Molecules and Cells

Intravenous Mesenchymal Stem Cell Administration Modulates Monocytes/ Macrophages and Ameliorates Asthmatic Airway Inflammation in a Murine Asthma Model

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Although asthma is a common chronic airway disease that responds well to anti-inflammatory agents, some patients with asthma are unresponsive to conventional treatment. Mesenchymal stem cells (MSCs) have therapeutic potential for the treatment of inflammatory diseases owing to their immunomodulatory properties. However, the target cells of MSCs are not yet clearly known. This study aimed to determine the effect of human umbilical cord-derived MSCs (hUC-MSCs) on asthmatic lungs by modulating innate immune cells and effector T cells using a murine asthmatic model. Intravenously administered hUC-MSCs reduced airway resistance, mucus production, and inflammation in the murine asthma model. hUC-MSCs attenuated not only T helper (Th) 2 cells and Th17 cells but also augmented regulatory T cells (Tregs). As for innate lymphoid cells (ILC), hUC-MSCs effectively suppressed ILC2s by downregulating master regulators of ILC2s, such as *Gata3* and *Tcf7*. Finally, regarding lung macrophages, hUC-MSCs reduced the total number of macrophages, particularly the proportion of the enhanced monocyte-derived macrophage population. In a closer examination of monocyte-derived macrophages, hUC-MSCs reduced the M2a and M2c populations. In conclusion, hUC-MSCs can be considered as a potential anti-asthmatic treatment given their therapeutic effect on the asthmatic airway inflammation in a murine asthma model by modulating innate immune cells, such as ILC2s, M2a, and M2c macrophages, as well as affecting Tregs and effector T cells.

Keywords: asthma, immunity, innate, macrophage activation, mesenchymal stem cells

INTRODUCTION

Asthma, characterized by airway hyperresponsiveness (AHR) and airway remodeling, is denoted as a prototypical T helper type 2 (Th2) inflammation, which usually shows a good re-

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sponse to inhaled corticosteroids (ICS) (Bosnjak et al., 2011). However, severe asthma is sometimes refractory to high doses of ICS, therefore alternative approaches may be required to reduce inflammation and improve corticosteroid response (Carr and Szefler, 2016).

While the pathogenesis of asthma has traditionally focused on adaptive immune responses to environmental stimuli, the innate immune system has recently been highlighted as a driver of asthma development. Among the diverse innate immune cells, innate lymphoid cells (ILCs) have been identified as linkers of innate and adaptive immunity. Previous reports have shown that ILC2s, which secrete interleukin (IL)-4, IL-5, and IL-13, contribute to eosinophilic inflammation in allergic asthma and coordinate adaptive immune responses (Doherty and Broide, 2019; Kim et al., 2016). ILC3s are mainly found in certain asthma endotypes, especially non-allergic and severe asthma, in which the release of IL-17 and/or IL-22 promotes neutrophilic inflammation. Macrophages are the most common immune cells in the airways. Depending on the stimulus, macrophages can be polarized into the M1 or M2 subtypes (Muraille et al., 2014). M1 macrophages are polarized by Th1 cytokines such as interferon-γ (IFN-γ). M2 macrophages, polarized by Th2 cytokines such as IL-4 and IL-13, are responsible for tissue repair, regeneration, and fibrosis (Bosco, 2019). Recent studies have revealed that M2 macrophage subtypes in murine asthma models are strongly associated with asthma pathophysiology (Kang et al., 2022; Lee et al., 2015). In particular, M2a cells triggered by IL-4, IL-13, and IL-33, initiate allergic responses by releasing IL-4 and IL-13. In addition, M2c cells activated by IL-10 and transforming growth factor-β (TGF-β) contribute to tissue remodeling (Jiang and Zhu, 2016). Taken together, the modulation of ILCs and hence macrophage polarization can be potential targets in asthma treatment (Fricker and Gibson, 2017).

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent, self-renewable stromal cells. These can differentiate into various cell types, such as osteoblasts, chondrocytes, myocytes, and adipocytes (Wang et al., 2014). In addition, it was recently confirmed that MSCs modulate innate immune cells. However, the exact role of MSCs in regulating innate immune cells has yet to be clarified in asthma (Abumaree et al., 2013; Mo et al., 2022). Therefore, we investigated the immunomodulatory effects of human umbilical cord MSCs (hUC-MSCs) on innate immune cells including ILCs and macrophages in an ovalbumin (OVA)-induced murine allergic asthma model.

MATERIALS AND METHODS

Preparation of hUC-derived MSCs

All procedures involving hUC or hUC-derived cells (CTI-195; Cell2in, Korea) were conducted in accordance with the guidelines of the Seoul National University College of Medicine and Hospital Institutional Review Board (IRB No. C-1708-083-878). The hUC tissues were obtained immediately after full-term births following cesarean section, with informed consent. The hUCs were washed with phosphate-buffered saline (PBS) to remove vessels and amnion. Wharton's jelly (WJ) tissues within the hUC were isolated and minced. These explants were digested for 3 h at 37°C using an umbilical cord dissociation kit (Miltenyi Biotec, Germany), filtered through a 100-μm cell strainer (BD Biosciences, USA), and pelleted by low-speed centrifugation at 200 × *g* for 10 min. The isolated WJ-MSCs were cultured in a CellCor™ CD medium (Xcell Therapeutics, Korea) supplemented with 2% human platelet lysates (StemCell Technologies, Canada) in a 37°C incubator under humidified conditions with 5% CO_2. The cells were harvested once they reached 90% confluence. High levels of glutathione (GSH) in hUC-MSCs (CTI-195; Cell2in) were isolated from cultured hUC-MSCs using a fluorescent real-time thiol tracer, which is a ratiometric probe capable of monitoring reactive oxygen species-induced GSH changes in living stem cells (Jeong et al., 2018).

Murine asthma model

Female 6-week-old BALB/c mice were purchased from Orient Bio (Korea). All the experiments were approved and conducted in accordance with the guidelines of the Seoul National University Institutional Animal Care and Use Committee (IACUC No. SNU-200302-2-2).

Mice were sensitized with intraperitoneal injections of 100 μg OVA and 2 mg aluminum hydroxide (Sigma-Aldrich, USA) on days 0 and 7, and challenged by intranasal injection of 50 μg OVA on days 14, 15, 16, 21, 22, and 23.

hUC-MSCs (1×10^5) were injected intravenously on day 17. The mice were divided into the following four groups (n = 4 per group): PBS (control group), hUC-MSC-treated (MSC group), OVA asthma (OVA group), and hUC-MSC-treated OVA asthma (OVA/MSC group).

Measurement of airway hyperresponsiveness and inflammation

On day 24, AHR was invasively measured using the FinePointe Resistance and Compliance System (Buxco®, USA) in response to aerosolized methacholine. After the mice were anesthetized with pentobarbital sodium (50 mg/kg), lung resistance (RL) was measured over 3 min. The measured RL values were subtracted from the baseline values and transformed into percentages.

After measuring the RL values, bronchoalveolar lavage fluid (BALF) and lung tissue were collected. BAL cells were stained with Diff-Quik (Sysmex, Japan) and at least 300 cells were counted to determine the differential cell count in each sample.

Histological analysis

Left lung tissues were fixed in 4% paraformaldehyde for H&E and periodic acid-Schiff (PAS) staining. H&E and PAS staining was performed by experts at the pathology core facility at the Seoul National University Hospital Biomedical Research Institute. The degree of inflammation and mucus production were semi-quantitatively measured histologically (Hu et al., 2017).

Cell analysis by flow cytometry

Minced lung tissue was incubated in RPMI1640 (Biowest, France) with 10% type IV collagenase (Worthington Biochemical Corporation, USA) at 37°C for 90 min and sorted

using a sterile 40-μm strainer for single-cell preparation.

Single-cell suspensions were treated with Fcγ receptor-blocking monoclonal antibodies (BD Biosciences) at 4°C for 5 min. To analyze intracellular cytokines and transcription factors at the protein level, single-cell suspensions were stimulated with PMA (Sigma-Aldrich), ionomycin (Sigma-Aldrich), and BD GolgiPlug (BD Biosciences) for 3 h on a cell plate. Subsequently, cell surface markers were stained, and BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences) were used to release accumulated intracellular cytokines. Finally, the released extracellular cytokines were stained in each cell subtype (Supplementary Table S1). The cells were analyzed using the LSR Fortessa X-20 (BD Biosciences) and FlowJo10 software (TreeStar, USA). After single-cell and $CD4^+$ lymphocyte gating, regulatory T cells (Tregs) were defined as those expressing Foxp3 and CD25, whereas cytokine-secreting effector Th cells were those expressing lineage, IL-5, IL-13, IFN-γ, and IL-17A (Supplementary Fig. S1). ILCs were defined as $CD45^+Lineage^-CD90.2^+$ cells and classified as ILC1, ILC2, and ILC3 according to the secretion of IFN-γ, IL-5, IL-13, and IL-17A (Supplementary Fig. S1B). The lineage markers are presented in Supplementary Table S1.

The gating strategy for macrophages and dendritic cells (DCs) was as follows. First, $SiglecF^+CD11c^-$ eosinophils were gated out among $CD45^+$ cells, then macrophages were defined as $CD45^+F4/80^+$ cells, and DCs were defined as $CD45^+F4/80^-CD11c^+$ cells (Supplementary Fig. S2). Using CD11c, CD11b, and SiglecF (Duan et al., 2016; Janssen et al., 2011), alveolar macrophages (AMs) were defined as $SiglecF^+CD11c^+CD11b^-$ resident AMs, $SiglecF^+CD11c^+CD11b^+$ transient AMs, and $SiglecF^-CD11b^+$ monocyte-derived macrophages (MoMs) (McQuattie-Pimentel et al., 2018) (Supplementary Fig. S2B). Also, subclassification was performed according to the expression of CD86, CD206, and MHC II in MoMs (Gautier et al., 2012); M1 and M2 were defined as $CD86^+SiglecF^-CD11c^+CD11b^+$ cells and $CD206^+SiglecF^-CD11c^-CD11b^+$ cells, respectively. M2 MoMs were further subdivided into M2a ($CD206^+MHCII^{hi}CD86^-$) and M2c ($CD206^+MHCII^{low}CD86^-$) subtypes (Klopfleisch, 2016; Liegeois et al., 2018). Independently, M2b was defined as $CD206^-MHCII^{mid}CD86^+$ (Swieboda et al., 2020).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Lung tissues were homogenized in 1 ml of Trizol (Thermo Fisher Scientific, USA) using a tissue homogenizer (TaKaRa, Japan) and total RNA was isolated with chloroform. Reverse transcription was performed using a SensiFAST cDNA Synthesis Kit (Bioline, UK). RT-qPCR was performed using SensiFAST™ SYBR® No-ROX Kit (Bioline) and measured with a 7500 real-time PCR system (Applied Biosystems, USA). The expression levels in each sample were normalized to the housekeeping gene *Gapdh* using the ΔΔCt method, which calculates the relative gene expression against the control sample. The primer sequences used were verified using PrimerBank (USA) (Supplementary Table S2).

Immune cell isolation

For Treg *ex vivo* experiments, naïve $CD4^+$ T cells were isolated from mouse spleens using the MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit (BioLegend, USA) and differentiated into Tregs using the CellXVivo Mouse Treg Cell Differentiation Kit (R&D Systems, USA). During the differentiation period, Tregs (1×10^5) were directly co-cultured with hUC-MSCs (1×10^4). Transwell assays were performed with equivalent amounts of Tregs and hUC-MSCs.

For *ex vivo* ILC experiments, ILCs were isolated from mouse lungs using an EasySep™ Mouse Pan-ILC Enrichment Kit (StemCell Technologies). The isolated ILCs were differentiated into ILC2s by treatment with mouse recombinant IL-2, IL-7, and IL-33 proteins (BioLegend). hUC-MSCs (1×10^4) were co-cultured with the differentiated ILC2s (1×10^5) for one day. Indirect contact transwell assays were performed to evaluate the effect of soluble factors secreted from hUC-MSCs using Transwell Permeable Support (Costar, USA).

Co-culture of hUC-MSCs with macrophages

For *in vitro* experiments on human peripheral blood mononuclear cell (PBMC)-derived macrophages, human peripheral blood was obtained from allergic rhinitis patients. Patient blood was diluted in PBS and centrifuged with Ficoll-Paque PLUS (Sigma-Aldrich). PBMCs were isolated and differentiated using 50 ng/ml human recombinant M-CSF (R&D Systems). All patients provided written informed consent, and the study was approved by the Seoul National University Hospital Institutional Review Committee (1610-062-799).

The murine AM cell line MH-S (ATCC, USA) was seeded under the same conditions and stimulated with 20 ng/ml recombinant IL-13 (R&D Systems), with or without hUC-MSCs. In addition, a transwell assay was performed using Transwell Permeable Support (Costar). In macrophage *in vitro* experiments, 1×10^5 macrophages were co-cultured with 1×10^4 hUC-MSCs.

Statistical analysis

The Mann–Whitney test was used to compare two groups, and one-way ANOVA with Tukey's post-hoc test was used to compare four or more groups. Data are represented as the mean ± SEM. Statistical significance was set at $P < 0.05$. All statistical analyses were performed with the Prism 10 software (GraphPad Software, USA).

RESULTS

Effect of hUC-MSCs on AHR and asthmatic airway changes

To determine the effect of hUC-MSCs on the asthmatic airways, an OVA-induced asthma model was treated with intravenous hUC-MSCs (Fig. 1A). hUC-MSCs significantly reduced AHR and inflammatory cell populations of macrophages, neutrophils, and eosinophils in BALF (Figs. 1B and 1C). In addition, the proportion of eosinophils in the lung tissue was lower in the OVA/hUC-MSC group than that in the OVA group, whereas the level of neutrophils was not significantly different between the two groups (Fig. 1D). Next, cytokines in BALF were analyzed. In the OVA model, increased IL-5 and IL-13 levels were alleviated by hUC-MSCs, whereas IL-17 and IFN-γ levels were not altered significantly (Fig. 1E).

Immune cell infiltration in the bronchi and the blood ves-

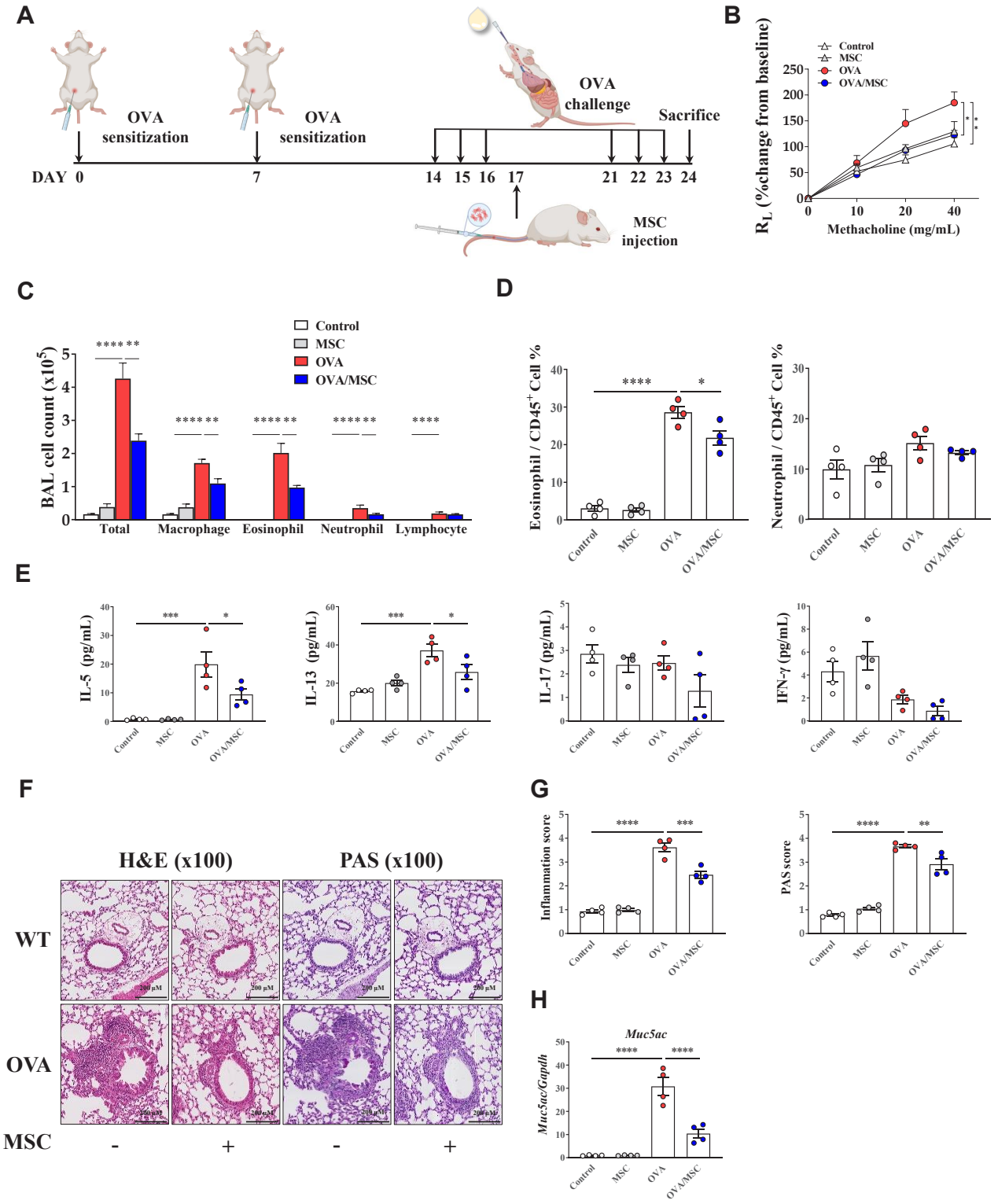

Fig. 1. Effect of hUC-MSCs on AHR and lung inflammation in a murine asthma model. (A) Establishment of a murine asthma model with OVA and Alum administration. (B) AHR was measured 24 h after the last challenge. (C) The number of total inflammatory cells, including macrophages, neutrophils, eosinophils, and lymphocytes in BAL fluid. (D) Proportion of eosinophils and neutrophils to $CD45^+$ cells in the lungs. (E) IL-5, IL-13, Il-17, and IFN-γ cytokine expression analysis by ELISA in BAL fluid. (F) H&E (100×) and PAS staining (100×) for lung histology. (G) Lung inflammation score and PAS score in a murine asthma model. (H) *Muc5ac* expression in lungs. n = 4 for each group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. All results are representative of at least three independent experiments. Alum, aluminum hydroxide; R_L, resistance of lung; BAL, bronchoalveolar lavage; WT, wild type.

sels and goblet cell hyperplasia were improved in the histological analysis of the OVA/hUC-MSC group (Fig. 1F). In addition, the histological quantification scores of inflammatory cell infiltration and the degree of inflammation in the tissues were significantly reduced after hUC-MSC injection (Fig. 1G). The increased expression of *Muc5ac* in the OVA group was reduced by hUC-MSCs (Fig. 1H).

Effect of hUC-MSCs on CD4$^+$ T cells

The total numbers of IL-5$^+$ or IL-13$^+$ CD4$^+$ T cells were significantly increased in the OVA group, whereas they were decreased in the hUC-MSCs group (Fig. 2A). Similarly, the number of IL-17A$^+$ CD4$^+$ T cells was markedly increased in the OVA group, but was significantly reduced in the hUC-MSC group (Fig. 2B). In contrast, the number of IFN-γ^+ CD4$^+$ T cells in the OVA group was noticeably increased relative to the hUC-MSC group (Fig. 2B).

Next, we analyzed the effect of hUC-MSCs on Tregs. The number of Tregs in the lungs and spleen was increased in the OVA group, but was further upregulated in the hUC-MSC group (Fig. 2C). In addition, hUC-MSC treatment further augmented *Foxp3* and *Il10* expression in the OVA group (Fig. 2D).

Subsequently, the hUC-MSC-mediated effect on the differentiation of spleen-derived naive CD4$^+$ T cells into Tregs was analyzed (Fig. 2E). Interestingly, during Treg differentiation, increased Treg population and *Foxp3* expression were observed in direct and indirect contact hUC-MSC co-culture systems (Fig. 2F).

Effect of hUC-MSCs on ILCs

Similar to the results observed in Th2 and Th17 cells, IL-5$^+$ or IL-13$^+$ ILC2s and IL-17A$^+$ ILC3s populations were increased in the OVA group but significantly reduced by hUC-MSC injection (Figs. 3A and 3B). However, hUC-MSC injection did not change the number of increased IFN-γ^+ ILC1s in the OVA group (Fig. 3B).

To analyze whether hUC-MSCs can directly regulate the polarization of ILC2s, hUC-MSCs were co-cultured with differentiated mouse ILC2 (Fig. 3C). hUC-MSCs significantly reduced ILC2s for both direct and indirect contact groups; direct contact was more effective (Fig. 3D). The expression of *Il5* and *Il13* in ILC2s were also reduced in both direct and indirect contact assays (Fig. 3E). However, *Il10* expression was increased only when indirectly co-cultured with hUC-MSCs (Fig. 3E). The expression of *Gata3* and *Tcf7*, the master regulators of ILC2s, had a similar pattern to that of type 2 cytokines (Fig. 3F). However, hUC-MSCs did not affect expression of ILC2 receptors, such as *Il1rl1* and *Il7r* (Fig. 3G).

Changes in mRNA expression induced by hUC-MSCs

The enhanced expression of Th2 cytokines (*Il5* and *Il13*) and master regulators (*Gata3* and *Rora*) in the OVA group was significantly reduced after hUC-MSC treatment (Fig. 4A). Similarly, the increased expression of *Il17a* and *Rorc* in the OVA group was also significantly reduced by hUC-MSC injection (Fig. 4B). However, no significant difference was observed in the elevated expression of Th1 cytokines, including *Ifng* and *Tbx21*, in the OVA group (Fig. 4C) by hUC-MSC injection.

In addition, intravenous hUC-MSC administration modulated the expression levels of macrophage activation markers, which were increased by OVA challenge (Fig. 4D). While M1 activation markers, such as *Cd86*, were significantly upregulated in the hUC-MSC administered OVA group (Fig. 4E), M2 activation markers, such as *Mrc1, H2-ab, Ym1, Relmα, Cd163, Arg1,* and *Pparg,* were significantly downregulated. The expression of genes related to monocyte chemotaxis, such as *Mcp1* and *Itgam*, encoding CD11b in the OVA group, was decreased by hUC-MSC treatment (Fig. 4F).

Changes in DCs and macrophage/monocyte population induced by hUC-MSCs

In DC population, hUC-MSC-treated mice showed a decreased number of CD11b$^+$ DCs and cDC2s compared with the OVA group (Supplementary Fig. S4).

In addition, in the OVA-induced asthma model, hUC-MSC-treated mice showed a reduced number of macrophages compared with the OVA group (Fig. 5A). Next, we subdivided the macrophages into subgroups according to SiglecF and CD11b (Fig. 5B).

hUC-MSC treatment did not affect the decreased SiglecF$^+$CD11c$^+$CD11b$^-$ resident AM population (Fig. 5C). However, hUC-MSC treatment replenished SiglecF$^+$CD11c$^+$CD11b$^+$ cells, which are transient AMs evolved from monocytes.

Meanwhile, hUC-MSC treatment significantly reduced the proportion of SiglecF$^-$CD11c$^-$CD11b$^+$ MoMs, especially Ly6c$^+$ MoMs (Fig. 5D).

Effect of hUC-MSCs on macrophage polarization

Interestingly, hUC-MSCs differentially regulated the MoM polarization in the OVA group; they enhanced M1 polarization but reduced M2 polarization (Fig. 6A). Among M2 MoMs, M2a (CD206$^+$MHCII^{hi}CD86$^-$) and M2c (CD206$^+$MHCII^{low}CD86$^-$) populations were increased in the OVA group and significantly reduced by intravenous hUC-MSC treatment, whereas variations in the M2b (CD206$^-$MHCII^{mid}CD86$^+$) population were non-significant (Fig. 6B).

To directly analyze the immunomodulatory effects of hUC-MSCs on M2 macrophages, we performed an *in vitro* differentiation experiment using a mouse AM cell line (Supplementary Fig. S4A). When hUC-MSCs were treated after M2 stimulation, M2 macrophage activation was affected not only by cell-to-cell contact but also by MSC-derived soluble factors (Fig. 6C).

Next, we conducted an *in vitro* macrophage polarization experiment using human PBMCs to determine whether hUC-MSCs also affected macrophage polarization in humans (Supplementary Fig. S4B). Despite IL-13 treatment, hUC-MSC-treated human PBMCs showed reduced *MRC1* and *HLA-DR* mRNA expression, suggesting decreased M2 polarization (Fig. 6D).

DISCUSSION

In this study, we demonstrated the anti-asthmatic effect of hUC-MSCs on innate immune cells, especially on ILC2 function and M2 macrophage polarization.

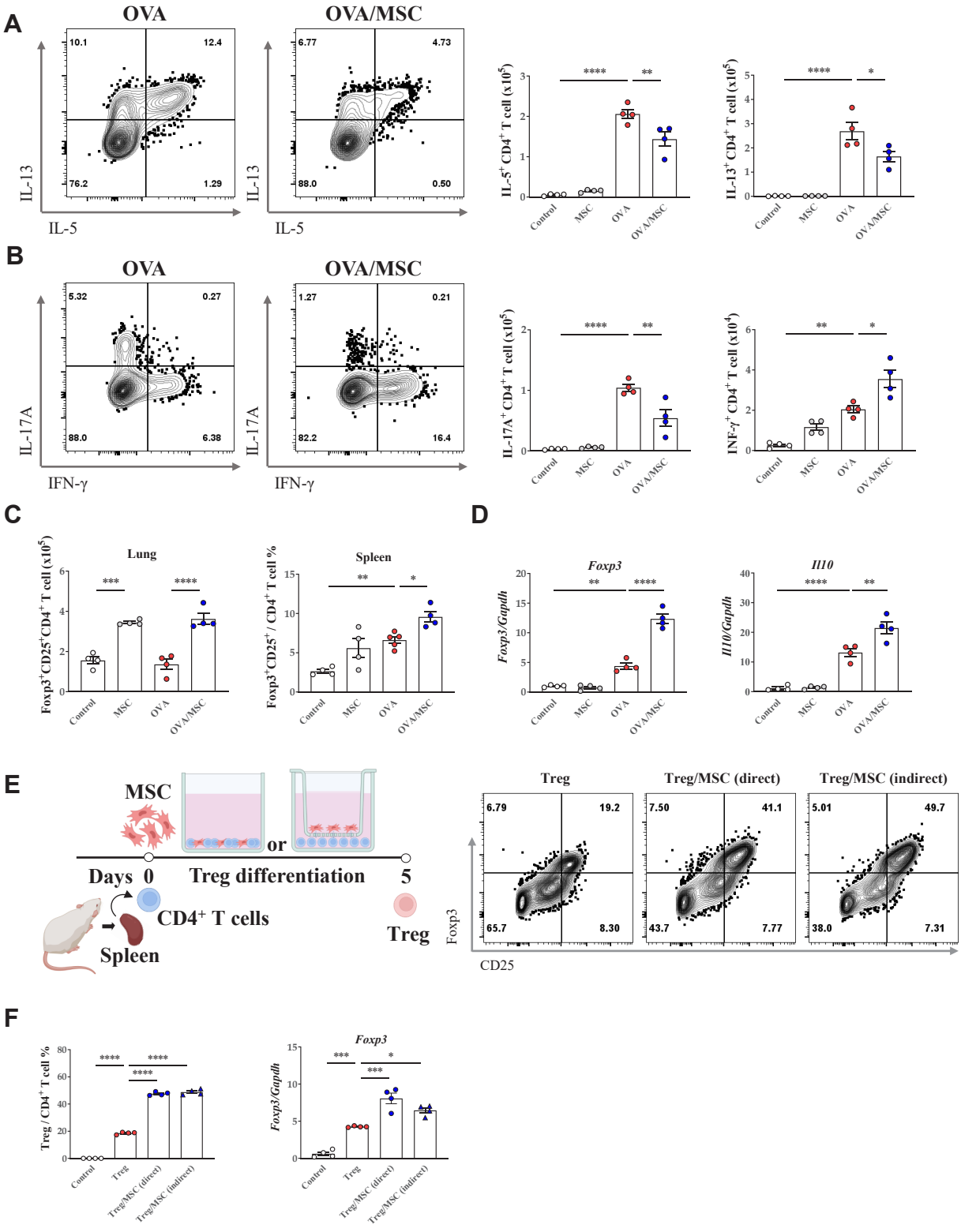

Fig. 2. Effect of hUC-MSCs on effector T cell activation *in vivo* and *ex vivo*. The number of (A) IL-5$^+$ CD4$^+$ T cells, IL-13$^+$ CD4$^+$ T cells, (B) IL-17A$^+$ CD4$^+$ T cells and IFN-γ$^+$ CD4$^+$ T cells in the lungs and their dot plots. (C and D) Foxp3$^+$CD25$^+$CD4$^+$ T cells among CD4$^+$ T cells in the lungs and the spleen, and the mRNA expression of *Foxp3* and *Il10* with and without hUC-MSCs treatment in the lungs. (E) Experimental scheme and dot plots showing Tregs induced by hUC-MSCs during differentiation from naïve CD4$^+$ T cells isolated from the spleen. (F) The effect of hUC-MSCs on *ex vivo* Tregs differentiated from naïve CD4$^+$ T cells isolated from the spleen (control). n = 4 for each group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. All results are representative of three independent experiments.

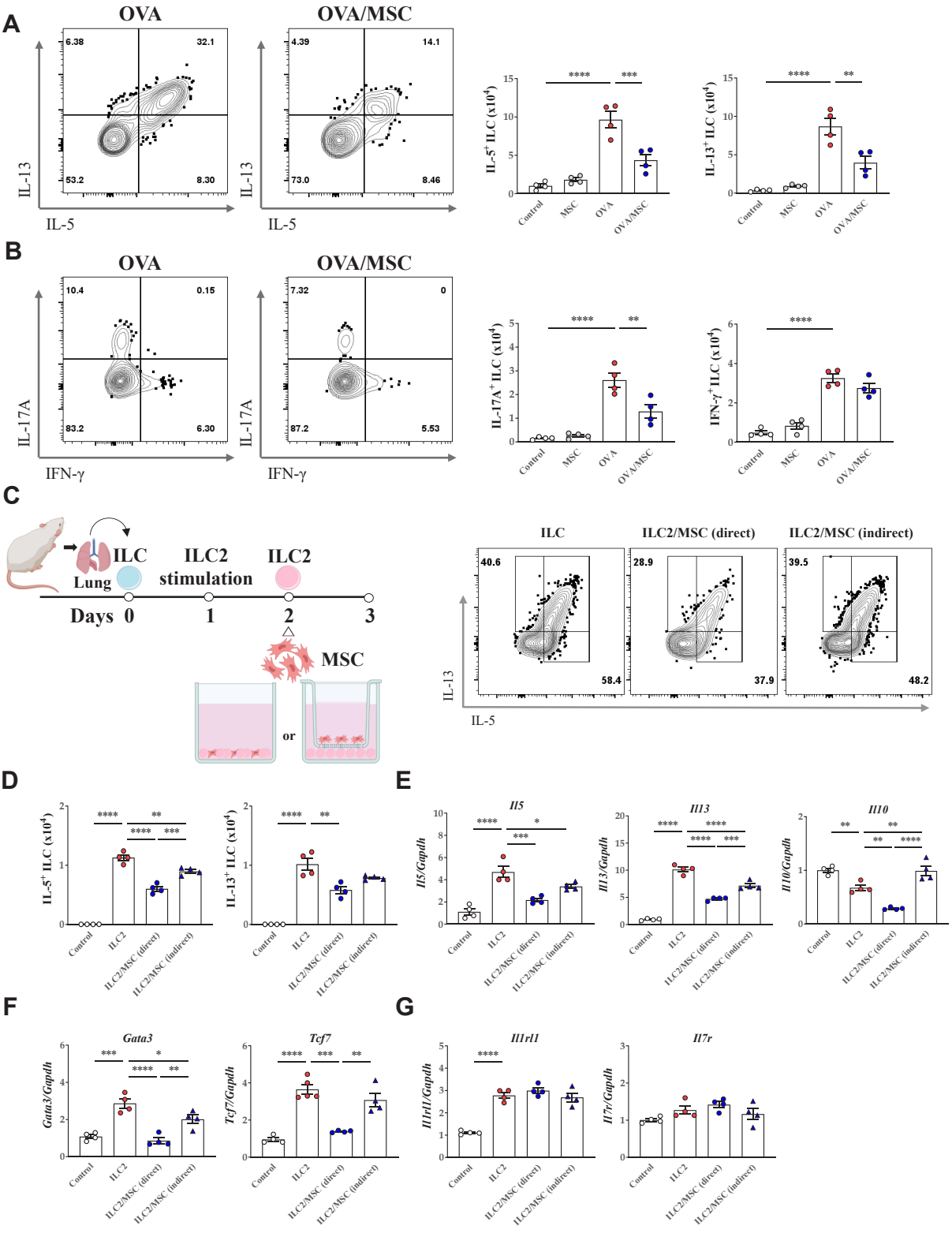

Fig. 3. Effect of hUC-MSCs on ILCs *in vivo* and *ex vivo*. (A) Dot plot and the number of IL-5$^+$ ILCs, IL-13$^+$ ILCs, (B) IL-17A$^+$ ILCs and IFN-γ$^+$ ILCs in the lung homogenate. (C) Protocol for differentiation of ILCs isolated from the lungs and co-culturing of hUC-MSCs *in vitro*, and mechanism-dependent changes in their activation. (D) The number of IL-5$^+$ ILCs and IL-13$^+$ ILCs due to hUC-MSCs treatment *ex vivo*. (E) *Il5*, *Il13*, *Il10*, (F) *Gata3*, *Tcf7*, (G) *Il1rl1* and *Il7r* gene expression in ILC2s. Naïve ILCs isolated from the mouse lungs were used as the control. n = 4 for each group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. All results are representative of at least three independent experiments.

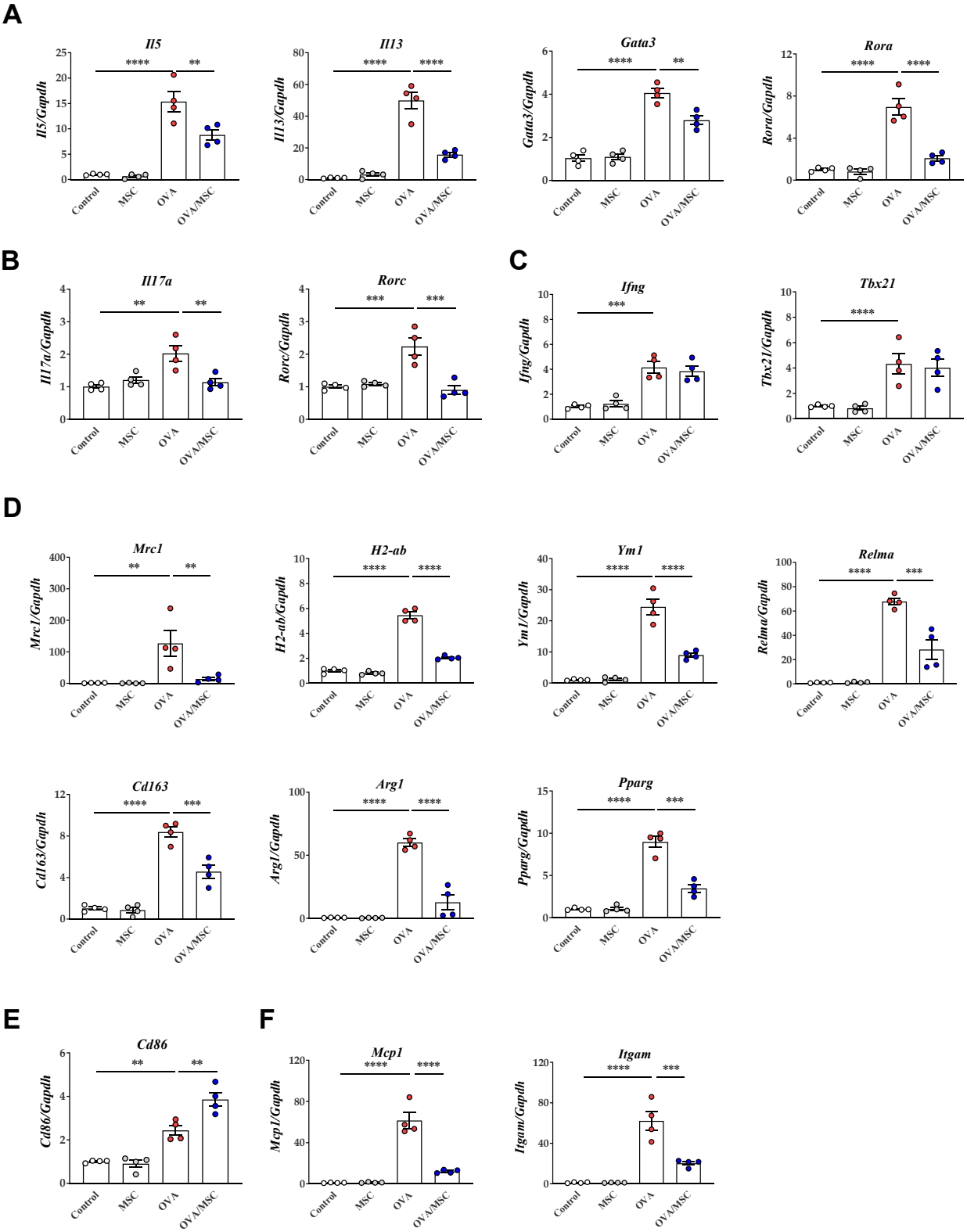

Fig. 4. Effect of hUC-MSCs on lung mRNA expression in a murine asthma model. (A) Changes in *Il5*, *Il13*, *Gata3*, and *Rora* gene expression in the lungs following hUC-MSC treatment. (B) Changes in *Il17a*, *Rorc*, (C) *Ifng* and *Tbx21* gene expression in the lungs following hUC-MSC treatment. (D and E) *Mrc1*, *H2-ab*, *Ym1*, *Relma*, *Cd163*, *Arg1*, *Pparg*, and *Cd86* gene expression in the lungs following hUC-MSC treatment. (F) *Mcp1* and *Itgam* gene expression in the lungs following hUC-MSC treatment. n = 4 for each group, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. All results are representative of at least three independent experiments.

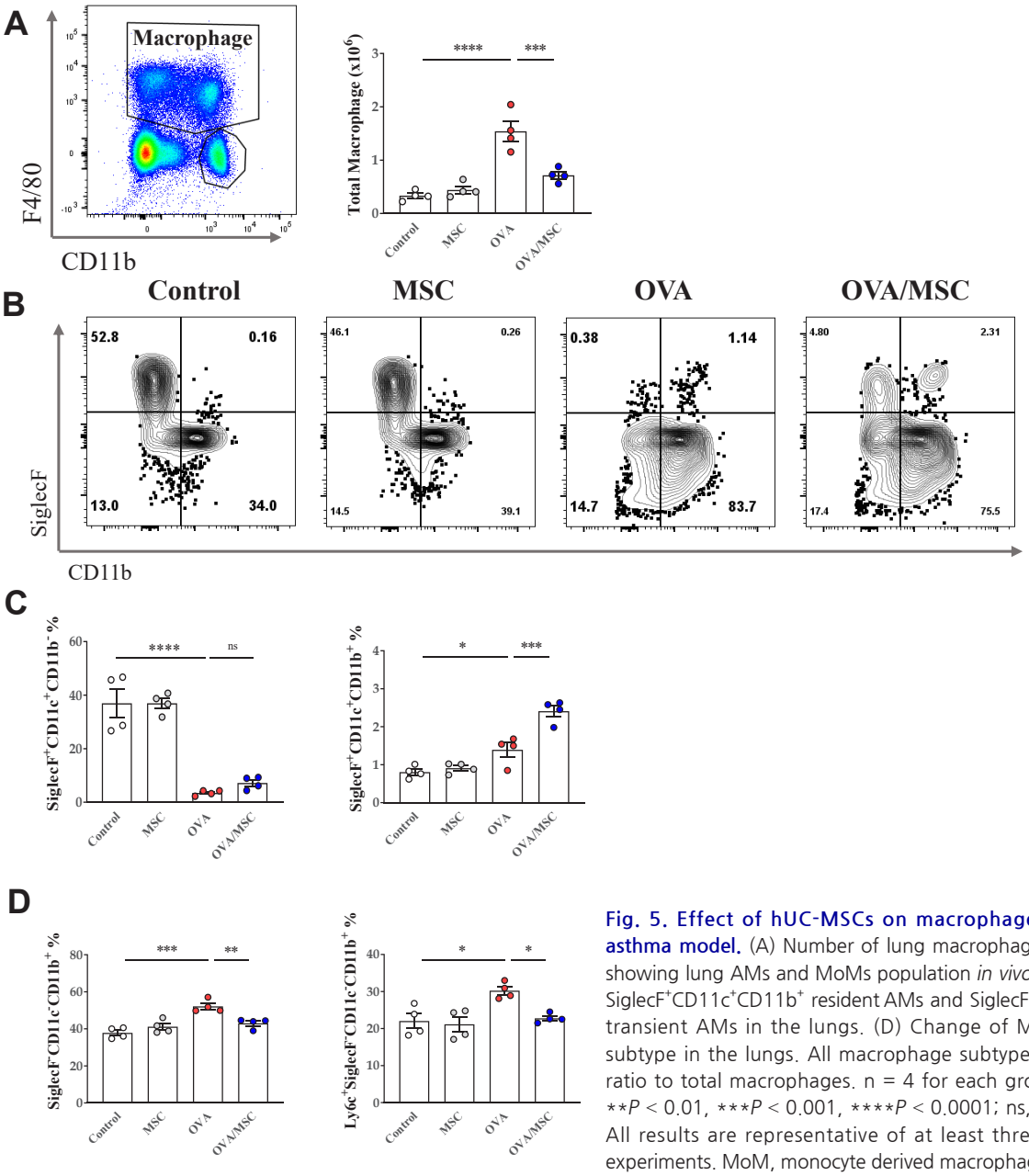

Fig. 5. Effect of hUC-MSCs on macrophages in a murine asthma model. (A) Number of lung macrophages. (B) Dot plot showing lung AMs and MoMs population *in vivo*. (C) Change of SiglecF$^+$CD11c$^+$CD11b$^+$ resident AMs and SiglecF$^+$CD11c$^+$CD11b$^+$ transient AMs in the lungs. (D) Change of MoMs and their subtype in the lungs. All macrophage subtypes represent the ratio to total macrophages. n = 4 for each group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant. All results are representative of at least three independent experiments. MoM, monocyte derived macrophage.

MSCs are known to exert widespread immunomodulatory effects on cells of the innate and adaptive immune system by both enhancing and inhibiting cell proliferation, and inducing cytokine production. However, the exact mechanisms underlying the immunosuppressive effects of MSCs are still not clear (Fan et al., 2020; Shi et al., 2010; Shin et al., 2021).

Compared with MSCs of other origins, MSCs derived from the umbilical cord have demonstrated superior immunosuppressive effects, and are not subject to ethical issues, as they can be harvested non-invasively in large amounts. Thus, they have been identified as a viable option for treatment in patients with severe asthma (Huang et al., 2012; Kim et al., 2022; Mathias et al., 2013). We investigated the immunomodulatory effect of hUC-MSCs in an experimental asthma model, and demonstrated that the intravenous injection of hUC-MSCs can influence both innate and adaptive immune cells. Intravenous administration of hUC-MSCs resulted in decreased Th2 cell differentiation in OVA-induced murine asthma. In contrast, the proportion of Tregs in the lung and spleen were increased, accompanied by the upregulation of IL-10. Previous reports have also demonstrated that MSCs activate Tregs. These cells secrete anti-inflammatory cytokines, such as IL-10 and TGF-β, which lead to a decrease in lung eosinophilic inflammation, Th2 cytokines, and IgE antibody production (Cho et al., 2014). In addition, pathways related to Treg soluble factors, such as IDO and TGF-β, prostaglan-

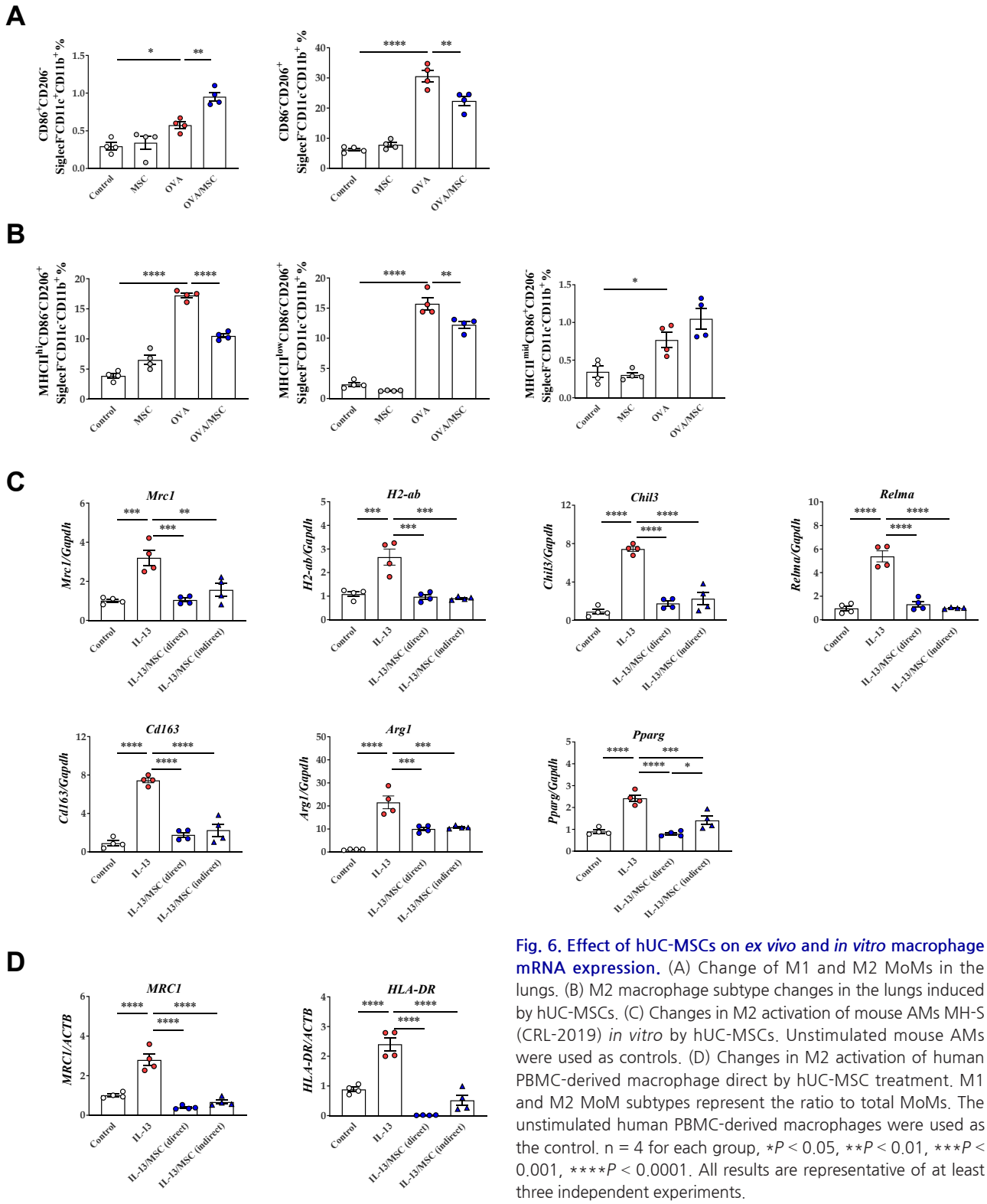

Fig. 6. Effect of hUC-MSCs on *ex vivo* and *in vitro* macrophage mRNA expression. (A) Change of M1 and M2 MoMs in the lungs. (B) M2 macrophage subtype changes in the lungs induced by hUC-MSCs. (C) Changes in M2 activation of mouse AMs MH-S (CRL-2019) *in vitro* by hUC-MSCs. Unstimulated mouse AMs were used as controls. (D) Changes in M2 activation of human PBMC-derived macrophage direct by hUC-MSC treatment. M1 and M2 MoM subtypes represent the ratio to total MoMs. The unstimulated human PBMC-derived macrophages were used as the control. n = 4 for each group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. All results are representative of at least three independent experiments.

din E2, extracellular vesicles, and the reprogramming of antigen-presenting cells to tolerogenic phenotypes, have emerged as possible mechanisms of immune-modulatory Treg cell proliferation (Negi and Griffin, 2020).

Recently, GSH has been proposed as a marker to evaluate stem cell function. In the asthma model, hUC-MSCs with high GSH levels showed elevated anti-asthmatic effect evident in the histological and immune cell analyses compared

to those of low GSH counterparts (Jeong et al., 2018).

In this study, high GSH hUC-MSCs especially decreased the ILC2 population, which is thought to play a crucial role in the pathogenesis of asthma, as ILC2s secrete large amounts of Th2 cytokines, such as IL-5 and IL-13, thereby contributing to eosinophilic inflammation in allergic asthma. Interestingly, hUC-MSC treatment resulted in decreased expression of *Gata3* and *Tcf7* mRNA in ILC2s, whereas no significant effect was observed for *Il1rl1* and *Il7r* mRNA. These results suggest that hUC-MSCs regulate ILC2 differentiation by direct cell–cell contact, which is unrelated to the regulation of *Il1rl1* and *Il7r* (Huang et al., 2017).

Macrophage activation is a dynamic process in which early macrophages react to environmental signals and differentiate into functionally distinct macrophages (Murray, 2017). We found that the administration of hUC-MSCs resulted in a pronounced decrease in total macrophage population; this finding is consistent with previous reports (Huang et al., 2016). Furthermore, macrophage subset populations were investigated because the roles of macrophage subsets in allergic asthma have been emphasized recently. Macrophages are classified into AM and MoM subsets based on their expression of CD11c and CD11b. AMs express SiglecF and are subdivided according to the expression of CD11b; $SiglecF^{+}CD11b^{-}$ resident AMs and $SiglecF^{+}CD11b^{+}$ transient AMs (McQuattie-Pimentel et al., 2018). The intravenous injection of MSC-derived exosomes was found to induce a decrease in M2 macrophages in a murine asthma model by regulating the differentiation of monocytes into macrophages (Fang et al., 2020a). Similarly, we found that intravenously injected hUC-MSCs modulate MoMs but not $SiglecF^{+}$ resident AMs.

Several studies have been conducted to functionally classify macrophages, and these studies have shown that macrophage subtypes are associated with asthma endotypes (Chakarov et al., 2019; Gibbings et al., 2017; Ural et al., 2020). Increased M2 macrophage polarization and activation have been observed in allergic asthma; furthermore, of M2 macrophage subsets, M2a and M2c are implicated in allergic asthma phenotypes. M2a macrophages secrete IL-5 and IL-13, which induce Th2 cell activation and initiate eosinophil infiltration in the lungs (Byers and Holtzman, 2011). M2a macrophages expressing CD206 and MHCII have been found to increase in accordance with asthma severity, suggesting that M2a macrophages are closely involved in the pathophysiology of severe asthma (Girodet et al., 2016). M2c macrophages have been reported to drive pulmonary fibrosis (Lu et al., 2013; Mann et al., 2011). Importantly, it has been demonstrated *in vitro* that M2c macrophage activation may be subject to regulation by adipose tissue-derived MSCs (Sun et al., 2019).

In this study, the reduction of M2a and M2c macrophages following intravenous administration of hUC-MSCs suggests that the therapeutic effects of hUC-MSCs may be attributed to the regulation of these M2 macrophage subtypes; hUC-MSCs facilitate the resolution of allergic inflammation and promote tissue remodeling and repair.

Through *ex vivo* and *in vitro* experiments, we have shown that hUC-MSC treatment decreases the expression of ILC2-, M2-, and Th2-related markers. We also found that hUC-MSCs can act either directly as demonstrated in co-culture or indirectly via the exchange of soluble factors as demonstrated in transwells on innate and adaptive immune cells. Even though hUC-MSCs were not separated from macrophages in the direct co-culture group, the contaminating effect of hUC-MSCs on the mRNA expression of macrophages was negligible (Supplementary Fig. S5).

Recent studies confirming the immunosuppressive properties of MSCs have proposed that paracrine secretion of soluble factors may be the main mechanism by which MSCs exert immunomodulatory, angiogenic, antiapoptotic, and antioxidative effects (Bernardo and Fibbe, 2013; Chen et al., 2010; Goodwin et al., 2011). Studies have reported that MSCs mediate immunosuppressive functions by secreting water-soluble agents, such as IL-6, IL-10, prostaglandin E2, and nitric oxide (Bernardo and Fibbe, 2013; Chen et al., 2010; Helal et al., 2016). In addition to paracrine effects, cell-to-cell contact makes MSCs modulate their immunosuppressive effects and promote cell viability. Recently, MSCs have been shown to reduce inflammation and increase cell proliferation by releasing exosomes that contain various molecular constituents, including proteins, peptides, mRNA, and microRNA (Tang et al., 2021).

The limitations in our study are as follows. We could not rigorously demonstrate the mechanistic link by which hUC-MSCs exert their immunomodulatory effects by inducing the development and differentiation of ILCs, Tregs, and macrophages. However, we have previously uncovered innate immune crosstalk in asthmatic airways by determining the interaction between ILC and macrophage subtypes (Kim et al., 2019). In this study, we confirmed that hUC-MSCs inhibited both ILC2 differentiation and M2 differentiation of lung macrophages. This suggests that the immune crosstalk of the innate immune system might be modulated by hUC-MSCs, resulting in more effective amelioration of the type 2 immune response (English et al., 2008; Fang et al., 2020a; 2020b). Taken together, our present results show that hUC-MSC treatment can favorably change the outcome of asthmatic inflammation, especially by targeting innate immune cells such as ILCs and macrophages. Based on the results of animal studies, we agree that the therapeutic potential of hUC-MSCs may be attributed to not only the regulation of both innate and adaptive immune cells, but also the cascade of molecular interactions. In-depth exploration is, however, required for clinical application.

In summary, our work provides evidence that intravenous delivery of hUC-MSCs leads to attenuation in AHR and type 2 inflammation, which may be mediated by innate and adaptive cell regulation in asthmatic lungs. In particular, hUC-MSC treatment has been demonstrated to reduce the levels of ILC2s and MoMs, especially M2a and M2c. Based on our findings, these regulatory properties of hUC-MSCs may render hUC-MSC to be a promising novel treatment for severe asthma refractory to conventional therapy.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS
Y.M. involved in all animal experiments and data collection, performed the statistical analysis, and drafted the manuscript. S.Y.K. drafted the manuscript. J.Y.B. interpreted the data. Y.K. and J.J. contributed to revising the manuscript. E.M.J., H.Y.K., and S.H.C. made substantial contributions to the conception of the study. H.R.K. contributed to the conception, designed the study, supervised the conduction of the study and drafting of the manuscript, and revised it critically. All authors read and approved the final manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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