Effect of Corneal Nerve Ablation on Immune Tolerance Induced by Corneal Allografts, Oral Immunization, or Anterior Chamber Injection of Antigens

Juan Mo, Sudha Neelam, Jessamee Mellon, Joseph R. Brown, and Jerry Y. Niederkorn

Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas, United States

Correspondence: Jerry Y. Niederkorn, Department of Ophthalmology, U.T. Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9057, USA; jerry.niederkorn@utsouthwestern.edu.

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PURPOSE. Severing corneal nerves during corneal transplantation does not affect first corneal transplants, but abolishes immune privilege of subsequent corneal allografts. This abrogation of immune privilege is attributable to the disabling of T regulatory cells (T regs) induced by corneal transplantation. The goal of this study was to determine if severing corneal nerves induces the development of contrasuppressor (CS) cells, which disable T regs that impair other forms of immune tolerance.

METHODS. Effect of corneal nerve ablation on immune tolerance was assessed in four forms of immune tolerance: anterior chamber-associated immune deviation (ACAID); oral tolerance; corneal transplantation, and intravenously (IV) induced immune tolerance. T regulatory cell activity was assessed by adoptive transfer and by local adoptive transfer (LAT) of suppression assays.

RESULTS. Corneal nerve ablation prevented ACAID and oral tolerance, but did not affect IV-induced immune tolerance. Contrasuppressor cells blocked the action of T regs that were generated by anterior chamber injection, oral tolerance, or orthotopic corneal transplantation. The neuropeptide substance P (SP) was crucial for contrasuppressor activity as CS cells could not be induced in SP−/− mice and the SP receptor inhibitor, Spantide II, prevented the expression of CS cell activity in vivo. Contrasuppressor cells expressed CD11c surface marker that identifies dendritic cells (DC).

CONCLUSIONS. The loss of immune privilege produced by corneal nerve ablation following corneal transplantation extends beyond the eye and also affects immune tolerance induced through mucosal surfaces and appears to be mediated by a novel cell population of CD11c+ CS cells that disables T regs.

Keywords: anterior chamber, cornea, immune privilege, keratoplasty, mucosal tolerance, transplantation, T regs

C orneal transplantation is the oldest, most common, and most successful form of solid tissue grafting. In the United States alone, over 40,000 corneal transplants are performed each year.1 With only the use of steroid eye drops, corneal transplants experience a 90% success rate, even though histocompatibility matching and systemic immunosuppressive drugs are not routinely used.2,3

The remarkable success of corneal allografts is attributed to a number of unique properties of the corneal allograft and graft bed. The conspicuous absence of lymph vessels in the corneal allograft bed limits the induction of a primary alloimmune response.4 Cell membranes of the corneal allograft are decorated with molecules such as FasL and PD-L1 that induce apoptosis of infiltrating activated T cells.5–7 Corneal allografts also have the unique capacity to induce CD4+CD25+Foxp3+ T regulatory cells (T regs) that are required for long-term corneal allograft survival.8,9 Although first-time corneal allografts enjoy a remarkably high success rate in keratoplasty patients, second or third corneal transplants have a 3-fold increase in the incidence of rejection.10 We recently found that a similar condition occurs in a mouse model of penetrating keratoplasty in which 50% of the first-time corneal allografts enjoyed long-term survival even in the absence of major histocompatibility complex (MHC) matching and without immunosuppressive drugs.11 However, >90% of subsequent corneal allografts underwent rejection even if the second corneal transplant was from a donor mouse strain entirely unrelated to the donor of the first corneal allograft.11 The heightened incidence and tempo of rejection for second corneal allografts was associated with a loss of T reg function. Further study revealed that it was the ablation of corneal nerves by circular incisions made in preparing the graft bed, and not the orthotopic transplant, that led to a disabling of corneal allograft-induced T regs and the loss of immune privilege for future corneal transplants.11 Remarkably, the loss of T reg function persisted for at least 100 days.

The present study sought to determine if the severing of corneal nerves that abolishes the function of corneal allograft-induced T regs is unique to corneal transplants or if T regs induced through other tissue sites would also be affected by corneal nerve ablation. We focused our attention on three well-established models of immune tolerance to test this hypothesis. Immune privilege in the anterior chamber (AC) of the eye is due in large part to a form of immune tolerance elicited when
any of a wide range of antigens are introduced into the AC. In particular, alloantigenic cells introduced into the AC induce an antigen-specific suppression of alloimmune responses called anterior chamber-associated immune deviation (ACAID) that enhances corneal allograft survival by inducing Tregs; these downregulate delayed-type hypersensitivity (DTH) responses to alloantigens that are expressed on the corneal allograft.\textsuperscript{12–14} Oral administration of alloantigens also induces Tregs that produce a similar downregulation of allospecific DTH and enhance corneal allograft survival by a process frequently referred to as oral tolerance.\textsuperscript{15,16} A form of immune tolerance called intravenous immune deviation is invoked when either soluble antigens or alloantigenic cells are injected intravenously.\textsuperscript{17} Intravenous injection of alloantigens induces Tregs that support a temporary form of immune tolerance and transient suppression of skin allograft rejection.\textsuperscript{17} The present study was an effort to determine if ablation of corneal nerves affects immune tolerance induced by antigens introduced intracamerally, intravenously, or orally.

\section*{Materials and Methods}

\subsection*{Animals}

C57BL/6 (H-2\textsuperscript{b}) and BALB/c (H-2\textsuperscript{d}) were purchased from the University of Texas Southwestern Mouse Breeding Facility. For grafting experiments, 8- to 10-week-old female BALB/c and C57BL/6 (B6) mice were purchased from Taconic Farms (Germantown, NY, USA). BALB/c D011.10 mice bearing the transgene for the T cell receptor for ovalbumin (OVA) and positive control mice were sensitized by one subcutaneous (SC) injection of either 1\texttimes\textsuperscript{10}\textsuperscript{6} C57BL/6 splenocytes in 100 \textmu L PBS) and 100 \textmu g OVA in 20 \textmu L HBSS was injected into the SC tissue of the right ear. The left ear served as a negative control and was injected with 20 \textmu L HBSS without cells. Results were expressed as antigen-specific ear-swelling response = (experimental ear 24-hour measurement – experimental ear 0-hour measurement) – (negative control ear 24-hour measurement – negative ear 0-hour measurement).

\subsection*{Corneal Nerve Imaging}

A 2.0-mm circular trephine was used to produce a shallow circular incision in the cornea of one eye. The incision penetrated the epithelial layer and the upper two-thirds of the stromal layer.

\subsection*{Oral Tolerance Induction}

Oral tolerance to alloantigens was induced as previously described.\textsuperscript{21} C57BL/6 spleen cells were conjugated with the Qiagen RNeasy Mini Isolation Kit (Germantown, MD, USA). Real-time PCR was performed using the RT2 QuantiTect Reverse Transcription kit and RT2 SYBR Green kits with prevalidated primers for Tacr1 and GAPDH (SA Biosciences, Frederick, MD, USA). The results were analyzed by the comparative threshold cycle method and normalized with GAPDH as an internal control.

\subsection*{Intravenous Induced Immune Deviation}

Intravenous immune deviation was induced by injecting with OVA (100 \textmu g in 100 \textmu L PBS) through tail vein injection on days 0, 2, 4, and 6. A single SC injection of a mixture of OVA (500 \textmu g in 100 \textmu L PBS) and 100 \textmu L CFA was given on day 7.

\subsection*{Oral Tolerance Induction}

Oral tolerance to alloantigens was induced as previously described.\textsuperscript{21} C57BL/6 spleen cells were conjugated with the nontoxic B subunit of cholera toxin (CTB, Sigma-Aldrich Corp.) by incubating 1 \times 10\textsuperscript{7} C57BL/6 spleen cells with 100 \textmu g CTB in 1.0 mL HBSS. The cell suspension was incubated for 2 hours at 37°C with frequent shaking followed by three washes in HBSS. For each oral immunization, 2 \times 10\textsuperscript{6} allogeneic spleen cells were administrated directly into the stomach using a gastric gavage tube for 5 consecutive days. Animals were immunized

\subsection*{Subcutaneous Immunization and SP Injections}

Positive control mice were sensitized by one subcutaneous (SC) injection of either 1 \times 10\textsuperscript{6} C57BL/6 splenocytes in 100 \textmu L Hank's balanced salt solution (HBSS) or 250 \textmu g OVA (Sigma-Aldrich Corp., St. Louis, MO, USA) emulsified 1:1 in Complete Freund's Adjuvant (CFA; Sigma-Aldrich Corp.). Each SC injection was in a total volume of 200 \textmu L. In some experiments, a single intravenous (IV) injection of 1 pg substance P (Sigma-
SC with \(1 \times 10^6\) spleen cells 1 day after the fifth oral immunization. Ear-swelling assays were performed 7 days after SC immunizations.

### Induction of ACAID

Anterior chamber-associated immune deviation was induced as described previously using microinjection of antigen into the AC of the eye. A Hamilton (Whittier, CA, USA) automatic dispensing apparatus was used to inject 6 \(\mu\)L 16.67 mg/mL OVA (Sigma-Aldrich Corp.) in PBS (100 \(\mu\)g OVA) into the AC. Seven days after the AC injection, animals were SC immunized with 200 \(\mu\)g OVA in an equal volume of CFA. Ear was challenged 7 days after SC immunization by injecting OVA (400 \(\mu\)g in 20 \(\mu\)L PBS). The opposite ear was injected with 20 \(\mu\)L PBS as a negative control. Ear swelling was measured 24 hours later to measure DTH.

### Local Adoptive Transfer Assay

The local adoptive transfer (LAT) assay was used as an in vivo test for T reg activity. Corneal allograft-induced CD4\(^+\)CD25\(^+\) T regs or ACAID CD8\(^+\) T regs (1 \(\times\) 10\(^6\)) were incubated with BALB/c antigen presenting cells (APC) pulsed with either C57BL/6 sonicated spleen cells (alloantigen) or OVA and immune CD4\(^+\) T cells from SC-immunized BALB/c mice. Cells were mixed in a 1:1:1 ratio. The right ears of naive BALB/c mice were injected with 20 \(\mu\)L of the mixed-cell population. The opposite ear was injected with 20 \(\mu\)L HBSS as a negative control. Ear swelling was measured 24 hours later to measure DTH. To block SP signaling, 72 \(\mu\)g Spantide II (Sigma-Aldrich Corp.) was included in the cell suspensions used in the LAT assays.

### Isolation of T Regs Using MACS Miltenyi Biotech Beads

**Corneal Allograft-Induced CD4\(^+\)CD25\(^+\) Regulatory T Reg.** Spleen cells were isolated from the BALB/c mice bearing clear C57BL/6 corneal allografts on day 21 post transplantation, as CD4\(^+\)CD25\(^+\) T regs are consistently detected in the spleens in corneal allograft receptor mice at this time point. The isolation was performed in a two-step procedure. First, the non-CD4\(^+\) T cells were magnetically labeled with a cocktail of biotin-conjugated antibodies, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. In parallel, the cells were also labeled with CD25-PE. The magnetically labeled cells were subsequently depleted by separation over a MACS Column, using the magnetic field of a MACS Separator (San Diego, CA, USA). The magnetically labeled non-CD4\(^+\) T cells are retained in the column, while the unlabeled CD4\(^+\) T cells run through. In the second step, the CD25\(^+\) PE-labeled cells were magnetically labeled with Anti-PE MicroBeads and isolated by positive selection from the pre-enriched CD4\(^+\) cell fraction by separation over a MACS Column. The magnetically labeled CD4\(^+\)CD25\(^+\) cells are retained in the column, while the unlabeled CD4\(^+\)CD25\(^-\) cells run through. We have found that this enrichment technique yields >95% CD4\(^+\)CD25\(^+\) T cells of which 90% to 95% are Foxp3\(^+\).

**ACAID CD8\(^+\) T Regs.** Spleen cells were isolated from BALB/c mice 10 days following AC injection of OVA for the isolation of CD8\(^+\) ACAID T regs. Spleen cells were isolated using magnetically labeled with CD8 (Ly-2) microbeads. The cell suspension was then loaded onto a MACS Column, which was placed in a magnetic field of the MACS Separator. The magnetically labeled CD8\(^+\) T regs were retained within the column and the unlabeled cells run through. The column was removed from the magnetic field and the retained CD8\(^+\) cells were eluted as the positively selected cell fraction. We have previously found that this enrichment technique yields >95% CD8\(^+\) T cells.

### Statistical Analysis

Results for DTH assays were evaluated by Student’s t-test. Results are expressed as mean ± SEM. Differences in all experiments were considered to be statistically significant if the \(P\) values were <0.05.

### RESULTS

**Trephining Transiently Ablates Corneal Nerves and Upregulates mRNA Levels of SP Receptor in Both Eyes**

We previously reported that the circular corneal incisions (trephining) that are made in preparing the graft bed for orthotopic corneal allografts ablate corneal nerves and stimulate a burst in SP production in the affected eye. The present study sought to determine if corneal nerves would undergo regeneration following severing with circular corneal incisions. Circular corneal incisions were introduced into the right eyes by trephining, and both eyes were removed 2, 4, 20, and 50 days later. Corneal nerves were stained in both eyes using anti-mouse \(\beta\) III tubulin. The immunostaining results showed that trephining transiently ablated corneal nerves, which returned to their original density as early as 4 days after trephining (Fig. 1A). The corneal nerve density was quantified using the ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA) (Fig. 1B).

Substance P is a neuropeptide that is released from injured nerves and is correlated with a steep increase in the incidence and tempo of orthotopic corneal allograft rejection. Interestingly, laser burns to the retina induce an upregulation in NK1-R, the receptor for SP, which is associated with the abrogation of ACAID. Accordingly, we examined the eyes of mice following trephining for the upregulation NK1-R message in trephined eyes and the contralateral nonmanipulated eyes of BALB/c mice. The results revealed a spike in NK1-R message 14 days following trephining and a return to baseline levels by day 21 (Fig. 1C). We have previously reported that a single IV injection of 0.1 pg SP can mimic the effects of trephining and results in the loss of immune privilege for corneal allografts that persists for over 100 days. The present results, along with our previous findings, indicate that trephining produces a transient ablation of corneal nerves and a short-lived upregulation of NK1-R in both the trephined eye and the opposite nonmanipulated eye. Together, these findings suggest that SP rapidly abolishes immune privilege, which persists long after the peptide and its receptor have dissipated.

**Corneal Nerve Ablation, SP Injection, or Corneal Transplantation Prevents the Induction of ACAID**

Previous studies have shown that coinjection of antigen and SP into the AC of the eye prevents the induction of ACAID. Similarly, laser burns to the retina elicit a 10-fold increase in the expression of NK1-R in the opposite eye and prevent the induction of ACAID in both eyes. With this in mind, we examined the effect of trephining, SP, and orthotopic corneal transplantation on the induction of ACAID in both eyes.
We hypothesized that trephining the cornea would prevent the induction of ACAID. Circular corneal incisions were placed in the right (OD) eye 4 days prior to injecting OVA into the AC of either the OD eye or the left (OS) eye. The results showed that trephining abolished ACAID if antigen was injected into either the OD eye or the nontrephined OS eye (Fig. 2A), suggesting that ablating corneal nerves by trephining elicits a systemic response that prevents the development of ACAID. As mentioned earlier, we have previously shown that trephining stimulates the production of SP and the loss of immune privilege for corneal allografts. Accordingly, experiments were performed to determine if a single IV injection of SP would produce a similar abrogation of ACAID. The results revealed that a single IV injection of 1 pg SP prevented the induction of ACAID (Fig. 2B). As expected, orthotopic corneal transplantation to one eye prevented the induction of ACAID when OVA was injected into the AC of either the grafted eye or the
contralateral nongrafted eye (Fig. 2C). Thus, disruption of corneal nerves, injection of SP, or orthotopic corneal transplantation exerts a systemic effect that robs both eyes of their immune privilege.

**Nerve Ablation, SP, or Corneal Transplantation Prevents the Induction of Oral Tolerance**

Antigens administered through mucosal routes (e.g., orally) induce an antigen-specific induction of immune tolerance that has many similarities to ACAID. Moreover, oral administration of alloantigenic cells from a corneal allograft donor mouse strain into mice that subsequently receive a corneal transplant from the same histoincompatible donor strain used for oral sensitization induces antigen-specific suppression of DTH responses and results in a significant enhancement of corneal allograft survival. Experiments were performed to determine if trephining, injection of SP, or orthotopic corneal transplantation affected the induction of oral tolerance. The right eyes of BALB/c mice were trephined 4 days prior to the oral administration of C57BL/6 spleen cells (2 × 10^6 cells/dose) that were conjugated with neutralized cholera toxin. Spleen cells were administered orally with a gavage tube for 5 consecutive days, and on the sixth day mice were immunized SC with 10^6 C57BL/6 spleen cells. Oral administration of alloantigenic C57BL/6 cells induced a significant downregulation of DTH responses (Fig. 3A). However, this induction of immune tolerance did not occur in mice that had been subjected to trephining prior to oral administration of alloantigenic cells. Similarly, injection of SP also prevented the induction of oral tolerance to C57BL/6 alloantigens (Fig. 3B). Like trephining and SP injection, application of orthotopic syngeneic corneal isografts also prevented the induction of oral tolerance (Fig. 3C). Thus, the effect of corneal nerve ablation, orthotopic corneal transplantation, or SP injection was not restricted to immune tolerance in the eye, but also extended to immune tolerance induced through mucosal tissues.

**Neither Nerve Ablation nor SP Prevents the Induction of Intravenous Tolerance**

Antigens introduced via the venous route induce a form of immune deviation that is reminiscent of ACAID and produces a transient prolongation of skin graft survival. We employed a well-characterized model of IV-induced immune deviation to determine if trephining, SP, or orthotopic corneal transplantation would have a similar effect in abrogating tolerance that was induced through a nonmucosal and nonocular route (i.e., intravenously). Immune deviation was induced with four IV injections of OVA prior to SC immunization with OVA suspended in CFA. Although IV injection of OVA prevented the induction of positive DTH responses, neither trephining nor injection of SP adversely affected the downregulation of DTH in either wild-type (WT) mice (Fig. 4A) or OVA TCR transgenic mice (Fig. 4B). Although nerve ablation and SP injection prevented the development of immune tolerance induced via the AC of the eye or through mucosal tissues, it had no effect on immune deviation elicited via IV administration of antigens.

**Severing Corneal Nerves Elicits the Generation of Leukocytes With CS Cell Activity**

The results reported here and elsewhere indicate that ablation of corneal nerves prevents the development of oral tolerance and ACAID and blocks the development of corneal allograft-induced T regs. A possible explanation for these curious findings is based on the notion that severing corneal nerves elicits the release of SP that promotes the generation of cells that block T reg activity. Such “contrasuppressor” (CS) cells were proposed by Suzuki et al., who found that mice that were genetically resistant to the development of oral tolerance to sheep red blood cells possessed CS cells that when adoptively transferred prevented the development of oral tolerance to sheep red blood cells in mice that were genetically amenable to oral tolerance induction. We used this approach to determine if either nerve ablation or IV injection of SP elicited the development of leukocytes with CS cell activity. BALB/c mice were subjected to either trephining of one eye or a single IV injection of SP. Spleen cells were collected 14 days later, and one donor-equivalent of spleen cells (~5 × 10^7 cells) was injected intraperitoneally into naïve BALB/c recipients. One day later, ACAID was induced by AC injection of OVA followed by SC immunization with OVA emulsified in CFA. Assessment of DTH responses 10 days later revealed that spleen cells collected from mice that were subjected to either trephining or
IV injection of SP when transferred to naive recipients prevented the induction of ACAID in recipient mice (Fig. 5A). By contrast, adoptive transfer of spleen cells from naive donors had no effect on the induction of ACAID. Similar experiments were performed to determine if putative CS cells would affect the induction of oral tolerance. Spleen cells were collected from BALB/c mice that were subjected to trephining or IV injection of SP and were adoptively transferred to mice prior to oral administration of antigen. The results indicated that adoptive transfer of spleen cells from trephined donors prevented the induction of oral tolerance (Fig. 5B). By contrast, spleen cells from naive mice did not affect the induction of oral tolerance (Fig. 5B). Since neither trephining nor injection SP prevented the induction of IV-induced immune deviation, it was not surprising to find that adoptive transfer of spleen cells from either trephined mice or mice treated with IV injection of SP had no effect on the induction of IV-induced immune deviation (Fig. 5C). These results indicate that either corneal nerve ablation or injection of SP induces the development of cells with CS activity, which prevent either the induction or the expression of T reg activity induced by AC or oral administration of antigens. We next turned our attention to the question of whether CS cells prevent the suppressive effects of T regs that have already been induced.

**FIGURE 4.** Effect of trephining and SP on IV-induced immune deviation. (A) WT BALB/c mice were either trephined or injected IV with SP 4 days before IV-induced immune deviation was initiated. Mice were injected IV with OVA on days 0, 2, 4, and 6. Mice were immunized SC with OVA emulsified in complete Freund’s adjuvant 7 days after the final IV injection of OVA. Ear-swelling responses were assessed 7 days after SC immunization. (B) Similar experiments were performed on OVA TCR transgenic mice subjected to trephining or IV injection of SP. The results represent the mean ± SEM (5 mice/group). Results were similar in two additional experiments.

**FIGURE 5.** Effect of CS cells induced by trephining or SP injection on induction of immune tolerance. BALB/c mice were either trephined or injected IV with SP. Fourteen days later, mice were killed and one donor-equivalent of spleen cells from either naive mice or treated mice was transferred to naive BALB/c mice. One day later, spleen cell recipients were tested for their capacity to develop ACAID, oral tolerance, or IV-induced immune deviation. (A) ACAID was induced with AC injection of OVA. Seven days later mice were immunized SC with OVA emulsified in CFA. Ear-swelling responses were assessed 10 days after SC immunization. (B) Oral tolerance was induced by five daily oral administrations of CTB-conjugated C57BL/6 spleen cells. Mice were immunized SC with C57BL/6 spleen cells 1 day after the final oral administration of C57BL/6 spleen cells. Ear-swelling responses were assessed 10 days after SC immunization. (C) Intravenous injection of OVA was initiated 1 day after adoptive transfer of spleen cells and administered 2, 4, and 6 days later. Mice were immunized SC with OVA emulsified in CFA 7 days after the final IV injection of OVA. Ear-swelling responses were assessed 7 days after SC immunization. The results represent the mean ± SEM (5 mice/group). Results were similar in two additional experiments.
CS Cells Express the CD11c Dendritic Cell Marker

A wide variety of cells including antigen-presenting dendritic cells (DC) express NK1-R, which is the only known receptor for SP.33–36 Dendritic cells stimulated via the NK1-R undergo accelerated maturation; display increased expression of co-stimulatory molecules CD80, CD86, CD40, and MHC class II; and produce increased amounts of IL-12.36 Moreover, DC stimulated with an NK1-R agonist inhibit IL-10 production and preferentially promote the generation of Th1 immune responses.36 With this in mind, we tested the hypothesis that the SP produced in response to trephining activated DC, which in turn acted as the CS cells that prevented the induction and expression of ACAID T reg. Accordingly, spleen cells were collected from BALB/c mice that had been subjected to corneal trephining. CD11c+ cells were isolated using a Miltenyl Biotec pan dendritic cells isolation kit and were tested for CS activity in a LAT assay. CD11c+ cells from nontrephined mice and CD11c-depleted spleen cells from trephined mice were also tested for CS activity in the LAT assay using CD8+ T reg cells from mice primed in the AC with OVA. The results indicated that CD11c+ DC from trephined mice inhibited the suppressive activity of CD8+ ACAID T reg (Fig. 8A). By contrast, neither

Role of SP in CS Cell Activity

 Substance P is known to prevent the induction of ACAID when it is cojected with antigen into the AC.29 We have previously shown that SP prevents the suppressive function of corneal allograft-induced T reg once they have been induced.11 Accordingly, we tested the hypothesis that CS cells release SP, which is responsible for the ablation of ACAID T reg activity in the LAT assays described above (Fig. 6B). The ears of naive mice were injected with a mixture of CD8+ ACAID T reg, spleen cells from mice immunized SC with OVA, and spleen cells from mice subjected to trephining 4 days earlier. To block SP activity in situ, Spantide II (an NK1-R antagonist that inhibits SP signaling) was cojected with the cell mixtures in some of the LAT assays. As expected, coinjection of spleen cells from trephined mice prevented the suppression of ear-swelling responses by ACAID T reg (Fig. 7A). In situ inhibition of SP by Spantide II blocked CS cell activity and prevented CS cells from interfering with the suppression of DTH by CD8+ ACAID T reg. Additional experiments were performed to confirm that the SP was produced by the CS cells. Spleen cells were collected from WT C57BL/6 mice and SP−/− mice 4 days after trephining the corneas in the right eyes. Local adoptive transfer assays were performed to determine if the absence of the SP gene would prevent the induction of CS cells by trephining. As before, trephining induced the generation of CS cells in WT mice that blocked the suppressive activity of ACAID T reg. However, spleen cells from trephined SP−/− mice failed to inhibit ACAID T reg activity (Fig. 7B).
The results represent the mean ± SEM (5 mice/group).

**FIGURE 7.** CS cells require SP for their induction and function. CS cells were induced by trephining the OD eyes of naïve BALB/c mice or SP−/− C57BL/6 mice. Fourteen days later, spleen cells were collected and tested in a LAT assay for contrasuppressive activity against CD8+ ACAID Tregs. Adoptive cell transfer experiments were performed using CD11c+ DC from trephined mice to determine if these cells would prevent the induction of ACAID when adoptively transferred to naïve mice prior to AC priming with OVA. Recipients of adoptively transferred CD11c+ DC from trephined donors failed to develop ACAID (Fig. 8B). Like the LAT assays, the adoptive transfer experiments demonstrated that neither CD11c-depleted spleen cells from trephined donors nor CD11c+ DC from naïve mice affected the induction and expression of ACAID Tregs.

**DISCUSSION**

We have previously shown that circular incisions to the corneal epithelium transiently ablate corneal nerves and abolishes immune privilege for future orthotopic corneal allografts.11 The loss of immune privilege persists for at least 100 days. Since the serum half-life of SP is less than 2 minutes, detecting subtle changes in SP production is problematic.37,38 However, we were able to detect a steep, albeit transient, upregulation of NK1-R, the only known receptor for SP, in the anterior segment of eyes subjected to trephining and, to a lesser degree, in the contralateral nonmanipulated eyes. These findings are reminiscent of a previous report indicating that retinal laser burns (RLB) to one eye evoked a sharp increase in NK1-R expression in both the manipulated and the contralateral nonmanipulated eye.20 Importantly, RLB prevented the induction of ACAID in either the burned eye or the nonmanipulated eye and persisted for at least 68 days.26 The present findings indicate that trephining the corneal surface leads to a transient ablation of corneal nerves, which return to their normal density within 4 days. Our results also indicate that three separate manipulations can prevent the induction of ACAID, that is, corneal nerve ablation, penetrating keratoplasty, or a single injection of SP. The abrogation of ACAID occurred even when antigens were injected into the opposite eye that was not subjected to either trephining or orthotopic corneal transplantation. These findings are similar to observations in an earlier study by Streilein and coworkers,39 who also reported that either circumferential incisions in the cornea or penetrating keratoplasty prevented the induction of ACAID. However, in that study, antigens were injected into the manipulated eye but not into the nonmanipulated eye, and we can only speculate as to the outcome if the investigators had injected antigen into the opposite nonmanipulated eye.

The capacity of a circular corneal incision or a single injection of SP to rob corneal allografts of their immune privilege led us to suspect that corneal nerve ablation and SP exerted their effects systemically. With this in mind, we explored the effect of corneal nerve ablation, penetrating keratoplasty, and SP injection on other forms of immune tolerance that were induced at body sites distant from the eye. The results showed that each of these manipulations prevented the induction of oral tolerance, confirming our suspicion that the effects of corneal nerve ablation extend beyond the eye. However, the possibility exists that the abolition of ocular immune privilege by corneal nerve ablation is due to an anatomic effect that is hardwired through the sympathetic or parasympathetic nerves of the eye. Previous reports have shown that surgical removal of the superior cervical ganglion, which provides sympathetic innervation of the eye, abolishes ACAID.40 Along similar lines, Li and coworkers41 reported that chemical sympathectomy prevented the induction of ACAID. It is noteworthy that the trigeminal and vagus nerves are in close proximity to the corneal nerves. Thus, these findings suggest that corneal nerve ablation and SP may affect immune privilege at body sites distant from the eye in a manner similar to the ablation of sympathetic ganglia.
proximity within the brain stem, and it is possible that the effect of trephining affects the vagus nerve via an “inflammatory reflex arc” in which inflammatory stimuli originating in tissue innervated by sensory nerves (i.e., the eye) are relayed via the brain stem nuclei and are transmitted via the vagus nerve and terminates a homeostatic anti-inflammatory immune circuit. This in turn could lead to the termination of the homeostatic anti-inflammatory circuits that are normally intact and dampen inflammatory responses. Since an intact spleen is required for maintaining the immune privilege of corneal tissue innervated by sensory nerves (i.e., the eye) are relayed via the brain stem nuclei and are transmitted via the vagus nerve and terminates a homeostatic anti-inflammatory immune circuit. This in turn could lead to the termination of the homeostatic anti-inflammatory circuits that are normally intact and dampen inflammatory responses.

FIGURE 8. CS cells express the CD11c+ dendritic cell marker. CS cells were induced by trephining the right eyes of BALB/c mice on day 0. Spleen cells were collected 14 days later, and CD11c+ DC-enriched and CD11c-depleted spleen cells were isolated and used as putative CS cells in a LAT assay using ACAID Tregs induced by AC injection of OVA. (A) LAT assay for detecting CS cell activity. CS cells were induced by trephining the OD eyes of naive BALB/c mice. Fourteen days later, CD11c-enriched, CD11c-depleted, and unfractionated spleen cells were isolated and used as CS cells in a LAT assay. Right ears of naive BALB/c mice were injected with a mixture of CS cells, T regs, OVA immune spleen cells, and syngeneic APCs pulsed with OVA, and used in a LAT assay for in situ suppression of DTH. The opposite ear was injected with HBSS and served as a negative control. Ear-swelling responses were measured 24 hours later. (B) Adoptive transfer of CS cells. CS cells were induced by trephining the OD eyes of naive BALB/c mice. Fourteen days later CD11c-enriched, CD11c-depleted, and unfractionated spleen cells were isolated and one donor-equivalent was adaptively transferred to naïve BALB/c mice. One day later, ACAID was induced with AC injection of OVA. Seven days later, mice were immunized SC with OVA emulsified in CFA. Ear-swelling responses were assessed 10 days after SC immunization. The results represent the mean ± SEM (5 mice/group).

It bears noting that either nerve ablation or a single bolus injection of SP also abolishes mucosal tolerance, also known as oral tolerance. Oral tolerance and ACAID share a number of similarities. Both of these forms of immune tolerance require the participation of γδ T cells, TGF-β, and CD103 expression. Moreover, CD4+CD25+ Tregs and CD8+ Tregs are generated in both oral tolerance and ACAID, and as shown here, both of these forms of immune tolerance are abrogated by either trephining the cornea or the IV injection of SP. It is also noteworthy that induction of either oral tolerance or ACAID using donor alloantigenic cells results in a dramatic enhancement in the long-term survival of subsequent corneal allografts prepared from the same donors used for oral immunization or ACAID induction.

In the mid-1980s, considerable controversy surrounded discussions about “suppressor cells,” and many questioned their very existence. It would take almost a decade until the seminal studies of Sakaguchi and coworkers resurrected suppressor cells, but this time around they were assigned the name “T regulatory cells.” The notion that suppressor cells might be cross-regulated by another cell population called “contrasuppressor” cells was proposed by Gershon and coworkers at about the same time that the “suppressor cell” controversy was being debated. Like, suppressor cells, CS cells have enjoyed a renewed legitimacy and have been demonstrated in a number of models of immune regulation. Our results suggest that nerve ablation induces the generation of leukocytes that display CS activity and inhibit Tregs induced either by AC injection of antigens or by orthotopic corneal allografts. It is noteworthy that the CS cells are not antigen specific and their emergence is not antigen driven. That is, trephining the corneas of naïve mice induces the appearance of CS cells that disable Tregs that suppress immune responses to two unrelated antigens, OVA and B6 alloantigens. Interestingly, the CS cells inhibited both CD4+CD25+ Tregs induced by corneal allografts and CD8+ Tregs induced by AC injection of antigen (i.e., ACAID). Two pieces of evidence strongly suggest that these CS cells exert their effects by elaborating SP. First, in the LAT assay, blockade of NK1-R with Spantide II prevented the expression of contrasuppressive activity by putative CS cells induced by trephining. Second, spleen cells collected from SP−/− mice that had been subjected to trephining did not display CS activity in the LAT assay using CD8+ ACAID Tregs. It is possible either that SP is required for the generation of CS cells induced by trephining or that the CS activity of spleen cells from trephined donors is due to the release of SP by the CS cells, and SP directly exerts its effects on Treg function. T cells, macrophages, and DC have the capacity to produce and
respond to SP.\textsuperscript{62–66} Recently, Janelsins and coworkers\textsuperscript{36} reported that stimulation of CD11c\textsuperscript{+} DC with an NK1-R agonist led to their accelerated maturation, increased expression of costimulatory molecules (e.g., CD80, CD86, CD40), and upregulation of MHC class II molecules. Moreover, antigen-pulsed DC activated via NK1-R produced increased amounts of IL-12, inhibited the production of immunosuppressive IL-10, and induced a robust Th1 immune response.\textsuperscript{36} The present findings are consistent with the notion that the transient release of SP following corneal nerve ablation “licenses” CD11c\textsuperscript{+} DC that act as CS cells that disable T regs induced via corneal transplantation, ACAID, or mucosal routes of antigen administration.

One of the most puzzling aspects of the SP-induced loss of immune privilege is its longevity. Substance P has an extraordinarily short serum half-life, yet the abrogation of immune privilege that results from a single injection of SP persists for at least 100 days in mice, which is the rough equivalent of 7 years in a human’s life. Moreover, the dose of SP used in our studies was well below the normal baseline serum level of SP (48 pg/mL) in mice and indicates that the injections of SP in these experiments did not represent overwhelming nonphysiological concentrations of SP.\textsuperscript{67} This suggests either that the precursor cells from which T regs are generated have been categorically deleted as a result of the nerve ablation or that a long-lived cell is imprinted with the capacity to disable T regs. We are attracted to the latter hypothesis. Amadesi et al.\textsuperscript{67} reported that ischemia induced release of SP following corneal nerve ablation ‘‘findings are consistent with the notion that the transient injury and damage inflicted by foreign bodies. In addition to mechanical and foreign body–evoked responses, infections of the ocular surface elicit protective immune responses that terminate immune privilege. In this regard, it bears noting that two important causes of infectious keratitis, \textit{Pseudomonas} infections and herpes simplex virus (HSV) stromal keratitis, are immune-mediated diseases that are associated with SP activation.\textsuperscript{21–24} Moreover, inhibition of SP ameliorates both \textit{Pseudomonas} keratitis and HSV stromal keratitis.\textsuperscript{72–74} We propose that the termination of immune privilege is a drastic, albeit necessary, response to corneal infections and trauma to the ocular surface. Corneal HSV infections in T cell–deficient nude mice do not produce stromal keratitis and instead leave the cornea intact; however, the mice succumb to viral encephalitis and die as a result of the relentless progression of the viral infection.\textsuperscript{75} Thus, the development of an adaptive immune response to HSV corneal infections preserves life, but the cost is blindness. We propose that injury to the cornea produced by trephining is perceived by the adaptive immune response in the same manner as \textit{Pseudomonas} and HSV infections and elicits a burst of SP in both eyes as a means of abolishing immune privilege and releasing the full array of immune elements protecting against infectious agents that pose a threat to the survival of the host.

In the case of corneal trephining, the threat is more perceived than real. Thus, the immunologic imperative of the eye is to control life-threatening infections even if the cost is blindness.

Much remains to be learned about the CS cells induced in response to corneal nerve ablation. Future studies will need to determine how SP disables both CD4\textsuperscript{+}CD25\textsuperscript{+} and CD8\textsuperscript{+} T regs. It will also be important to determine if the CS cells are long-lived or if they promote the development of second-generation CS cells. Finally, it is tempting to speculate that CS cells might have an application for tumor immunotherapy.

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