Corneal Inflammation After Miniature Keratoprosthesis Implantation

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Purpose. To compare corneal inflammation after syngeneic and allogeneic penetrating keratoplasty (PK) with miniature Keratoprosthesis (m-KPro) implantation in mice.

Methods. BALB/C (syngeneic) or C57BL/6 (allogeneic) corneas were transplanted onto BALB/C host beds as part of PK or m-KPro implantation. Corneal inflammation was assessed by determining the frequencies of $CD45^+$ leukocytes, $CD4^+$ T cells, $CD11b^+$ cells, and $Gr\text{-}1^+$ granulocytes/monocytes by flow cytometry at 2, 4, and 8 weeks post transplantation. In addition, expression levels of the proinflammatory cytokines TNF-α and IL-1β were analyzed using real-time qPCR at 8 weeks post transplantation.

Results. Cell frequencies in the syngeneic (syn) and allogeneic (allo) m-KPro groups were higher compared with the syngeneic and allogeneic PK groups, respectively, at all time points. However, after week 4, frequencies of all analyzed immune cells were higher in the alloPK group as compared with synKPro group. At 8 weeks, the expression of TNF-α was higher in synKPro, alloPK, and alloKPro groups compared with the naïve and synPK groups. The expression of IL-1β was significantly higher in both KPro groups as compared with PK groups.

Conclusions. Although the m-KPro device augments the inflammatory response in the cornea after its implantation, allogenicity (of the carrier tissue) is also a significant contributor to corneal inflammation. These data suggest that using syngeneic or decellularized corneal tissue as a Boston-KPro carrier could reduce the postoperative inflammation response.

Keywords: Boston Keratoprosthesis, penetrating keratoplasty, cornea inflammation, syngeneic, allogeneic

Penetrating keratoplasty (PK) is the mainstay of sight restoration in most severe corneal diseases,[1–3] and possesses a good survival rate in low-risk transplants, but has a significantly higher rejection rate in high-risk grafts performed in inflamed host beds.[4] When grafts fail, due to rejection or other causes, regrafting yields a poor prognosis for visual rehabilitation, with a survival rate of under 10% after the fourth regraft.[5–11] A promising and well-established alternative to regrafting and high-risk transplantation is the Boston Keratoprosthesis (B-KPro), an artificial cornea typically used after multiple PK failures or when PK is determined to be unlikely to succeed. Retention for B-KPro is 80% to 100% at 1 to 2 years[12–16] and 2/3 at 7 years, with 50% of patients retaining useful vision.[17] Today, glaucoma, optic neuropathy, epiretinal membrane, macular edema, and retinal detachment remain threats to good long-term visual outcomes after B-KPro surgery[18–20] and it has been hypothesized that chronic postoperative corneal inflammation may be a contributing factor to these complications.

The immunoinflammatory response after syngeneic and allogeneic PK has been investigated before, showing an early innate immune response that is present in both syngeneic and allogeneic PK and is mostly induced by the surgical procedure, as well as a late immune and inflammatory response seen only in allogeneic PK.[21–23] We have recently established a novel murine miniature-KPro (m-KPro) model well suited to investigate corneal inflammation.[24] We have used this model in the present study to compare the magnitude and kinetics of the corneal immune response following both syngeneic and allogeneic PK and B-KPro implantation. For the first time, we shed light on potential sources of corneal inflammation after KPro implantation, specifically the relative contribution of an allogeneic graft (or carrier tissue) versus the presence of the KPro device itself.

Methods

Animals

A total of 306 male mice (8- to 10-weeks old; Jackson Laboratory, Bar Harbor, ME, USA; 162 BALB/C used as recipients and 72 as donors, and 72 C57BL/6 used as donors) were used in this study. They were housed in a climate-controlled animal facility at the Schepens Eye Research Institute (Massachusetts Eye and Ear, Harvard Medical School, Boston, MA, USA) and kept under cyclic light conditions (12 hours on/off). All animal experiments were approved by the Institutional Animal Care and Use Committee, and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

For this study mice were divided into the following groups: (1) naïve, (2) syngeneic penetrating keratoplasty (SynPK; BALB/

C donor to BALB/C recipient), (3) allogeneic penetrating keratoplasty (AlloPK; C57BL/6 donor to BALB/C recipient), (4) syngeneic m-KPro implantation (SynKPro; BALB/C carrier graft to BALB/C recipient), and (5) allogeneic m-KPro implantation (AlloKPro; C57BL/6 carrier graft to BALB/C recipient).

Penetrating Keratoplasty (PK)

Surgeries were performed under a surgical microscope (Zeiss, Jena, Germany). All procedures were performed by a single investigator (MO). After euthanasia of the donor mice, corneas were marked with a 2-mm diameter biopsy punch (Miltex, Plainsboro, NJ, USA). To maintain a deep anterior chamber, viscoelastic (Viscoat; Alcone, Irvine, CA, USA) was injected through the groove with a 30-G cannula (Rumex, Clearwater, FL, USA). The cornea was excised with Vannas scissors (Storz Instruments Company, San Damis, CA, USA) and placed in PBS until the host corneal bed had been prepared.

Recipient mice were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (20 mg/kg). A drop of 1% Tropicamide (Bausch & Lomb, New York, NY, USA) and a drop of 0.5% phenylephrine hydrochloride (Altaire Pharmaceuticals, New York, NY, USA) were applied to the right eye to dilate the pupil 15 minutes before the procedure. The cornea was marked with a 2-mm diameter biopsy punch (Harris UniCore punch, Ted Pella, Inc., Redding, CA, USA). Before opening the eye viscoelastic (Viscoat; Alcon) was injected via a 30-G cannula (Rumex). The cornea was excised with Vannas scissors (Storz Instruments Company). Throughout the procedure viscoelastic was used to maintain the depth of the anterior chamber. The host lens was removed as previously described.[25] Briefly, a curvilinear continuous capsulorrhexis was performed with jewellers forceps (Katena, Denville, NJ, USA) and a hydrodissection was performed with a 30-G cannula and PBS to deliver the lens en bloc. Visible cortex remnants were carefully aspirated through the cannula. The donor cornea was then placed in the recipient bed and secured with eight interrupted 11-0 nylon sutures (Sharpoint; Angiotech Pharmaceuticals, Vancouver, Canada) in standard penetrating keratoplasty fashion.

Miniature KPro (m-KPro) Implantation

A miniature keratoprosthesis (m-KPro; JG Machine, Wilmington, MA, USA), minimized, but otherwise identical to the B-KPro device used in humans,[26] was used. It consists of a poly[methyl methacrylate] (PMMA) front plate with central stem and a titanium (Ti) back plate with eight peripheral holes. The surgical procedure was performed similarly to B-KPro implantation in human and has been described previously.[24]

Briefly, to prepare for the assembly of the m-KPro device, excised donor corneas were trephined centrally with a 0.5-mm diameter punch (Harris UniCore punch; Ted Pella, Inc.), and then slid over the stem of the PMMA front plate. The titanium back plate was positioned and locked on the stem such that the donor cornea became securely sandwiched between the front and back plates. The donor-device complex was then placed in PBS until the host corneal bed had been prepared as described above. After preparation the donor cornea-m-KPro complex was placed in the recipient bed and secured with eight interrupted 11-0 nylon sutures (Sharpoint, Angiotech Pharmaceuticals) in standard penetrating keratoplasty fashion.

Postoperative Treatment

Postoperative treatment was identical after PK and m-KPro implantation. One drop of 1% corticosteroid (Pred Forte; Allergan, Irvine, CA, USA) and antibiotic ointment (AK-POLY-BAC; Akorn, Lake Forest, IL, USA) were applied immediately after the procedure and a tarsorrhaphy was performed using 8-0 nylon sutures (Sharpoint; Angiotech Pharmaceuticals). Corticosteroid was administered once a day for 2 weeks and then every other day for 2 weeks. The tarsorrhaphy was removed 48 hours after the surgery, and corneal sutures were removed 1 week following surgery. As per standard institutional animal care and use protocols, 0.1 mg/kg Buprenorphine (Reckitt Benckiser Healthcare [UK] Ltd., Hull, England) was applied subcutaneously after the procedure and every 12 hours for 48 hours postoperatively.

The mice were euthanized 2, 4, and 8 weeks after the procedure for further analyses.

Flow Cytometry

Grafts and host corneal beds and corneas from naïve mice were collected 2, 4, and 8 weeks post transplantation and digested with DNase I (0.2 mg/mL; Roche, Basel, Switzerland) and collagenase D (0.4 mg/mL; Roche) to create single cell suspensions. Samples from individual animals were prepared and analyzed separately. All cell suspensions were incubated with an Fc-blocking agent (R&D Systems, Minneapolis, MN, USA) before staining. Corneal cells were stained with PE/Cy5-conjugated anti-CD45 (eBioscience, San Diego, CA, USA), Alexa Fluor 488-conjugated anti-CD11b (BD Pharmingen, San Jose, CA, USA), Brilliant Violet 421-conjugated anti-CD4 (BioLegend, San Diego, CA, USA), and PE-conjugated anti-Gr-1 (BioLegend) antibodies. Appropriate isotype matched control antibodies were used in all experiments. Cells were analyzed using a LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Summit v4.3 Software (DAKO Colorado, Inc., Fort Collins, CO, USA). The analysis for each time point was performed twice with $n = 3$ eyes/group.

Reverse Transcription and Real-Time PCR

Corneas were harvested at 8 weeks post surgery. Ribonucleic acid was isolated with the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and reverse transcribed using Superscript III Kit (Invitrogen, Grand Island, NY, USA). Real-time qPCR was performed using Taqman Universal PCR Mastermix and preformulated primers for murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TNF-α, and IL-1β (Applied Biosystems, Foster City, CA, USA). The results were normalized to the expression level in naive mice and analyzed by the comparative threshold cycle method, using GAPDH as an internal control. Real-time PCR was repeated four times for each cytokine. Corneas of three mice were pooled and duplicates for each group were analyzed.

Statistics

The two-tailed ANOVA test was employed to analyze flow cytometry data for all time points and one-tailed ANOVA test was used to analyze real-time qPCR data at week 8. *P* less than or equal to 0.05 was considered statistically significant. Results are presented as the mean ± SEM.

Results

Cellular Corneal Inflammation After Syngeneic or Allogeneic PK and m-KPro Implantation

To analyze the magnitude and kinetics of the corneal immune response after PK and m-KPro surgery, we analyzed the frequencies of $CD45^+$ leukocytes, $CD4^+$ T cells, $CD11b^+$ cells,

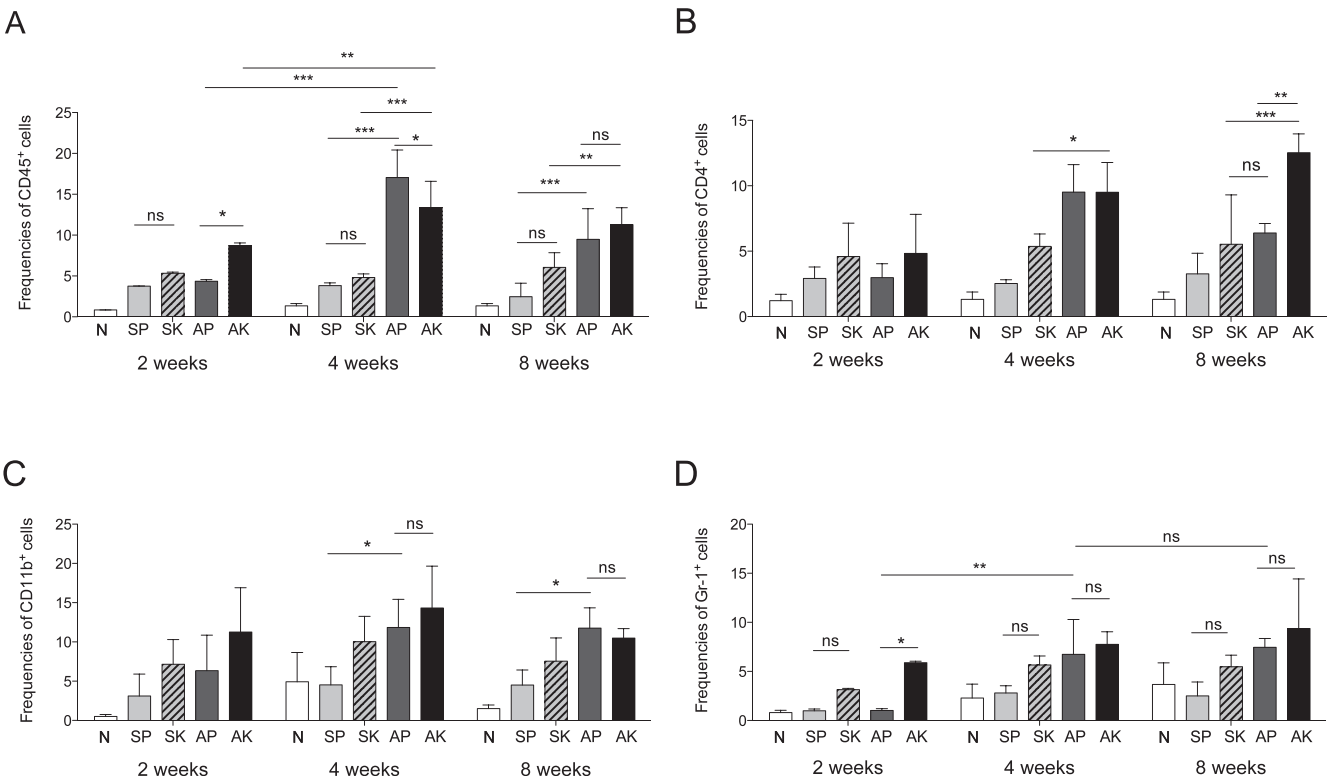

Figure 1. Quantification of graft-infiltrating immune cells. Transplant recipients from all groups (syngeneic [syn] and allogeneic [allo] PK and m-KPro) were euthanized at 2, 4, and 8 weeks post surgery and corneal grafts plus host corneal beds were collected and analyzed for the presence of CD45+ (**A**), CD4+ (**B**), CD11b+ (**C**), and Gr1+ (**D**) cells using flow cytometry. $n = 3$ eyes/group; $^{***}P < 0.001$, $^{**}P < 0.005$, $^{*}P < 0.05$. Data from one experiment of two is shown. N, naïve; SP, syn PK; SK, syn KPro; AP, allo PK; AK, allo KPro.

and Gr-1$^+$ granulocytes/monocytes in the cornea of mice after SynPK, AlloPK, SynKPro, AlloKPro, and naïve mice at 2, 4, and 8 weeks post transplantation.

At week 2, we found higher frequencies of all analyzed immune cells in the syngeneic and allogeneic m-KPro groups as compared with the syngeneic and allogeneic PK groups, respectively (Figs. 1A–D). At week 4, cornea infiltration of CD45$^+$ and CD4$^+$ cells significantly increased in allogeneic PK and m-KPro groups as compared with syngeneic PK and m-KPro groups, respectively, and as well as when compared with the allogeneic PK and m-KPro group at 2 weeks, respectively (Figs. 1A, 1B). Additionally, CD11b$^+$ and Gr1$^+$ cells showed a tendency to increase in allogeneic PK and m-KPro groups compared with syngeneic PK and m-KPro groups, respectively. Both cell types also increased in allogeneic PK and m-KPro groups at week 4 compared with week 2, respectively, although the differences were not significant (Figs. 1C, 1D). At week 8, frequencies of most analyzed immune cells decreased or remained similar to week 4, except CD45$^+$ and CD4$^+$ cells increased in the SynKPro group and AlloKPro group, respectively (Figs. 1A–D). Based on flow cytometry data, chronic inflammation 8 weeks after surgery was enhanced in the AlloPK group compared with the SynKPro group.

Inflammatory Cytokine Expression After Syngeneic or Allogeneic PK and m-KPro Implantation

To evaluate inflammation at the cytokine level following PK and m-KPro, we quantified mRNA expression of the proinflammatory cytokines TNF-α and IL-1β in the cornea at week 8 post surgery. We detected increased TNF-α expression in SynKPro, AlloPK, and AlloKPro groups compared with naïve and SynPK group (Fig. 2A). The expression of IL-1β was

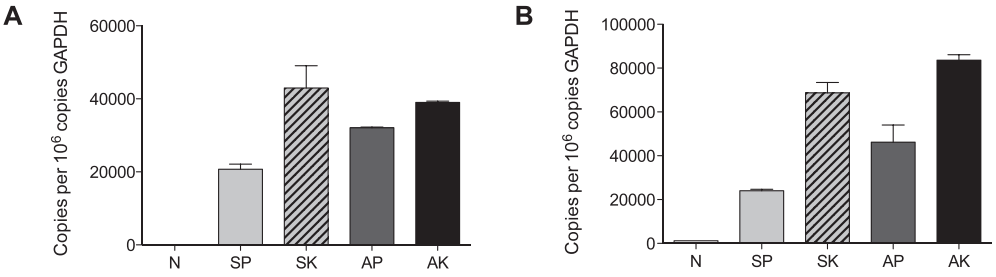

Figure 2. Quantification of proinflammatory cytokines in the graft. Transplant recipients from all groups (syn and allo PK and m-KPro) were euthanized at 2, 4, and 8 weeks post surgery and corneal grafts plus host corneal beds were collected and analyzed for the expression of TNF-α (**A**) and IL-1β (**B**) by real-time qPCR. $^{*}P < 0.01$. Data from one representative experiment of two is shown.

significantly higher in syngeneic and allogeneic m-KPro groups compared with syngeneic and allogeneic PK groups, respectively, and expression was also significantly higher in the SynKPro as compared with AlloPK group (Fig. 2B).

Discussion

In a recently published retrospective study about glaucoma in B-KPro patients, we reported that 7% of the eyes with normal or low IOP had progressive cupping of the optic nerve.[27] The etiology of the optic nerve damage in those eyes is unclear. Likewise, the mechanisms inducing other chronic complications after B-KPro surgery such as optic neuropathy, epiretinal membrane, macular edema, and retinal detachment remain unknown. However, it is known that corneal inflammation can induce posterior segment damage, for example, massive apoptosis of the retinal ganglion cells can occur within 24 hours after alkali burns of the cornea.[28] This is most likely caused by posterior diffusion of inflammatory cytokines from the injured anterior segment, indicating that corneal inflammation can have wide-reaching effects.[28] With this in mind, we sought to analyze the extent and kinetics of the corneal inflammatory response following PK and m-KPro surgery, and perhaps shed light on a mechanism by which postoperative complications arise after B-KPro implantation. Comparing corneal inflammation after syngeneic and allogeneic PK and m-KPro implantation, we found that the use of an allogeneic carrier graft contributes to chronic inflammation to a greater degree than the presence of the KPro device itself.

Using flow cytometry, we assessed the frequencies of corneal immune cells at 2, 4, and 8 weeks after surgery and observed similar kinetics for $CD45^+$ leukocytes, $CD4^+$ T cells, $CD11b^+$ cells, and $Gr\text{-}1^+$ granulocytes/monocytes. The frequencies of all investigated immune cells in the cornea were higher after syngeneic and allogeneic m-KPro implantation compared with syngeneic and allogeneic PK, respectively, at 2 weeks post transplantation. At week 4, the frequencies of all observed immune cells increased after allogeneic PK and m-KPro, and were higher in comparison to syngeneic PK and m-KPro implantation, respectively. Finally, at week 8, the highest frequencies of all analyzed inflammatory cells were found after AlloKPro implantation, followed by AlloPK, SynKPro, and SynPK groups.

Given our data on cellular corneal inflammation, we conclude that the KPro device contributes more to acute inflammation than chronic inflammation. Moreover, at weeks 4 and 8, we found immune cell infiltration to be higher after AlloPK than after SynKPro, suggesting that in the long-term, allogeneic immunity contributes to corneal inflammation to a greater extent than the KPro device. This suggests using patient's own cornea as a carrier for the B-KPro if possible. When an auto graft carrier is not an option, methods to reduce the allogenicity of the carrier graft should be considered, such as decellularization through gamma irradiation or cross-linking.[29–31] Cross-linking has an additional beneficial effect: it makes the cornea more resistant to keratolysis.[32,33]

In our study, we detected increased TNF-α expression in the cornea after SynKPro, AlloPK, and AlloKPro implantation compared with naïve and SynPK at 8 weeks. The expression of IL-1β was significantly higher after syngeneic and allogeneic m-KPro implantation compared with syngeneic and allogeneic PK, respectively. Because inflammatory cytokine expression was higher in the m-KPro groups compared with the PK groups at 8 weeks, but infiltrating immune cells were higher after allogeneic PK and KPro compared with syngeneic PK and m-KPro, we speculate that infiltrating immune cells as well as corneal cells (such as epithelial cells and keratocytes) secrete TNF-α and IL-1β as shown by Grabner et al.[34] Cytokine production by corneal epithelial and stromal cells may also enhance inflammation in the m-KPro group possible through higher mechanical stress from the KPro as compared with the PK group.

Interleukin-1 and TNF-α are critical in mediating corneal inflammation, and blockade of IL-1 and TNF-α has been shown to decrease corneal inflammation and promote graft survival in standard PK.[35] In a murine model of corneal inflammation the effect of IL-1 blockade was similar to corticosteroids, and anti–TNF-α treatment was less effective. It has also been reported that blocking the IL-1 pathway inhibits $CD11b^+$ monocytic cell infiltration in the corneal stroma.[35,36] Additionally, TNF-α inhibitors have been shown to have a protective effect on retinal ganglion cell apoptosis after alkali burn of the cornea.[28] Because TNF-α and IL-1β are expressed higher after m-KPro compared with PK, we can speculate that blocking these cytokines will be efficacious in preventing chronic inflammation after KPro. Further studies should be performed to assess the damage done to the posterior segment of the eye caused by chronic corneal inflammation, and to determine whether cytokine antagonism can be effective in suppressing this damage.

In summary, chronic inflammation due to allogenicity in the cornea is profound and is augmented by the m-KPro device. These results support the use of strategies aimed at decreasing carrier graft allogenicity such as use of syngeneic or decellularized corneal tissue.

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