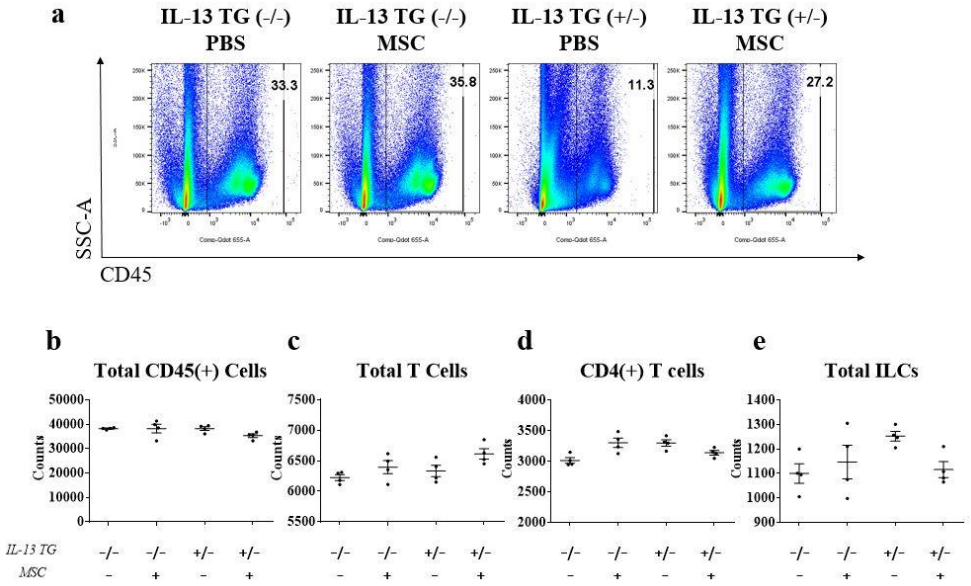

Figure 6. IL-13 TG mice showed no sign of proliferated CD45$^+$ leukocytes, CD3$^+$ T cells, or ILCs.

(a) represents a flow cytometric visualization of decreased percentages of CD45$^+$ leukocytes. (b-d) represents the absolute counts of CD45$^+$ leukocytes (b), CD3$^+$ T cells (c), CD4$^+$ T cells (d), or total ILCs (e). No significant statistic was found. *ILC stands for innate lymphoid cells.*

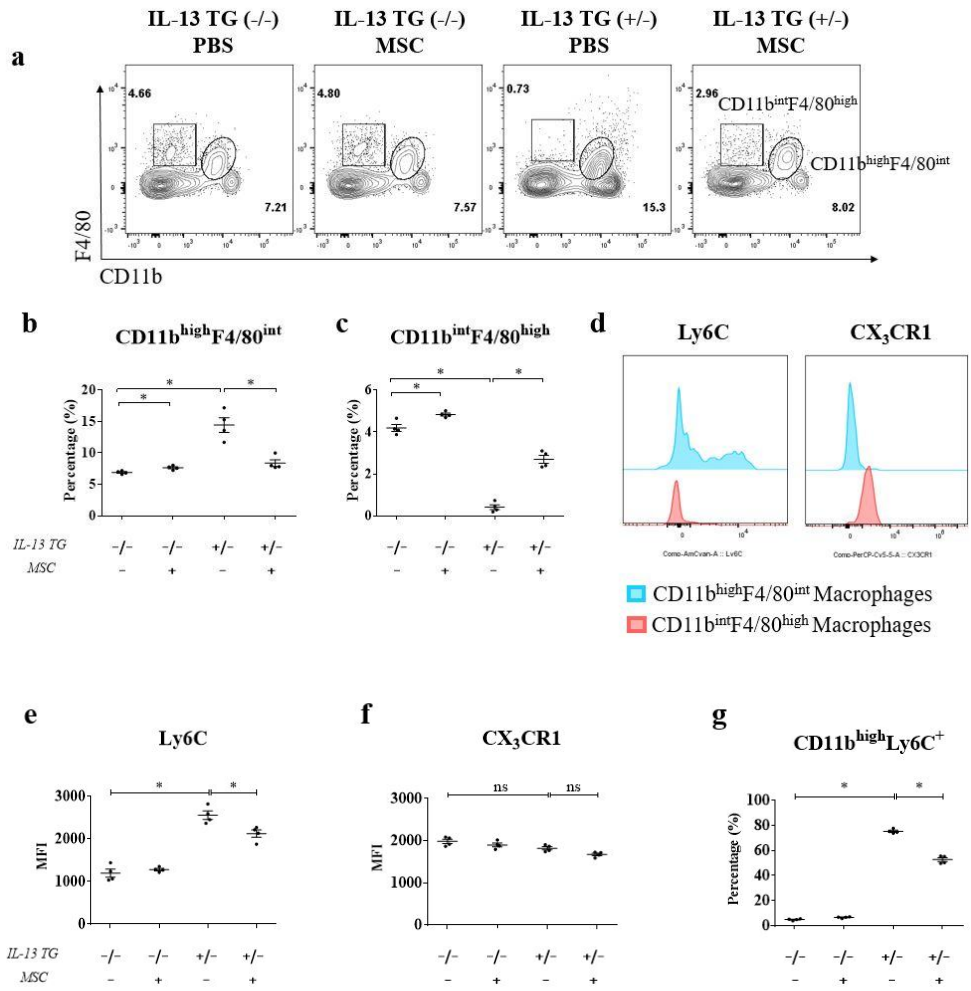

Figure 7. Liproxstatin-1-primed hUC-MSCs altered macrophage populations in the lung.

(a) Flow cytometry data showing two distinct macrophage populations that differ by the levels of CD11b and F4/80 expressions. Numbers represent percentages among total $CD45^+$ leukocytes excluding $SiglecF^+CD11c^-$ eosinophils. Based on the flow cytometry representations shown in (a),

percentages of $CD11b^{high}F4/80^{int}$ macrophages (b) and $CD11b^{int}F4/80^{high}$ macrophages (c) were each portrayed in graphs. (d) represent a histogram of Ly6C and CX_3CR1 expression levels of $CD11b^{int}F4/80^{high}$ macrophages and $CD11b^{high}F4/80^{int}$ macrophages. (e) represents the MFI of Ly6C of $CD11b^{high}F4/80^{int}$ macrophages and (f) shows the MFI of CX_3CR1 of $CD11b^{int}F4/80^{high}$ macrophages. (g) shows the percentages of $Ly6^{+}$ macrophages among $CD11b^{high}F4/80^{int}$ macrophages. Mann-Whitney t-test, $*p < 0.05$, and n=4.

Liproxstatin-1-primed hUC-MSCs reduced the number of Ly6C^{+}M2 macrophages

Next, the population shifts between M1 and M2 macrophages were investigated. To avoid a possible misleading interpretation, the CD11c^{+}F4/80^{+} alveolar macrophages were excluded, as they express surface markers for both M1 and M2 macrophages. The disease group that received the MSC treatment showed a significant reduction in the CD86^{-}CD206^{+} M2 macrophages compared to the disease control group, while showing no difference in the CD86^{+}CD206^{-} M1 macrophage populations (**Figure 8a-c)**. M1 and M2 macrophages were distributed unbiasedly among the CD11b^{int}F4/80^{high} macrophage and CD11b^{high}F4/80^{int} macrophage regions (data not shown), suggesting that the macrophage polarization occurrences are unaffected by their origins.

Next, the Ly6C expression levels of M1 and M2 macrophages were compared between all four groups. Neither M1 nor M2 macrophages from the healthy WT groups showed upregulated Ly6C expressions. The disease groups, on the other hand, showed inflated Ly6C expression levels in the M2 macrophages, but not in the M1 macrophages (**Figure 8d**). The results suggest that the M2 macrophage adapt Ly6C expressions in IL-13 enriched condition. When the percentages of the Ly6C^{+}M2 macrophages were evaluated among the total M2 macrophages, it was found that the ratio of the

Ly6C^{+}M2 macrophages was lower in the disease group with the MSC injection than the disease group without the MSC injection (**Figure 8e**). The data implies that the Ly6C^{+}M2 macrophages may be highly associated with the pathogenesis of asthma and the therapeutic mechanism of the hUC-MSCs.

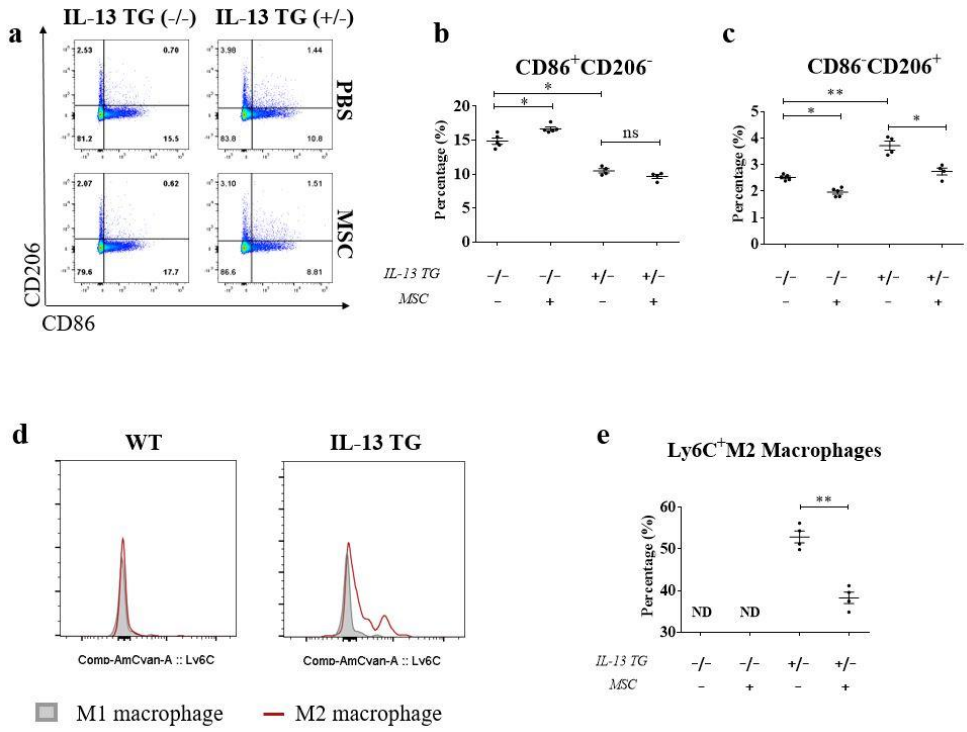

Figure 8. Liproxstatin-1-primed hUC-MSCs reduced M2 macrophages only in asthmatic conditions and they are characterized by high expression levels of Ly6C.

(a) Flow cytometric data on M1 and M2 macrophages. Numbers at each quadrant represent percentages of total macrophages excluding alveolar macrophages. (b-c) shows graphs of the changes in M1 and M2 populations among groups. (d) shows Ly6C expression levels in M1 and M2 macrophages of WT and IL-13 TG mice. (e) shows the ratio of Ly6C^{+}M2 macrophage populations among the total M2 macrophages, multiplied by a hundred. Mann-Whitney t-test was used and error bars represent SEM. *p < 0.05, **p

< 0.01, and $n = 4$

Liproxstatin-1-primed hUC-MSCs caused a reduction in the numbers of $CD11b^{+}CD11c^{+}$ pro-inflammatory macrophages.

The $CD11b^{int}$ to $CD11b^{high}$ macrophages were then subdivided by their CD11c expressions, resulting in three distinct populations; $CD11c^{+}CD11b^{-}$, $CD11c^{+}CD11b^{+}$, and $CD11c^{-}CD11b^{+}$ macrophages. In the two wild type mice groups, the $CD11c^{+}CD11b^{-}$ macrophages constituted approximately 20% to 25% of the total macrophages, in contrast to the two disease groups where they constituted less than 2% of the population (**Figure 9a-b).** The majority of the macrophages in the disease groups were found to be expressing both CD11c and CD11b, which showed a remarkable decrease in population after the MSC injection (**Figure 9a, 9c**). When the MFI of Ly6C of the $CD11c^{+}CD11b^{+}$ macrophages were analyzed, a remarkable reduction was noted (**Figure 9d**), suggesting that the number of $CD11c^{+}CD11b^{+}$ $Ly6C^{high}$ macrophages were decreased upon the MSC injection. Both $CD11c^{+}CD11b^{+}$ macrophages and $CD11c^{-}CD11b^{+}$ macrophages showed opposite trends between the WT and the IL-13 TG mice that did not received the MSC treatment (**Figure 9a, 9c, 9e**). It was demonstrated that the intratracheal administration of Liproxstatin-1-primed hUC-MSCs into the IL-13 mice partially reverted the composition ratios of the macrophage populations to those of the WT mice. Although the $CD11c^{-}CD11b^{+}$ macrophage populations of IL-13 TG mice seemed to hike with the largest growth rate upon MSC

administration in Figure 9a, it was revealed that the absolute counts of the population showed no significant change (**Figure 9f**). The result can be explained with the reduced numbers of total macrophages in the MSC-treated IL-13 TG mice (**Figure 9g**).

The increased $CD11c^+CD11b^+$ macrophages in the IL-13 TG mice showed an extremely strong correlation with the lung eosinophil population, with a correlation coefficient of 0.96507 (**Figure 9h**). Among $CD11c^+CD11b^+$ macrophages, Ly6C-expressing populations also showed a remarkable correlation with lung eosinophil counts ($r^2 = 0.94744$) (**Figure 9i**), suggesting that $CD11c^+CD11b^+Ly6C^+$ macrophages have a correlation with T_H2 responses.

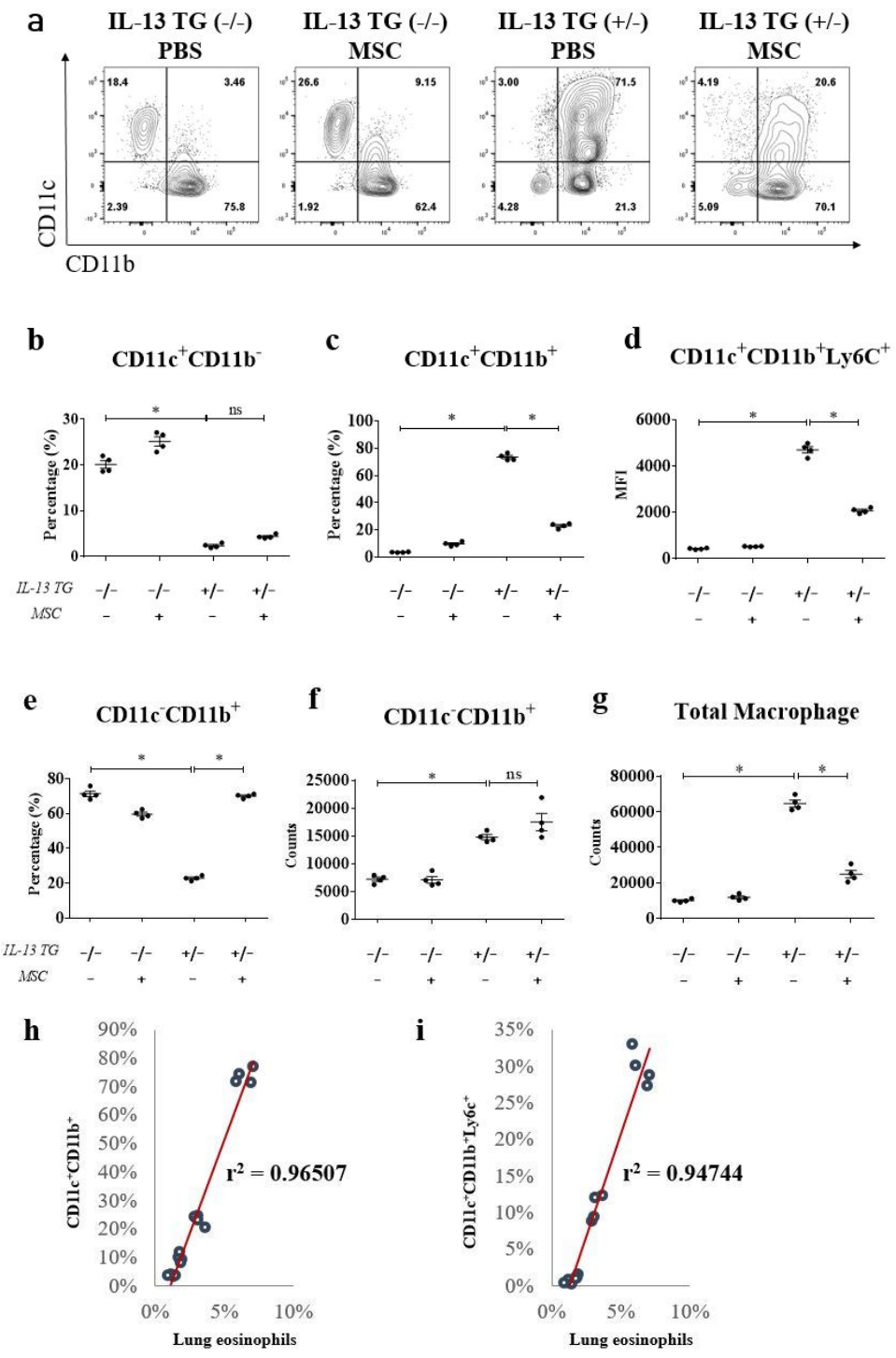
a
IL-13 TG (-/-) PBS
IL-13 TG (-/-) MSC
IL-13 TG (+/-) PBS
IL-13 TG (+/-) MSC
18.4
3.46
2.39
75.8
26.6
9.15
1.92
62.4
3.00
71.5
4.28
21.3
4.19
20.6
5.09
70.1
CD11c
CD11b
b
CD11c+CD11b-
c
CD11c+CD11b+
d
CD11c+CD11b+Ly6C+
Percentage (%)
MFI
ns
IL-13 TG
MSC
-/-
+/-
e
CD11c-CD11b+
f
CD11c-CD11b+
g
Total Macrophage
Counts
h
CD11c-CD11b+
r2 = 0.96507
Lung eosinophils
i
CD11c-CD11b+Ly6c+
r2 = 0.94744
Lung eosinophils

Figure 9. **Liproxstatin-1-primed hUC-MSCs caused a reduction in the numbers of $CD11b^+CD11c^+$ pro-inflammatory macrophages.**

(a) shows a flow cytometric representation of macrophage populations divided into four groups by their expressions of CD11b and CD11c. (b-c) show percentages of $CD11c^+CD11b^-$ (b) and $CD11c^+CD11b^+$ macrophages (c) among total macrophages. (d) represents the MFI of Ly6C of $CD11c^+CD11b^+$ macrophages. (e) shows the percentages of $CD11c^-CD11b^+$ macrophages of total macrophages and (f) represents the absolute counts of $CD11c^-CD11b^+$ macrophages. (g) is a graph of absolute counts of total macrophages among groups. The correlation between $CD11c^+CD11b^+$ macrophages and lung eosinophil is represented in (h) and that between $CD11c^+CD11b^+Ly6C^+$ macrophages and lung eosinophil are shown in (i). Mann-Whitney t-test was used and error bars represent SEM. *$p < 0.05$, and n =4 *MFI; mean fluorescence index*

The characteristics of $CD11c^{+}CD11b^{+}$ macrophages were altered in asthmatic conditions, and Liproxstatin-1-primed hUC-MSCs reverted their characteristics to those in healthy conditions

To further characterize the three distinct groups categorized with CD11b and CD11c expressions, their expression levels of SiglecF were compared. $CD11c^{+}CD11b^{+}$ macrophages of both wild type mice groups showed high expressions of SiglecF, whereas those of the disease control group showed a low expression. In the disease group that was injected with the Liproxstatin-1-primed UC MSCs, a co-existence of $SiglecF^{+}$ and $SiglecF^{-}$ macrophages was discovered. Although the observation was not supported by a statistical significance, the phenomenon was clearly represented by the two distinct peaks in the histograms analyzed by flow cytometry (**Figure 10a-b**). The conspicuous difference of SiglecF expressions between the WT and the IL-13 TG mice was not seen in $CD11c^{-}CD11b^{+}$ macrophage populations. For $CD11c^{+}CD11b^{-}$ and $CD11c^{-}CD11b^{-}$ macrophages, it was challenging to compare between the groups, as there were nearly none $CD11c^{+}CD11b^{-}$ macrophages in the IL-13 TG groups and only a few $CD11c^{-}CD11b^{+}$ macrophages in the WT groups.

The polarization status of $CD11c^{+}CD11b^{+}$ macrophages were assessed based on the existence of CD86 and CD206. Unlike the trends seen in $CD11b^{high}F4/80^{int}$ macrophages, both M1 and M2 macrophages of

$CD11c^+CD11b^+$ macrophages were upregulated in the disease control group (**Figure 10c-e**). The IL-13 TG mice that received hUC-MSCs showed reductions in both M1 and M2 macrophages. $CD86^+CD206^+$ populations were noted within the $CD11c^+CD11b^+$ macrophages of IL-13 TG mice, which also decreased dramatically after the MSC injection (**Figure 10f**). Collectively, the findings of the study indicated that Liproxstatin-1-primed UC MSCs leads a shift in macrophages in the asthmatic conditions towards the status of healthy wild type mice.

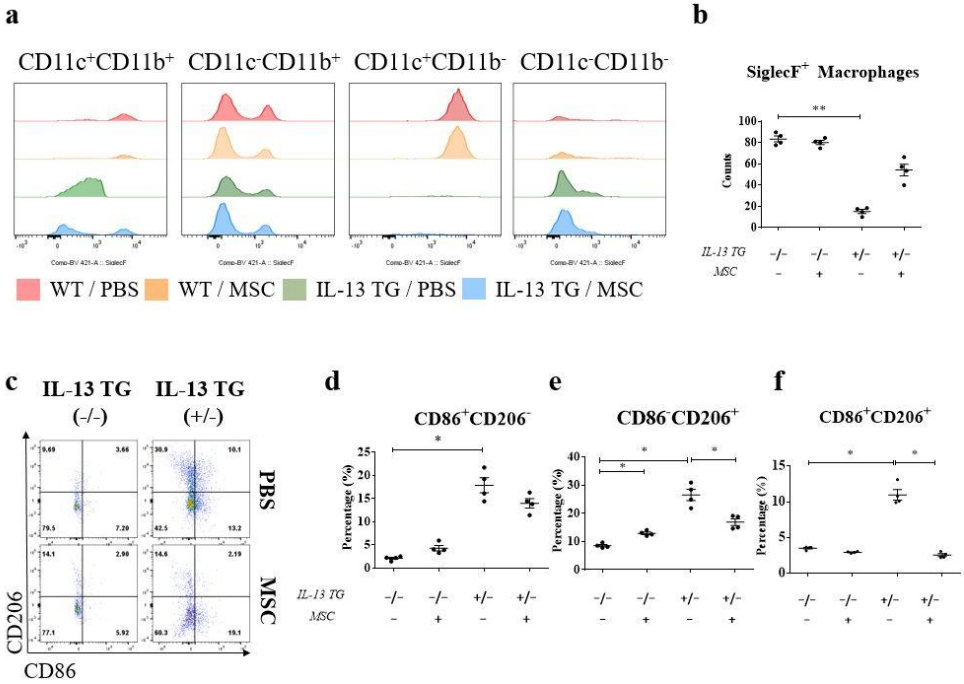

Figure 10. The characteristics of CD11c^{+}CD11b^{+} macrophages. (a) represents a histogram of SiglecF expressions of CD11c^{+}CD11b^{+}, CD11c^{-}CD11b^{+}, CD11c^{+}CD11b^{-}, and CD11c^{-}CD11b^{-} macrophages. The percentages of CD11c^{+}CD11b^{+}SiglecF^{+} macrophages are represented in a graph in (b). Flow cytometric representations of CD86^{+}CD206^{-} M1 and CD86^{-}CD206^{+} M2 macrophages along with CD86^{+}CD206^{+} macrophages are shown in (c), and scattered plots of each population is represented in (d-f). Mann-Whitney t-test was used and error bars represent SEM. *$p < 0.05$, and $n = 4$

Discussion

In this study, I evaluated the therapeutic abilities of Liproxstatin-1-primed hUC-MSCs against chronic asthmatic conditions and elucidated how their effects can partially be explained by modulation of macrophage phenotypes.

Asthma is a pulmonary disorder that is characterized by a T_H2 cell-driven inflammation, which results in airway inflammatory responses, bronchial hyperresponsiveness, and mucus hyperproduction (49). Induction of Treg cells, the subset of T cells responsible for maintaining homeostasis, are often appreciated as a therapeutic target due to their ability to inhibit the function of effector T cells (50-52). However, due to the lack of a complete understanding of the mechanisms underlying the pathogenesis of asthma, there exists a limitation in choosing the most effective therapeutic target.

Asthma is one of the most common chronic diseases and is estimated to affect an additional 100 million by 2025 (53). There have been numerous attempts to treat asthma using diverse therapies, including therapies using monoclonal anti-IgE antibodies and leukotriene receptor antagonists. The current treatment mostly depends on inhaled corticosteroids, which is known to ameliorate airway inflammation. However, there remains a significant number of patients still suffering from exacerbations of the disease, even with the inhaled corticosteroid treatments. Consequently, MSC-based stem cell

therapies were developed, utilizing regenerative and immunosuppressive potentials of MSCs. In addition to their accessibility and low requirements for maintenance in laboratory settings (54), their highly preserved self-renewal capabilities (55) were greatly appreciated. Despite the numerous attempts to utilize these advantages of the MSCs to treat asthma, however, only a single clinical study using intravenously administered bone marrow-derived MSCs was successful at entering the phase 1 investigation for clinical trials as of 2019 (56).

There has been a great number of previous studies on the development of stem cell therapies using MSCs with various origins, dosages, and frequencies of injection. Among the other tissue-derived MSCs, hUC-MSCs, in particular, are known to exhibit a higher colony-forming efficiency (57) and self-renewal rates (58). They were proven to assuage acute bacteria-induced respiratory distress syndrome (59) and bleomycin-induced pulmonary fibrosis in the lung (60). As the research targets human applications, the hUC-MSCs were chosen for the study.

The majority of the MSC studies on asthma injected the cells intravenously (8-12), though there were also a few cases that used intratracheal administration (61-63). Proven that the intravenous injection of the MSCs result in a significant cell loss (14), the intratracheal administration method was chosen to augment the delivery efficiency by delivering the MSCs more

locally to the lung. As for the concentration of the MSCs, both 10^5 and 10^6 counts were tested through a pilot study, and the result indicated that an injection of 10^5 is sufficient for a substantial reduction in the airway inflammation (data not shown). Therefore, the protocol for the study was finalized as administering 10^5 of hUC-MSCs intratracheally.

The immunoregulation of the MSCs is known to be further enhanced by a process called priming, otherwise known as licensing or pre-conditioning. The idea was developed from the inconsistent responses of the MSCs depending on the surrounding milieu (64). It was found that the immunoregulatory behaviors of the MSCs start at a minimal level and become educated when they are exposed to various environmental cues, such as inflammation (64-66). Interferon-γ (IFN-γ), the most extensively studied priming factor (65-68), and IL-13, a cytokine upregulated during asthmatic conditions, were tested as priming factors *in vitro*, but neither was successful at downregulating the mRNA expressions of asthma-related markers (data not shown). Another candidate examined was Liproxstatin-1 which has been proven to enhance functionalities of the tissues when used as a priming factor (21).

Starting from an *in vitro* experiment with human PBMCs, the study proved that both naïve and Liproxstatin-1-primed hUC-MSCs show anti-asthmatic effects by reducing T_H2 and T_H17 cytokines and upregulating $Foxp3^+$ Treg

cells. When primed with Liproxstatin-1, however, the degrees of reduction or increment became even greater than those of the naïve MSCs, corroborating that Liproxstatin-1-primed hUC-MSCs hold a greater therapeutic potential for treating asthma.

Before entering *in vivo* experiments with mice, I examined whether the reduction in T_H2 cells is either extended to, or in any kind of relation with, a reduction in the alternatively activated M2 macrophage ratios that are found be elevated in asthma patients along with T_H2 cytokines. Although M2 macrophages are generally believed to be involved in wound healing (69), some cases have reported that M2 macrophages can lead to disease conditions, such as tissue fibrosis (70) or gastric adenocarcinoma (71), and asthma has also been found to be associated with an upregulation of M2 macrophages (72, 73). In responses to the MSCs, the macrophage immunomodulation has been shown to ameliorate experimental bronchopulmonary dysplasia, restore lung function, decrease septal fibrosis (74), and alleviate allergic asthma (4). When the changes in the macrophage modulation were assessed by co-culturing M2-stimulated murine macrophages with either naïve or Liproxstatin-1-primed hUC-MSCs, it was discovered that the macrophages co-cultured with Liproxstatin-1-primed hUC-MSCs showed a greater downregulation in the mRNA levels of M2-related genes.

The experiment was then taken to the next step with a murine chronic asthma model, represented by a genetic modification which lead to an overexpression of lung-specific IL-13 levels. The IL-13 TG mice portrayed a majority of the symptoms seen in chronic asthma patients, which include the inflammatory cell recruitments in the respiratory tissues, hyperproduction of mucus, and subepithelial fibrosis. Intratracheal injection of human-derived MSCs into a wild type mice did not cause noteworthy inflammatory responses, despite the heterogeneity in species between the donor and the recipient. A slight increase in the numbers of macrophages was detected, but it was inferred to be a part of the natural clearance process against foreign substances, as the degree of the increase was not significant. In the IL-13 TG mice, the intratracheal injection of Liproxstatin-1-primed hUC-MSCs resulted in a remarkable decrease in eosinophil and other immune cell infiltrations into the respiratory tissues. Reduced mucus production and assuaged airway remodeling further substantiated the abilities of Liproxstatin-1-primed hUC-MSCs to mitigate asthma *in vivo*.

Generally, the focus is placed on the subsets of T cells and ILCs when discussing the pathogenesis or the progression index of asthma (8-12). The IL-13 TG mice used for the study, however, did not show an increase in T cell or ILC populations. Therefore, the apparent anti-asthmatic effects of Liproxstatin-1-primed hUC-MSCs could not be explained in terms of T cells

and ILCs in this model. As the mice maintained elevated IL-13 levels since birth, instead of being repeatedly sensitized and challenged to allergens, I assumed that the T cells and ILCs did not recognize the overexpressed IL-13 as an unusual condition that necessitate immune reactions. Given that neither the proliferation nor the activation of T cells and ILCs were observed, I presumed that there must be other cell types that are activated by the excess IL-13. Macrophages, as their polarization statuses are known to be altered under asthmatic conditions and are also highly associated with T_H2 cells (35), was one of the top candidates. Here, I hypothesized that the changes in the macrophage modulation could partially explain the anti-asthmatic responses of Liproxstatin-1-primed hUC-MSCs that cannot be answered by T_H2 cells or ILCs.

First, the modulation of macrophages in asthmatic conditions was probed. A pronounced difference in the origins of the macrophages was noted between the healthy and the disease groups. The $CD11b^{int}F4/80^{high}$ macrophages were mostly diminished, in contrast to the nearly doubled $CD11b^{high}F4/80^{int}$ macrophages in the disease control group. The MSC injection to the IL-13 mice caused a partial reversal of the $CD11b^{high}F4/80^{int}$ and $CD11b^{high}F4/80^{int}$ population compositions by downregulating $CD11b^{high}F4/80^{int}$ macrophages while upregulating $CD11b^{int}F4/80^{high}$ macrophages. CD11b is associated with leukocyte migration (75), and often, $CD11b^{high}F4/80^{int}$ macrophages are

considered as monocyte-derived macrophages (42, 76) and $CD11b^{int}F4/80^{high}$ macrophages as resident macrophages in the alveolar spaces. The monocyte-derived macrophages, however, are generally referred to the recruited pro-inflammatory macrophages that come from the blood vessels in response to the asthmatic stimuli. There also exists $CD11b^{high}F4/80^{int}$ macrophages in steady states, which are called interstitial macrophages, that are monocyte-derived but are not pro-inflammatory (77). The interstitial macrophages are often misinterpreted as pro-inflammatory due to their high CD11b expressions, but they are rather involved in maintaining homeostasis and preventing allergic inflammations in truth (77, 78). The interstitial macrophages are known to differ from the pro-inflammatory infiltrated macrophages in their Ly6C expression levels; anti-inflammatory interstitial macrophages express low levels of Ly6C, and infiltrated pro-inflammatory monocyte-derived macrophages express high levels.

Along with the levels of Ly6C, which are known to be pro-inflammatory and pro-fibrotic (79), the expression levels of leukocyte migration-related CX_3CR1 were compared among $CD11b^{int}F4/80^{high}$ and $CD11b^{high}F4/80^{int}$ macrophages. All $CD11b^{int}F4/80^{high}$ macrophages of the wild type mice showed low expressions of Ly6C and high levels of CX_3CR1, which showed a correspondence to the phenotypes of the patrolling nonclassical monocytes. On the contrary, $CD11b^{high}F4/80^{int}$ macrophages showed two distinct

populations that are either $Ly6C^{low}$ or $Ly6C^{high}$, and both populations were expressing low levels of CX_3CR1. The $CD11b^{high}F4/80^{int}Ly6C^{high}CX_3CR1^{low}$ macrophages are thought to be derived from the pro-inflammatory classical monocytes, which differentiate into macrophages upon detecting an extravasation of pro-inflammatory cytokines (80, 81), and are recruited to the lung to take on an active pro-inflammatory roles in asthmatic conditions. The $CD11b^{high}F4/80^{int}Ly6C^{low}CX_3CR1^{low}$ macrophages, therefore, can be understood as interstitial macrophages. It was found that the IL-13 TG mice have a higher MFI of Ly6C among $CD11b^{high}F4/80^{int}$ macrophages than the WT mice, and an intratracheal injection of the Liproxstatin-1-primed hUC-MSCs into the IL-13 mice resulted in a significantly lowered MFI of Ly6C. The result suggests that the numbers of $CD11b^{high}F4/80^{int}Ly6C^{high}CX_3CR1^{low}$ macrophages may reflect, at least to some extent, the severity of asthma.

It is well known that the monocyte-derived macrophages migrate into alveolar spaces and adapt alveolar macrophage-like morphologies (78, 82), but there has not been any evidence published supporting that the alveolar macrophages migrate into the interstitium. In this study, an apparent evidence suggested that $CD11b^{int}F4/80^{high}$ macrophages in alveolar spaces also have ability to migrate into the interstitium, where I inferred that they become a part of the $CD11b^{high}F4/80^{int}$ macrophage population. To the best of my understanding, $CD11b^{high}F4/80^{int}$ macrophages therefore consist of three

distinct populations $CD11b^{int}F4/80^{high}$ macrophage-derived macrophages, infiltrated monocyte-derived macrophages, and interstitial macrophages. Further studies would be necessary to validate my inference and to reveal the associated mechanisms.

Using fluorochromed CD86 and CD206 antibodies, the polarization status of the macrophages was assessed. Prior to analyzing, $CD11c^{+}F4/80^{+}$ alveolar macrophages were gated out, as their autoflorescence and co-expressions of CD86 and CD206 may lead to an inaccurate result. Upon the exclusion of $CD11c^{+}F4/80^{+}$ alveolar macrophages, it was noted that the majority of the $CD11b^{int}F4/80^{high}$ macrophages were also removed, indicating that the alveolar macrophages are mostly the $CD11b^{int}F4/80^{high}$ macrophages that resemble the stationary, patrolling characteristics of nonclassical monocytes. It was demonstrated that the CD206 single-positive M2 macrophages were upregulated in the disease control group, which was consistent with the trends seen in the clinical data of the asthma patients (32, 35). The number of the $CD86^{+}CD206^{-}$ M1 macrophages, on the contrary, was downregulated. The administration of the Liproxstatin-1-primed hUC-MSCs resulted in a significant reduction in the M2 macrophage population, while causing no notable change in the M1 macrophage population. I understand that this could be controversial, as M2 macrophages are generally considered to play regulatory roles. However, recent studies have revealed that M2 macrophages

promote tumor progression (83) and fibrogenesis (84). In relation to asthma, M2 macrophages are known to secrete FGF, TGF-β, and MMP that are highly associated with airway remodeling (47, 85, 86) seen in chronic asthma patients.

By characterizing the M2 populations by their Ly6C expression levels, it was found that the M2 macrophages exhibited high Ly6C expressions only under the disease conditions. As $Ly6C^{high}$ monocytes have been shown to acquire both inflammatory and regulatory phenotypes depending on the exposure to the milieu (87), $Ly6C^{-}$M2 macrophages of the wild type mice may be devoted to maintaining homeostasis (88), whereas the $Ly6C^{+}$M2 macrophages of the IL-13 TG mice may have adapted pro-inflammatory and pro-fibrotic phenotypes in response to the asthmatic environment. The IL-13 TG mice that received the MSC injections showed a decrease in the populations of $Ly6C^{+}$M2 macrophages, suggesting that the $Ly6C^{+}$M2 macrophages may be associated with the pathogenesis of asthma.

The expressions of CD11b among the total macrophages were evaluated, together with their expressions of CD11c. $CD11c^{+}CD11b^{-}$ macrophages are often defined as the alveolar macrophages, and $CD11c^{-}CD11b^{+}$ as interstitial macrophages (77). Both populations corresponded each of the $CD11b^{int}F4/80^{high}$ and $CD11b^{high}F4/80^{int}$, respectively, which were specified earlier in this study (data not shown). The $CD11c^{+}CD11b^{+}$ macrophages are

also known as pro-inflammatory macrophages that secrete IL-6, IL-12, and tumor necrosis factor (TNF)-α (89, 90). The disease control group showed a significant upregulation of the $CD11c^{+}CD11b^{+}$ macrophages. When the $Ly6C^{+}$M2 macrophages of the IL-13 TG mice were back-traced, it was found that the majority were expressing both CD11c and CD11b (data not shown). A strong correlation between this upregulation and the lung eoscinophil counts substantiated that the $CD11c^{+}CD11b^{+}$ macrophages may be highly involved in the pathogenesis of eosinophilic asthma. Although it seemed that the $CD11c^{-}CD11b^{+}$ macrophages in the IL-13 TG mice were also upregulated, the absolute count revealed that the population did not experience any significant changes in numbers. The MFI of the $CD11c^{+}CD11b^{+}Ly6C^{+}$ macrophages was enormously elevated in the disease control mice, suggesting that the $CD11c^{+}CD11b^{+}$ macrophages are the key regulator of the asthmatic phenotypes. When the Liproxstatin-1-primed hUC-MSCs were administered to the disease control mice, the percentages of the total $CD11c^{+}CD11b^{+}$ macrophages were decreased. Both $CD11c^{+}CD11b^{+}$ and $CD11c^{-}CD11b^{+}$ macrophages from the disease control group obtained the population distribution patterns of the wild type groups when injected with the Liproxstatin-1-primed hUC-MSCs.

Furthermore, the SiglecF expression levels of the $CD11c^{+}CD11b^{+}$ macrophages elaborated the ability of the Liproxstatin-1-primed UC MSCs to

cause shifts towards the wild type phenotypes. The $CD11c^{+}CD11b^{+}$ macrophages of the healthy and the disease control mice showed an opposing trend; those of the healthy control mice expressed high SiglecF expressions, while those of the disease control mice showed low expressions. SiglecF is often used as a marker for immunoregulation in inflammatory environment (91, 92), and therefore, the low SiglecF expressions exhibited by the $CD11c^{+}CD11b^{+}$ macrophages of the disease control group confirm that they are highly inflammatory. The disease group with the MSC treatment showed two distinct populations that are either $SiglecF^{-}$ and $SiglecF^{+}$. The changes in SiglecF expression levels among the groups were not observed in any of the $CD11c^{-}CD11b^{+}$ macrophages, $CD11c^{+}CD11b^{-}$ macrophages, or $CD11c^{-}CD11b^{-}$ macrophages.

The polarization status of the $CD11c^{+}CD11b^{+}$ macrophages were discovered to be different from those observed in the $CD11b^{high}F4/80^{int}$ macrophages, as the M1 macrophages were also upregulated in the disease control mice. The Liproxstatin-1-primed hUC-MSCs resulted in a reduction of both M1 and M2 $CD11c^{+}CD11b^{+}$ macrophage population sizes. Additionally, $CD86^{+}CD206^{+}$ populations showed the same trends with both M1 and M2 macrophages, that was upregulated in the disease control group and downregulated upon the MSC injection. I inferred this population to be alveolar macrophage-derived populations as they express bothCD86 and CD206, but further studies are

needed to investigate their origins. Together with the phenotype alterations observed in $CD11c^{-}CD11b^{+}$ macrophages, it can be suggested that the MSCs stimulate phenotype shifts among macrophages towards the healthy status.

In conclusion, this study proves the anti-asthmatic effects of Liproxstatin-1-primed hUC-MSCs and spotlighted the $Ly6C^{+}$ M2 macrophage- and $CD11c^{+}CD11b^{+}$-directed explanations on describing the pathogenesis of asthma. The study elucidates the altered modulation in macrophage populations resulting from the MSC treatment could partially answer the pathogenesis of asthma, and therefore, I concluded that macrophages may be considered as a new therapeutic target for asthma.

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국문초록

만성 천식 마우스모델에서 대식세포 조절을 통한 중간엽줄기세포의 항천식 효과 연구

서울대학교 의과대학 중개의학교실

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천식은 호흡 곤란, 기도 개형 및 기도 과민성의 증가 그리고 점액의 과다 분비를 특징으로 하는 기도내 만성 염증 질환이다. 천식의 병인 기전은 많은 노력에도 불구하고 아직까지 명확히 알려지지 않았으며, 현재까지 진행된 연구들은 주로 천식을 Th2 도움세포의 관점으로만 설명하고 있다는 한계점이 존재한다. 따라서 천식의 병인기전을 완벽하게 파악하기 위해서는T_H2 도움세포를 제외한 또 다른 세포와 천식 간의 상관관계를 확인하는 연구가 필요한 상황이다.

줄기세포를 이용한 세포치료제 개발은 현재 전 세계적으로 주목을 받고 있다. 다양한 조직에서 줄기세포의 재생효과가 검증되었으며, 그 효과는 프라이밍 기법을 통하여 더욱 향상된다고 알려져 있다. 그 중에서도 무한 증식이 가능하며 다양한 계열의 세포로 분화할 수 있는 장점을 가진

중간엽줄기세포(MSC)는 천식과 같은 난치성 질병의 연구에 많이 이용되고 있다. 지금까지 다양한 MSC의 항천식 효과는 보고된 바 있다. 하지만 이를 입증한 연구는 대부분 정맥 내로 MSC를 투여하는 방법을 사용하고 있으며 기도 투여 방법이나 프라이밍된 중간엽 줄기세포를 이용한 연구는 아직 많이 수행되지 않았다.

본 연구에서는 프라이밍 된 MSC를 기도투여 하였을 때의 항천식 효과를 확인하고 그 기전을 T_H2도움세포가 아닌 대식세포의 관점에서 설명하고자 하였다. 먼저, 인터루킨-13 (IL-13)을 폐 특이적으로 과발현 시킨 만성 천식 마우스 모델을 이용하여, ferroptosis 억제제로 알려진 Liproxstatin-1으로 프라이밍한 인간 탯줄유래 중간엽줄기세포(UC-MSC)를 기도투여 하였을 때의 항천식 효과를 확인하였다. 다음으로, 천식환경에서 대식세포의 변화를 관찰함으로써 대식세포의 조절이 천식에 미치는 영향을 규명하고 MSC가 가지는 항천식 효과의 기전을 설명하였다. 또한, 대식세포가 가지는 천식 치료의 새로운 타겟 세포로서의 가능성을 확인하였다.

만성 천식을 구현한 IL-13과발현 마우스에서 호산구 증가를 중심으로 전체적인 기도와 폐의 염증이 증가하고, 점액의

분비량이 많아지며, 기도개형이 일어나는 것이 확인되었다. 또한, T 세포와 선천성 림프구 세포의 변화는 관찰되지 않았으나 뚜렷한 대식세포의 성격 변화가 확인되었는데, $CD11b^{int}F4/80^{hgih}$ 대식세포의 감소와 더불어 $CD11b^{high}F4/80^{int}$ 대식세포의 증가가 대표적이었다. 유입된 대식세포 중에서는 대식세포의 아형 중 하나인M2의 증가가 확인되었는데, M2 대식세포 중에서도 염증반응을 유도한다고 알려져 있는 Ly6C가 높게 발현된 세포가 증가하였음을 알 수 있었다. 뿐만 아니라, 호산구와 높은 상관관계를 보이는 $CD11c^{+}CD11b^{+}$ 대식세포의 증가도 만성 천식 마우스에서 두드러지게 관찰되었다.

만성 천식 마우스에 Liproxstatin-1으로 프라이밍 된 hUC-MSC를 기도 투여하였을 때, 기도폐포세척액 내 대식세포, 호산구, 호중구, 림프구를 포함한 전체 세포 수가 감소하였으며, 폐 조직 내의 염증세포 군집이 확연하게 감소한 것을 확인하였다. 점액 생성 및 만성 천식에서 나타나는 폐 조직의 섬유화 정도도 유의미하게 감소하였다. 간질조직으로 유입된 대식세포 수의 감소와 더불어서 Ly6C를 높게 발현하는 M2 대식세포의 수 또한 유의미하게 감소하였으며, CD11c와 CD11b를 모두 발현하는 대식세포의 수가

크게 감소하였다. 그 중에서도 Ly6C를 발현하는 $CD11b^{+}CD11c^{+}$ 대식세포의 감소가 가장 두드러졌다.

야생형 마우스에서 CD11c와 CD11b를 모두 발현하는 대식세포는 SiglecF를 높게 발현하고 있지만 만성 천식 마우스에서는 SiglecF가 낮게 발현된다는 특징이 관찰되었다. Liproxstatin-1으로 프라이밍 된 UC-MSC를 투여한 만성 천식 마우스에서는 SiglecF를 높게 발현하는 대식세포와 그렇지 않은 대식세포가 모두 존재함에 따라 야생형과 천식 모델의 특징이 공존한다는 것이 확인되었다. $CD11b^{+}CD11c^{+}$ 대식세포 중 각각 M1과 M2의 성격을 띄는 대식세포의 비율은 모두 MSC를 투여하지 않은 천식모델보다 MSC를 투여한 천식모델에서 낮게 나타났다.

본 실험 결과를 통해, Liproxstatin-1으로 프라이밍 된 hUC-MSC는 기도로 투여하였을 때도 항천식 효과를 나타내는 것을 입증하였다. 또한, MSC는 대식세포를 조절하여 항천식 효과를 나타낼 수 있으며, 대식세포는 천식의 새로운 치료 표적으로서의 가능성이 있음을 확인하였다.

키워드: 천식, 중간엽줄기세포, 세포치료제, IL-13, 대식세포