

Mesenchymal Stem Cells Home to Inflamed Ocular Surface and Suppress Allosensitization in Corneal Transplantation

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Submitted: August 6, 2014
Accepted: September 5, 2014

Citation: Omoto M, Katikireddy KR, Rezazadeh A, Dohlman TH, Chauhan SK. Mesenchymal stem cells home to inflamed ocular surface and suppress allosensitization in corneal transplantation. *Invest Ophthalmol Vis Sci*. 2014;55:6631-6638. DOI:10.1167/iovs.14-15413

PURPOSE. To investigate whether systemically injected syngeneic mesenchymal stem cells (MSCs) can home to the transplanted cornea, suppress induction of alloimmunity, and promote allograft survival.

METHODS. Mesenchymal stem cells were generated from bone marrow of wild-type BALB/c or GFP (green fluorescent protein)⁺ C57BL/6 mice, and 1×10^6 cells were intravenously injected to allografted recipients 3 hours after surgery. Mesenchymal stem cells homing to the cornea were examined at day 3 post transplantation by immunohistochemistry. MHC (major histocompatibility complex) II⁺CD11c⁺ cells were detected in the cornea and lymph nodes (LNs) 14 days post transplantation using flow cytometry. Cytokine expression of bone marrow-derived dendritic cells (BMDCs) was determined using real-time PCR. ELISPOT assay was used to assess indirect and direct host T cell allosensitization, and graft survival was evaluated by slit-lamp biomicroscopy weekly up to 8 weeks.

RESULTS. Intravenously injected GFP⁺ MSCs were found in abundance in the transplanted cornea, conjunctiva, and LNs, but not in the ungrafted (contralateral) tissue. The frequencies of mature MHC II⁺CD11c⁺ antigen-presenting cells (APCs) were substantially decreased in the corneas and draining LNs of MSC-injected allograft recipients compared to control recipients. Maturation and function of in vitro cultured BMDCs were decreased when cocultured with MSCs. Draining LNs of MSC-injected allograft recipients showed lower frequencies of IFN γ -secreting Th1 cells compared to the control group. Allograft survival rate was significantly higher in MSC-injected recipients compared to non-MSC-injected recipients.

CONCLUSIONS. Our data demonstrate that systemically administered MSCs specifically home to the inflamed ocular surface and promote allograft survival by inhibiting APC maturation and induction of alloreactive T cells.

Keywords: mesenchymal stem cells, homing, cornea transplantation, allosensitization, antigen-presenting cells

Corneal transplantation is the most commonly performed form of solid tissue transplantation worldwide. As reported by the Eye Bank Association of America, nearly 40,000 cases are performed annually in the United States alone.¹ Immune rejection of corneal graft remains the single most important cause of corneal graft failure.^{2,3} Transplantations performed in uninflamed or “low-risk” graft beds experience a survival rate greater than 70% at 10 years^{4,5}; however, each year, more than 4000 to 6000 first-time recipients suffer from graft rejection,¹ most often due to an alloimmune response. A significant proportion of corneas are transplanted onto inflamed or “high-risk” graft beds and yield rejection rates between 50% and 90% even with maximal local and systemic immune suppression.^{4,5} Given that immunosuppressive treatment is complicated by several side effects and that allorejection remains a substantial clinical problem, investigating new immunomodulatory strategies is crucial to improve corneal transplant survival.

Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent nonhematopoietic stem cells that have been linked to a variety of anti-inflammatory and tissue repair functions.⁶ Along with their capacity to differentiate into cells of multiple

lineages,⁷⁻¹⁰ MSCs have generated great interest because of their ability to display unique anti-inflammatory and immunomodulatory properties.⁶ In a previous study we demonstrated that systemically administered MSCs promote corneal epithelial regeneration in a mouse model of corneal injury. We showed that MSCs exert their proregenerative function in part via increasing the expression of anti-inflammatory cytokines (TGF- β and IL-1Ra) in injured corneas.¹¹ Similar anti-inflammatory effects of MSCs have also been demonstrated in a rat corneal chemical burn model, where MSCs are administered to the site of inflammation and prevent the expression of proinflammatory cytokines.^{12,13} Though the aforementioned properties of tissue regeneration and suppression of nonspecific inflammation have been studied extensively, the potential and underlying mechanisms by which MSCs modulate antigen-specific immunity such as corneal transplant immunity are not well understood.

Mesenchymal stem cells used in other solid organ transplantations have been shown to prolong graft survival via their regenerative abilities and immunomodulatory effect on host lymphocytes.¹⁴⁻¹⁷ Previous studies in corneal transplantation have shown that MSCs can promote allograft survival by

inhibiting early innate immune responses,¹⁸ activation and migration of antigen-presenting cells (APCs),¹⁹ and the function of effector T cells.²⁰ However, critical aspects of MSC-mediated immunomodulation in corneal transplantation, including the site of action and the suppressive mechanisms, are still not clear. Thus in the present study, using a well-characterized mouse model of corneal transplantation,^{21,22} we aimed to discern whether MSCs have the ability to home to the inflamed graft site and suppress allosensitization. Our data demonstrate that systemically administered syngeneic MSCs specifically home to the inflamed ocular surface as well as draining lymph nodes (LNs). Furthermore, MSCs promote allograft survival by suppressing both direct and indirect pathways of allosensitization.

MATERIALS AND METHODS

Mice

Six- to 8-week-old male wild-type BALB/c and C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) or green fluorescent protein (GFP) transgenic C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were used in these experiments. Mice were housed in a specific pathogen-free environment at the Schepens Eye Research Institute animal facility. All procedures were approved by the Institutional Animal Care and Use Committee, and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation, Expansion, and Characterization of Mesenchymal Stem Cells

Bone marrow (from femurs) of BALB/c or GFP⁺ C57BL/6 mice were harvested. Using the previously described plastic adherence method of MSC cultivation,¹¹ we cultured bone marrow cells (2×10^6 cells/mL) at 37°C in mouse mesenchymal stem cell medium (Stem Cell Technologies, Inc., Vancouver, Canada). Cells were passaged every third to fifth day. At passage 5 to 6, MSCs were harvested to use in experiments. Before using MSCs in indicated experiments, MSCs were characterized phenotypically for their expression of MSC markers (CD45⁻CD34⁺SCA1⁺CD29⁺) by flow cytometry as well as functionally by differentiating MSCs into adipocytes as described before.¹¹

Orthotopic Corneal Transplantation

Orthotopic corneal transplantation is a well-established procedure and was carried out as previously described.²² Briefly, central 2-mm-diameter corneal grafts were excised from C57BL/6 mice and transplanted onto prepared BALB/c host beds with eight interrupted 11-0 nylon sutures. Host beds were prepared by excising 1.5-mm pieces of tissue from the central cornea. Sutures were removed 7 days post transplantation.

Three hours after corneal transplantation, 10^6 MSCs, generated from the bone marrow of BALB/c mice, were intravenously injected to transplanted mice. Saline injections served as controls. However, to study MSC homing, corneal grafts from BALB/c mice were transplanted onto C57BL/6 host beds, and GFP⁺ C57BL/6 MSCs were injected 3 hours after surgery. Mice were euthanized for *in vitro* studies 3 or 14 days after transplantation, or they were followed for up to 8 weeks to study graft survival. Mice followed for survival studies received either no MSC injection, or one MSC injection 3 hours after transplantation, or two MSC injections 3 hours and 7 days after transplantation. Syngeneic graft-transplanted mice served

as controls. To evaluate graft rejection, corneas were visualized using slit-lamp biomicroscopy (Topcon SL-D4, Tokyo, Japan) once a week for 8 weeks and graded according to an established scoring scale,²³ with a score of 2+ taken to indicate graft rejection.

Generation of Bone Marrow–Derived Dendritic Cells

Bone marrow–derived dendritic cells (BMDCs) were generated as previously described.²⁴ Briefly, bone marrow cells collected from BALB/c mice were treated with red blood cell lysis buffer; they were then plated at a density of 10^6 /mL in petri dishes in 10% fetal calf serum (FCS) RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/mL; PeproTech, Rocky Hill, NJ, USA), and medium was changed every other day. After 6 days, loosely adherent immature CD11c⁺ BMDCs were collected and used for functional assays.

Fluorescence Microscopy

Freshly excised corneas were washed in PBS and fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 for 10 minutes. Whole corneas were then immunostained with Alexa Fluor 488-conjugated anti-GFP (Life Technologies) to detect GFP⁺ MSCs and mounted onto slides with 4',6-Diamidino-2-Phenylindole (DAPI)-containing mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). Mesenchymal stem cells were visualized using a confocal microscope (Leica TCS-SP5; Buffalo Grove, IL, USA).

Flow Cytometry

We performed multicolor flow cytometry to phenotypically characterize BMDCs (CD11c) and their maturation status (major histocompatibility complex [MHC] II and CD80) using fluorochrome-conjugated mAbs (Biolegends, San Diego, CA, USA) and matched isotype controls. Flow cytometry was also used to determine the frequencies of GFP⁺ MSCs in the ipsilateral and contralateral LNs of transplanted mice. Lymph nodes were excised 3 days after transplantation and MSC injection, and a single cell suspension was prepared and analyzed. To analyze stained cells, we used an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and Summit v4.3 software (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

RNA Isolation, RT-PCR, and Quantitative Real-Time PCR

Bone marrow–derived dendritic cells were snap frozen in liquid nitrogen, and mRNA was isolated using the RNeasy Micro Kit (Qiagen, Germantown, MD, USA). We reverse transcribed isolated total RNA to cDNA using oligo (dT) primer and Superscript TM III (Invitrogen, Grand Island, NY, USA). Then we performed quantitative real-time PCR using Taqman Universal PCR Mastermix and preformulated Taqman primers for murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-10, IL-12, and IL-6 (Applied Biosystems, Foster City, CA, USA). The results were analyzed by the comparative threshold cycle method and normalized to GAPDH as an internal control.²¹

ELISPOT Assay

The ELISPOT assay was used to measure the frequencies of directly and indirectly primed IFN- γ -producing T cells, as previously described.²⁵ Briefly, 96-well ELISPOT plates (Whatman Polyfiltronics, Rockland, MA, USA) were coated with 4 μ g/

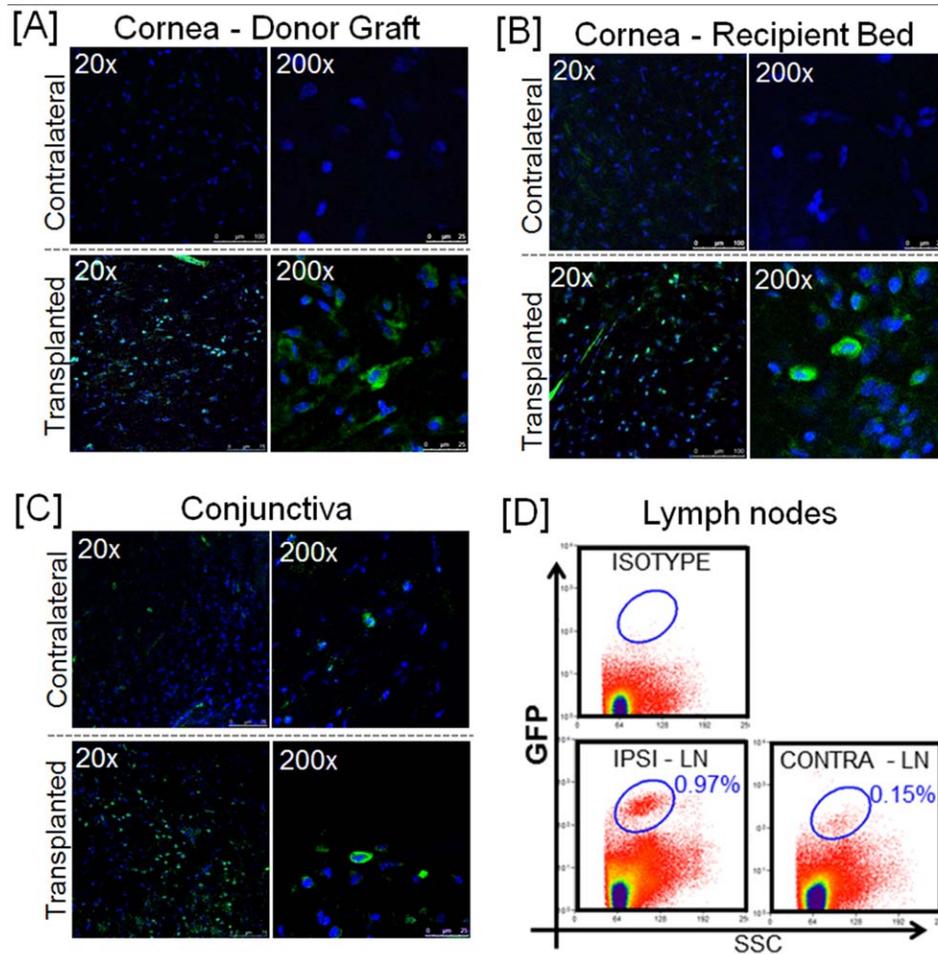


FIGURE 1. Mesenchymal stem cells specifically home to the inflamed ocular surface and lymphoid tissue. Grafts from BALB/c mice were transplanted onto C57BL/6 graft beds, and 3 hours later, 10^6 GFP⁺ MSCs (C57BL/6) were intravenously injected to the recipient mice. Three days after surgery, the transplanted and contralateral cornea, graft bed, and conjunctiva were harvested and analyzed via confocal microscopy. Fluorescence micrographs of transplanted and contralateral (A) cornea donor grafts, (B) cornea recipient beds, and (C) conjunctiva are shown. (D) Flow cytometric dot plots showing frequencies of GFP⁺ MSCs in the ipsilateral (IPSI) and contralateral (CONTRA) lymph nodes of corneal transplanted mice. Representative data from two independently performed experiments are shown, and each group consists of four to six mice.

mL primary anti-IFN- γ antibody (AN-18; BD Pharmingen, San Jose, CA, USA) in sterile PBS for 48 hours at 4°C. Then, the plates were washed with sterile PBS and blocked for 1.5 hours with PBS containing 1% BSA. CD4⁺ T cells from LNs of naïve mice or transplanted mice (BALB/c) with or without MSC injection were isolated 14 days post transplantation using magnetic cell sorting (Miltenyi Biotech, Bergisch-Gladbach, Germany). To quantify the frequencies of directly allosensitized T cells, BALB/c T cells (5×10^5) were incubated in triplicates with C57BL/6 splenic donor-derived APCs (1×10^6). To quantify frequencies of indirectly allosensitized T cells, BALB/c T cells (5×10^5) were incubated in triplicates with BALB/c splenic host-derived APCs (1×10^6) and sonicated donor C57BL/6 antigen. After 48 hours of culture, plates were washed three times with PBS, then four times with PBS containing 0.025% Tween 20. Biotinylated anti-IFN- γ detection mAbs were added at 2 μ g/mL (BD Pharmingen) and incubated for 2 hours at room temperature. The washing steps were repeated; and after 1 hour of incubation with avidin-horseradish peroxidase, the plates were washed again three times with PBS/0.025% Tween 20 and then three times with PBS alone. The spots were developed by the addition of aminoethylcarbazole staining solution (Sigma-Aldrich Corp., St.

Louis, MO, USA). The resulting spots were counted and analyzed on a computer-assisted ELISPOT image analyzer (Cellular Technology Ltd., Shaker Heights, OH, USA).

Statistical Analysis

Mann-Whitney *U* test was used to analyze cell frequencies (%), and two-tailed Student's *t*-test was performed to analyze changes in mRNA expression and ELISPOT assay. The Kaplan-Meier survival curve was analyzed using the log-rank test. *P* values less than 0.05 were regarded as statistically significant. Results are presented as the mean \pm standard error of the mean (SEM).

RESULTS

Mesenchymal Stem Cells Home Specifically to the Inflamed Ocular Surface and Draining Lymphoid Tissue

We used a well-established corneal transplantation model to investigate the homing potential of intravenously injected syngeneic MSCs, and to discern a difference (if any) between

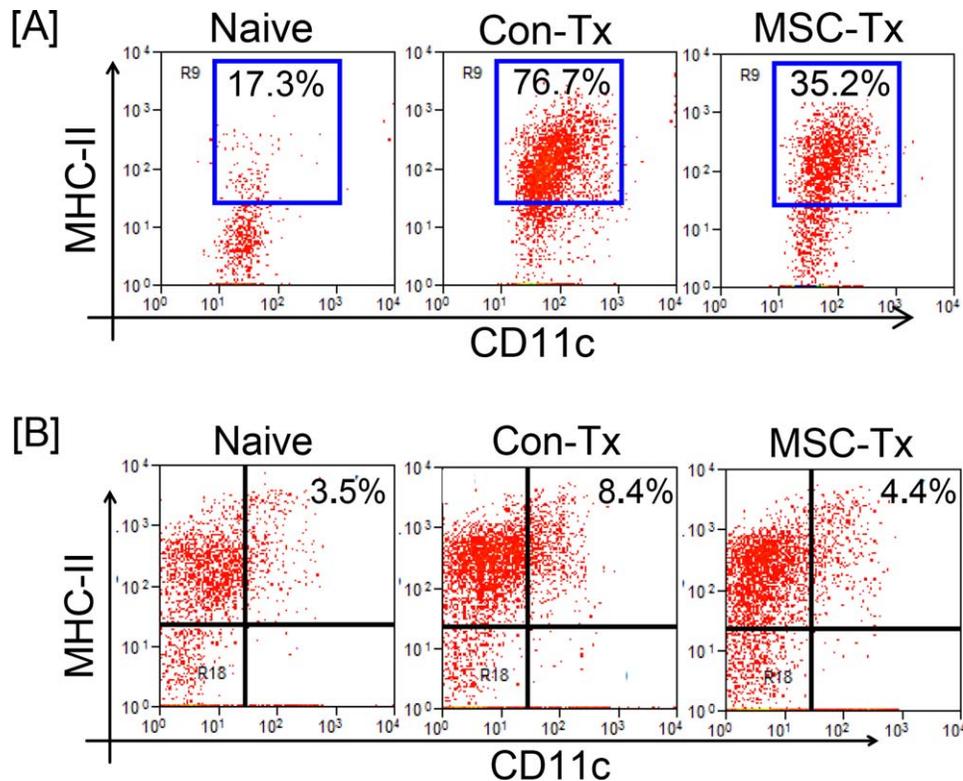


FIGURE 2. Mesenchymal stem cells suppress corneal and lymphoid APC maturation in vivo. We determined the maturation status of antigen-presenting cells (APCs) of the cornea and lymph nodes 14 days after corneal transplantation (C57BL/6 graft onto BALB/c recipient) with or without mesenchymal stem cell (BALB/c MSC) injection. Representative dot plots of flow cytometric data showing frequencies of MHC II⁺CD11c⁺ mature APCs in the cornea (A) and lymph node (B). Representative data from two independent experiments are shown, and each group consists of four to six mice.

the inflamed (transplanted) and contralateral (i.e., healthy) corneas. First, the corneal grafts were harvested from BALB/c mice and transplanted onto C57BL/6 mice. Three hours after corneal transplantation, 10⁶ GFP⁺ MSCs, generated from the bone marrow of GFP⁺ C57BL/6 mice, were intravenously injected into transplanted mice. Three days after surgery, ipsilateral (transplanted) and contralateral (not transplanted) eyes were harvested and analyzed via confocal microscopy to detect injected GFP⁺ MSCs. Substantial numbers of GFP⁺ MSCs were visible in transplanted eyes, including the corneal graft (Fig. 1A), recipient bed (Fig. 1B), and conjunctiva (Fig. 1C). In contrast, in the contralateral uninflamed eye, GFP⁺ cells were not detectable in the cornea, and only few GFP⁺ cells were found in the conjunctiva. To detect GFP⁺ MSCs in lymphoid tissue, we harvested draining LNs from the same mice. GFP⁺ MSCs were detected at a 6-fold higher frequency (1.2 ± 0.4%) in the ipsilateral LNs (on the side of the transplanted cornea) compared to the contralateral LNs (0.15 ± 0.06%; *P* = 0.029) (Fig. 1D). These results show that systemically administered host MSCs preferentially home to the inflamed ocular surface and draining LNs.

Mesenchymal Stem Cells Inhibit Antigen-Presenting Cell Maturation and Function

Antigen-presenting cells present on the ocular surface capture alloantigens and migrate to the draining LNs to advance the immune response by presenting alloantigen to naïve T cells. We thus investigated whether MSCs inhibit the activation and maturation of APCs (CD11c⁺ dendritic cells). First, C57BL/6 corneal grafts were transplanted onto BALB/c recipient mice, and then 10⁶ MSCs (BALB/c) were administered intravenously

3 hours after corneal transplantation. We harvested the corneas and respective LNs 14 days later and determined the frequencies of mature MHC II⁺CD11c⁺ dendritic cells (DCs) in the cornea and LNs of nontransplanted and transplanted mice (with or without MSCs) using flow cytometry. The frequencies of MHC II⁺CD11c⁺ mature DCs were significantly increased in inflamed corneas of transplanted mice without MSC injection (78 ± 8%) compared to uninflamed corneas of nontransplanted mice (15 ± 3%). However, transplanted mice receiving MSC injection displayed a significant ~50% decrease of mature DCs (37 ± 6%; *P* = 0.03) in the corneas compared to transplanted mice that had not received MSCs (Fig. 2A). Similarly, in the LNs of transplanted mice that had not received MSCs (Con-Tx), we detected increased frequencies of MHC II⁺CD11c⁺ DCs (8 ± 5%) but found only 4 ± 2% MHC II⁺CD11c⁺ DCs in transplanted mice that had received MSCs; that is, the percentages of mature DCs in the corneas and LNs were halved in the mice receiving MSCs (*P* = 0.029) (Fig. 2B).

To investigate whether decreased DC maturation in vivo was directly due to MSC injection, we next performed an in vitro experiment using C57BL/6 BMDCs and cocultured them with MSCs. Bone marrow-derived dendritic cells were stimulated with IL-1β with or without MSCs. After 24 hours we analyzed the maturation status of CD11c⁺ BMDCs, assessing their relative expression level of the maturation marker MHC II and the costimulatory molecule CD80 using flow cytometry. Bone marrow-derived dendritic cells cultured alone, without IL-1β stimulation, showed relatively low expression levels for MHC II and CD80. Interleukin-1β stimulation promoted BMDC maturation and led to increased expression of MHC II and CD80. However, additional administration of MSCs significantly inhibited this BMDC maturation and MHC II as well as CD80

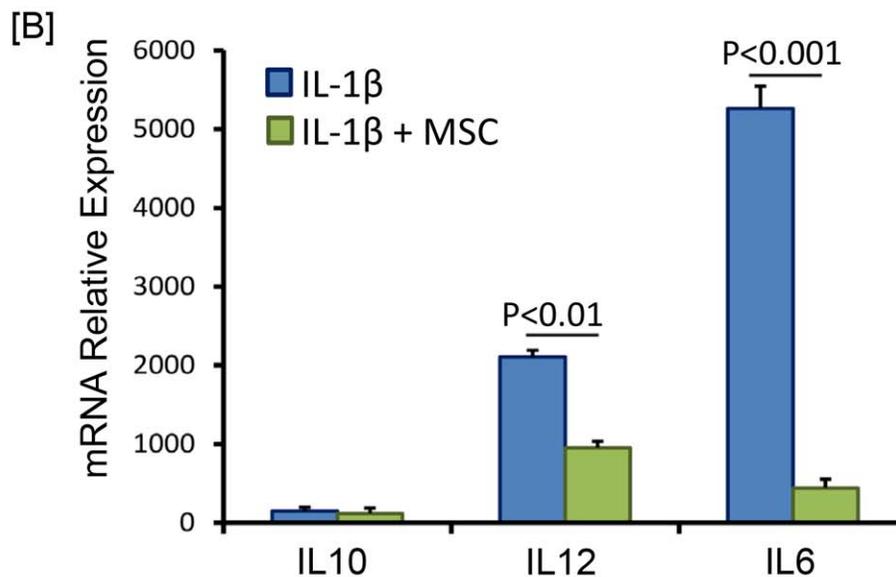
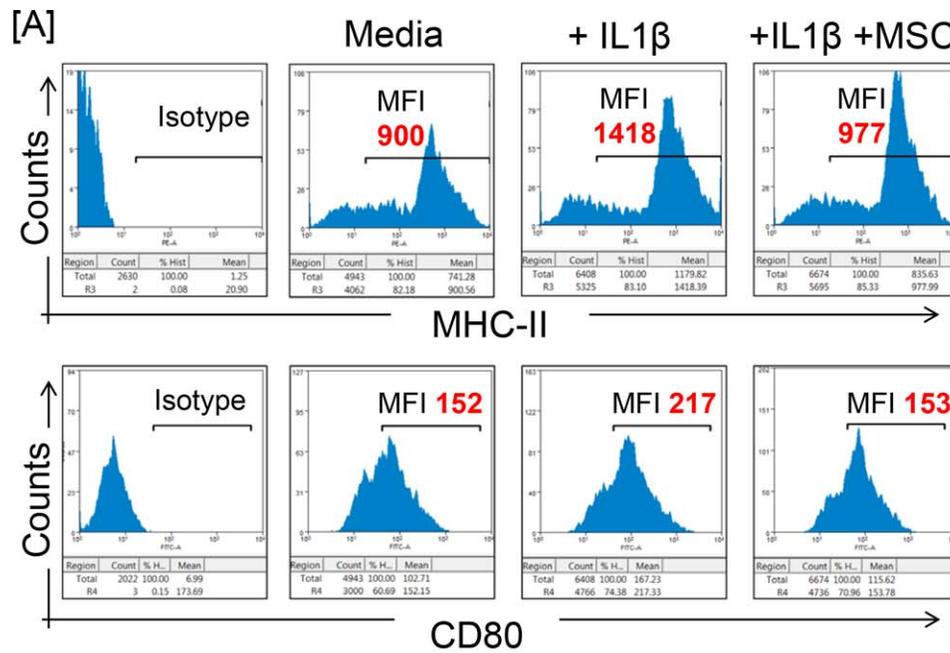


FIGURE 3. Mesenchymal stem cells suppress APC maturation and function in vitro. **(A)** Representative histograms of flow cytometric data showing MHC II and CD80 expression of BDMCs cultured (1) alone (Media), (2) with IL-1β (+IL-1 β), or (3) with IL-1β and MSCs (+IL-1β + MSC) for 24 hours. **(B)** Relative mRNA expression of IL-10, IL-12, and IL-6 in BDMCs when stimulated by IL-1β alone or in the presence of MSC is shown. Experiments were repeated twice, and data in each group are from triplicate wells.

expression, confirming a direct effect of MSCs on APC (BMDC) maturation (Fig. 3A).

Using the same coculture system, we investigated whether MSCs could inhibit the function of BMDs. We measured mRNA expression levels of IL-10, IL-12, and IL-6 using real-time PCR (Fig. 3B). We found no significant difference in the expression of the immunoregulatory cytokine IL-10. However, the relative mRNA expression of IL-12, which polarizes naïve T cells to IFNγ⁺CD4⁺ T cells (Th1), was reduced ~2-fold in the presence of MSCs ($P = 0.01$). The relative expression of IL-6, a proinflammatory cytokine that inhibits the induction and function of regulatory T cells, was significantly reduced 10-

fold in the presence of MSCs ($P = 0.001$). These results show that MSCs reduce APC maturation in vivo and in vitro, and also that MSCs diminish the expression of proinflammatory BMDc-secreted cytokines and thereby suppress APC function.

Mesenchymal Stem Cells Suppress Allosensitization

Because IFNγ⁺ Th1 cells are the principal mediators of acute corneal graft rejection,²⁶ and are primed either directly by donor APCs or indirectly by host APCs,²⁷ we analyzed whether MSCs were able to inhibit T cell allosensitization using the

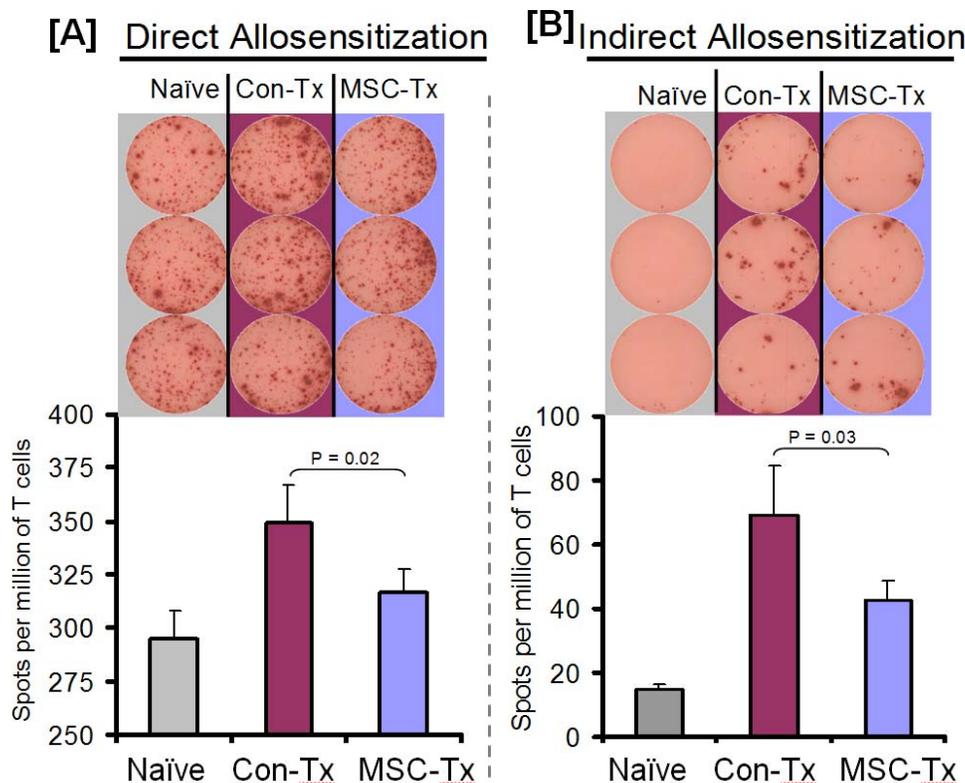


FIGURE 4. Mesenchymal stem cells suppress both direct and indirect allosensitization. ELISPOT assay of T cells harvested from lymph nodes of mice 14 days after corneal transplantation (C57BL/6 graft onto BALB/c recipient) with or without MSC (BALB/c) injection. IFN- γ ⁺ T cells were primed by either donor antigen-presenting cells (direct) (A) or donor antigen-pulsed host antigen-presenting cells (indirect) (B). Experiments were repeated twice, and data in each group are from triplicate wells.

ELISPOT assay. Fourteen days following transplantation (C57BL6 grafts onto BALB/c recipients), we harvested CD4⁺ T cells from the draining LNs of transplant recipients that had received no cells or syngeneic MSCs, and then assessed the positive spots of directly (Fig. 4A) and indirectly (Fig. 4B) primed Th1 cells. Transplant recipients with MSC administration displayed a significant decrease in directly ($P = 0.02$) and indirectly ($P = 0.03$) allosensitized Th1 cells compared to transplant recipients that had not received MSCs. These findings indicate that MSCs inhibit the activation of IFN- γ -producing T cells via both the direct and the indirect pathway.

Mesenchymal Stem Cells Promote Allograft Survival

Finally, we investigated whether immune suppression via systemic administration of MSCs promotes the survival of corneal grafts (Fig. 5). After we had performed corneal transplantation (C57BL/6 grafts onto BALB/c recipients), we compared the following four groups: (1) MSCs (BALB/c) intravenously injected 3 hours after surgery (MSC: D0); (2) MSCs injected 3 hours and 7 days after surgery (MSC: D0, D7); (3) a group in which saline was injected (control); and (4) mice that had received syngeneic transplants (syngeneic). These groups were examined postoperatively for 8 weeks using slit-lamp microscopy to monitor graft survival. While the non-MSC control group exhibited 35% survival ($n = 10$), 60% of the MSC: D0 group and 80% of the MSC: D0, D7 group survived ($n = 12$) (~2-fold higher survival than in the control group), demonstrating that graft survival is promoted significantly by MSCs ($P = 0.03$) and that the survival rate attributable to MSC administration is dose dependent. In the syngeneic group, all

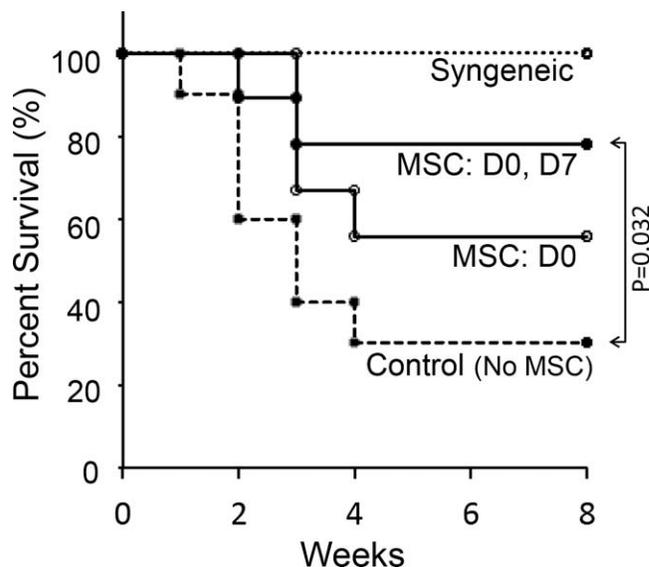


FIGURE 5. Mesenchymal stem cells promote the survival of allograft tissue. Transplanted corneal grafts (C57BL/6 graft onto BALB/c recipient) were monitored weekly for 8 weeks post surgery. MSCs (BALB/c) were injected 3 hours (D0) and/or 7 days (D7) post transplantation. Graft survival rate was 80% ($n = 12$) in the group with MSC injection at 3 hours and 7 days post transplantation (MSC: D0, D7), significantly higher than in the non-MSC control group (35%, $n = 10$, $P = 0.032$). Syngeneic mice received grafts from their genetic counterparts. Kaplan-Meier analysis, $n = 9$ to 12 mice per group.

of the corneas retained their transparency through the 8-week postsurgical period. These results indicate that systemically injected MSCs promote corneal allograft survival due to decreased immune rejection.

DISCUSSION

The current study provides novel evidence that systemically administered MSCs migrate to the inflamed graft site and helps in understanding MSC-mediated modulation of alloimmunity in corneal transplantation. We show that intravenously injected syngeneic MSCs specifically home to the inflamed ocular surface, as well as draining LNs, and suppress APC function, which in turn leads to decreased T cell allosensitization and enhanced allograft survival.

A previous study has indicated that human MSCs exert their anti-inflammatory effect through soluble immunomodulatory molecules from a distant site and do not home to the site of inflammation in a mouse model of corneal transplantation.¹⁸ However, several reports on corneal injury models have demonstrated that MSCs directly administered to the eye effectively suppress inflammation,^{12,13,28-30} and we have further shown that MSCs intravenously injected into a corneal injury model specifically migrate to injured corneas.¹¹ To investigate the homing abilities of MSCs to the cornea in a transplant setting, we intravenously injected syngeneic GFP⁺ MSCs in corneal allograft recipients, and found that these MSCs specifically homed to inflamed corneas and ipsilateral draining LNs but not to contralateral uninflamed corneas and LNs. Though this is in contrast to the findings of Oh et al.,¹⁸ one potential reason could be that they injected human MSCs into a mouse model; even though human MSCs are considered immune privileged, it is possible that this xeno-species barrier hindered the process of MSC migration and integration at the graft site. In our model, we injected syngeneic MSCs into transplant recipient mice, which could be the necessary factor in inducing MSC homing to the graft site. Our results are supported by previous data from a study in which we injected mouse MSCs (BALB/c) into a mouse model of corneal injury.¹¹ Moreover, we have used a suture mouse model to induce corneal inflammation and injected GFP-labeled MSCs; 3 days post surgery, we detected GFP⁺ MSCs only in sutured corneas but not in contralateral corneas (data not shown). Together these data suggest that specific homing of MSCs to the graft site is dependent on inflammation per se, which can be caused by injury, suture placement, or transplantation. Further investigation is still required to elucidate the necessary factors in MSC homing in these experiments, as the mechanism and requirements for this process remain contested.³¹

After transplantation, corneal DCs capture alloantigen(s) and acquire a mature phenotype by upregulating the expression of MHC II and costimulatory molecules.³² These mature antigen-bearing MHC II⁺ DCs then migrate to draining LNs and prime T cells to promote further immune responses; thus they are critical in induction of alloimmunity and transplant rejection. In this study, MSC administration post transplantation diminished APC maturation in vivo. In vitro we observed similar results for BMDC maturation when cocultured with MSCs. Further, the expression of the cytokines IL-12 and IL-6 was reduced when cocultured with MSCs, demonstrating that MSCs diminish the intrinsic inflammatory function of APCs. Th1 cells, promoted by IL-12, and regulatory T cells, negatively controlled by IL-6, both have a crucial role in transplant immunity.^{33,21} Thus, we propose that MSCs exert their immunomodulatory function by indirectly diminishing

effector Th1 cells and facilitating regulatory T cell differentiation.

The inhibitory effect of MSCs on effector T cell proliferation and IFN- γ production by corneal and LN cells has been shown before.^{18,20} Here we used a standardized ELISPOT assay²⁵ to show the capacity of MSCs to diminish host T cell allosensitization via both the direct (donor APC-primed host T cells) and the indirect (host APC-primed host T cells) sensitization pathway. The indirect pathway is primarily associated with "low-risk" cornea transplantation, while the direct pathway is mainly associated with "high-risk" transplantation.²⁵ Suppression of both pathways of allosensitization suggests that MSCs may have the potential to suppress corneal alloimmunity in both high-risk and low-risk graft recipients.

Previous studies have shown enhanced allograft survival using human MSCs in murine cornea transplantation.^{18,20} Here, we used mouse MSCs in a mouse model for cornea transplantation and report a positive and dose-dependent effect on transplant survival. Dose dependency is a benefit for future systemic therapies using MSCs post transplantation, as treatment dosage can be modulated depending on the transplant model. Given the fact that there are studies showing no or less effect of MSCs in corneal transplantation and studies reporting controversial results on the homing capacity of MSCs,^{34,35} our data suggest that suppression of corneal transplant rejection is dependent on the type of MSCs used as well as the frequency and route of administration.

In conclusion, these findings provide new insights on the immunomodulatory function of MSCs in corneal transplantation. Mesenchymal stem cells home to the inflamed graft site and draining LNs, and successfully suppress APC function and T cell allosensitization, which leads to enhanced allograft survival in corneal transplantation. Mesenchymal stem cells are a promising tool for future therapies to specifically suppress inflammation while additionally promoting tissue regeneration following corneal transplantation.

Acknowledgments

The authors thank Susanne Eiglmeier, PhD, Schepens Eye Research Institute, Boston, Massachusetts, for helpful discussion and for providing editorial assistance in the preparation of this manuscript. Supported in part by grants from the U.S. Department of Defense, Massachusetts Lions Eye Research Award, and the Eleanor and Miles Shore Fellowship for Scholars in Medicine, Harvard Medical School.

Disclosure: **M. Omoto**, None; **K.R. Katikireddy**, None; **A. Rezazadeh**, None; **T.H. Dohlman**, None; **S.K. Chauhan**, None

References

1. Eye Bank Association of America. *2012 Eye Banking Statistical Report*. Available: http://www.restoreight.org/wp-content/uploads/2013/04/2012_Statistical_Report_FINAL-reduced-size-4-10.pdf. Accessed July 28, 2014.
2. Lechler RI, Sykes M, Thomson AW, Turka LA. Organ transplantation—how much of the promise has been realized? *Nat Med*. 2005;11:605–613.
3. Lakkis FG, Lechler RI. Origin and biology of the allogeneic response [published online ahead of print August 1, 2013]. *Cold Spring Harb Perspect Med*. 2013;3. doi:10.1101/cshperspect.a014993.
4. Ing JJ, Ing HH, Nelson LR, Hodge DO, Bourne WM. Ten-year postoperative results of penetrating keratoplasty. *Ophthalmology*. 1998;105:1855–1865.
5. Inoue K, Amano S, Oshika T, Sawa M, Tsuru TA. 10-year review of penetrating keratoplasty. *Jpn J Ophthalmol*. 2000;44:139–145.

6. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol*. 2008;8:726-736.
7. Du Y, Funderburgh ML, Mann MM, SundarRaj N, Funderburgh JL. Multipotent stem cells in human corneal stroma. *Stem Cells*. 2005;23:1266-1275.
8. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. 1968;6:230-247.
9. Prockop DJ. Marrow stromal cells as stem cells for continual renewal of nonhematopoietic tissues and as potential vectors for gene therapy. *J Cell Biochem Suppl*. 1998;30-31:284-285.
10. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.
11. Lan Y, Kodati S, Lee HS, Omoto M, Jin Y, Chauhan SK. Kinetics and function of mesenchymal stem cells in corneal injury. *Invest Ophthalmol Vis Sci*. 2012;53:3638-3644.
12. Oh JY, Kim MK, Shin MS, et al. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells*. 2008;26:1047-1055.
13. Ma Y, Xu Y, Xiao Z, et al. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. *Stem Cells*. 2006;24:315-321.
14. Casiraghi F, Azzollini N, Cassis P, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semi-allogeneic heart transplant through the generation of regulatory T cells. *J Immunol*. 2008;181:3933-3946.
15. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol*. 2002;30:42-48.
16. Zhang W, Qin C, Zhou ZM. Mesenchymal stem cells modulate immune responses combined with cyclosporine in a rat renal transplantation model. *Transplant Proc*. 2007;39:3404-3408.
17. Hong Z-F, Huang X-J, Yin Z-Y, Zhao W-X, Wang X-M. Immunosuppressive function of bone marrow mesenchymal stem cells on acute rejection of liver allografts in rats. *Transplant Proc*. 2009;41:403-409.
18. Oh JY, Lee RH, Yu JM, et al. Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. *Mol Ther*. 2012;20:2143-2152.
19. Lee HJ, Ko JH, Ko AY, Kim MK, Wee WR, Oh JY. Intravenous infusion of mesenchymal stem/stromal cells decreased CCR7(+) antigen presenting cells in mice with corneal allotransplantation. *Curr Eye Res*. 2014;39:780-789.
20. Jia Z, Jiao C, Zhao S, et al. Immunomodulatory effects of mesenchymal stem cells in a rat corneal allograft rejection model. *Exp Eye Res*. 2012;102:44-49.
21. Chauhan SK, Saban DR, Lee HK, Dana R. Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation. *J Immunol*. 2009;182:148-153.
22. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med*. 2002;195:259-268.
23. Sonoda Y, Streilein JW. Orthotopic corneal transplantation in mice—evidence that the immunogenetic rules of rejection do not apply. *Transplantation*. 1992;54:694-704.
24. Inaba K, Inaba M, Deguchi M, et al. Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc Natl Acad Sci U S A*. 1993;90:3038-3042.
25. Jin Y, Chauhan SK, Saban DR, Dana R. Role of CCR7 in facilitating direct allosensitization and regulatory T-cell function in high-risk corneal transplantation. *Invest Ophthalmol Vis Sci*. 2010;51:816-821.
26. Boisgérault F, Liu Y, Anosova N, Ehrlich E, Dana MR, Benichou G. Role of CD4+ and CD8+ T cells in allorecognition: lessons from corneal transplantation. *J Immunol*. 2001;167:1891-1899.
27. Huq S, Liu Y, Benichou G, Dana MR. Relevance of the direct pathway of sensitization in corneal transplantation is dictated by the graft bed microenvironment. *J Immunol*. 2004;173:4464-4469.
28. Reinshagen H, Auw-Haedrich C, Sorg RV, et al. Corneal surface reconstruction using adult mesenchymal stem cells in experimental limbal stem cell deficiency in rabbits. *Acta Ophthalmol*. 2011;89:741-748.
29. Roddy GW, Oh JY, Lee RH, et al. Action at a distance: systemically administered adult stem/progenitor cells (MSCs) reduce inflammatory damage to the cornea without engraftment and primarily by secretion of TNF- α stimulated gene/protein 6. *Stem Cells*. 2011;29:1572-1579.
30. Yao L, Li Z, Su W, et al. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. *PLoS One*. 2012;7:e30842.
31. Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell*. 2009;4:206-216.
32. Hamrah P, Huq SO, Liu Y, Zhang Q, Dana MR. Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. *J Leukoc Biol*. 2003;74:172-178.
33. Niederkorn JY. Immune mechanisms of corneal allograft rejection. *Curr Eye Res*. 2007;32:1005-1016.
34. Oh JY, Kim MK, Ko JH, Lee HJ, Wee WR. Rat allogeneic mesenchymal stem cells did not prolong the survival of corneal xenograft in a pig-to-rat model. *Vet Ophthalmol*. 2009;12(suppl 1):35-40.
35. Yao L, Bai H. Review: mesenchymal stem cells and corneal reconstruction. *Mol Vis*. 2013;19:2237-2243.