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Original Article

Interleukin-6 neutralization prolongs corneal allograft survival

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ABSTRACT

The purpose of this study is to determine the effect of systemic blockade of Interleukin-6 (IL-6) on allosensitization, regulatory T cell frequencies and suppressive phenotype, and allograft survival rates in a mouse model of corneal transplantation. Allogeneic corneal transplantation was performed using C57BL/6 mice as donors and BALB/c mice as recipients. Graft recipients were injected daily with either anti-IL-6 antibody or an isotype control antibody (1.25 mg/ml) for the first 7 days and on alternate days thereafter until week 8 after transplantation. Allograft survival was evaluated for 8 weeks using Kaplan-Meier survival analysis. Draining lymph nodes (DLN) were harvested at week 3 after transplantation, and proliferation of isolated recipient T cells through direct and indirect pathways was determined using mixed lymphocyte reaction assay. Frequencies of $CD4^+CD25^+Foxp3^+$ regulatory T cells, their expression of Foxp3, and frequencies of $IFNy^+CD4^+$ Th1 cells were determined in DLN using flow cytometry. Finally, $CD4^+$ T cells were cultured with bone marrow-derived dendritic cells from either C57BL/6 or BALB/c mice in the presence of IL-6-blocking antibody to determine Treg induction through direct and indirect pathways, respectively. Treatment with anti-IL-6 antibody suppressed both the direct and indirect pathways of allosensitization in graft recipients and significantly improved allograft survival rates. Furthermore, *in vivo* blockade of IL-6 enhanced Foxp3 expression by Tregs in graft recipients undergoing rejection, but did not exert a significant effect on Treg frequencies. Finally, IL-6 neutralization *in vitro* enhanced the differentiation of Tregs from $CD4^+$ T cells through both direct and indirect pathways. Our results demonstrate that systemic administration of IL-6-blocking antibody to corneal allograft recipients suppresses direct and indirect routes of allosensitization, is associated with increased expression of Foxp3 by Tregs, and improves allograft survival rates.

KEYWORDS: Interleukin-6, corneal transplantation, allograft survival, regulatory T cells, allosensitization.

INTRODUCTION

Corneal transplantation is the most successful and prevalent form of solid organ transplantation, with approximately 40,000 procedures performed annually in the United States [1]. The 2-year survival rate of low-risk allografts placed in first-time avascular and non-inflamed hosts is approximately 90% [2]. However, immune-mediated rejection remains the most common cause of corneal allograft failure, and survival rates significantly decline to below 50% in grafts placed in inflamed or vascularized host beds despite maximal topical and systemic immunosuppressive therapy [3, 4].

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$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) account for up to 10% of peripheral $CD4^+$ T cells and have a critical role in maintaining allograft tolerance [5]. Previous work by our group has demonstrated a correlation between expression of Foxp3 by Tregs and their potential to suppress alloimmunity in corneal graft recipients [6]. Given the established impact of Treg functionality on corneal transplant outcomes, many studies have focused on strategies to induce tolerance by improving Treg function, thus potentially eliminating the need for immunosuppressive therapies in graft recipients.

The pro-inflammatory cytokine, Interleukin-6 (IL-6), possesses a broad range of functions and is involved in neutrophil recruitment, antigen-presenting cell maturation, and B cell differentiation [7]. Furthermore, IL-6 has been implicated as an important regulator of effector T cell/Treg balance. In the presence of TGF-β, IL-6 inhibits Treg differentiation, favoring Th17 development [8]. Blockade of IL-6 signaling is emerging as a promising strategy for the treatment of autoimmune conditions [9, 10]. In organ transplantation, the effect of IL-6 neutralization on transplant survival has mostly been studied in cardiac transplantation [11, 12], and no studies to date have been undertaken to study the efficacy of anti-IL-6 therapy in corneal transplantation. This is despite the fact that increased levels of IL-6 in the aqueous humor have been reported after transplantation and have been linked with the innate immune response in earlier stages of corneal allograft rejection [13, 14].

Herein, we investigated the effect of systemic IL-6 blockade on corneal transplantation outcomes. Using a well-established mouse model of corneal transplantation, we assessed the effect of systemic IL-6 blockade on allosensitization, Treg induction and corneal allograft survival. Our results demonstrate that IL-6 neutralization suppresses both direct and indirect pathways of allosensitization in graft recipients and significantly improves allograft survival rates. Furthermore, we show that *in vivo* blockade of IL-6 enhances Foxp3 expression in Tregs of graft recipients undergoing rejection, and induces the differentiation of Tregs from $CD4^+$ T cells *in vitro*.

MATERIALS AND METHODS

Animals

Six to eight-week old male BALB/c and C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Mice were housed in the animal facility at the Schepens Eye Research Institute in a pathogen-free environment. All animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Institutional Animal Care and Use Committee (IACUC) approval was obtained for all relevant experimental procedures. Ketamine (120 mg/kg) and Xylazine (20 mg/kg) were injected *via* the intraperitoneal route for the induction of anesthesia.

Allogeneic corneal transplantation

Mouse corneal transplants were performed as previously outlined [15]. In brief, 2-mm donor corneal buttons were harvested from C57BL/6 mice using vannas scissors, and were transplanted orthotopically onto a 1.5 mm recipient bed of age- and gender-matched BALB/c mice. Eight interrupted 11-0 nylon sutures were used to secure the donor graft to the host bed and sutures were removed 7 days after the procedure. Corneal allografts were examined twice weekly using a slit lamp biomicroscope for 8 weeks, and corneal graft opacity was graded using a standardized opacity grading system (range, 0–5+), as described previously [16]. Corneas with an opacity score of 2+ for two consecutive examinations were considered rejected.

Systemic administration of anti-IL-6 antibody

Rat anti-IL-6 antibody or rat IgG1 isotype antibody (Bioxcell, New Hampshire, USA) was diluted in phosphate-buffered saline (PBS) to yield a concentration of 1.25 mg/ml. 20 uL of anti-IL-6 or isotype control antibody was intraperitoneally injected to mice on the day of transplantation (day 0) and daily thereafter for 7 days. Injections were subsequently administered on alternate days until week 8 after transplantation.

Flow cytometry

A single-cell suspension was prepared from isolated draining submandibular and cervical LNs

of graft recipients. To quantify IFNγ-secreting $CD4^+$ cells (Th1) single-cell suspension was prepared from harvested lymph nodes. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich Corp.) in the presence of GolgiStop (BD Biosciences) for 6 hours, and subsequently stained with an anti-CD4 fluorescein isothiocyanate (FITC) antibody (Biolegend). After fixation and permeabilization (buffers from eBioscience, San Diego, CA, USA), cells were stained with an anti-IL-17A PE antibody (eBioscience) or anti-IFNγ APC antibody. For quantification of $CD4^+CD25^+$ T regulatory cell (Treg) frequencies, cells were stained with anti-CD4 FITC (eBioscience), anti-CD25 (Biolegend) and anti-Foxp3 PECy5 (eBioscience) antibodies. Similar to the intracellular cytokine staining procedure described above, surface staining with anti-CD4 and anti-CD25 was performed prior to the addition of fixation and permeabilization buffers (eBioscience, San Diego, CA, USA) and anti-Foxp3 antibody. Appropriate isotype-matched control antibodies were used in all experiments. Stained cells were analyzed using a Beckman Coulter flow cytometer (Beckman Coulter, Inc., Pasadena, CA, USA) and Summit v4.3 software (DAKO Colorado Inc., Fort Collins, CO).

Mixed lymphocyte reaction (MLR)

The draining ipsilateral cervical and submandibular lymph nodes were harvested from recipient mice 3 weeks post penetrating keratoplasty. CD90.2-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) were used to positively sort T cells. For the direct MLR, antigen-presenting cells (APCs) were obtained from the spleens of naive C57BL/6 mice. Briefly, splenocytes were incubated in RBC lysis buffer, washed, and re-suspended, and APCs were negatively sorted using anti-CD90.2 magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). APCs were then washed and re-suspended at 1×10^5 cells/ml in 10% Fetal Bovine Serum (FBS)-supplemented Roswell Park Memorial Institute medium (RPMI) 1640 (BioWhittaker, Walkersville, MD, USA) and co-cultured with purified T cells from BALB/c recipients at a 1:1 ratio in round-bottom 96-well plates in a reaction volume of 200 ul. For the indirect MLR, T cells from BALB/c recipients were isolated and co-cultured with BALB/c APCs and C57BL/6 antigens. The C57BL/6 antigen mixture was prepared from a 2 x 10^7 /ml suspension of CD 90.2-depleted splenocytes. The cell suspension was sonicated on ice and exposed to multiple freeze-thaw cycles using liquid nitrogen. The cells were incubated in triplicates at 37 °C and 5.0% CO_2 for 5 days. Twelve hours before termination of the cultures, BrdU reagent (Millipore, USA) was added and absorbance quantified according to the manufacturer's instructions. The combination of naïve T cells and syngeneic APCs was used as the control for both the direct and indirect MLRs. Proliferation was determined by assessing absorbance relative to the control reactions.

***In vitro* Treg induction**

Bone marrow-derived dendritic cells (BMDCs) were generated as previously described [15]. Briefly, bone marrow was harvested from the tibia and femur of male C57BL/6 mice. Cells were lysed using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), plated at a concentration of 0.2 x 10^6 /ml in cell culture petri dishes (BD Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and cultured in the presence of 20 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (BioLegend, San Diego, CA, USA) for 7 days. For the direct pathway of Treg induction, TNF-α (10 ng/ml) was added as a maturation stimulus 24 hours prior to the termination of cultures. Naïve effector T cells ($CD4^+CD25^-$ cells) were isolated from the lymph nodes and spleens of naïve BALB/c using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). Mature C57BL/6 dendritic cells (1 x 10^5) were co-cultured with naïve effector T cells (5.0 x 10^4) in the presence of TGF-β (5 ng/ml) and either anti-IL-6 or isotype control antibody (10 μg/ml) for 5 days. For the indirect pathway of Treg induction, BALB/c-derived BMDCs were generated, as described above, and a C57BL/6 alloantigen mixture was added 48 hours prior to the termination of cultures. The alloantigen stimulus was prepared in a similar fashion to the antigen mixture used in the indirect MLR. Mature BALB/c dendritic cells (1 x 10^5) were subsequently co-cultured with naïve syngeneic effector T cells (5.0 x 10^4) in the presence of TGF-β (5 ng/ml) and either anti-IL-6 or isotype control antibody (10 μg/ml) for 5 days.

Statistical analysis

Student's t test was used to compare means between two groups. Kaplan-Meier survival curves were used to compare corneal allograft survival between the two treatment groups, and log-rank test was used to assess the statistical difference. Data are presented as mean ± standard error of mean and considered statistically significant at $p < 0.05$.

RESULTS

IL-6 neutralization prolongs corneal allograft survival

First, we evaluated the effect of systemically administered anti-IL-6 antibody on corneal allograft survival. Graft recipients were injected daily with either anti-IL-6 antibody or an isotype control antibody (1.25 mg/ml) for the first 7 days and on alternate days thereafter until week 8 after transplantation. Corneal allografts were examined by slit lamp biomicroscopy and scored biweekly until week 8 post transplantation. Graft recipients treated with anti-IL-6 antibody demonstrated significantly lower graft opacity scores compared to control-treated mice (Figure 1A; $p < 0.0001$). While the survival rate in the control group was approximately 30% by week 8 after transplantation, 70% of grafts in the anti-IL-6-treated group survived (Figure 1B; $p < 0.05$).

IL-6 blockade suppresses direct and indirect allosensitization

To examine the effect of IL-6 blockade on allosensitization, direct and indirect mixed lymphocyte reactions (MLR) were performed using T cells isolated from the draining cervical and submandibular lymph nodes of transplanted mice at week 3 as responder cells. T cell proliferation was quantified using BrdU incorporation assay and spectrometric analysis. T cells derived from anti-IL-6-treated allograft acceptors and rejectors demonstrated decreased proliferation compared to T cells harvested from isotype-treated mice (Figure 2A). However, this difference was only statistically significant in graft rejectors (direct MLR, $p < 0.02$; indirect MLR, $p < 0.05$). To further investigate the effect of IL-6 neutralization on allosensitization, frequencies of IFNγ-secreting $CD4^+$ T cells (Th1 cells), the principal effector cells involved in allograft rejection, in the draining lymph nodes of

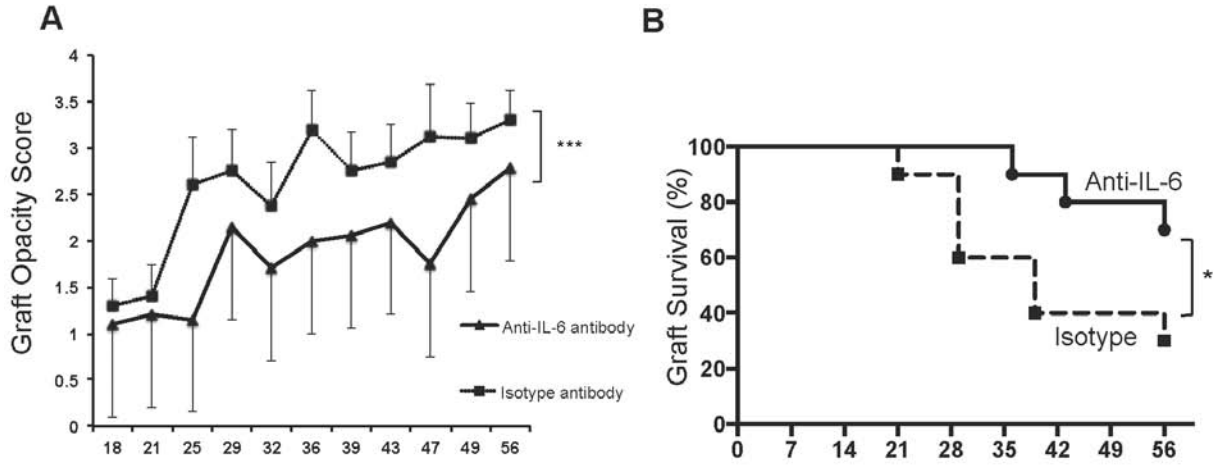

Figure 1. Systemic blockade of IL-6 prolongs corneal allograft survival. Corneal transplantation was performed (N = 10/group) and graft recipients were injected daily with either anti-IL-6 antibody (1.25 mg/ml) or an isotype control antibody for the first 7 days and on alternate days thereafter until week 8 after transplantation. Grafts were examined by slit lamp biomicroscopy and scored biweekly until week 8 post-transplantation. A. Mean graft opacity scores were significantly lower in anti-IL-6-treated graft recipients compared to controls (***$p < 0.0001$). Error bars represent mean +/- SEM of three experiments. B. Kaplan–Meier survival graph demonstrating significantly higher graft survival in anti-IL-6-treated mice compared to mice treated with isotype control antibody (70% vs. 30%, *$p < 0.05$; Log-rank test).

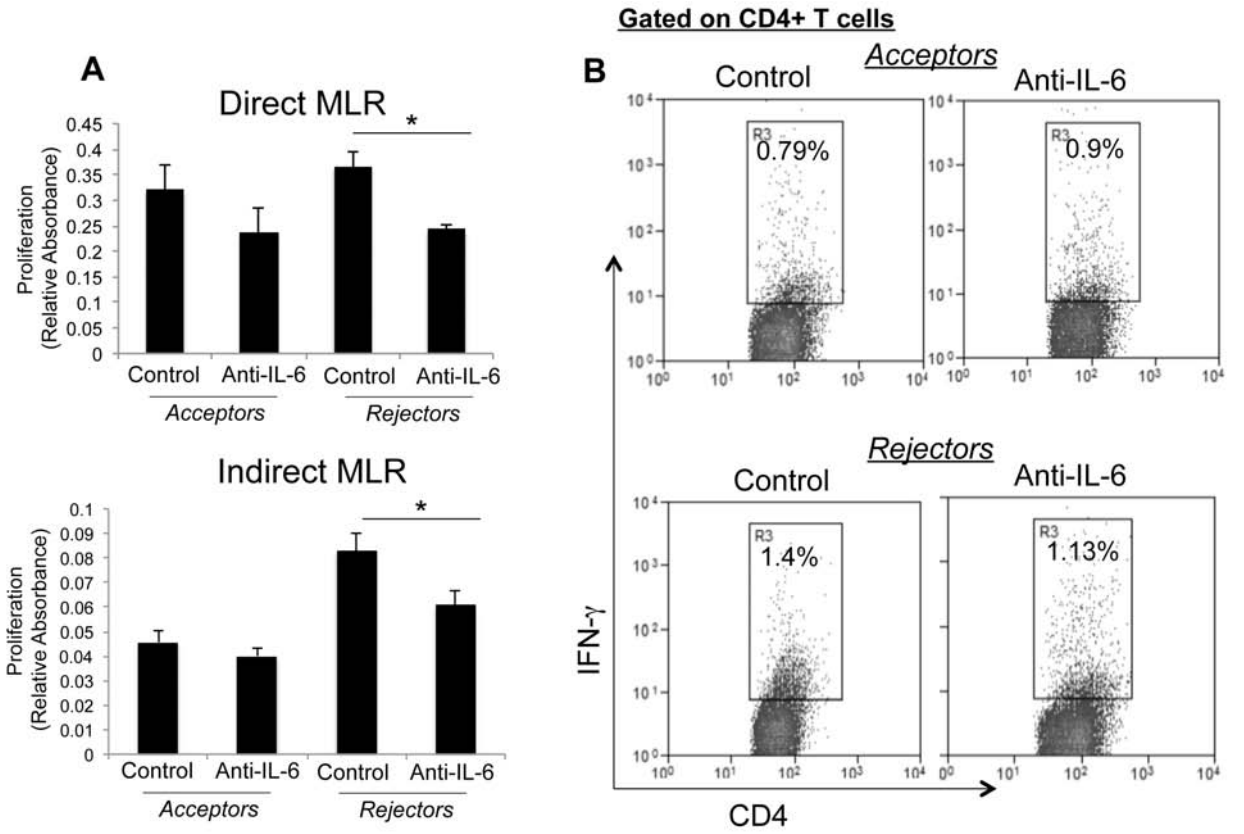

Figure 2. Reduced allosensitization in anti-IL-6-treated graft recipients. A. Responder T cells were isolated from ipsilateral draining lymph nodes of allograft acceptor and rejector BALB/c mice treated with anti-IL-6 antibody or control 3 weeks after transplantation. T cells were subsequently cultured with APCs derived from either naïve C57BL/6 or BALB/c mice to perform direct and indirect mixed lymphocyte reaction (MLR), respectively. T cell proliferation was quantified using BrdU incorporation assay. T cells derived from anti-IL-6-treated acceptors demonstrated significantly lower proliferation compared to T cells harvested from isotype-treated mice (direct MLR: $*p < 0.02$; indirect MLR: $*p < 0.05$). B. Frequencies of $IFN\gamma^+CD4^+$ Th1 cells in DLN of acceptor and rejector mice treated with anti-IL-6 antibody or control were determined using flow cytometry. Representative flow cytometric plots demonstrate similar Th1 cell frequencies in anti-IL-6-treated allograft rejectors and isotype-treated rejector mice. P values were calculated using the Student's t-test and error bars represent mean +/- SEM of three experiments. $*p < 0.05$.

anti-IL-6 and isotype-treated recipients were quantified using flow cytometry. A minimal reduction in Th1 frequencies was noted in the anti-IL-6-treated graft rejectors compared to isotype-treated rejectors (Figure 2B).

IL-6 blockade enhances Treg suppressive phenotype

Foxp3-expressing $CD4^+CD25^+$ T regulatory cells (Tregs) have been demonstrated to play a critical role in inducing tolerance to corneal allografts [5]. Given the demonstrated effect of IL-6 blockade on decreasing the ratio of effector T cells to Tregs [8, 17] and our findings of reduced allosensitization in anti-IL-6-treated graft recipients, we next investigated the effect of IL-6 neutralization on Treg frequencies and their expression of Foxp3 at week 3 after transplantation. Although no difference was detected in Treg frequencies in the ipsilateral draining lymph nodes (DLN) of anti-IL-6-treated acceptors and rejectors compared to isotype-treated controls, significantly higher expression of Foxp3 was seen in Tregs derived from the ipsilateral DLN of graft rejectors treated with anti-IL-6 compared to control-treated rejectors (Figure 3A).

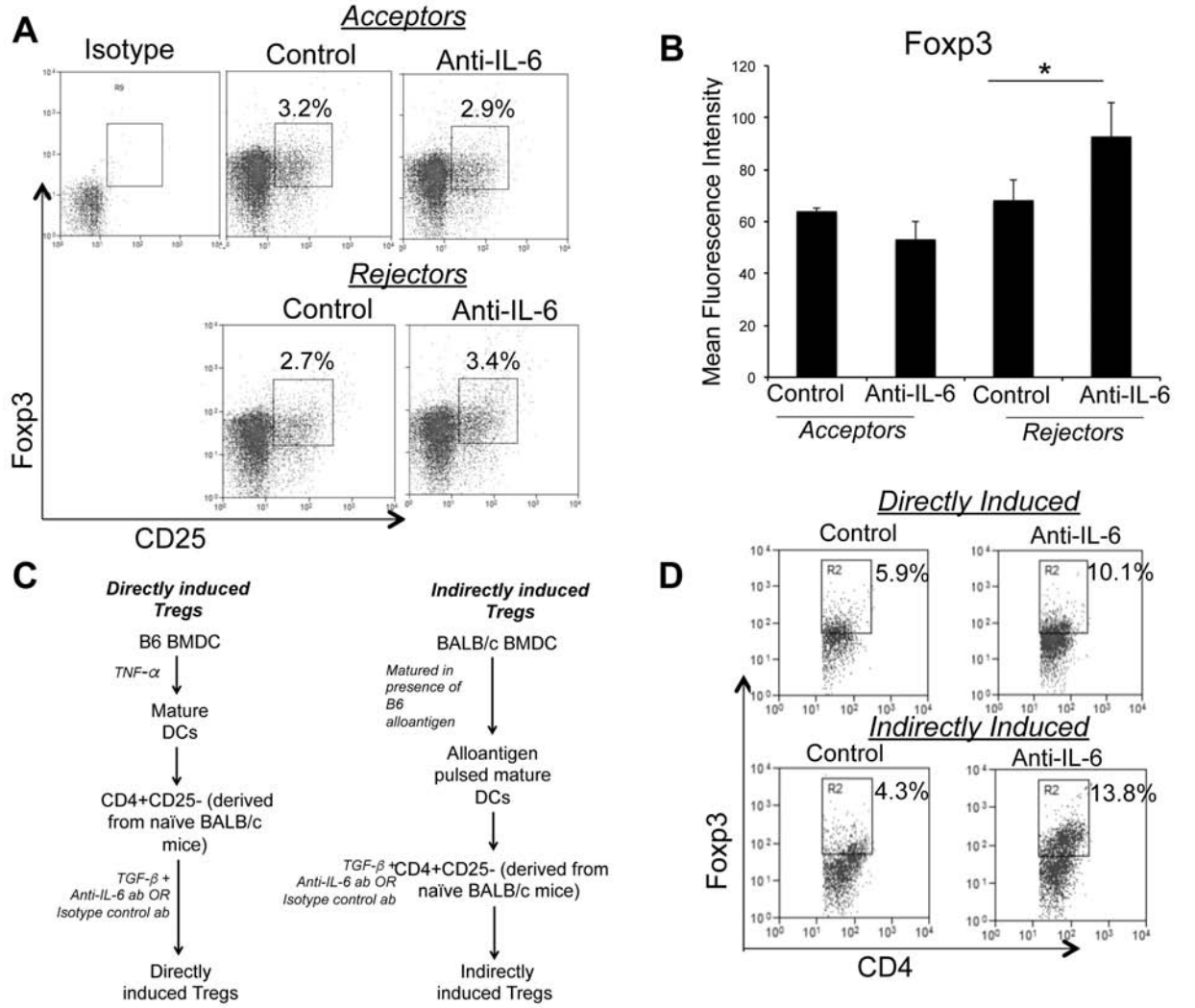

Figure 3. IL-6 blockade induces Tregs. A. Representative flow cytometric plots showing frequencies of $CD4^+CD25^{hi}Foxp3^+$ Tregs in anti-IL-6 or control isotype-treated graft acceptors and rejectors. Plots are gated on $CD4^+$ T cells. B. Bar graph demonstrating mean fluorescence intensity (MFI) of Foxp3 expression in Tregs derived from the ipsilateral DLN of graft recipients treated with anti-IL-6 antibody compared to isotype-treated recipients. C. Schematic diagram outlining the experimental design of direct and indirect Treg induction *in vitro*. For the direct pathway, bone marrow-derived dendritic cells (BMDCs) from C57BL/6 mice were co-cultured with BALB/c-derived $CD4^+CD25^-$ effector T cells in the presence of 5 ng/ml TFG-β and either anti-IL-6 (10 µg/ml) or isotype antibody. Indirectly induced Tregs were generated using BALB/c-derived BMDCs matured in the presence of alloantigen. Alloantigen-pulsed BALB/c BMDCs were subsequently co-cultured with TGF-β and either anti-IL-6 or isotype antibody. D. Representative flow cytometric plots demonstrating an increase in both directly and indirectly induced Tregs with IL-6 neutralization. P values were calculated using the Student's t-test and error bars represent mean +/- SEM of three experiments. *$p < 0.05$.

IL-6 neutralization promotes Treg development from $CD4^+$ T cells *in vitro*

Next, we utilized an *in vitro* system to directly evaluate the effect of IL-6 neutralization on Treg induction. To examine the effect of Treg induction *via* the direct pathway, bone marrow-derived dendritic cells (BMDCs) were harvested from C57BL/6 mice and matured in the presence of 5 ng/ml TFG-β. Mature C57BL/6-derived DCs were co-cultured with BALB/c-derived $CD4^+CD25^-$

effector T cells in the presence of TGF-β and either anti-IL-6 (10 μg/ml) or isotype control antibodies (Figure 3C). Subsequent flow cytometric analyses showed a 1.7-fold increase in the frequencies of $CD4^+$ $CD25^{hi}Foxp3^+$ Tregs in anti-IL-6-treated cultures compared to isotype-treated cultures (Figure 3D). For the indirect pathway of Treg induction, BALB/c-derived BMDCs were matured in the presence of C57BL/6 alloantigen. These alloantigen-pulsed mature BMDCs were subsequently co-cultured with syngeneic $CD4^+CD25^-$ effector T cells in combination with TGF-β and either anti-IL-6 or isotype control antibodies (Figure 3B). As shown in Figure 3D, a 3.2-fold increase was observed in Treg frequencies in cultures in which IL-6 was neutralized compared to control-treated cultures.

DISCUSSION

Interleukin-6 (IL-6) is a pleiotropic immunomodulatory cytokine that has been described to have a diverse range of functions in neutrophil recruitment, antigen-presenting cell maturation, and B cell differentiation [7]. IL-6 has also been implicated in regulating the T-helper 17 (Th17)/ Treg axis; in the presence of TGF-β, IL-6 has been demonstrated to inhibit Treg differentiation, favoring Th17 development [8]. IL-6 is also shown to work in concert with other inflammatory cytokines such as IL-1β to dampen Tregs' suppressive function against effector T cells [18]. The immunomodulatory effects of anti-IL-6 therapy have been further confirmed by the promising results obtained in clinical trials using anti-IL-6R monoclonal antibody (tocilizumab) in the treatment of rheumatoid arthritis [19, 20]. In addition, lower rates of acute graft versus host disease (GVHD) have been demonstrated in recipients of human leukocyte antigen (HLA)-matched stem cell transplantation when tocilizumab is added to the GVHD prophylaxis regimen [21].

In order to assess the functional significance of IL-6 in corneal allograft rejection, we assessed corneal allograft survival in recipient mice treated systemically with anti-IL-6 antibody. Our findings demonstrate significantly improved allograft survival in anti-IL-6-treated mice compared to isotype-treated controls. These findings are consistent with previous reports examining the effect of IL-6 neutralization in other forms of organ transplantation [22-24]. One such study by Lei and colleagues demonstrated prolonged cardiac allograft survival in major histocompatibility complex (MHC)-mismatched recipient mice treated with anti-IL-6 antibody, a finding which was correlated with induction of Tregs and diminished T cell proliferation capacity in response to alloantigens [22]. In another study by Booth and colleagues, prolonged cardiac allograft survival after administration of anti-IL-6 was found to be associated with skewed Th2-dominant response and suppressed immune cell infiltration to the graft [25].

Direct and indirect pathways of allosensitization are operational in the pathogenesis of graft immune rejection. In grafts placed in uninflamed host beds, the indirect pathway plays a more dominant role in induction of $CD4^+$ T cell response [26]. Our results show a significant reduction in both direct and indirect allosensitization in the anti-IL-6-treated rejecter mice compared to control-treated rejecters. The observed effect on allosensitization may in part be due to the effect of IL-6 blockade on Tregs. A significant increase in the expression of Foxp3 by Tregs was noted in our study in the draining lymph nodes of anti-IL-6-treated mice. This observation is in accord with other studies reporting an amplified Treg response in MHC-mismatched cardiac allograft recipient mice receiving IL-6 blocking antibody [22], and in IL-6 knockout renal allograft recipient mice compared to wild type recipients [27]. However, contrary to our findings, both studies reported higher frequencies of Tregs infiltrating the grafts with IL-6 blockade. Previous work by our group has shown that Foxp3 expression by Tregs, rather than Treg frequencies, is the most critical functional aspect of Treg immune suppression and tolerance induction in the corneal transplant setting [6]. The differences between our results and the above-cited studies in terms of altered Treg frequencies with IL-6 neutralization could partly be due to differences in potency of the allogeneic stimulus arising in major organ transplantation compared to corneal transplants.

To further evaluate the effect of IL-6 blockade on Tregs induced by an alloantigen stimulus, we utilized an *in vitro* system where isolated naïve

Tregs from BALB/c mice were cultured with APCs derived from C57BL/6 and BALB/c mice to assess direct and indirect Treg induction, respectively. Interestingly, we show a more pronounced increase in frequencies of Foxp3-expressing Tregs in anti-IL-6-treated cultures compared to the insignificant increase seen in our *in vivo* experiments. This increase is evident in Tregs induced through both direct and indirect alloantigen recognition, implicating the involvement of both pathways in enhanced differentiation of Tregs from $CD4^+$ T cells after IL-6 neutralization. Expanded Treg population after blockade of IL-6 has been reported by Lei and colleagues in their study evaluating the use of tocilizumab as a component of the immunotherapy regimen in a primate model of islet cell transplantation [28]. The authors show that administration of tocilizumab, an anti-IL-6 receptor monoclonal antibody, results in significant expansion of Treg population among peripheral blood mononuclear cells and prolongs islet cell graft survival [28].

CONCLUSION

In summary, our study is the first report to show that systemic treatment with anti-IL-6 antibody significantly promotes corneal allograft survival. Our findings demonstrate that IL-6 blockade suppresses both direct and indirect routes of allosensitization. Furthermore, we show that IL-6 neutralization expands alloantigen-induced Tregs through both direct and indirect pathways, and increases Tregs' suppressive function through enhancing Foxp3 expression levels. Further studies are needed to further elucidate the mechanisms by which IL-6 neutralization enhances corneal allograft survival.

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CONFLICT OF INTEREST STATEMENT

The authors have no financial conflicts of interest.

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