Mesenchymal stem/stromal cells precondition lung monocytes/macrophages to produce tolerance against allo- and autoimmunity in the eye

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Intravenously administered mesenchymal stem/stromal cells (MSCs) engraft only transiently in recipients, but confer long-term therapeutic benefits in patients with immune disorders. This suggests that MSCs induce immune tolerance by long-lasting effects on the recipient immune regulatory system. Here, we demonstrate that i.v. infusion of MSCs preconditioned lung monocytes/macrophages toward an immune regulatory phenotype in a TNF-α–stimulated gene/protein (TSG)-6–dependent manner. As a result, mice were protected against subsequent immune challenge in two models of allo- and autoimmune ocular inflammation: corneal allotransplantation and experimental autoimmune uveitis (EAU). The monocytes/macrophages primed by MSCs expressed high levels of MHC class II, B220, CD11b, and IL-10, and exhibited T-cell–suppressive activities independently of FoxP3$^+$ regulatory T cells. Adoptive transfer of MSC-induced B220$^+$CD11b$^+$ monocytes/macrophages prevented corneal allograft rejection and EAU. Deletion of monocytes/macrophages abrogated the MSC-induced tolerance. However, MSCs with TSG-6 knockdown did not induce MHC II$^+$B220$^+$CD11b$^+$ cells, and failed to attenuate EAU. Therefore, the results demonstrate a mechanism of the MSC-mediated immune modulation through induction of innate immune tolerance that involves monocytes/macrophages.

corneal allotransplantation | experimental autoimmune uveitis | immune tolerance | mesenchymal stem/stromal cell | monocyte/macrophage

Intravenous administration of mesenchymal stem/stromal cells (MSCs) has emerged as a promising cell-based immunotherapy for autoimmune diseases, graft-vs.-host disease, and transplantation (1–3). A significant body of data from preclinical and clinical studies has demonstrated remarkable immunosuppressive capacities of MSCs in various diseases, but the mechanisms are still difficult to explain.

One key observation is that therapeutic benefits of MSC administration in animal models are achieved without significant engraftment of the cells; after i.v. infusion, most MSCs reside transiently within the lung and disappear rapidly with a $t_{1/2}$ of approximately 24 h in mice (4, 5). Therefore, direct suppressive effects of MSCs on the immune system are short-lived, and do not explain the long-term therapeutic effects observed with MSCs in clinical and animal studies. In this context, it has been suggested that MSCs trigger a state of immune tolerance, for example, through the induction of regulatory T cells (Tregs) (6, 7).

Classically, lymphoid cells such as Tregs have been known to play a major role in regulating immune responses and maintaining immune tolerance. Recently, however, myeloid cells, including monocytes/macrophages, have gained attention as important mediators of immune regulation and tolerance (8, 9). In line with this, a few studies demonstrated that MSCs induce the expansion of myeloid cells with immunosuppressive activity and modulate monocytes/macrophages to an antiinflammatory phenotype, thereby inhibiting excessive inflammatory responses (10–12).

However, it is not clear whether the MSC-educated myeloid cells can induce significant immune tolerance to repress adaptive immune responses in a setting of allo- or autoimmunity. Moreover, little is known about the mechanism(s) whereby MSCs induce immune tolerance through myeloid cells.

In this study, we demonstrate that i.v. injection of MSCs into naive mice before immune challenge induces a significant immune tolerance in TNF-α–stimulated gene/protein (TSG)-6–dependent manner, and prevents the development of immune responses in two models of allo- and autoimmune ocular inflammation: corneal allotransplantation and experimental autoimmune uveitis (EAU). The MSC-induced tolerance involves a distinct subset of suppressive monocytes/macrophages in the lung, and is transferable independently of FoxP3$^+$ Tregs. These data suggest a mechanism of MSCs in regulating adaptive immunity through induction of nonspecific innate tolerance.

Results

MSC Pretreatment Improves Corneal Allograft Survival. We first tested whether i.v. infusion of MSCs would induce tolerance to prevent alloimmunity. To this end, we injected 1×10^6 human bone marrow-derived MSCs (hMSCs) into tail vein of BALB/c

Significance

Mesenchymal stem/stromal cells (MSCs) are the focus of intensive efforts directed at developing cell-based therapies in immunologic disorders. However, one of the paradoxical observations made so far is that MSCs engraft transiently in the recipient after exogenous infusion, but achieve long-term therapeutic benefits. Here we demonstrate that MSCs induce the immune tolerance by activating endogenous immune regulatory system of the recipient. Specifically, i.v. administered MSCs induce a population of regulatory monocytes/macrophages in the lung, which are capable of suppressing allo- and autoimmune responses independently of regulatory T cells. The data provide a mechanistic insight into the action of MSCs in immunologic disorders, one of the most frequent indications of diseases for clinical trials using stem cells.

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The authors declare no conflict of interest.

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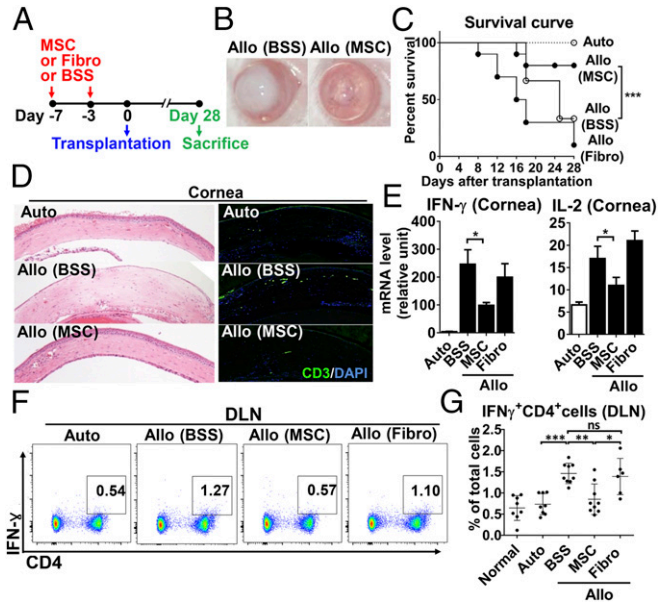

Fig. 1. MSC pretreatment prolongs corneal allograft survival. (*A*) Schema of MSC pretreatment in a model of corneal allotransplantation. (*B*) Representative photographs of the cornea at day 28. (*C*) Survival curve of corneal grafts. Allo, allogeneic grafts; Auto, syngeneic autografts. (*D*) H&E staining and CD3 immunostaining of corneal grafts (original magnification of 100×). (*E*) Real-time RT-PCR assays of the cornea. Data indicate the mRNA levels as the relative ratio to the levels in normal corneas. (*F* and *G*) Representative and quantitative flow cytometry results for IFN-γ and CD4 expression in cells from DLNs. Data depict the percentage of IFN-γ^+CD4$^+$ cells among the total lymph node cells. Each dot represents an individual animal, and results are presented as mean ± SD from three independent experiments, each experiment with three mice per group (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; ns, not significant).

(H-2^d) mice at days −7 and −3, and performed corneal allotransplantation at day 0 using C57BL/6 mice (H-2^b) as donors (Fig. 1*A*). We elected to inject hMSCs 7 and 3 d before transplantation because previous studies indicate that tolerance induced by cyclosporine or endotoxin can be sustained for 3–7 d (13, 14). For positive controls, vehicle (HBSS) or 1×10^6 human skin fibroblasts (Fibro) were injected in place of hMSCs. For negative controls, syngeneic corneal autografts were performed in BALB/c mice using BALB/c mice as donors. As a result, the survival of corneal allografts was significantly prolonged in the hMSC-pretreated mice compared with the HBSS- or Fibro-pretreated mice (Fig. 1 *B* and *C*). Histological examination of corneal allografts at day 28 demonstrated marked corneal stromal swelling with CD3$^+$ cell infiltration in the HBSS-pretreated group, indicating corneal allograft rejection (Fig. 1*D*). In contrast, corneal thickness was normal with well-preserved corneal endothelium and few CD3$^+$ infiltrates in the hMSC-pretreated group, similar to syngeneic autografts. Molecular assay showed that the levels of IFN-γ and IL-2 were markedly increased in the corneas of HBSS- or Fibro-pretreated mice, but significantly reduced in those of hMSC-pretreated mice (Fig. 1*E*). Similarly, flow cytometric analysis of draining cervical lymph nodes (DLNs) revealed that the number of IFN-γ^+CD4$^+$ cells was significantly lower in hMSC-pretreated mice than in HBSS- or Fibro-pretreated mice (Fig. 1 *F* and *G* and Fig. S1). Together, results indicate that i.v. injection of hMSCs before corneal transplantation suppressed the immune rejection and prolonged the corneal allograft survival.

To examine the possibility that hMSCs directly suppress the immune rejection, we evaluated whether hMSCs were present in the lung, DLNs, or cornea at day 0 (at the time of corneal transplantation) after i.v. injections of the cells at days −7 and −3. We carried out quantitative RT-PCR assays at day 0 for human-specific GAPDH as previously described (4, 5), and detected less than 10 hMSCs in the tissues, an observation consistent with previous studies (4, 5). Therefore, the beneficial effects of hMSC pretreatment in corneal allografts were not attributed to direct immunosuppression by hMSCs, and suggest induction of immune tolerance by hMSCs.

MSC Pretreatment Prevents EAU Development. In parallel, we tested whether hMSC pretreatment would be effective in preventing autoimmunity. For this purpose, we used a mouse model of EAU, a well-established model for human autoimmune intraocular inflammation (15). We injected 1×10^6 hMSCs, 1×10^6 Fibro, or the vehicle (HBSS) into the tail vein of C57BL/6 mice at days −7 and −3 before EAU induction. At day 0, the mice were immunized by s.c. injection of the retina-specific antigen interphotoreceptor retinal binding protein (IRBP) into footpads (Fig. 2*A*). The retinal cross-sections at day 21 showed severe disruption of retinal photoreceptor layer in mice pretreated with HBSS or Fibro (Fig. 2*B*). In contrast, there was little structural damage with few inflammatory infiltrates in the retina of hMSC-pretreated mice, similar to normal retina without EAU. The disease score assigned by retinal pathologic examination was significantly lower in hMSC-treated mice compared with HBSS- or Fibro-treated mice (Fig. 2*C*). The transcript levels of proinflammatory cytokines, IFN-γ, IL-2, IL-1β, IL-6, and TNF-α in the whole eyeball were dramatically decreased in the hMSC-pretreated group compared with the HBSS- or Fibro-pretreated group (Fig. 2*D*). In addition, flow cytometric assays of DLNs revealed a significant reduction in the number of IFN-γ^+CD4$^+$ and IL-17$^+$CD4$^+$ cells in hMSC-pretreated vs. HBSS-pretreated mice (Fig. 2 *E* and *F* and Fig. S1).

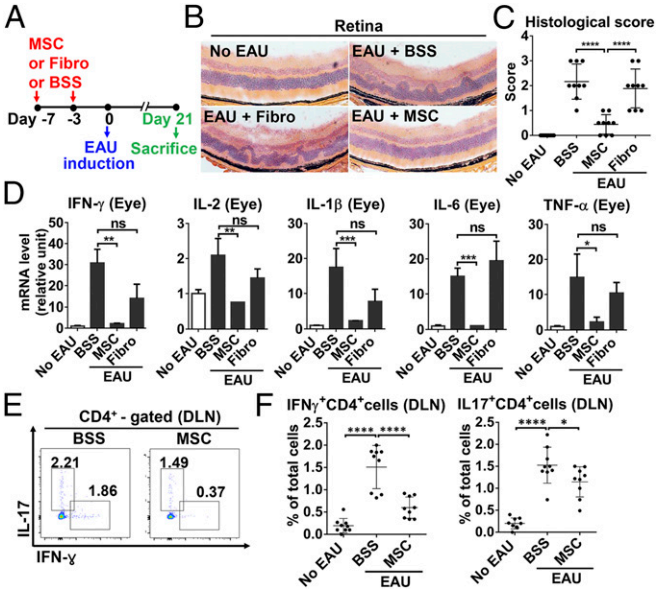

Fig. 2. MSC pretreatment suppresses EAU development. (*A*) Schema of MSC pretreatment in a model of EAU. (*B*) H&E staining of retinal cross-sections. A marked disruption of the photoreceptor layer was observed in the retinas in HBSS- and Fibro-treated mice indicating EAU induction (original magnification of 100×). (*C*) Disease score assigned by retinal histology. (*D*) Real-time RT-PCR assays of the whole eye. Shown are relative values of mRNA levels to the levels in normal eyes. (*E* and *F*) Representative and quantitative flow cytometry results for CD4, IFN-γ, and IL-17 in DLNs. Data depict the percentage of IFN-γ^+CD4$^+$ cells and IL-17$^+$CD4$^+$ cells among the total lymph node cells. Each dot represents an individual animal. Results are presented as mean ± SD from three independent experiments, each experiment with three mice per group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; ns, not significant).

Therefore, the results suggest that hMSC pretreatment prevented EAU development.

We also examined whether the beneficial effects of hMSCs in corneal allograft rejection and EAU might be a result of nonspecific effects of apoptotic cells. However, i.v. injections of hMSCs that were made apoptotic by repeated cycles of freezing and thawing (16) did not have any effects on corneal graft survival and EAU development (Fig. S2).

Collectively, the data demonstrate that i.v. injections of hMSCs into naive mice induce a state of immune tolerance, and thereby protect the eye against allo- and autoimmune inflammation.

A Distinct Population of Myeloid Cells Appears in the Lung After MSC Infusion. Next, we sought to identify the immune cell population (s) responsible for the hMSC-induced tolerance observed. We collected the lung, peripheral blood, spleen, and DLNs from the mice at day 0 after i.v. injection of hMSCs, Fibro, or HBSS into naive mice at days −7 and −3 (Fig. 3*A*). The cells from each tissue were analyzed by flow cytometry for the expression of myeloid lineage markers (MHC class II, CD11b, CD11c, F4/80, Ly6C, B220), lymphoid lineage markers (CD4, CD8, CD19, CD25, FoxP3), and immune regulatory cytokine IL-10. First, we evaluated live cells for MHC class II expression, and found that the percentage of MHC class II^+ cells was markedly higher in the lung of hMSC-pretreated mice compared with HBSS-pretreated mice (46.3 ± 4.8% vs. 29.5 ± 6.4%; $P = 0.001$). Next, we analyzed MHC class II^+ cells, and found that there was a significant difference in MHC class II^+ cell populations based on differential expression of B220 and CD11b between hMSC- and HBSS-pretreated groups (Fig. 3*B*). Quantitative analysis revealed a higher percentage of MHC class $II^+B220^+CD11b^+$ cells and a lower percentage of MHC class $II^+B220^-CD11b^+$ cells in the lung of hMSC-pretreated mice compared with HBSS- or Fibro-pretreated mice (Fig. 3*C*). Similarly, a significant increase was observed in MHC class $II^+B220^+CD11b^+$ cells in peripheral blood, spleen, and DLNs of hMSC-pretreated mice (Fig. 3 *B* and *C*). Further examination of phenotypic markers revealed that MHC class $II^+B220^+CD11b^+$ cells expressed high levels of IL-10, F4/80, and Ly6C and a moderate level of CD11c (Fig. 3*D*). The cells were negative for classical lineage markers of T cells (CD4 and CD8) and B cells (CD19). A number of studies reported that MSCs up-regulate $FoxP3^+$ Tregs (6, 7). Likewise, we observed a significant increase in the number of $CD4^+CD25^+FoxP3^+$ cells in the lung, peripheral blood, and DLNs in mice pretreated with hMSCs (Fig. S3). However, the number of $CD4^+CD25^+FoxP3^+$ cells was markedly reduced in the spleen of hMSC-pretreated mice.

Next, to determine the kinetics of IL-10, MHC class $II^+B220^+CD11b^+$ cells, and $CD4^+CD25^+FoxP3^+$ cells, we analyzed the lung, peripheral blood, and DLNs at days 0, 1, 4, and 7 after i.v. injection of hMSCs into naive mice at days −7 and −3. Time course showed that the plasma level of IL-10 remained significantly elevated until day 4 (i.e., 7 d after hMSC injection; Fig. 3*E*). Consistent with the change in IL-10 levels, the percentage of MHC class $II^+B220^+CD11b^+$ cells was significantly increased in the lung, peripheral blood, and DLNs until day 4, indicating that the cells persist in mice until at least 7 d after hMSC injection (Fig. 3*F*). On the contrary, $CD4^+CD25^+FoxP3^+$ cells were elevated in the lung, blood, and DLNs until day 1 (i.e., 4 d after hMSC injection) in hMSC-pretreated mice, but not different from controls at day 4 and thereafter (Fig. S3).

Together, the results demonstrate that a distinct population of MHC class $II^+B220^+CD11b^+$ cells is induced in the lung by i.v. hMSCs and circulates for at least 7 d postinjection.

MSC-Primed Lung Monocytes/Macrophages Are T-Cell Suppressive. From the aforementioned observations, we hypothesized that the subset of lung myeloid cells defined as MHC class $II^+B220^+CD11b^+$ cells might be involved in the hMSC-induced tolerance. To better characterize the cells, we sorted $B220^+CD11b^+$ and $B220^-CD11b^+$ cells from the lung of mice at day 0 after treatment with i.v. hMSCs at days −7 and −3. Giemsa staining showed that $B220^+CD11b^+$ cells were a morphologically homogenous population of monocytes/macrophages, and $B220^-CD11b^+$ cells were a heterogeneous mixture of monocytes and granulocytes (Fig. 4*A*). To investigate the effects of $B220^+CD11b^+$ cells on T cells, we cocultured $B220^+CD11b^+$ cells that were isolated from the lung of hMSC-pretreated mice with $CD4^+$ cells that were purified from blood of naive mice. To assay T-cell proliferation, $CD4^+$ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with anti-CD3/anti-CD28 monoclonal antibodies. Flow cytometric

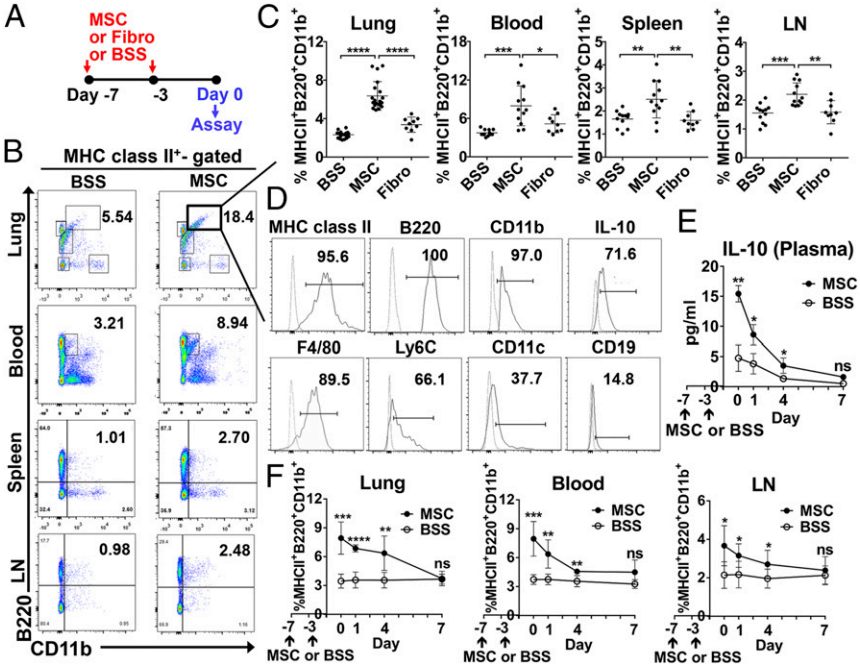

Fig. 3. A distinct population of MHC class $II^+B220^+CD11b^+$ cells is induced by MSC pretreatment. (*A*) Schema of assays. At days −7 and −3, MSC, Fibro, or HBSS vehicle (BSS) were injected into naive BALB/c mice. At day 0, the lung, peripheral blood, spleen, and DLNs were collected for assays. (*B* and *C*) Representative and quantitative flow cytometry results for expression of CD11b and B220 after gating on MHC class II^+ cells. Each dot represents an individual animal. (*D*) Representative flow cytometry plots of phenotypic markers on MHC class $II^+B220^+CD11b^+$ subset in the lung of MSC-pretreated mice. (*E*) Time course of plasma IL-10 levels in mice pretreated with MSC or HBSS. (*F*) Kinetics of MHC class $II^+B220^+CD11b^+$ cells in tissues. Data (mean ± SD) are representative of at least four independent experiments (each with three mice per group) in MSC and HBSS groups and three independent experiments (each with three mice) in Fibro group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; ns, not significant).

analysis of CFSE dilution revealed that $CD4^+$ cell proliferation was significantly repressed by coculture with $B220^+CD11b^+$ cells compared with coculture with $B220^-CD11b^+$ cells or without coculture (Fig. 4*B*). To assay T-cell differentiation, the anti-CD3/anti-CD28–activated $CD4^+$ cells were primed with Th1- or Th17-polarizing conditions and cocultured with $B220^+CD11b^+$ or $B220^-CD11b^+$ cells. The percentage of IFN-γ^+CD4^+ and IL-17^+CD4^+ cells was significantly lower in $CD4^+$ cells cocultured with $B220^+CD11b^+$ cells than in those cocultured with $B220^-CD11b^+$ cells or without coculture (Fig. 4*C*). However, $B220^+CD11b^+$ or $B220^-CD11b^+$ cells did not promote the expansion of $CD4^+CD25^+FoxP3^+$ cells (Fig. S4). Therefore, the data indicate that $B220^+CD11b^+$ monocytes/macrophages from the hMSC-primed lung inhibit T-cell proliferation and Th1/Th17 differentiation.

MSC-Primed Lung Monocytes/Macrophages Induce Tolerance. To confirm the role of the hMSC-induced $B220^+CD11b^+$ monocytes/macrophages in vivo, we transferred $B220^+CD11b^+$ or $B220^-CD11b^+$ cells isolated as described earlier into naive BALB/c or C57BL/6 mice through a tail vein. Corneal allotransplantation was then performed in BALB/c mice or EAU was induced in C57BL/6 mice (Fig. 5). The infusion of $B220^+CD11b^+$ cells significantly prolonged the survival of corneal allografts, whereas $B220^-CD11b^+$ cells did not (Fig. 5 *A* and *B*). The percentage of IFN-γ^+CD4^+ cells in DLNs was significantly reduced in the mice receiving $B220^+CD11b^+$ cells (Fig. 5*C*). Similarly, the expression of IFN-γ and IL-2 in the cornea was markedly decreased in the mice treated with $B220^+CD11b^+$ cells (Fig. 5*D*). To further determine whether monocytes/macrophages are necessary for the MSC-induced tolerance, we depleted mice of monocytes/macrophages by i.v. administration of clodronate-encapsulated liposome at days −8 and −4 and treated them with hMSCs at days −7 and −3. As the maximum monocyte/macrophage depletion occurs 24 h after clodronate liposome delivery (17), the mice were depleted of monocytes/macrophages at the time of hMSC injection. Corneal allotransplantation was performed at day 0. The hMSC pretreatment was not effective in preventing allograft rejection in

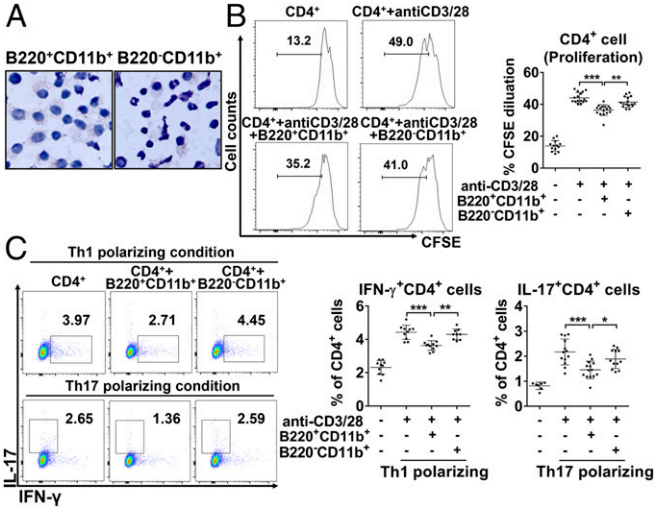

Fig. 4. Impact of lung $B220^+CD11b^+$ vs. $B220^-CD11b^+$ cells on $CD4^+$ cells in vitro. (*A*) Giemsa staining of $B220^+CD11b^+$ vs. $B220^-CD11b^+$ cells isolated from the lung tissue of mice at day 0 after i.v. injections of MSC at days −7 and −3. (*B*) Flow cytometry results for CFSE dilution in $CD4^+$ cells purified from blood of naive mice upon coculture with $B220^+CD11b^+$ or $B220^-CD11b^+$ cells. (*C*) Flow cytometric analysis for expression of IFN-γ and IL-17 on $CD4^+$ cells under Th1 or Th17 polarizing condition. Data depict the percentage of IFN-γ^+CD4^+ cells and IL-17^+CD4^+ cells among $CD4^+$ cells. Each dot indicates a single animal, and the bar indicates the mean ± SD (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

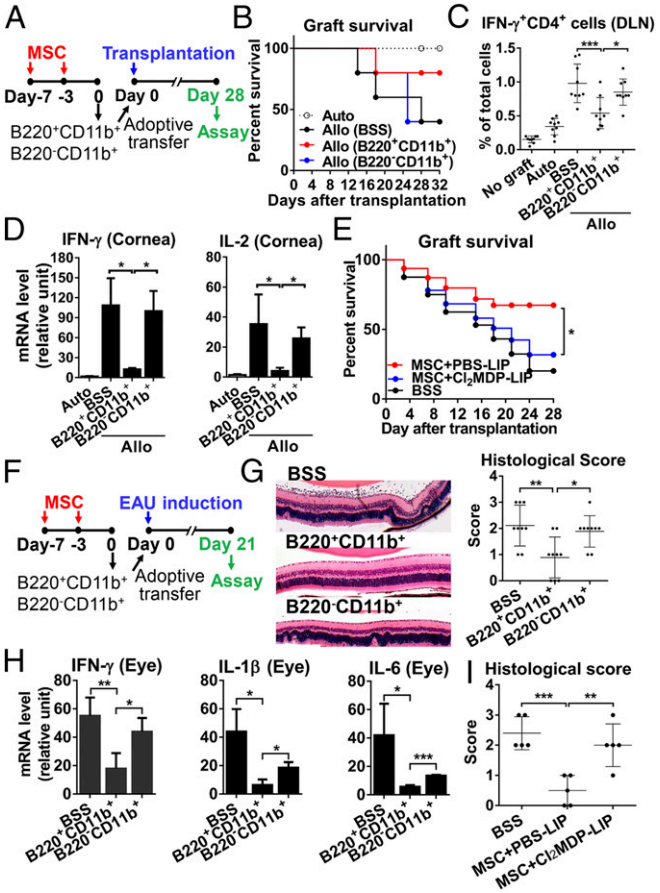

Fig. 5. Adoptive transfer of the lung $B220^+CD11b^+$ cells induced by MSC ameliorates corneal allograft rejection and EAU. (*A*) Schema of $B220^+CD11b^+$ or $B220^-CD11b^+$ cell transfer in a corneal transplant model. (*B*) Survival curve of corneal grafts. Allo, allogeneic grafts; Auto, syngeneic autografts. (*C*) Quantitative flow cytometry results for IFN-γ^+CD4^+ cells in DLNs. (*D*) Real-time RT-PCR assays of the cornea. (*E*) Effects of depletion of monocytes/macrophages on graft survival. PBS-LIP, PBS solution-encapsulated liposome; Cl_2MDP-LIP, clodronate-encapsulated liposome. (*F*) Schema of $B220^+CD11b^+$ or $B220^-CD11b^+$ cell transfer in an EAU model. (*G*) H&E staining of retinal cross-sections (original magnification of 100×). Disease score assigned by retinal histologic examination. (*H*) Real-time RT-PCR assays of the whole eye. (*I*) Effects of depletion of monocytes/macrophages on EAU development as assessed by histological disease score. Dot represents an individual animal, and data are presented as mean ± SD from three independent experiments, each experiment with three mice per group (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

mice in which macrophages were depleted at the time of hMSC injection (Fig. 5*E*).

In a parallel experiment, we found that adoptive transfer of $B220^+CD11b^+$ cells markedly suppressed the development of EAU (Fig. 5 *F–H*). However, hMSC pretreatment did not suppress EAU development in mice depleted of monocytes/macrophages (Fig. 5*I*).

Together, results indicate that monocytes/macrophages are required for the hMSC-mediated tolerance, and $B220^+CD11b^+$ monocytes/macrophages induced by hMSCs are capable of suppressing corneal allograft rejection and EAU.

MSCs Prime Monocytes/Macrophages Within the Lung in Situ in a TSG-6–Dependent Manner. A number of studies indicate that therapeutic effects of MSCs are associated with the secretion of paracrine factors. We therefore examined whether the factor(s) secreted by MSCs might modulate the lung monocytes/macrophages toward a

regulatory phenotype. To this end, we used an in vitro transwell system to coculture the lung cells of naive mice with hMSCs. As a result, we found that the hMSC coculture significantly increased the percentage of $B220^+$ cells among lung $CD11b^+$ cells (Fig. 6*A*). The $B220^+CD11b^+$ cells expressed higher levels of IL-10 compared with $B220^-CD11b^+$ cells (Fig. 6*B*). Next, to identify the paracrine factor(s) of hMSCs associated with an increase in $B220^+CD11b^+$ cells, we performed microarrays to detect changes in the hMSC transcriptome following incubation with the mouse lung cells. Of interest were the gene(s) that encode secreted proteins and were up-regulated by more than twofold in hMSCs upon coculture with lung cells (Table S1). Of the 11 up-regulated genes for secreted proteins, the following four genes have been previously shown to be associated with immunomodulatory and antiinflammatory effects of MSCs: stanniocalcin-1 (STC-1) (18), TSG-6 (4, 5, 11, 19), TGF-β (7, 20, 21), and prostaglandin endoperoxidase synthase 2 (PTGS-2) (10, 12, 21, 22). To examine whether any of these genes are indeed increased in the lung of mice in vivo after i.v. hMSC injection, we performed human-specific real-time RT-PCR assays to analyze the lungs for expression of the four genes 12 h after i.v. infusion of hMSCs in mice. Among the four genes, the most highly up-regulated human transcript was TSG-6 (125-fold; Fig. S5). To confirm whether TSG-6 expression of hMSCs is required for the induction of $B220^+CD11b^+$ cells, we cocultured lung $CD11b^+$ cells with hMSCs transfected with TSG-6 siRNA- or control scrambled (SCR) siRNA (siRNA). The TSG-6 siRNA-transfected hMSCs did not increase the percentage of $B220^+CD11b^+$ cells, whereas SCR siRNA-transfected hMSCs significantly enhanced $B220^+CD11b^+$ cells (Fig. 6*C*). Similarly, infusions of hMSCs with TSG-6 knockdown into mice at days −7 and −3 did not induce MHC class $II^+B220^+CD11b^+$ cells in the lung (Fig. 6 *D* and *E*).

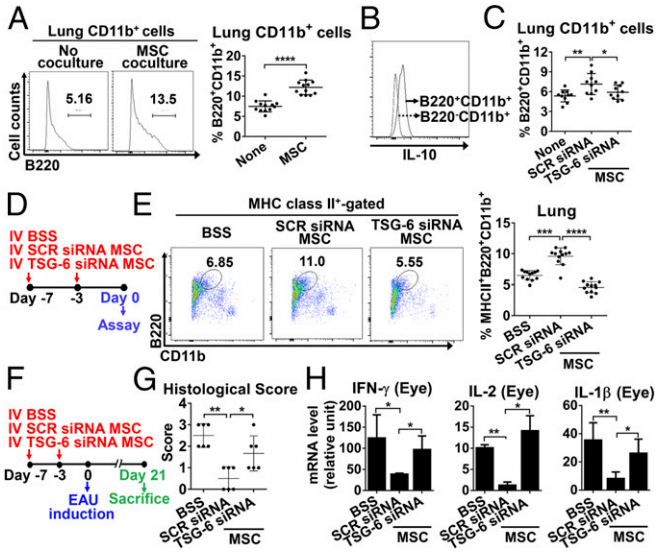

Fig. 6. MSCs with TSG-6 knockdown neither induce MHC class $II^+B220^+CD11b^+$ cells nor prevent EAU. (*A*) Flow cytometry results for B220 expression in mouse lung $CD11b^+$ cells that were cocultured with MSCs. (*B*) Representative flow cytometry plot of IL-10 intracellular staining in $B220^+CD11b^+$ cells vs. $B220^-CD11b^+$ cells. (*C*) The percentage of $B220^+CD11b^+$ cells in mouse lung $CD11b^+$ cells that were cocultured with TSG-6 siRNA- or SCR-transfected MSCs. (*D*) Schema of experiments. (*E*) Flow cytometry results for MHC class $II^+B220^+CD11b^+$ cells in the lung. Each dot indicates a single animal, and the bar represents the mean ± SD. (*F*) Schema of TSG-6 siRNA MSC pretreatment in an EAU model. (*G*) Histologic disease score. (*H*) Real-time RT-PCR assays of the eye. Data are presented as mean ± SD from two independent experiments, each with three mice per group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$).

TSG-6 Expression of MSC Is Required for Tolerance in Vivo. To confirm that TSG-6 expression of hMSCs contributes to tolerance induction by hMSCs, we injected TSG-6 siRNA- or SCR siRNA-transfected hMSCs into naive mice at days −7 and −3 and induced EAU at day 0 (Fig. 6*F*). The pretreatment with TSG-6 knockdown hMSCs was not effective in suppressing intraocular inflammation and preventing retinal damage in mice with EAU, whereas SCR siRNA-transfected hMSCs ameliorated EAU (Fig. 6 *G* and *H*).

Discussion

In this study, we tested the hypothesis that MSCs induce immune tolerance by activating the endogenous immune regulatory system of the recipient. To exclude the effects of direct immunosuppression by MSCs, we injected MSCs into mice at least 72 h before immune challenge to the eye. As a result, we found that the mice were protected from allo- and autoimmune attacks to the eye even though MSCs no longer existed in the system. Because our findings are similar to an experimental strategy of preexposing a tissue to stimuli to diminish the vulnerability of the tissue to a subsequent attack ("preconditioning") (23), we term the induced protection observed with hMSCs as "MSC preconditioning." Similar protection has been observed in ischemia or endotoxin tolerance, in which preexposure to ischemia or lipopolysaccharide results in attenuated tissue injury during subsequent insults (23–25). The nature of the induced protection and underlying mechanisms of preconditioning are not yet clear, but studies suggest that innate immune cells such as monocytes/macrophages play a central role (26–28). Here we found that the MSC preconditioning involved a distinct subset of suppressive MHC $II^+B220^+CD11b^+$ monocytes/macrophages in the lung.

Studies previously suggested that MSCs induce immune tolerance by up-regulating $CD4^+CD25^+FoxP3^+$ Tregs (6, 7). An elegant study by Akiyama et al. showed that i.v. infusion of mouse bone marrow MSCs into naive mice induced T-cell apoptosis in peripheral blood and bone marrow from 6 to 72 h after infusion (6). The apoptotic T cells triggered macrophages in the spleen to produce TGF-β that subsequently resulted in the up-regulation of $CD4^+CD25^+FoxP3^+$ Tregs in peripheral blood at 24 and 72 h. Consistent with this report, we observed an increase in $CD4^+CD25^+FoxP3^+$ cells in peripheral blood that lasted until 96 h after MSC infusion. However, a distinct type of suppressive MHC $II^+B220^+CD11b^+$ monocytes/macrophages was induced at higher levels in the lung by hMSCs and lasted longer in mice than $CD4^+CD25^+FoxP3^+$ Tregs. These monocytes/macrophages were effective in protecting against EAU and corneal allograft rejection independently of $FoxP3^+$ Tregs, indicating a direct role of monocytes/macrophages in the MSC-induced tolerance.

Modulation of monocyte/macrophage function is a crucial component of the host immune response. Recent studies showed that monocytes/macrophages can reprogram their responses toward immunogenic or tolerogenic phenotypes depending on the type and concentration of ligands they encountered, in the phenomenon termed "trained immunity and tolerance" (27, 28). In this context, our study provides a possibility that MSCs use the plasticity of monocytes/macrophages and prime them to produce tolerance. The involvement of monocytes/macrophages in mediating therapeutic effects of MSCs has been previously demonstrated (29). Nemeth et al. showed that i.v. administration of mouse MSCs before or shortly after sepsis induction reprogrammed the lung monocytes/macrophages to increase IL-10 production and reduced mortality in mice (10). The beneficial effects of mouse MSCs on macrophages were caused by the release of prostaglandin E_2 (PGE_2) by MSCs. Another study by Choi et al. demonstrated that i.p. injection of hMSCs modulated peritoneal macrophages to suppress the production of proinflammatory cytokines and attenuated zymosan-induced peritonitis in mice (11). The effects of hMSCs on macrophages were mediated by TSG-6 secreted by hMSCs.

Similarly, we found that the exposure of hMSCs to lung monocytes/macrophages activated hMSCs to produce TSG-6, which led to reprogramming of monocyte/macrophage toward a tolerogenic phenotype that is potent in preventing auto- and alloimmune responses. However, unlike the study by Nemeth et al., we did not observe any change in the expression of $PTGS_2$ in hMSCs in vivo, suggesting that TSG-6, not PGE_2, might be a main factor mediating the MSC action in our models.

TSG-6 is a 30-kDa glycoprotein expressed by many cells in response to proinflammatory cytokines, and has multiple actions related to modulation of inflammation and stabilization of the extracellular matrix (30, 31). Recently, observations by our group and others identified TSG-6 as being responsible for the beneficial effects of MSCs in the treatment of disease models of the heart (4), cornea (5, 19, 32), brain (33), lung (34), and pancreas (35). TSG-6 exerts its antiinflammatory actions through multiple mechanisms, one of which is the modulation of myeloid cells into a tolerogenic phenotype. In the study by Lee et al., TSG-6 treatment of bone marrow-derived myeloid precursors increased the number of $CD11c^+B220^+CD8\alpha^+$ cells of plasmacytoid dendritic subtype, and transfer of myeloid cells cultured with TSG-6 delayed the development of autoimmune diabetes in nonobese diabetic mice (35). Therefore, MSCs and their secreted protein TSG-6 may modulate the endogenous immune system through actions on myeloid cells. Further research would be needed to investigate the detailed mechanism by which MSCs and TSG-6 reprogram monocytes/macrophages.

In conclusion, our study provides a mechanistic insight into how an exogenous infusion of MSCs modulates immune responses despite transient engraftment. The MSCs precondition the recipient immune system toward a state of tolerance through induction of nonspecific innate tolerance involving a distinct subset of suppressive lung monocytes/macrophages.

Materials and Methods

Detailed information on study materials and methods is provided in *SI Materials and Methods*. The experimental protocols were approved by the institutional animal care and use committee of Seoul National University Hospital Biomedical Research Institute (nos. 12–0247-C1A0 and 13–0104-C3A0). EAU was induced as we previously reported (36). Corneal transplantation was performed as we previously described (5).

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