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

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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

Corneal 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 T regs; these downregulate delayed-type hypersensitivity (DTH) responses to alloantigens that are expressed on the corneal allograft.[12–14] Oral administration of alloantigens also induces T regs that produce a similar downregulation of allospecific DTH and enhance corneal allograft survival by a process frequently referred to as oral tolerance.[15,16] A form of immune tolerance called intravenous immune deviation is invoked when either soluble antigens or alloantigenic cells are injected intravenously.[17] Intravenous injection of alloantigens induces T regs that support a temporary form of immune tolerance and transient suppression of skin allograft rejection.[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.

Materials and Methods

Animals

C57BL/6 (H-2^b) and BALB/c (H-2^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 substance P (SP) knockout (KO) mice on a C57BL/6 background were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). The animal studies were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Animals were housed and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Orthotopic Corneal Transplantation

BALB/c mice received full-thickness orthotopic corneal grafts from C57BL/6 as previously described.[18] Corneal grafts were examined two or three times a week for opacity, neovascularization, and edema with a slit-lamp biomicroscope (Carl Zeiss, Oberkochen, Germany). The degree of opacification ranged between 0 and 4+, with 0 = clear; 1+ = minimal superficial opacity; 2+ = mild deep stromal opacity with pupil margin and iris visible; 3+ = moderate stromal opacity with pupil margin visible but iris structure obscured; and 4+ = complete opacity, with pupil and iris totally obscured. Clinical scores based on opacity were assessed until the allograft was determined as rejected. Corneal grafts were considered rejected upon two successive opacity scores of 3+ or greater.[19] No immunosuppressive drugs or topical corticosteroids were used in any of these experiments. Median survival times (MSTs) were used to determine statistical significance between groups.

Subcutaneous Immunization and SP Injections

Positive control mice were sensitized by one subcutaneous (SC) injection of either 1×10^6 C57BL/6 splenocytes in 100 μL Hank's balanced salt solution (HBSS) or 250 μg OVA (Sigma-Aldrich Corp., St. Louis, MO, USA) emulsified 1:1 in Complete Freud's Adjuvant (CFA; Sigma-Aldrich Corp.). Each SC injection was in a total volume of 200 μL.[20] In some experiments, a single intravenous (IV) injection of 1 pg substance P (Sigma-Aldrich Corp.) was administered to BALB/c mice prior to their use in either ACAID or oral tolerance investigations.

Delayed-Type Hypersensitivity Assay

Delayed-type hypersensitivity was measured using a conventional ear-swelling assay. An eliciting dose of 4×10^6 mitomycin C–treated (400 μg/mL) C57BL/6 spleen cells or 400 μg OVA in 20 μL HBSS was injected into the SC tissue of the right ear. The left ear served as a negative control and was injected with 20 μL 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).

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.

Trephined eyes of BALB/c mice were assessed for the presence and integrity of corneal nerves. Enucleated eyes from mice were treated with Dispase II for 2 hours at room temperature and then fixed in 4% paraformaldehyde, washed with PBS, permeabilized, and blocked in 1% BSA in PBS and 0.2% Triton X-100 for 2 hours at room temperature. Corneas were incubated with rabbit anti-mouse β III tubulin primary antibody (TUJ1) IgG (Covance, Richmond, CA, USA), washed in PBS, and incubated with propidium iodide and secondary antibody (Alexa 488 goat anti-rabbit IgG; Invitrogen, Carlsbad, CA, USA) for 2.5 hours at room temperature. Images were captured on a Leica TCS SP8 (Buffalo Grove, IL, USA).

Quantitative Real-Time PCR (q-RT-PCR)

The anterior segment of the eyes of trephined mice or the unmanipulated contralateral eyes from five donors were excised and placed on ice in 700 mL complete RPMI (cRPMI). The tissue was homogenized and total RNA was isolated using 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 preformulated 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.

Intravenously Induced Immune Deviation

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

Oral Tolerance Induction

Oral tolerance to alloantigens was induced as previously described.[21] C57BL/6 spleen cells were conjugated with the nontoxic B subunit of cholera toxin (CTB; Sigma-Aldrich Corp.) by incubating 1×10^7 C57BL/6 spleen cells with 100 μg 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, 2×10^6 allogeneic spleen cells were administered directly into the stomach using a gastric gavage tube for 5 consecutive days. Animals were immunized

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SC with 1×10^6 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.[22] A Hamilton (Whittier, CA, USA) automatic dispensing apparatus was used to inject 6 μL 16.67 mg/mL OVA (Sigma-Aldrich Corp.) in PBS (100 μg OVA) into the AC. Seven days after the AC injection, animals were SC immunized with 200 μg OVA in an equal volume of CFA. Ear was challenged 7 days after SC immunization by injecting OVA (400 μg in 20 μL PBS). The opposite ear was injected with 20 μL 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.[23] Corneal allograft–induced $CD4^+CD25^+$ T regs or ACAID $CD8^+$ T regs (1×10^6) 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 naïve BALB/c mice were injected with 20 μL of the mixed-cell population. The opposite ear was injected with 20 μL HBSS as a negative control. Ear swelling was measured 24 hours later to measure DTH. To block SP signaling, 72 μg 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.[9] 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 $Foxp3^+$.[9]

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.[24] 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.[25]

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.[11] 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 β 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.[11] Interestingly, laser burns to the retina induce an upregulation in NK1-R, the receptor for SP, which is associated with the abrogation of ACAID.[26,27] 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.[11] 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.[28] 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.[26] With this in mind, we examined the effect of trephining, SP, and orthotopic corneal transplantation on the induction of ACAID in both eyes.

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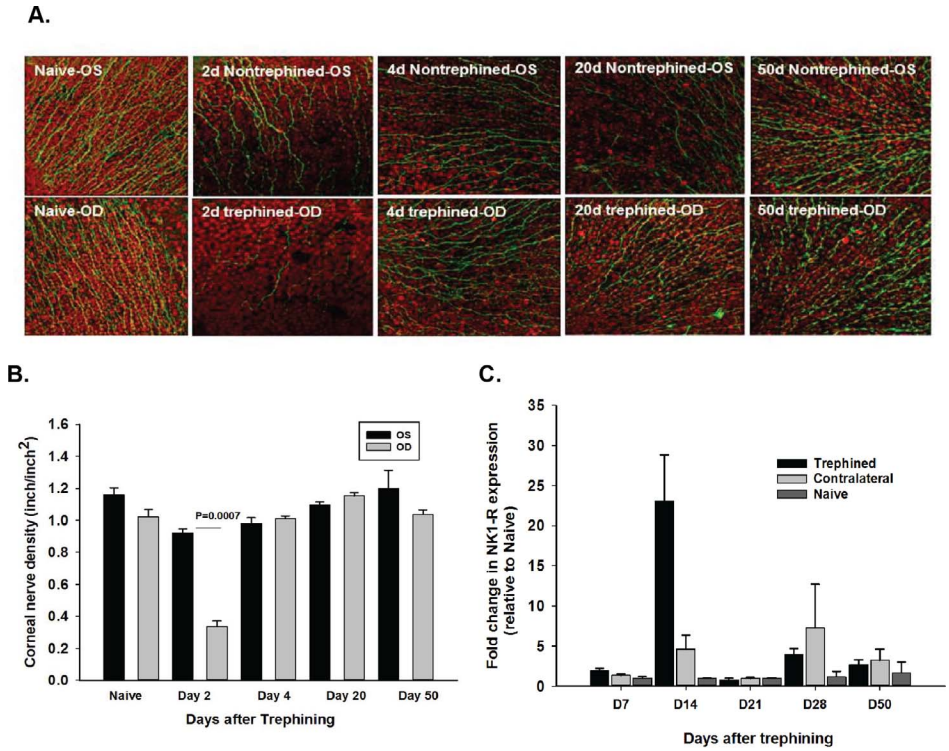

Figure 1. Effect of trephining on integrity of corneal nerves. (**A**) Corneas of the right eyes of BALB/c mice were trephined and the corneas of both eyes were removed 2, 4, 20, and 50 days later and were stained with rabbit anti-mouse β III tubulin primary antibody. (**B**) Corneal nerve density was quantified using ImageJ analysis. (**C**) The anterior segment (cornea, iris, and ciliary body) of the trephined or unmanipulated contralateral eye was excised and assessed by q-PCR for NK1-R message at the times indicated. The results represent the mean ± SEM (5 mice/group). Results were similar in two additional experiments.

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.[11] 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

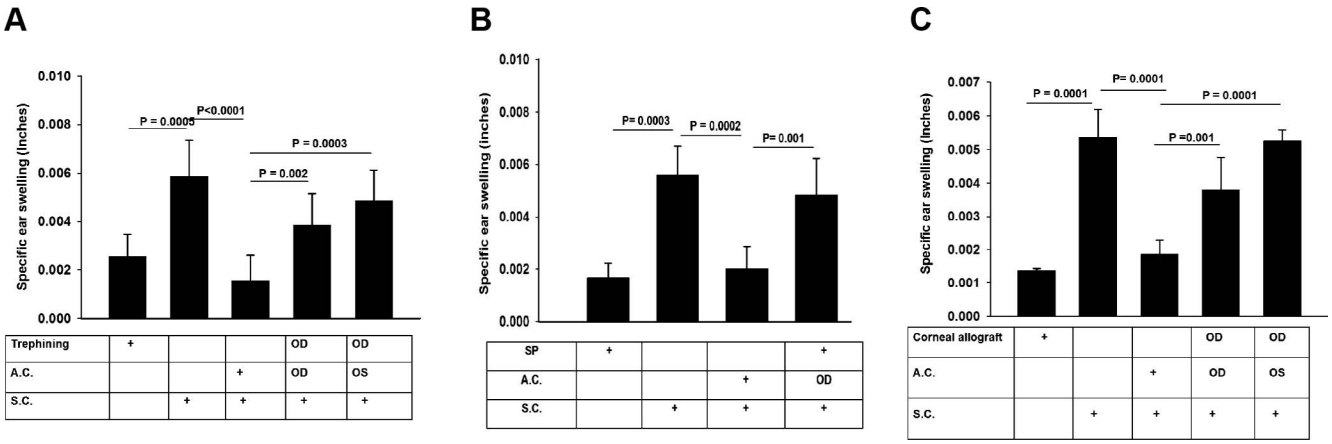

Figure 2. Effect of trephining, SP injection, or penetrating keratoplasty on the induction of ACAID. (**A**) Corneas of the right eye (OD) were trephined 4 days prior to AC injection of OVA into either the OD or OS eyes. (**B**) SP (1.0 pg in 100 μL PBS) was injected IV 4 days prior to AC injection of OVA into either the OD or OS eyes. (**C**) Orthotopic C57BL/6 corneal allografts were transplanted to the OD eye 21 days prior to AC injection of OVA into either the OD or OS eyes. Mice were immunized SC with OVA emulsified in CFA 10 days after AC injection of OVA. Ear-swelling responses were assessed 7 days after SC immunization with OVA. The results represent the mean ± SEM (5 mice/group). Results were similar in two additional experiments.

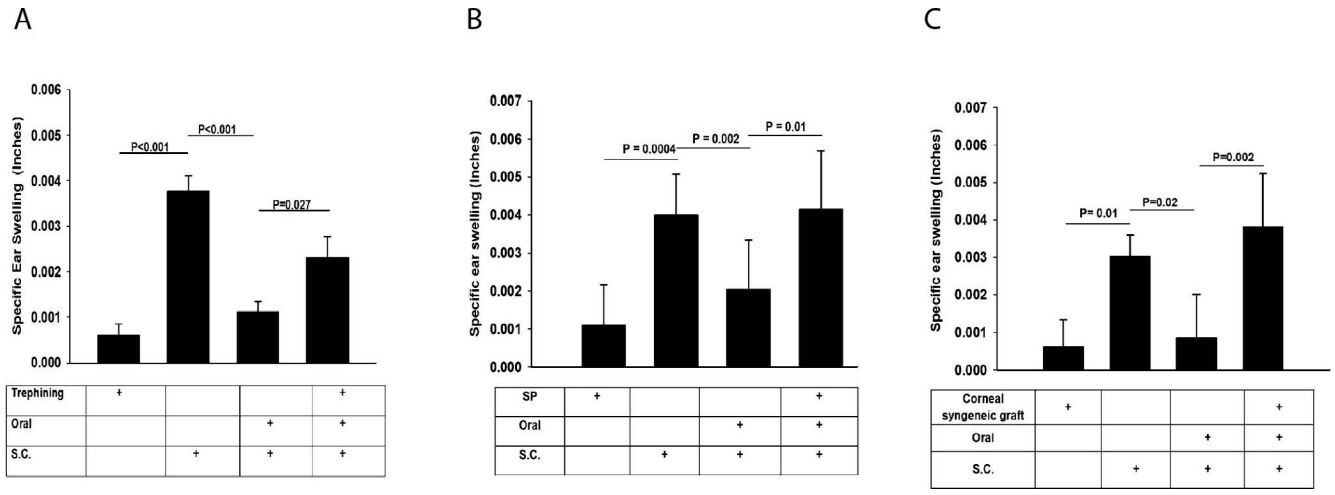

Figure 3. Effect of trephining, substance P, and orthotopic corneal transplantation on oral tolerance. Oral tolerance with OVA was initiated in BALB/c mice (**A**) 4 days after trephining the corneas of the right eyes; (**B**) 4 days after IV injection of SP; or (**C**) 21 days after applying an orthotopic syngeneic BALB/c corneal transplant to the right eye. BALB/c mice received CTB-conjugated C57BL/6 spleen cells for 5 consecutive days. One day after the fifth oral immunization, mice were immunized SC with C57BL/6 spleen cells. Ear-swelling responses were assessed 10 days after SC immunization. The results represent the mean ± SEM (5 mice/group). Results were similar in two additional experiments.

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.[29,30] 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.[29,30] 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 1×10^6 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 privilege 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.[31] 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.,[32] 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 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 ($\sim 5 \times 10^7$ 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

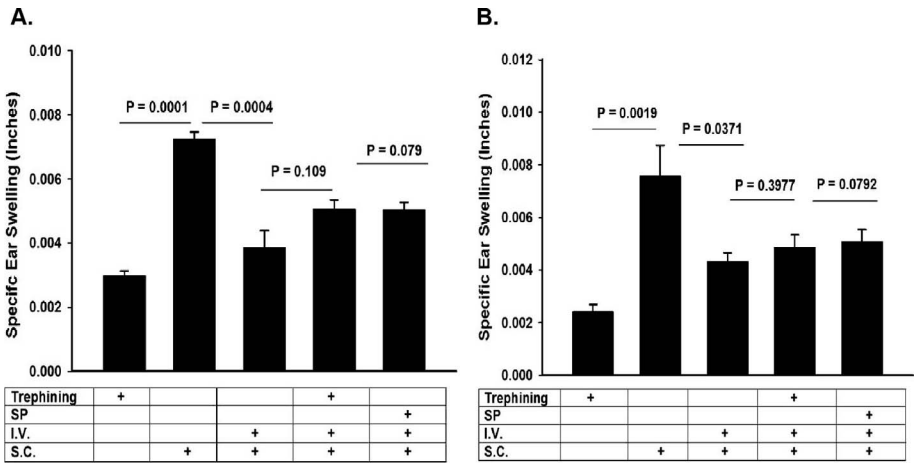

Figure 4. Effect of trephining and SP on IV-induced immune deviation. (**A**) WT BALB/c mice were either trephined or injected IV with SP 4 days before IV-induced immune deviation was initiated. Mice were injected IV with OVA on days 0, 2, 4, and 6. Mice were immunized SC with OVA emulsified in complete 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 of 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.

IV injection of SP when transferred to naïve recipients prevented the induction of ACAID in recipient mice (Fig. 5A). By contrast, adoptive transfer of spleen cells from naïve 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 naïve 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.

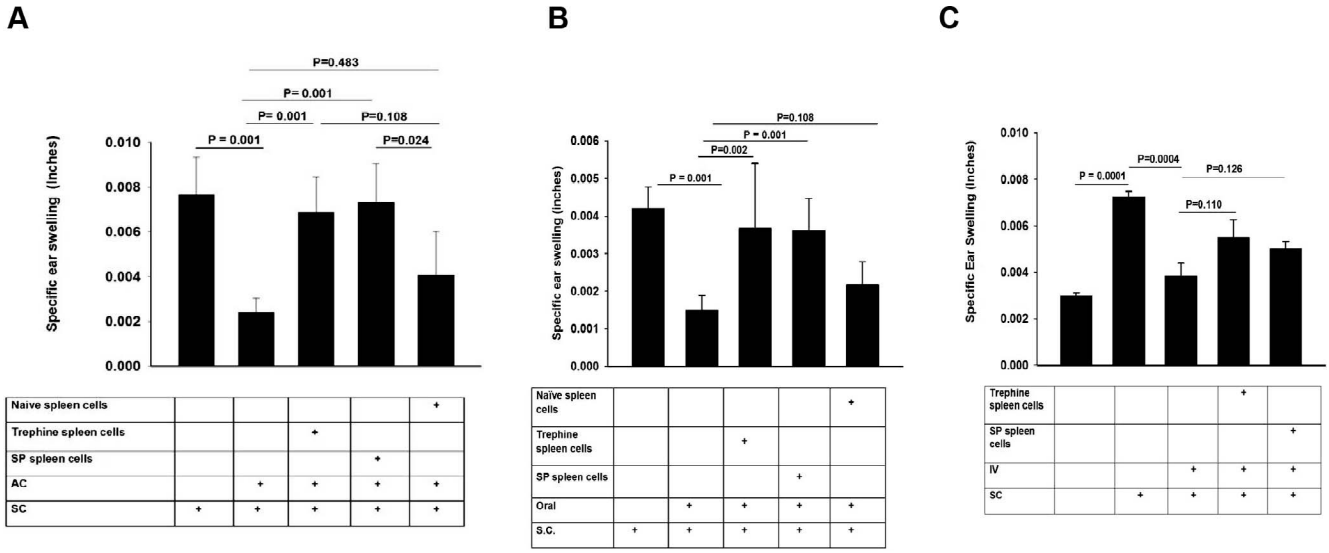

Figure 5. Effect of CS cells induced by trephining or SP injection on induction of immune tolerance. BALB/c mice were either trephined or injected IV with SP. Fourteen days later, mice were killed and one donor-equivalent of spleen cells from either naïve mice or treated mice was transferred to naïve 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.

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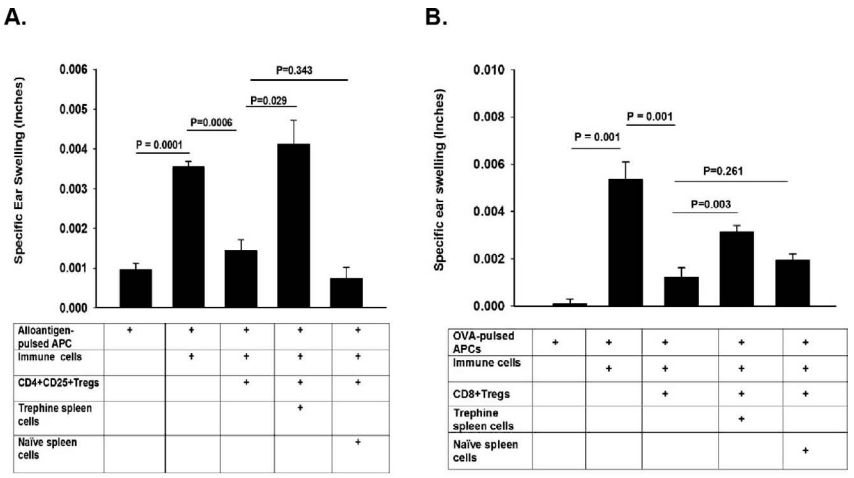

Figure 6. In situ blockade of T reg activity by CS cells induced by trephining. CS cells were induced by trephining the right eyes of BALB/c mice. Fourteen days later, spleen cells were collected and used in a LAT assay for in situ suppression of DTH. Right ears of naïve BALB/c mice were injected with a mixture of CS cells, T regs, immune spleen cells (OVA immunized or C57BL/6 alloimmunized), and BALB/c APCs pulsed with either C57BL/6 alloantigens or OVA. The opposite ear was injected with HBSS and served as a negative control. Ear-swelling responses were measured 24 hours later. (**A**) Contrasuppression of corneal allograft–induced $CD4^+CD25^+$ T regs and (**B**) $CD8^+$ ACAID T regs. The results represent the mean ± SEM (5 mice/group). Results were similar in two additional experiments.

The LAT assay is a convenient tool for demonstrating in situ suppressive activity of T regs that have already been induced. Immune T cells coinjected with antigen-pulsed APC into the ears of naïve mice produce significant antigen-specific ear-swelling DTH responses. However, coinjection of T regs from hosts with longstanding corneal allografts or from mice primed in the AC with antigens prevents these ear-swelling responses and is a highly sensitive assay for detecting T reg activity in situ.[23] To determine if CS cells blocked T regs that had already been induced, a LAT assay was performed using T regs that were isolated from either BALB/c mice harboring longstanding C57BL/6 corneal allografts or mice primed in the AC with OVA. T regulatory cells were coinjected with BALB/c APC pulsed with either C57BL/6 alloantigens or OVA, along with immune cells from mice subcutaneously immunized with either C57BL/6 alloantigenic cells or OVA, respectively. Local adoptive transfer assays demonstrated that C57BL/6 corneal allograft–induced T regs prevented ear-swelling responses produced by lymphocytes from mice immunized with C57BL/6 alloantigens (Fig. 6A). However, coinjection of spleen cells from trephined donor mice prevented the suppressive activity of corneal allograft–induced T regs. Importantly, spleen cells from naïve mice did not affect the suppressive activity of corneal allograft–induced T regs. Similar results were found using T regs isolated from mice primed in the AC with OVA. Coinjection of spleen cells from trephined mice, but not spleen cells from naïve mice, blocked the suppressive activity of T regs from AC-primed donors (Fig. 6B). Since the CS cell activity was generated by trephining alone, the blockade of T reg activity is not antigen specific.

Role of SP in CS Cell Activity

Substance P is known to prevent the induction of ACAID when it is coinjected with antigen into the AC.[28] We have previously shown that SP prevents the suppressive function of corneal allograft–induced T regs 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 naïve mice were injected with a mixture of $CD8^+$ ACAID T regs, 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 coinjected 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 regs (Fig. 7A). In situ inhibition of SP signaling by Spantide II blocked CS cell activity and prevented CS cells from interfering with the suppression of DTH by $CD8^+$ ACAID T regs. 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 regs. However, spleen cells from trephined $SP^{-/-}$ mice failed to inhibit ACAID T reg activity (Fig. 7B).

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 regs. 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 regs 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 regs (Fig. 8A). By contrast, neither

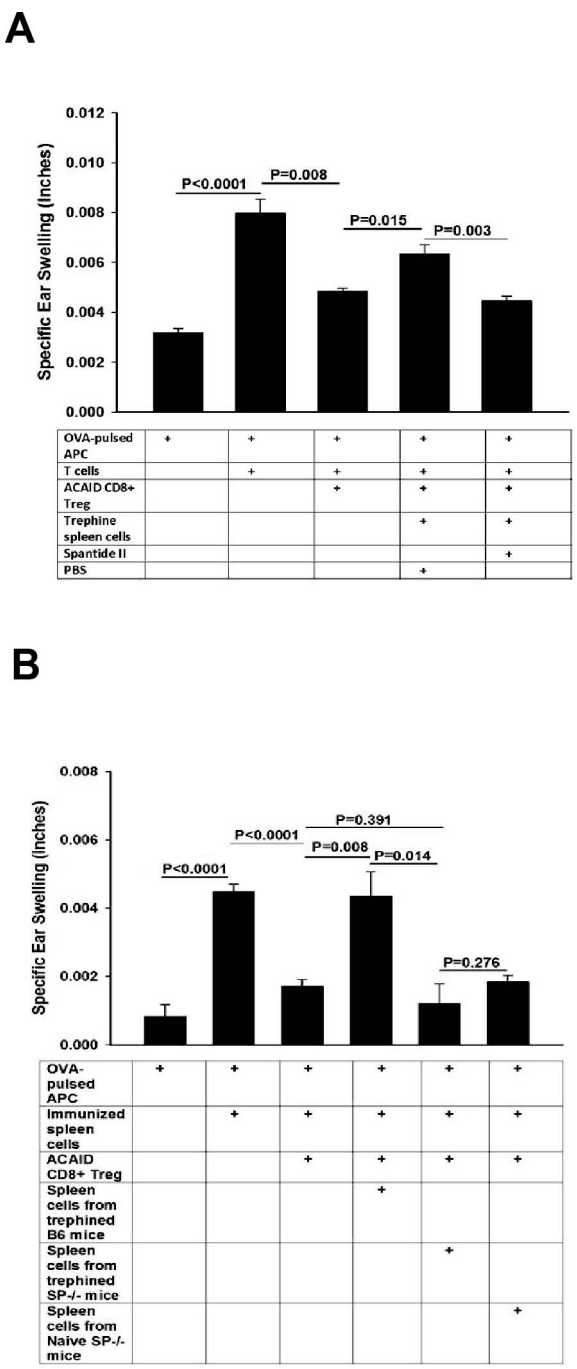

Figure 7. CS cells require SP for their induction and function. CS cells were induced by trephining the OD eyes of naïve BALB/c mice or $SP^{-/-}$ C57BL/6 mice. Fourteen days later, spleen cells were collected and tested in a LAT assay for contrasuppressive activity against CD8+ ACAID T regs induced with OVA. Right ears of naïve BALB/c mice were injected with a mixture of CS cells, T regs, OVA immune spleen cells, syngeneic APCs pulsed with OVA, and either HBSS or 72 μg Spantide II. The opposite ear was injected with HBSS and served as a negative control. Ear-swelling responses were measured 24 hours later. (**A**) LAT assays using splenic CS cells induced by trephining the eyes of BALB/c mice in the presence or absence of 72 μg Spantide II. (**B**) LAT assays were also performed with putative CS cells isolated from the spleens following trephining the eyes of either $SP^{-/-}$ or C57BL/6 WT mice. The results represent the mean ± SEM (5 mice/group).

CD11c-depleted spleen cells from trephined mice nor $CD11c^+$ DC from nontrephined naïve mice affected the suppressive activity of $CD8^+$ ACAID T regs. 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 T regs.

Discussion

We have previously shown that circular incisions to the corneal epithelium transiently ablate corneal nerves and abolish 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.[26] 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 coworkers[41] reported that chemical sympathectomy prevented the induction of ACAID. It is noteworthy that the trigeminal and vagus nerves are in close

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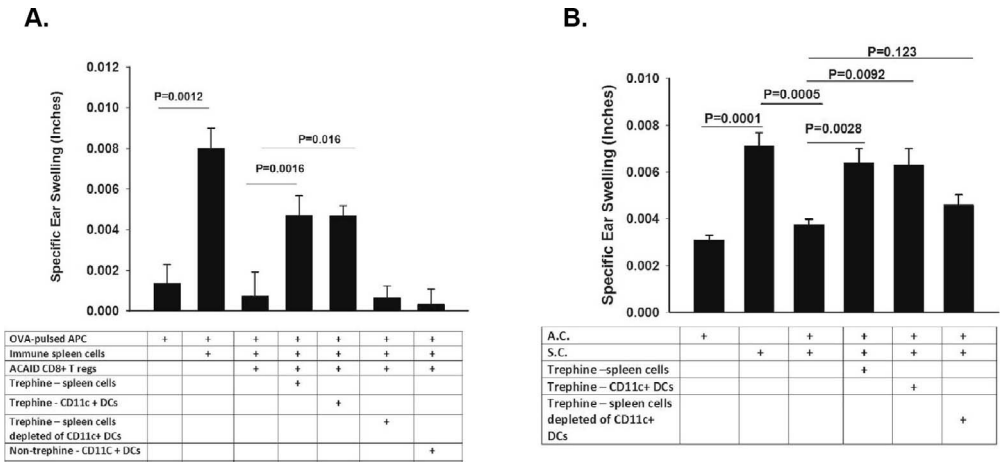

Figure 8. CS cells express the CD11c+ dendritic cell marker. CS cells were induced by trephining the right eyes of BALB/c mice on day 0. Spleen cells were collected 14 days later, and CD11c+ DC-enriched and CD11c-depeleted spleen cells were isolated and used as putative CS cells in a LAT assay using ACAID T regs induced by AC injection of OVA. (**A**) LAT assay for detecting CS cell activity. CS cells were induced by trephining the OD eyes of naïve 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 naïve 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 naïve BALB/c mice. Fourteen days later CD11c-enriched, CD11c-depleted, and unfractionated spleen cells were isolated and one donor-equivalent was adoptively 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).

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.[42,43] Since an intact spleen is required for maintaining the immune privilege of corneal allografts[44,45] and for the induction of ACAID,[46] it is tempting to speculate that the effect of trephining on immune privilege is due in part to a disruption of this "anti-inflammatory reflex arc."

Intravenously induced immune deviation has been recognized for over 30 years, and its discovery predated the first reports of ACAID.[47] The conspicuous absence of patent lymph vessels draining the AC of the eye led some investigators to suspect that ACAID was merely another form of IV-induced immune deviation and was the result of antigens escaping the AC via the trabecular meshwork, which drains directly into the venous circulation. However, subsequent studies have demonstrated numerous fundamental differences between IV-induced immune deviation and ACAID.[48] The results reported here add to the list of differences between IV-induced immune deviation and ACAID and reveal that neither corneal nerve ablation nor IV injection of SP prevents IV-induced immune deviation.

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[49,24]; TGF-β[50–52]; and CD103 expression.[53,54] Moreover, $CD4^+CD25^+$ T regs and $CD8^+$ T regs are generated in both oral tolerance and ACAID,[55–57] and as shown here, both of these forms of immune tolerance are abrogated by either trephining the cornea or the IV injection of 1.0 pg SP. It 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.[5,16,30,12–14]

In the mid-1980s, considerable controversy surrounded discussions about "suppressor cells," and many questioned their very existence.[58] It would take almost a decade until the seminal studies of Sakaguchi and coworkers[59] 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[60] 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.[61] Our results support the notion that nerve ablation induces the generation of leukocytes that display CS activity and inhibit T regs 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 T regs that suppress immune responses to two unrelated antigens, OVA and B6 alloantigens. Interestingly, the CS cells inhibited both $CD4^+CD25^+$ T regs induced by corneal allografts and $CD8^+$ T regs 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 T regs. 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 T reg function. T cells, macrophages, and DC have the capacity to produce and

respond to SP.[62–66] Recently, Janelsins and coworkers[36] reported that stimulation of CD11c$^+$ 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.[36] The present findings are consistent with the notion that the transient release of SP following corneal nerve ablation "licenses" CD11c$^+$ 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.[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.[67] reported that ischemia induced by limb ligation in mice elicited the secretion and localization of SP in the areas of ischemic necrosis. Interestingly, NK1R$^+$ bone marrow–derived cells with progenitor cell markers (e.g., c-Kit$^+$, Sca1$^+$, Lin$^-$) migrated to and preferentially accumulated in the areas of necrosis where SP levels were elevated. Coinciding with the accumulation of these putative progenitor cells in the necrotic area was a remarkable increase in arteriogenesis and improved blood flow in the ischemic limb. We favor the hypothesis that trephining elicits the production of SP, which in turn elicits the migration of bone marrow–derived hematopoietic progenitor cells that are self-sustaining and transform into CS cells. This is in keeping with previous findings indicating that DC respond to stimulation with NK1-R agonists, such as SP, with increased cell survival and enhanced migration to regional lymph nodes[68]—and is also consistent with the observation that the trephined-induced CS cell activity was demonstrable in a CD11c$^+$ cell population within the spleen (i.e., DC or macrophages). The accumulation of CS cells in the spleen in trephined mice is also consistent with the aforementioned "anti-inflammatory immune reflex arc" that has been shown in other systems to be hardwired through the vagus nerve and involves the participation of the spleen.[42,43] The notion that SP elicits the generation of a long-lived or self-sustaining progenitor cell population imprinted with CS activity would explain how a molecule such as SP with an ephemeral half-life could lead to a permanent loss of immune privilege.

One wonders how the elaboration of SP following injury to corneal nerves would benefit the host and why it would be advantageous to terminate immune privilege in the eye. We and others have suggested that immune privilege is an adaptation to limit unbridled immune-mediated inflammation in an organ, such as the eye, that has a limited capacity for regeneration.[69,70] We propose that the dense innervation of the cornea is an adaptation to provide sensitive and swift responses to injury to the ocular surface. The threshold for stimulating corneal nerves is low, and even minor mechanical stimuli evoke avoidance responses, blinking, and the secretion of tears, all of which protect the eye from mechanical 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, *Pseudomonas* infections and herpes simplex virus (HSV) stromal keratitis, are immune-mediated diseases that are associated with SP activation.[71–74] Moreover, inhibition of SP ameliorates both *Pseudomonas* keratitis and HSV stromal keratitis.[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.[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 *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$^+$CD25$^+$ and CD8$^+$ 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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