


Mesenchymal Stromal Cells Modulate Corneal Alloimmunity via Secretion of Hepatocyte Growth Factor

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ABSTRACT

Mesenchymal stromal cells (MSCs) are multipotent stem cells that participate in tissue repair and possess considerable immunomodulatory potential. MSCs have been shown to promote allograft survival, yet the mechanisms behind this phenomenon have not been fully defined. Here, we investigate the capacity of MSCs to suppress the allogeneic immune response by secreting the pleiotropic molecule hepatocyte growth factor (HGF). Using an in vivo mouse model of corneal transplantation, we report that MSCs promote graft survival in an HGF-dependent manner. Moreover, our data indicate that topically administered recombinant HGF (a) suppresses antigen-presenting cell maturation in draining lymphoid tissue, (b) limits T-helper type-1 cell generation, (c) decreases inflammatory cell infiltration into grafted tissue, and (d) is itself sufficient to promote transplant survival. These findings have potential translational implications for the development of HGF-based therapeutics. *STEM CELLS TRANSLATIONAL MEDICINE* 2019;8:1030–1040

SIGNIFICANCE STATEMENT

Corneal transplantation is the most common form of tissue grafting, with more than 45,000 surgeries performed in the United States alone each year. Graft recipients often have ocular inflammation and increased corneal blood vessels, and these features are associated with >50% transplant failure despite maximal treatment with immunosuppressive therapy. Mesenchymal stem cells have substantial immunoregulatory and tissue reparative functions at the ocular surface, but cell-based therapeutics are currently limited by the regulatory and financial challenges of manufacturing clinical-grade cells. Here, the authors show that mesenchymal stem cells' promotion of corneal graft survival depends on their expression of hepatocyte growth factor. Furthermore, the study demonstrates that hepatocyte growth factor alone is sufficient to limit the alloimmune response and promote corneal graft survival.

INTRODUCTION

Corneal transplantation is the definitive treatment for a variety of end-stage ocular surface diseases [1]. In clinical practice, graft recipients commonly exhibit inflamed and vascularized host beds, in which case failure rates exceed 50% despite maximal treatment with nonspecific immunosuppressive medications [2, 3]. Given that conventional immunosuppressive agents are associated with severe ocular side effects (including cataract, glaucoma, and increased susceptibility to infections) [4], there is an unmet need for novel immunomodulatory strategies to promote corneal transplant survival.

Corneal graft rejection occurs due to the activation of proinflammatory immune pathways directed against specific alloantigens [5]. Following

transplantation, the localized upregulation of proinflammatory cytokines promotes antigen-presenting cell (APC) maturation (i.e., acquisition of major histocompatibility complex (MHC) class II and other costimulatory molecules) [6]. Subsequently, APCs migrate via lymphatic vessels to the draining lymph nodes (DLNs), where they prime naïve T cells by presenting alloantigens, which differentiate and proliferate into interferon- γ (IFN γ)-secreting CD4⁺ T helper type 1 (Th1) cells [5]. The efferent arm of the alloimmune response involves the egress of Th1 cells from the lymph nodes and their migration via blood vessels toward the graft site along a chemokine gradient [7]. At the cornea, Th1 cells promote graft rejection through the production of proinflammatory cytokines [5]. Importantly, the alloimmune response is limited by cellular

and molecular immunoregulatory mechanisms that suppress the effector response and foster graft tolerance [8].

Mesenchymal stromal cells (MSCs) are multipotent stem cells that are capable of differentiating into a variety of cell types [9]. MSCs also have substantial immunoregulatory potential and have been reported to modulate both innate and adaptive immune responses [9, 10]. Previous work from our group, using a murine model of corneal transplantation, has established that systemically administered MSCs delivered postoperatively specifically home to the inflamed ocular tissues [11]. Moreover, we have demonstrated that MSCs promote allograft survival through the inhibition of APC maturation and suppression of alloreactive T-cell induction [11]. However, the exact mechanisms by which MSCs modulate the immune response in allogeneic tissue transplantation have not been fully delineated.

Hepatocyte growth factor (HGF) is a pleiotropic growth factor that acts via the receptor *c-met* [12]. Renowned for promoting epithelial motility and proliferation, HGF is also recognized as an important immunoregulatory factor in both acute and chronic inflammation [13]. In a previous study, we established that corneal injury induces an inflammatory milieu that promotes the secretion of high levels of HGF by MSCs [14]. Furthermore, we have shown that topical administration of HGF following corneal injury suppresses ocular inflammation [15]. In this study, we systematically investigate the capacity of HGF to promote corneal transplant survival by suppressing generation of the alloimmune Th1 effector response and decreasing inflammatory cell infiltration. In our well-characterized murine model of corneal transplantation, approximately 50% of grafts are rejected within the first three postoperative weeks, with the remaining grafts surviving indefinitely [16–18]. Unlike other models of transplantation, this system allows for continuous assessment of the alloimmune response (i.e., the evaluation of graft opacity) without necessitating sacrifice of the recipient.

Our data demonstrate that MSCs promote corneal transplant survival in an HGF-dependent manner. Moreover, we establish that topical application of HGF limits the generation of Th1 cells, inhibits graft infiltration of immune cells, and is itself sufficient to increase corneal allograft survival.

MATERIALS AND METHODS

Animals

Seven- to eight-week-old BALB/c and C57BL/6 wild-type male mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed in the animal vivarium of the Schepens Eye Research Institute. The mice were treated in accordance with the Association of Research in Vision and Ophthalmology's "Statement for the Use of Animals in Ophthalmic and Vision Research." The Institutional Animal Care and Use Committee of the Schepens Eye Research Institute reviewed and approved all animal procedures.

Corneal Transplantation

Allogeneic corneal transplantation was performed as described previously [19, 20]. Briefly, 2-mm-diameter corneal buttons were excised from the central cornea of C57BL/6 mice and were grafted onto similarly prepared 1.5-mm host beds of BALB/c mice using eight interrupted 11-0 nylon sutures. Recipient eyelids were closed for 3 days postoperatively using a single 8-0 nylon suture. Corneal sutures were removed 7 days after

surgery. Syngeneic corneal transplantation was performed in a similar fashion but using BALB/c donors and BALB/c hosts.

Evaluation of Corneal Graft Survival

Graft opacity was graded using a slit-lamp microscope on a weekly basis for 8 weeks postoperatively, according to an established 0–5+ scale [21, 22]. Allograft rejection was determined according to corneal opacity scores, with scores of >2 (i.e., a level of opacity that conceals iris details) for more than two consecutive weeks considered to be immune rejected. Eyes that became opaque in the first two postoperative weeks and never became clear were excluded from the analysis, as were eyes that underwent either intraoperative or postoperative complications (e.g., hemorrhage, infection, cataract, or synechia).

MSC and HGF Administration

For experiments investigating the effects of MSC administration, *in vitro* expanded and characterized MSCs were treated with either HGF-specific small interfering RNA (siRNA) or control siRNA and injected into the tail veins of mice at 3 hours post-transplantation (0.5×10^6 cells suspended in 100 μ l of sterile saline) as previously described [11, 14, 23]. For experiments investigating the effects of HGF administration, 5 μ l of either 0.1% murine recombinant HGF protein (R&D systems, Minneapolis, MN) in phosphate-buffered saline (PBS) or protein control (mouse serum, Sigma-Aldrich, St. Louis, MO) was applied topically to the ocular surface twice daily for 14 days following corneal transplantation.

MSC Purification and Expansion

Bone marrow was harvested from the femurs of euthanized BALB/c mice. MSCs were phenotypically and functionally characterized according to the criteria set forth by The International Society for Cellular Therapy [24]. The previously described plastic adherence technique of MSC cultivation was used [25, 26]. Bone marrow cells were cultured in murine MSC-specific MesenCult medium with supplement (STEMCELL Technologies, Vancouver, Canada). Nonadherent cells were removed every 48 hours by changing the medium. MSCs were harvested at passage 2 for use in experiments. Detailed characterizations of MSCs were performed prior to using them in experiments, as previously described [11, 14, 23].

Cell Culture Assays

For our *in vitro* experiments, dendritic cells (DCs) were differentiated from bone marrow cells harvested from the femurs of BALB/c mice using a protocol described previously [27]. Briefly, bone marrow cells were cultured in media containing granulocyte-macrophage colony-stimulating factor (20 ng/ml) for 6 days. At this time, cultures routinely contain >85% CD11c^{high} cells. For the coculture assays, DCs were cultured alone or on a monolayer of MSCs either in the presence or absence of IL1 β (100 ng/ml) for 24 hours. In our HGF neutralization assays, cocultures were treated with soluble HGF receptor (s-HGFR) at the doses of 1 and 10 μ g/ml. Two mice were used in each experiment, and each experiment was repeated three times.

siRNA Transfection

MSCs (1.5×10^6 cells) were plated and incubated in a 75-cm² culture flask for 18–24 hours in order to reach 60%–70% confluence. Subsequently, the cells were washed and transfected

with 4.8 μg of *Hgf*-specific or nonspecific control siRNA using transfection reagent in siRNA transfection medium as per the manufacturer's protocol (Santa Cruz Biotechnology, Dallas, TX). Following overnight incubation, the transfection medium was replaced with standard MSC growth culture medium, and the cells were cultured for an additional 48 hours. Real-time polymerase chain reaction (PCR) using *Hgf*-specific primers was used to validate the knockdown efficiency of siRNA after 3 days of transfection.

Real-Time PCR

A commercially available kit was used to isolate total RNA (RNeasy Micro Kit; Qiagen, Valencia, CA). Reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA) was used to reverse transcribe RNA into cDNA. Following this, quantitative real-time PCR was performed using preformulated Taqman-based probes for murine HGF (*Hgf*, Mm01135184_m1) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1; Taqman Universal PCR Mastermix, Thermo Fisher Scientific, Waltham, MA) in a Mastercycler Realplex2 (Eppendorf, Hamburg, Germany). The results were analyzed by the comparative threshold cycle method and normalized to GAPDH as an internal control.

Flow Cytometry

Corneal single cell suspensions were prepared as previously described [23]. In brief, corneas were digested in Roswell Park Memorial Institute (RPMI)- 1640 media (Lonza, Walkersville, MD) containing 2 mg/ml collagenase type IV (Sigma Aldrich, St. Louis, MO) and 2 mg/ml DNase I (Roche, Basel, Switzerland) for a duration of 45 minutes at a temperature of 37°C. Following this, cells were filtered through a 70 μl cell strainer. Single-cell suspensions were stained with fluorochrome-conjugated monoclonal antibodies. Cell surface staining was conducted using antibodies against CD11c, MHC class II, CD80, CD4, CD45, CD3, and CD11b. To evaluate the intracellular expression of IFN γ in Th1 cells, single cell suspensions derived from lymph nodes were stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma-Aldrich) and ionomycin (1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) for a duration of 4 hours in the presence of Golgistop (0.1 $\mu\text{l}/100 \mu\text{l}$ media; BD Biosciences, San Jose, CA) at 37°C, as described previously [19, 28]. Intracellular staining was performed in order to evaluate the expression of IFN γ in Th1 cells and Foxp3 in CD4⁺ cells (regulatory T cell [Tregs]). Antibodies and isotype controls were sourced from Biolegend (San Diego, CA). Stained cells were analyzed using an LSR II flow cytometer (BD Biosciences) and Summit software (Dako Colorado Inc., Fort Collins, CO).

Enzyme-Linked Immunosorbent Assay

Protein levels of HGF in MSC culture supernatants and corneas were analyzed using commercially available murine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions. Levels of HGF in culture supernatants were normalized according to the number of MSCs (100,000 cells/200 μl) included in each group.

Statistical Analysis

Unpaired two-tailed Student's *t* tests were used to compare means between two groups, and *p* values <.05 were considered statistically significant. Survival curves were constructed using Kaplan-Meier analysis, and the log-rank test was used to compare rates of corneal graft survival. Data are presented as

mean \pm SD of mean of three independent experiments. Samples sizes were estimated on the basis of previous corneal transplantation studies [29, 30].

RESULTS

MSC-Derived HGF Suppresses the Maturation of APCs

For the efficient presentation of antigens to naïve T cells, APCs undergo a process of maturation with increased surface expression of MHCII and CD80 [11, 27]. In view of our previously published data demonstrating that MSCs suppress allosensitization [11], we first sought to clarify whether MSCs suppress the activation and maturation of APCs in the setting of corneal transplantation. To this aim, DCs were cocultured with MSCs, and the acquisition of MHCII and CD80 expression by DCs was evaluated by flow cytometry. Given the limited number of MSCs and DCs at the ocular surface, murine bone marrow was harvested for both the isolation of MSCs and the generation of CD11c⁺ DCs. Detailed characterization of MSCs was performed as described previously [11, 14, 23], with flow cytometric analysis of MSCs revealing positive expression of the mesenchymal cell marker CD29 and negative expression of hematopoietic cell markers CD45 and CD34 (Fig. 1A). Increased expression of HGF by MSCs was observed in response to stimulation with the inflammatory cytokine interleukin-1 β (IL1 β) (*p* = .008; Fig. 1B).

Subsequently, DCs were stimulated with IL1 β , with or without MSCs, for 24 hours. Unstimulated DCs exhibited low expression of MHCII and CD80, but stimulation with IL1 β dramatically increased the expression of MHCII and CD80 by DCs (*p* < .001 for both MHCII and CD80; Fig. 1C, 1D). Notably, this increased expression was significantly abrogated by MSCs (*p* = .003 for MHCII, *p* = .006 for CD80), suggesting that MSCs suppress APC maturation. In view of our result showing increased HGF expression by MSCs in inflammatory conditions (Fig. 1B), we next investigated whether MSC-derived HGF could itself inhibit IL1 β -induced DC maturation. To evaluate this, we neutralized HGF using s-HGFR and showed that neutralization of HGF abrogated the suppressive potential of MSCs (Fig. 1C, 1D). Moreover, this effect was confirmed at two different dosages of s-HGFR: 1 $\mu\text{g}/\text{ml}$ (*p* = .021 for MHCII, *p* = not significant for CD80) and 10 $\mu\text{g}/\text{ml}$ (*p* = .014 for MHCII, *p* = .015 for CD80). Our results clearly demonstrate that MSCs inhibit the maturation of APCs by the secretion of HGF.

MSCs Inhibit Corneal Leukocyte Infiltration and Maturation of APCs, and Promote Allograft Survival in an HGF-Dependent Manner

Next, we investigated whether in vivo administration of MSCs promotes increased levels of HGF at the graft site. Transplant recipients received i.v. injection of MSCs at 3 hours post-transplantation. Corneas were harvested from MSC-treated and untreated hosts on day 7 and day 14 following transplantation. ELISA analysis demonstrated significant, two-fold increases in the level of HGF in corneal lysates sourced from MSC-treated hosts relative to untreated hosts at both day 7 and 14 post-transplantation (Fig. 2A).

To determine whether MSC-derived HGF regulates allo-immune responses at the graft site, HGF expression was silenced using siRNA, which reduced expression of HGF by MSCs by approximately 80% (*p* < .001; Fig. 2B). Corneal

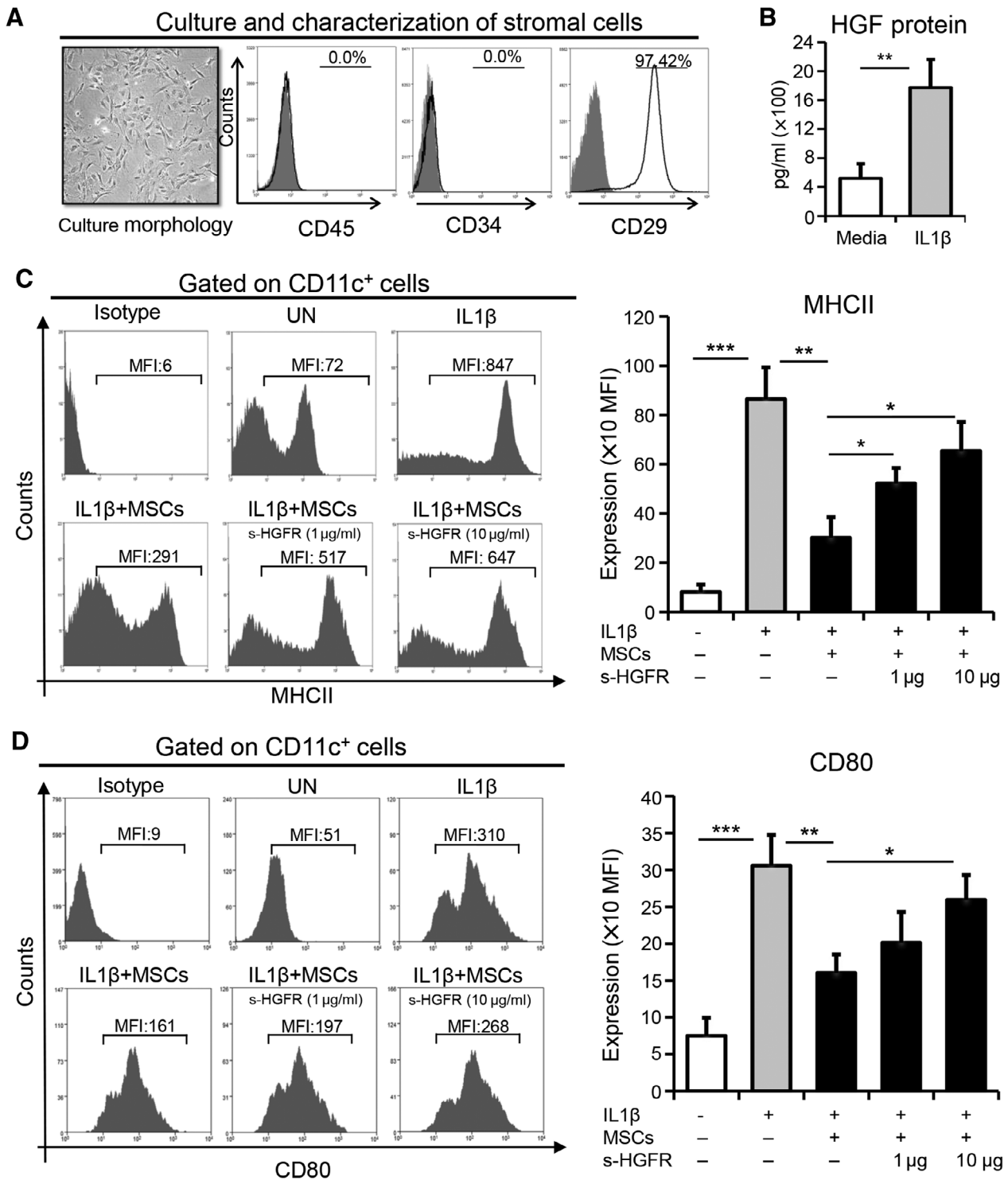


Figure 1. MSC-derived HGF suppresses the maturation of antigen presenting cells. **(A):** MSCs were purified from bone marrow and expanded in vitro using MSC-conditioned culture media via the plastic adherence method. Representative microscopic image exhibiting the morphology of expanded MSCs at passage two (left). Flow cytometry histograms showing negative expression of CD45 and CD34, as well as positive expression of CD29 (right). **(B):** Protein levels of HGF in the supernatants of MSCs cultured in media or stimulated with IL1β for 24 hours using enzyme-linked immunosorbent assay. Representative flow cytometry plots (left) and quantitative bar charts (right) demonstrating the expression of **(C)** MHCII and **(D)** CD80 on CD11c⁺ cells cocultured with MSCs in the presence or absence of IL1β (100 ng/ml) with or without s-HGFR (1 or 10 μg/ml) for 24 hours. Each experiment was repeated three times. Data are represented as mean ± SD. *t* test: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: HGF, hepatocyte growth factor; MSC, mesenchymal stromal cell; UN, untreated; s-HGFR, soluble hepatocyte growth factor receptor; MFI, mean fluorescence intensity.

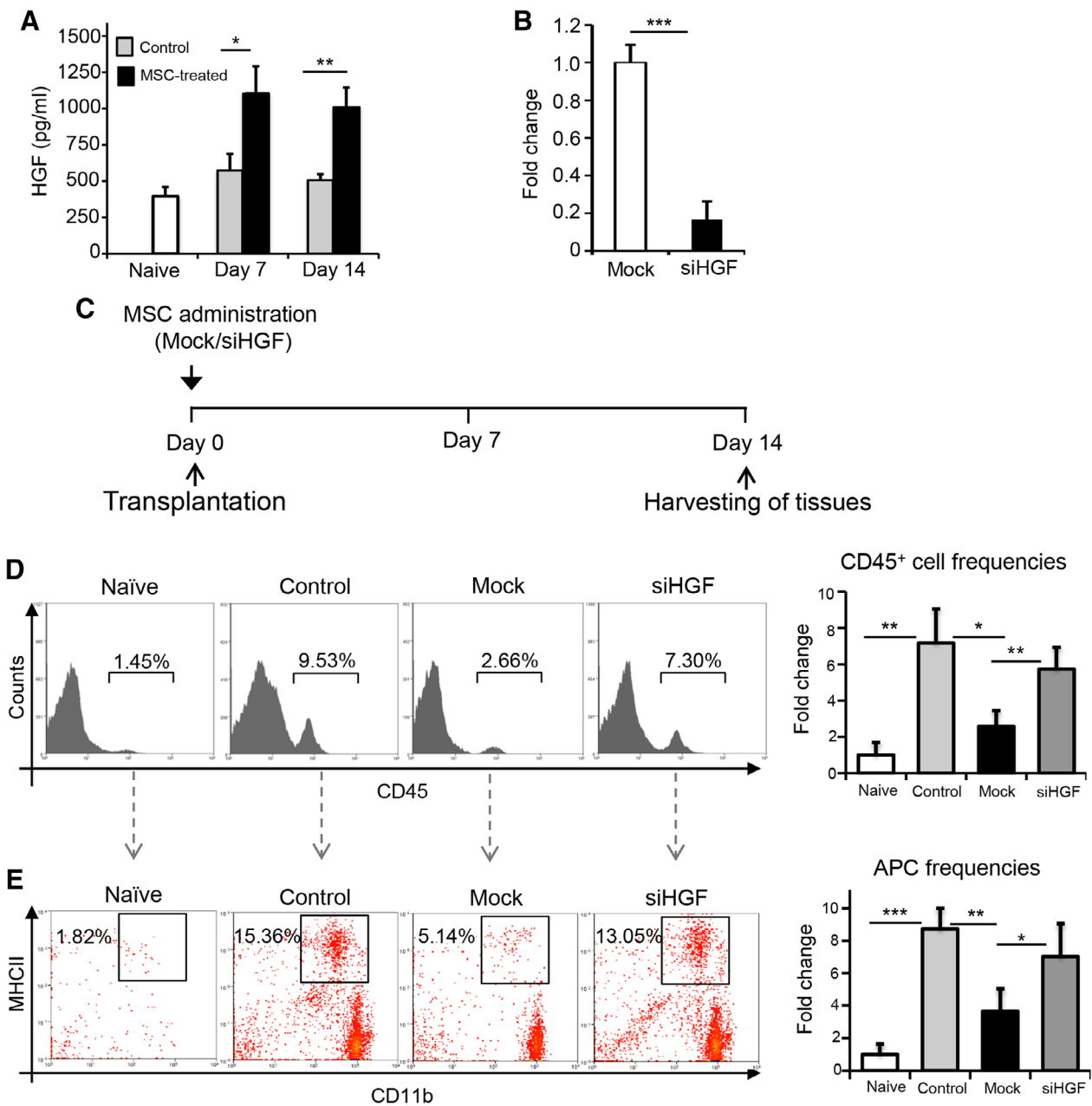


Figure 2. MSCs inhibit corneal infiltration of leukocytes and mature APCs in an HGF-dependent manner. **(A):** Corneas were harvested from untreated and MSC-treated allograft recipients at day 7 and day 14 post-transplantation. Bar chart depicts enzyme-linked immunosorbent assay data of HGF expression in corneal lysates. **(B):** Real-time analysis showing reduced expression of Hgf mRNA at 72 hours after treatment of MSCs with siHGF compared with control small interfering RNA (siRNA; Mock). **(C):** Schematic of experimental design showing time points for MSC administration and tissue harvesting for flow cytometry. **(D):** Representative flow cytometric histograms (left) and cumulative bar chart (right) showing the relative frequencies of CD45⁺ immune cells in the corneas of naïve mice, untreated allograft recipients (Control), allograft recipients injected with control siRNA-treated MSCs (Mock), or Hgf-silenced MSCs at 2 weeks post-transplantation. **(E):** Representative flow cytometric histograms (left) and cumulative bar chart (right) showing the relative frequencies of mature APCs (CD11b⁺MHCII⁺; gated on CD45⁺ cells) in the corneas of naïve mice, untreated allograft recipients (Control), allograft recipients injected with control siRNA-treated MSCs (Mock), or Hgf-silenced MSCs at 2 weeks post-transplantation. Experiment was repeated two times, with four animals per group in each experiment. Data are represented as mean \pm SD. *t* test: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: APC, antigen presenting cell; HGF, hepatocyte growth factor; MSC, mesenchymal stromal cell; siHGF, HGF-specific small interfering RNA.

transplantation was performed, and recipient mice were either not treated with MSCs or received i.v. injection of either Hgf-silenced MSCs or control siRNA-treated MSCs (Mock; Fig. 2C). Corneas were harvested at 14 days post-transplantation, and single-cell suspensions were prepared and flow cytometry was performed. Our data show that administration of control

siRNA-treated MSCs reduced corneal infiltration of CD45⁺ cells by ~65% relative to untreated allograft recipients ($p = .018$), but administering Hgf-silenced MSCs substantially reduced this effect ($p = .007$; Fig. 2D). Consistent with this observation, flow cytometry analysis of corneal single cell suspensions demonstrated significantly reduced frequencies of CD11b⁺MHCII⁺ APCs

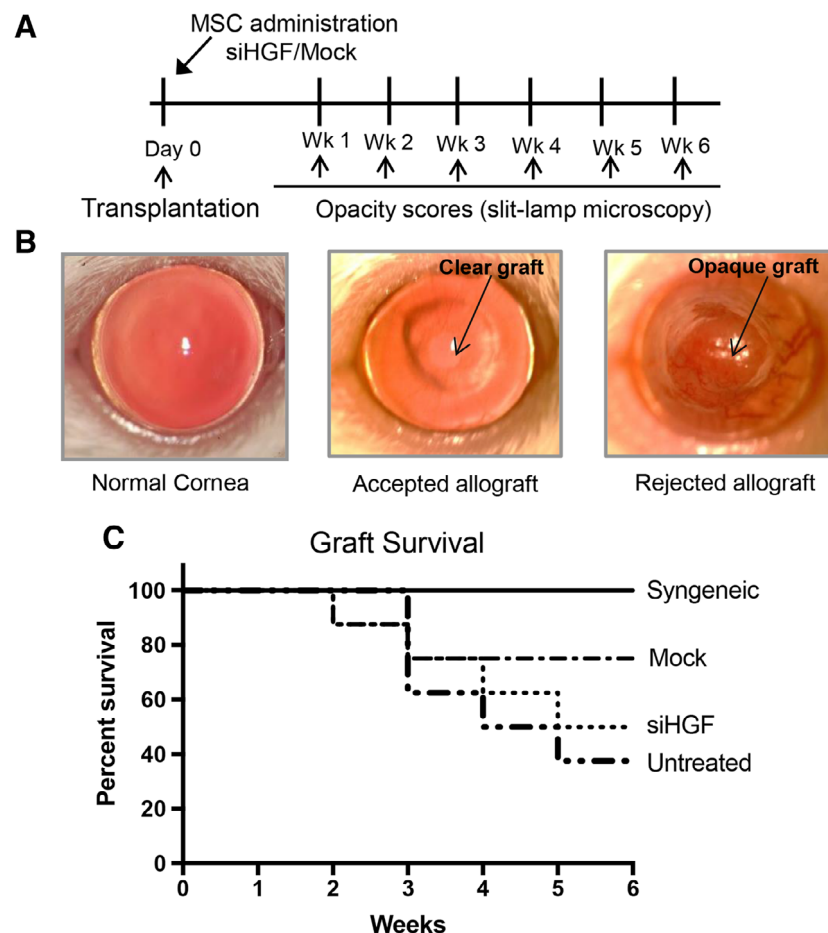


Figure 3. MSCs promote corneal graft survival in an hepatocyte growth factor (HGF)-dependent manner. **(A):** Schematic of experimental design detailing the time points of MSC administration as well as graft opacity evaluation via slit-lamp biomicroscopy. **(B):** Representative slit lamp photographs of a normal murine cornea, an accepted corneal allograft, and a rejected corneal allograft. **(C):** Line diagram showing Kaplan-Meier graft survival analysis of (i) untreated syngeneic graft recipients, (ii) allogeneic graft recipients i.v. injected with control small interfering RNA-treated (Mock) MSCs, (iii) allogeneic graft recipients i.v. injected with Hgf-silenced MSCs, or (iv) untreated allogeneic graft recipients ($n = 8-10$ in each group; log-rank test; $p = .026$). Abbreviations: MSC, mesenchymal stromal cell; siHGF, HGF-specific small interfering RNA.

in hosts that were administered control siRNA-treated MSCs compared with untreated hosts ($p = .009$), yet this effect was abrogated in hosts that received Hgf-silenced MSCs ($p = .031$; Fig. 2E). These data show that HGF expressed by MSCs inhibits the alloimmune response following corneal transplantation.

Next, we sought to determine whether suppression of HGF expression by MSCs reduces their capacity to promote graft survival [11]. Following corneal transplantation, graft survival was compared in four treatment groups: (a) allogeneic graft recipients i.v. injected with control siRNA-treated (Mock) MSCs; (b) allogeneic graft recipients i.v. injected with Hgf-silenced MSCs; (c) untreated allogeneic graft recipients; and (d) untreated syngeneic graft recipients. In order to assess transplant survival, grafts were examined by slit lamp biomicroscopy once weekly for 6 weeks following transplantation (Fig. 3A, 3B). Our data demonstrate a two-fold increase in graft survival in recipients i.v. injected with control siRNA-treated MSCs (78%) compared with untreated controls (Fig. 3C). However, recipients i.v. injected with Hgf-silenced MSCs showed reduced survival (48%) compared with recipients that were administered control siRNA-treated MSCs. These results clearly demonstrate that HGF expressed by MSCs promotes graft survival following corneal transplantation.

Treatment with HGF Alone Suppresses APC Activation and Limits the Generation of Th1 Cells in Allograft Recipients

To investigate whether HGF alone can inhibit the allogeneic immune response, we evaluated the *in vivo* effect of HGF on APC maturation and the generation of IFN γ -producing CD4 $^+$ T cells (Th1 cells) in the DLNs. Five microliters of HGF in PBS (0.1%) or protein control was applied topically to host eyes, twice daily for 2 weeks post-operatively (Fig. 4A). For our flow cytometry analyses, DLNs were harvested at day 14 following transplantation, and single-cell suspensions were prepared. Our data revealed significantly increased frequencies of CD11c $^+$ MHCII $^+$ mature APCs in control-treated transplant recipients compared with naive controls ($p = .015$; Fig. 4B). However, treatment with HGF significantly reduced frequencies of CD11c $^+$ MHCII $^+$ mature APCs relative to the control-treated group ($p = .006$). A six-fold increase in CD4 $^+$ IFN γ^+ Th1 cell frequencies was observed in the DLNs of vehicle-treated recipients after corneal transplantation, relative to the naive group ($p = .002$; Fig. 4C). However, this increase was substantially reduced following HGF treatment. Specifically, HGF-treated mice showed a significant 41% decrease in the generation of IFN γ -secreting Th1 cells compared with control-treated controls ($p = .033$; Fig. 4C). As CD4 $^+$ Foxp3 $^+$

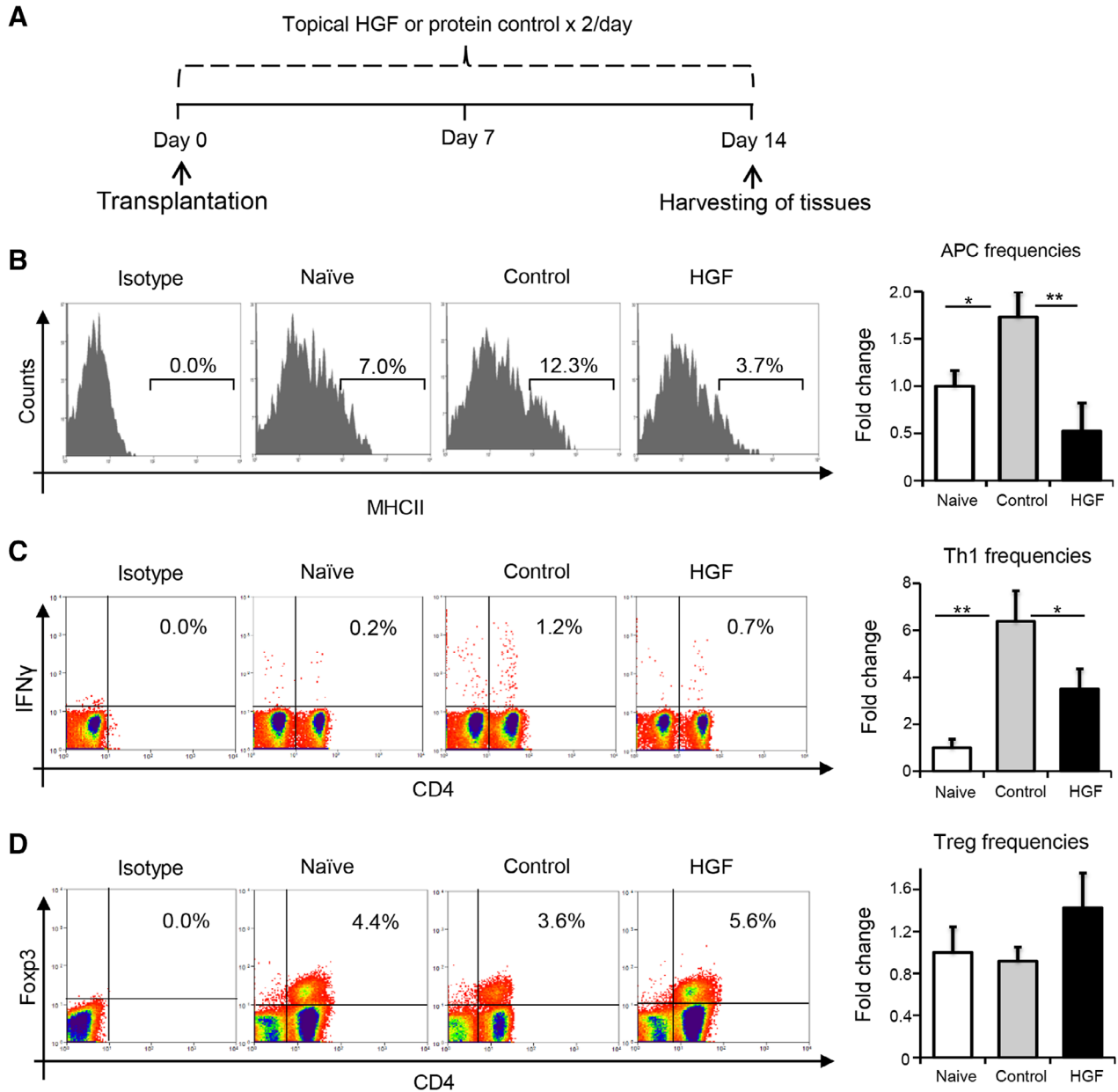


Figure 4. HGF treatment suppresses induction of alloimmunity in transplant recipients. **(A):** Schematic of experiment detailing the time points of topical administration of HGF (0.1%) or protein control twice daily for 2 weeks post-transplantation. Following week 2, ipsilateral draining lymph nodes were harvested to prepare single cells suspension for flow cytometry analysis. **(B):** Representative flow cytometric histograms (left) and cumulative bar chart (right) showing the relative frequencies of CD11c⁺MHCII⁺ mature APCs in the draining lymph nodes of naïve, control-treated, and HGF-treated groups at 2 weeks post-transplantation. **(C):** Representative flow cytometric dot plots (left) and quantification chart (right) of frequencies of IFN γ ⁺CD4⁺ Th1 cells in the draining lymph nodes of naïve, protein control, and HGF-treated groups at 2 weeks post-transplantation. **(D):** Representative flow cytometric analysis (left) and cumulative bar chart (right) showing the relative frequencies of CD4⁺Foxp3⁺ Tregs in the draining lymph nodes of naïve, control-treated, and HGF-treated groups at 2 weeks post-transplantation. Representative data from four independent experiments are shown, and each experiment consisted of four to six animals. Data are represented as mean \pm SD (error bar). *t* test: *, $p < .05$; **, $p < .01$. Abbreviations: APC, antigen presenting cell; HGF, hepatocyte growth factor.

Treg have been shown to inhibit the development of the Th1 immune response, we investigated whether HGF treatment affects Treg frequencies following transplantation and observed a moderate increase in the frequencies of CD4⁺Foxp3⁺ Tregs in draining lymphoid tissues (Fig. 4D). Collectively, our results demonstrate that HGF suppresses the alloimmune response primarily by inhibiting APC maturation and subsequent Th1 generation.

Topical Application of HGF Inhibits Immune Cell Infiltration into Corneal Grafts

Having observed HGF-mediated inhibition of APC maturation and Th1 cell generation in the DLNs, we evaluated the alloimmune effector response at the graft site. Following transplantation, hosts were administered topical HGF or control eye drops as described previously (Fig. 4A). Corneas were harvested at day

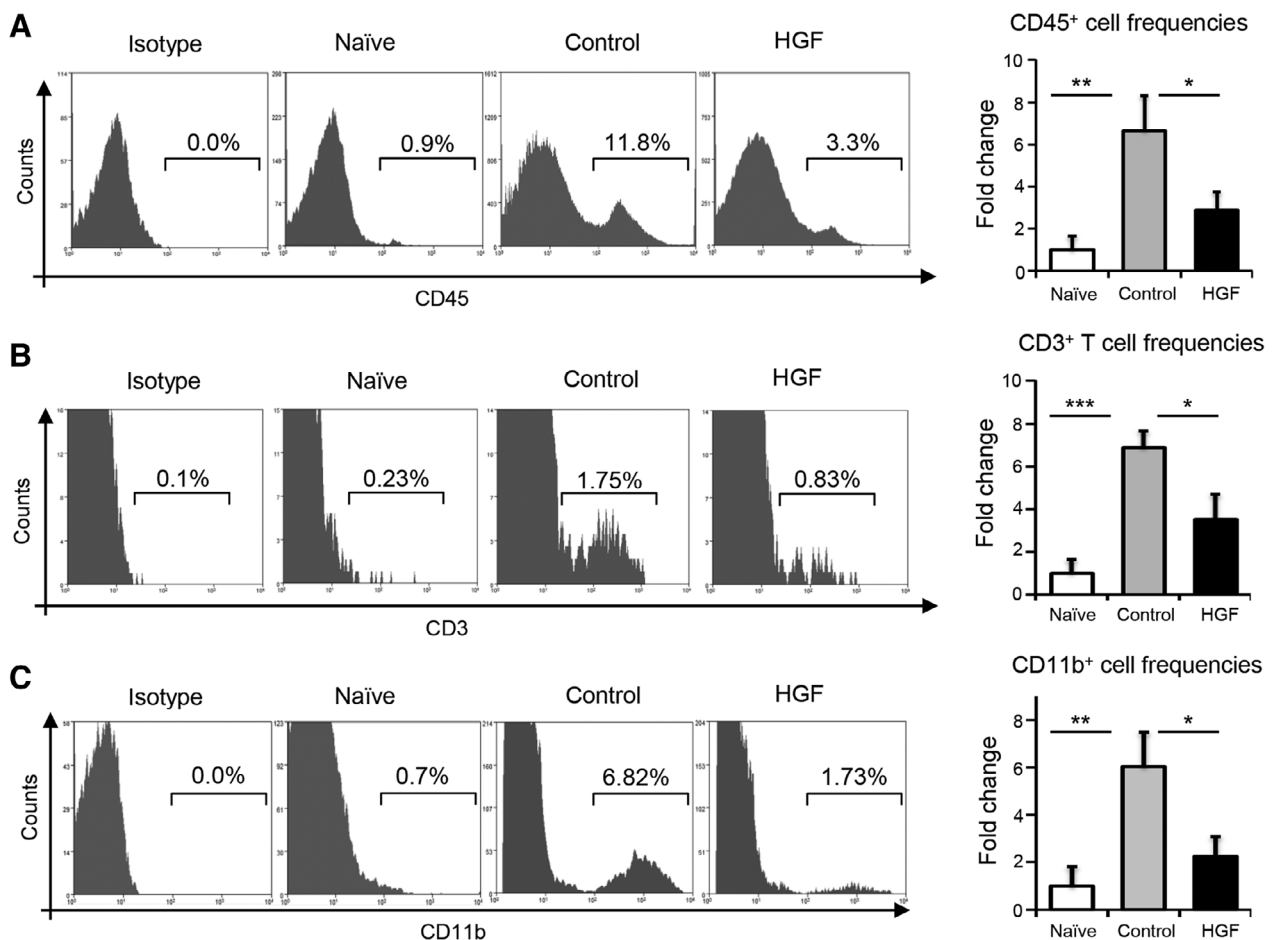


Figure 5. Topical application of HGF inhibits immune cell infiltration into corneal grafts. At 2 weeks post-transplantation, corneal grafts were harvested and digested to prepare single cell suspensions. **(A):** Representative flow cytometry histograms and quantitative bar charts showing the infiltration of CD45⁺ inflammatory cells into corneal grafts of control- and HGF-treated groups, relative to naïve controls. **(B):** Histogram plot (left) and bar graphs (right) demonstrating the frequencies of CD3⁺ T cells in corneas sourced from naïve mice and transplant recipients treated with either protein control or HGF. **(C):** Representative plots (left) and frequency data (right) of CD11b⁺ macrophages in corneal grafts of indicated groups at 2 weeks post-transplantation. Four independent experiments were performed, with each experiment consisting of four to six animals. Data are represented as mean \pm SD (error bar). *t* test: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: HGF, hepatocyte growth factor.

14 following transplantation, and the infiltration of CD45⁺ inflammatory cells, CD3⁺ T cells, and CD11b⁺ macrophages was evaluated using flow cytometry. Our data demonstrate an eight-fold increase in the infiltration of CD45⁺ cells into the cornea in the control-treated allogeneic group compared with the naïve group ($p = .005$; Fig. 5A). Notably, frequencies of CD45⁺ inflammatory cells were significantly reduced in the HGF-treated group relative to the control-treated group (52%; $p = .026$). Relative to control-treated graft recipients, HGF-treated hosts exhibited decreased corneal infiltration of CD3⁺ T cells by 48% ($p = .016$; Fig. 5B) and CD11b⁺ cells by 73% ($p = .018$; Fig. 5C). Taken together, these results demonstrate that HGF inhibits the alloimmune effector response at the graft site.

HGF Alone Is Sufficient to Promote Graft Survival

Finally, taking account of the observed *in vivo* effect of HGF on the suppression of alloimmune responses, we evaluated whether topical HGF administration promotes corneal allograft survival. To determine this, once weekly slit-lamp biomicroscopy was performed to evaluate graft survival in four groups: (a) allogeneic grafts treated with HGF; (b) allogeneic grafts treated with protein

control; (c) untreated allogeneic grafts; and finally (d) untreated syngeneic grafts. Treatment was administered for 2 weeks postoperatively (Fig. 6A). Control-treated and untreated mice exhibited approximately 40% graft survival at 6 weeks (Fig. 6B). However, HGF treatment increased graft survival, as indicated by 67% survival at week 6. Syngeneic controls exhibited 100% survival at week 6. Thus, in addition to inhibiting the alloimmune response, our data indicate that topical HGF increases graft survival following corneal transplantation.

DISCUSSION

Current research suggests that there is considerable plasticity in the immunosuppressive capacity of MSCs and that the immunoregulatory factors produced by MSCs are markedly influenced by the local inflammatory microenvironment [9]. The current study deepens our understanding of the immunoregulatory mechanisms of MSCs in the setting of corneal allotransplantation. Specifically, our data demonstrate that: (a) HGF expression by MSCs is upregulated in an inflammatory milieu; (b) the secretion of HGF by MSCs suppresses corneal leukocyte infiltration and APC maturation and prolongs

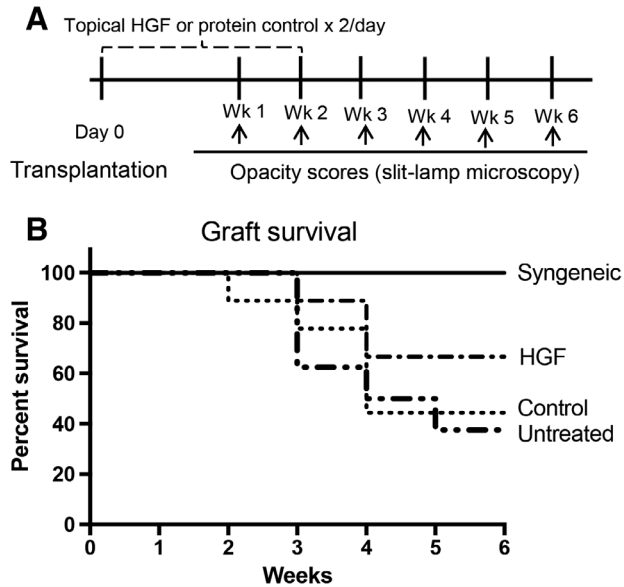


Figure 6. HGF alone is sufficient to prolong graft survival. **(A):** Schematic of experimental design. After transplantation, grafts were treated with HGF eye drops (0.1%) or control eye drops twice daily for 2 weeks. Slit-lamp microscopy was used to evaluate the corneal opacity of naïve, control-treated, and HGF-treated mice. **(B):** Kaplan-Meier analysis showing graft survival of HGF-treated allogeneic graft recipients, control-treated allogeneic graft recipients, untreated allogeneic graft recipients, and untreated syngeneic graft recipients ($n = 8-10$ in each group; log-rank test; $p = .022$). Abbreviation: HGF, hepatocyte growth factor.

corneal graft survival; (c) topical HGF treatment limits APC maturation, Th1 generation, and inflammatory cell infiltration of the graft site; and finally (d) topical HGF alone (in the absence of MSCs) promotes corneal allograft survival.

The immunosuppressive function of MSCs is enhanced in the presence of inflammatory cytokines [31]. Our data indicate that stimulation of MSCs with IL1 β substantially increases expression of HGF, corroborating our results from a previous study [14]. Prior to initiating the alloimmune response, APCs undergo a maturation process with the acquisition of MHCII and other costimulatory molecules including CD80 [5, 32]. The capacity of MSCs to inhibit APC maturation has previously been established [33, 34]. Indeed, Oh and colleagues have reported that peritransplant i.v. infusion of MSCs prevents the rejection of allogeneic corneal transplants and observed reduced APC activation [35]. Our data, obtained using a soluble HGF receptor acting as an HGF “sink,” indicate that the suppression of APC maturation by MSCs is mediated primarily via secretion of HGF. Moreover, our findings demonstrate that the promotion of corneal graft survival by MSCs is dependent on their secretion of HGF. Hosts that received i.v. injection of MSCs transfected with control siRNA exhibited 75% graft survival at 6 weeks compared with 37.5% in the untreated group. However, this survival benefit with MSC therapy was diminished when MSCs transfected with *Hgf* siRNA were administered, with a survival rate of only 50%. These data elucidate an immunoregulatory mechanism underlying our previous observations of suppressed allosensitization and improved corneal allograft survival in MSC-treated mice [11]. Specifically, our previous study established that GFP⁺ MSCs i.v. injected at 3 hours post-transplantation home to the inflamed ocular surface, resulting in decreased APC maturation and reduced frequencies of IFN γ -secreting Th1 cells [11]. Of note,

we have previously shown that systemically administered GFP⁺ MSCs specifically home to inflamed corneal tissue, where they exhibit long-term survival (i.e., more than 50 days) [25].

HGF has been shown to be protective against a variety of inflammatory and autoimmune diseases [13]. In a murine model of inflammatory bowel disease, Oh and colleagues transfected mice intramuscularly with human HGF cDNA and demonstrated that HGF treatment suppressed inflammation [36]. The authors reported decreased intestinal mRNA expression of Th1 cytokines (including IL1 β , IFN γ , and tumor necrosis factor- α [TNF- α]), as well as decreased CD4⁺ T cells and decreased neutrophils in the intestinal epithelium [36]. In a rat model of experimental autoimmune myocarditis, Futamatsu and colleagues injected human HGF gene with hemagglutinating virus of the Japan-envelope vector directly into the myocardium and demonstrated reduced antigen-specific T-cell proliferation and production of IFN γ by CD4⁺ T cells isolated from mice treated with HGF [37]. The immunomodulatory properties of HGF have also been reported in murine studies of allergic airway disease [38] and collagen-induced arthritis [39].

In the field of transplantation, HGF has been reported to promote the survival of heterotopic cardiac allografts in a murine model [40]. Yamaura and colleagues demonstrated that treatment with HGF decreased cellular infiltration of the cardiac graft, reduced myocardial necrosis, and suppressed IFN γ expression [40]. The authors attribute the enhanced allograft survival to the immunomodulatory potency of HGF. Decreased IFN γ and TNF- α expression has been reported in the liver and intestinal mucosa of mice transfected with HGF cDNA following bone marrow transplantation in a model of acute graft-versus-host disease (GVHD), with decreased donor T-cell infiltration observed in the host liver [41]. In a model of chronic GVHD, HGF gene transfer has been shown to suppress MHC class II expression by host B cells and downregulate DC immunogenicity, resulting in the amelioration of murine lupus, cholangitis, and sialoadenitis [42]. Although these studies have indicated the translational potential of HGF in transplantation, further research is required to formally establish the precise immunoregulatory mechanisms of HGF in this setting.

In our study, we demonstrate that HGF inhibits APC maturation both in vitro and in vivo. We show that topical application of HGF suppresses Th1 generation in the DLNs. Given that the fate of an allograft is determined by the interactions between proinflammatory and immunoregulatory arms of the immune response [5], it is interesting to note that our data also indicate a moderate (but not significant) increase in the frequencies of Tregs following topical application of HGF.

CONCLUSION

The tissue regenerative and immunomodulatory activities of MSCs have been established [43–45], yet as a cell-based therapy, their therapeutic potential is constrained by the technical challenge of consistently producing clinical-grade cells, as well as the expense (both financial and temporal) of meeting high regulatory standards. MSCs are known to exert their immunoregulatory activity via a diverse array of mechanisms, with the majority of these involving the secretion of soluble factors [46]. In this study, we demonstrate the immunomodulatory function of MSC-derived HGF, showing that the promotion of corneal graft survival by MSCs is partly dependent on their expression of HGF. Furthermore, we establish that topical HGF suppresses APC maturation

and the generation of a Th1 immune response in the lymphoid tissue, limits immune cell infiltration into corneal grafts, and is itself sufficient to promote graft survival. In doing so, our work suggests the translational potential of topical HGF in the setting of corneal transplantation, offering an alternative to cell-based therapeutic approaches.

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AUTHOR CONTRIBUTIONS

S.K.M.: conception/design, collection of data, data analysis and interpretation, manuscript writing; W.F.: data analysis and interpretation, manuscript writing; S.S., E.E., M.O.: collection of data, data analysis and interpretation, revising the manuscript; S.K.C.: conception/design, data analysis and interpretation, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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