Protective Role of ICOS and ICOS Ligand in Corneal Transplantation and in Maintenance of Immune Privilege

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Purpose. The interaction between the inducible costimulatory molecule (ICOS) and ICOS ligand (ICOSL) has been implicated in the differentiation and functions of T cells. The purpose of the present study was to determine the role of ICOS-ICOSL in the immune privilege of corneal allografts.

Methods. Expression of ICOS and ICOSL mRNA from mouse eyes was assessed by RT-PCR. Corneas of C57BL/6 mice were orthotopically transplanted into the eyes of $ICOS^{-/-}$ BALB/c recipients and BALB/c wild-type (WT) recipients treated with anti-ICOSL mAb, and graft survival was assessed. A separate set of WT and $ICOS^{-/-}$ BALB/c mice received an anterior chamber injection of C57BL/6 splenocytes, and induction of allospecific anterior chamber–associated immune deviation (ACAID) was assessed. In vitro, cornea was incubated with T cells from WT and $ICOS^{-/-}$ BALB/c mice, and destruction of corneal endothelial cells (CECs) and the population of $Foxp3^{+}$ $CD25^{+}$ $CD4^{+}$ T cells was assessed.

Results. Inducible costimulatory molecule ligand mRNA was constitutively expressed in the cornea, iris-ciliary body, and retina. Allograft survival in $ICOS^{-/-}$ recipients and WT recipients treated with anti-ICOSL mAb was significantly shorter than in control recipients. Anterior chamber–associated immune deviation was induced less efficiently in $ICOS^{-/-}$ mice. Destruction of CECs by alloreactive $ICOS^{-/-}$ T cells was enhanced compared with WT T cells. After coincubation with allogeneic corneal tissue, the proportion of regulatory T cells was significantly greater among WT T cells than in $ICOS^{-/-}$ T cells.

Conclusions. The expression of ICOSL in the cornea and the ICOS-mediated induction of $Foxp3^{+}$ $CD4^{+}$ regulatory T cells may contribute to successful corneal allograft survival.

Keywords: ICOS, immune privilege, ACAID

The eye is an immune-privileged organ, similar to the brain and reproductive organs.[1,2] Inflammation is automatically controlled by the immune privilege, and as a result, homeostasis in the eye is maintained. Rejection after corneal transplantation is less frequent compared with after transplantation of other organs, and this is generally attributable to immune privilege.[1,2] However, if corneal transplantation is performed on eyes that have been deprived of immune privilege, the rejection rate becomes as high as with heart and skin transplantation.[1–3] Thus, immune privilege is important for successful corneal transplantation. In recent years, through analysis focused on animal models of corneal transplantation, the mechanism of immune privilege in the eye has become clear. Currently, the molecular mechanism of the immune privilege in the eye is due to three factors: anatomical barrier, anterior chamber–associated immune deviation (ACAID), and the immune-suppressive ocular microenvironment.[1,4]

Our group has reported some of the molecules that contribute to ACAID and the immune-suppressive ocular microenvironment for establishment of immune privilege for corneal transplantation. B7-H1,[5] glucocorticoid-induced tumor necrosis factor receptor family–related protein ligand (GITR-L),[6] and galectin-9[7] are involved in immune suppression in the cornea and are essential for immune privilege for corneal transplantation. However, the molecular mechanisms of immune privilege in the eye are not completely clear. To further clarify the mechanism of immune privilege for corneal transplantation, further immunologic analysis of new molecules is important.

A third member of the CD28/CTLA4 family of costimulatory molecules, inducible costimulator (ICOS), was identified.[8] Inducible costimulator is expressed on activated T cells and resting memory T cells.[8–10] Inducible costimulator ligand (ICOSL), which has homology to B7 molecules and is called B7-related protein-1 (B7RP-1), is expressed on B cells and macrophages.[9,11] Inducible costimulator stimulation is important for T-cell activation, and ICOS may play a particularly important role in the development of Th2 cells.[12] Inducible costimulator blockade has opposite effects on immune responses depending on disease models and on the timing of the blockade. Inducible costimulator–ICOSL signal blockade during antigen priming impairs Th2 development and leads to Th1 polarization, hence exacerbating autoimmune diseases, such as experimental autoimmune encephalomyelitis, as well as

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acute graft-versus-host disease.[13,14] Also, the timing of ICOS blockade significantly influences therapeutic effects.[15] In a cardiac allograft model, delayed ICOS blockade prolongs allograft survival, whereas early ICOS blockade accelerates rejection.[16,17]

Various studies have investigated the relationship between regulatory T cells (Tregs) and ICOS. Blockade of ICOS-ICOSL results in a decrease in the generation of $CD4^+$ Tregs, suggesting that ICOS-ICOSL is essential for the generation of $CD4^+$ Tregs.[18] Antibody (Ab)-mediated blockade or genetic deficiency of ICOS selectively abrogates Treg-mediated functions.[19] Inducible costimulator is part of a key costimulatory pathway that restrains islet autoimmunity by specifically affecting the homeostasis and function of $Foxp3^+$ Tregs in prediabetic islets of nonobese diabetic mice.[19]

The involvement of ICOS-ICOSL in immune privilege of the eye is still not well understood. Here we examined the role of ICOS-ICOSL in immune privilege of the eye using a well-established murine model of corneal transplantation.

Materials and Methods

Mice and Anesthesia

Male BALB/c, C57BL/6, and C3H/He mice were purchased from Sankyo Lab Service (Tokyo, Japan) and used at 8 to 10 weeks of age. The Science University of Tokyo (Noda City, Chiba, Japan) provided $ICOS^{-/-}$ mice on the BALB/c background, which have been described previously.[20] Mice were treated according to the ARVO guidelines on the use of animals in research. The protocol of this animal study was reviewed and approved by our institutional review committee. Before all surgical procedures, each mouse was anesthetized by intramuscular injection of a mixture of 3.75 mg ketamine and 0.75 mg xylazine.

Antibodies and Flow Cytometry

For immunofluorescent staining and flow cytometry, mAbs against CD4 (GK1.5, rat IgG2b, κ), CD25 (PC61.5, rat IgG1), Foxp3 (FJK-16s, rat IgG2a), and ICOS (C398.4A, Armenian hamster IgG) were used. All FITC-, phycoerythrin (PE)-, or biotin-conjugated mAbs and isotype control IgGs were obtained from eBioscience (San Diego, CA, USA). Culture supernatant from the 2.4G2 hybridoma (anti-CD16/CD32 mAb) was used to block nonspecific binding. Stained cells were analyzed with a flow cytometer (BD FACSCanto 2, CellQuest software; BD Biosciences).

Reverse-Transcription PCR

Cornea, iris-ciliary body (CB), and neural retina were isolated from a total of 10 normal BALB/c and C57BL/6 mouse eyes. Total RNA was extracted from each tissue using STAT 60 (TEL-Test, Inc., Friendswood, TX, USA). First-strand cDNA was prepared by using the SuperScript First Strand Synthesis System (Invitrogen Life Technologies, Waltham, MA, USA). Standardization of cDNA samples was based on the content of beta-actin cDNA. Primers for mouse beta-actin cDNA were 5′-ACAATGAGCTGCGTGTGGCC-3′ and 5′-ACGGCCAGGTCAT CACTATTG-3′. Polymerase chain reaction was performed by using mouse ICOS- and ICOSL-specific primers. The sequences of sense and antisense primers were as follows: ICOS, 5′-GAAATGCGGTGTCCATCAAGAA-3′ and 5′-TATCACTTATGTA CAAGTACCGC-3′; and ICOSL, 5′-GATCAATGTGGACAGTTCC TAC-3′ and 5′-GTCTCGGGTTGGACATAACCT-3′. Polymerase chain reaction was performed in a total volume of 20 μL in PCR buffer in the presence of 0.2 mM dNTP, 1 μM each primer, and 1 U Taq DNA polymerase (Advanced Biotechnologies, Eldersburg, MD, USA). After 32 cycles of amplification, the PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. The PCR conditions were as follows: 94°C for 5 minutes (60°C for 2 minutes, 72°C for 1 minute, 94°C for 1 minute) × 32 cycles, 60°C for 2 minutes, and 72°C for 10 minutes.

Orthotopic Corneal Transplantation and Treatment

Penetrating keratoplasty was performed as previously described.[21] C57BL/6 mice were used as donors, and wild-type (WT) or $ICOS^{-/-}$ BALB/c mice were used as recipients. Normal corneas of C57BL/6 mice were transplanted orthotopically into normal eyes of BALB/c mice. Donor corneas (2-mm diameter) were placed in the same-sized recipient bed with eight interrupted 11-0 nylon (Mani, Tochigi, Japan) sutures, which were removed 7 days after grafting. After the grafting procedure, WT recipients were intraperitoneally administered 0.25 mg anti-ICOSL mAb (HK5.3)[22] or control rat IgG (Sigma-Aldrich Corp., St. Louis, MO, USA) three times a week for 3 weeks. Syngeneic transplantation using WT BALB/c donor corneas into an $ICOS^{-/-}$ BALB/c recipient was also performed as a control.

Evaluation of Corneal Allografts

Grafts were evaluated by slit lamp microscopy and scored for opacity twice a week. A masked assessment of orthotopic corneal grafts was performed by a single observer (H.J.), who evaluated each graft for survival according to a previously reported scoring system that defines graft survival. Grafts with a score of 2+ (mild stromal opacity with iris structure visible) or greater after 3 weeks were considered rejected.[21,23]

Quantification of mRNA by Quantitative Real-Time PCR

Corneas were isolated from the syngeneic graft and allograft. Total RNA was extracted from each tissue using STAT 60. Reverse transcription was performed using a ThermoScript RT-PCR System (Invitrogen Life Technologies). The sequences of sense and antisense primers were as follows: ICOS, 5′-GAAAT GCGGTGTCCATCAAGAA-3′ and 5′-TATCACTTATGTACAAGTA CCGC-3′; and ICOSL, 5′-GATCAATGTGGACAGTTCCTAC-3′ and 5′-GTCTCGGGTTGGACATAACCT-3′. The amount of total ICOS and ICOSL mRNA amplified with each primer pair was quantified using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously[22] with TaqMan gene expression products (Applied Biosystems) and TaqMan Universal Master Mix (Applied Biosystems). The expression level of ICOS and ICOSL was defined as the ratio to that of beta-actin by calculation of cycle threshold values in amplification plots using 7500 SDS software (Applied Biosystems, Waltham, MA, USA). Quantitative PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and then 50 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Histology and Immunohistochemistry

For immunohistochemistry, graft-bearing eyes were removed and frozen in optimum cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) in acetone–dry ice and stored at −80°C. Cryostat sections (5 μm; approximately 20 sections per graft-bearing eye) were fixed in cold acetone, followed by immunofluorescent staining for the detection of mouse ICOS, CD4, Foxp3, and rat IgG (eBioscience). Other sections were

stained with hematoxylin-eosin (H&E). Briefly, after blocking with 2% BSA, the sections were incubated with FITC-, PE-, or allophycocyanin (ACP)-conjugated primary Ab diluted to 4 μg/mL overnight at 4°C. After washing with PBS, sections were mounted with 4, 6 diamidino-2-phenylindole (DAPI)-containing mounting medium and observed under a confocal microscope (LSM710; Zeiss, Jena, Germany).

Evaluation of Alloantigen-Specific ACAID

In vivo experimental models including ACAID were used as described previously.[5] Wild-type or ICOS$^{-/-}$ BALB/c mice underwent anterior chamber injection of 5×10^5 C57BL/6 splenocytes 2 weeks before subcutaneous immunization. Recipients were immunized by subcutaneous injection of 1×10^7 C57BL/6 spleen cells. Induction of allospecific ACAID was assessed by ear challenge with 1×10^6 C57BL/6 splenocytes at 1 week after immunization. At 24 hours after the ear challenge, ear thickness was measured using a low-pressure micrometer (Mitsutoyo; MTI, Kanagawa, Japan).

In Vitro Assessment of Corneal Endothelial Cell (CEC) Destruction by Alloreactive T Cells

In vitro experimental models of the efferent phase of corneal rejection in culture dishes were established as described previously.[5] CD4$^+$ T cells (91%–95% pure as estimated by flow cytometry) were purified from the spleens of WT or ICOS$^{-/-}$ BALB/c mice that had been presensitized by subcutaneous immunization with 1×10^7 C57BL/6 spleen cells or third-party (C3H/He) spleen cells, or from the spleens of naïve BALB/c or C57BL/6 mice, using the MACS magnetic cell sorting and separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) with CD4$^+$ T-Cell Isolation Kit 2. Corneas from C57BL/6 were incubated with 2.5×10^5 T cells for 6 hours at 37°C. Unfixed corneal samples were incubated with 50 μg/mL propidium iodide (PI) for 30 minutes to stain the nuclei of dead endothelial cells. Using confocal microscopy (magnification ×40), PI-positive cells were counted in three randomly selected areas in the corneal endothelium of each corneal sample, as previously described.[5] Flow cytometry was used to analyze the proportion of Foxp3$^+$ CD25$^+$ T cells (Tregs) before and after the coculture.

Statistical Analyses

Corneal graft survival rates were compared using Kaplan-Meier survival curves and the Breslow-Gehan-Wilcoxon test. Relative quantification (RQ), numbers of ICOS$^+$ Foxp3$^+$ CD4$^+$ cells and ICOS$^+$ Foxp3$^-$ CD4$^+$ cells, ear-swelling measurements, CEC death, and percentage of Foxp3$^+$ CD25$^+$ cells among CD4$^+$ T cells before and after coculture were analyzed by using the 2-tailed Student's *t*-test. Probability (*P*) values less than 0.05 were considered statistically significant.

Results

Inducible Costimulator Ligand, But Not ICOS, Is Expressed in Normal Retina and Cornea

Reverse-transcription PCR revealed that ICOSL mRNA was constitutively expressed in freshly isolated cornea, retina, and iris-CB of normal mouse eyes (Fig. 1). The expression of ICOS was low in the cornea, but absent in the iris-CB and retina (Fig. 1).

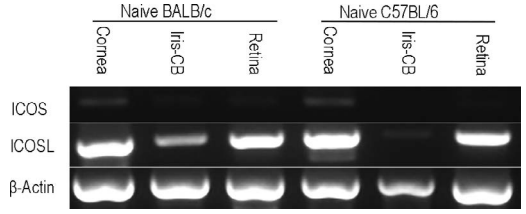

Figure 1. Inducible costimulatory ligand, but not ICOS, is expressed in normal cornea. Inducible costimulator and ICOSL mRNAs were extracted from freshly isolated cornea, iris-CB, and neural retina of normal eyes from naïve BALB/c and C57BL/6 mice, and then reverse transcribed and amplified by PCR. Polymerase chain reaction products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

Accelerated Corneal Allograft Rejection in ICOS$^{-/-}$ Mice and WT Mice Treated With Anti-ICOSL mAb

Normal corneas of C57BL/6 mice were transplanted orthotopically into normal eyes of WT or ICOS$^{-/-}$ BALB/c mice. Normal corneas from WT BALB/c mice were also transplanted orthotopically into ICOS$^{-/-}$ BALB/c mice as a syngeneic transplantation control. In WT recipients, 0.25 mg anti-ICOSL mAb or control rat IgG was administered intraperitoneally three times per week for 3 weeks after grafting. Graft survival was clinically assessed periodically. Approximately 50% of allografts survived 8 weeks in the control IgG-treated recipients (Fig. 2). We previously reported that approximately 50% of corneal allografts from C57BL/6 donors survive in untreated BALB/c recipients,[5] and thus, administration of control IgG did not affect corneal allograft survival. All syngeneic grafts transplanted into ICOS$^{-/-}$ mice survived more than 8 weeks (Fig. 2). Notably, all allografts were rejected in ICOS$^{-/-}$ or anti-ICOSL mAb-treated WT recipient mice. Allograft survival times in the ICOS$^{-/-}$ recipients and the anti-ICOSL mAb-treated WT recipients were significantly shorter than those of the control IgG-treated WT mice ($^*P < 0.01$; Fig. 2).

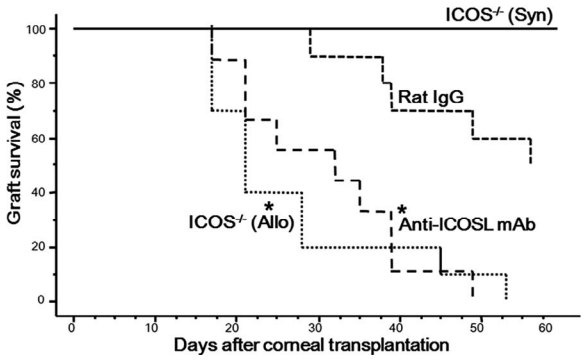

Figure 2. Inducible costimulator deficiency and blockade of ICOSL accelerated corneal allograft rejection. Normal corneas from C57BL/6 mice were transplanted orthotopically into normal eyes of WT or ICOS$^{-/-}$ BALB/c mice. Normal corneas from WT BALB/c mice were also transplanted orthotopically into ICOS$^{-/-}$ BALB/c mice as a syngeneic transplantation model. In WT recipients, 0.25 mg anti-ICOSL mAb or control rat IgG was administered intraperitoneally three times per week for 3 weeks after grafting. Graft survival was clinically assessed and compared. Corneal graft survival rates were compared using Kaplan-Meier survival curves and the Breslow-Gehan-Wilcoxon test ($n = 9$–10 per group, $^*P < 0.01$).

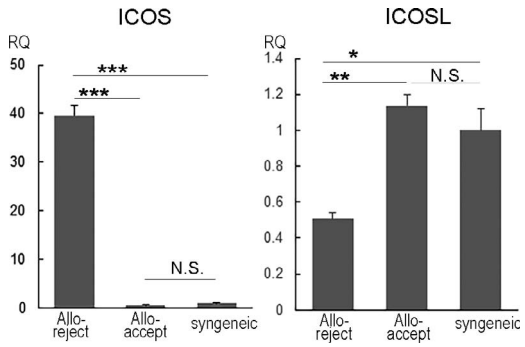

FIGURE 3. Rejected corneas express more ICOS and less ICOSL than accepted corneas. Corneas from recipients bearing accepted or rejected allografts and syngeneic grafts at 3 ± 1 weeks were analyzed with quantitative real-time PCR. Relative quantification was derived from the difference in cycle threshold (Ct) between ICOS and ICOSL after normalization to beta-actin. Data are presented as the mean ± SD of three recipients in each group. Experiments were repeated three times and analyzed using the 2-tailed Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Expression of ICOS and ICOSL in Grafted Corneas as Determined With Quantitative Real-Time PCR and Immunofluorescent Staining

Next, we analyzed the expression of ICOS and ICOSL in mouse corneas after corneal transplantation with quantitative real-time PCR. Syngeneic grafts (BALB/c to BALB/c), rejected allografts (C57BL/6 to BALB/c), and accepted allografts (C57BL/6 to BALB/c) were assessed at 2 to 4 weeks ($n = 3$ each). The expression of ICOSL remained significantly higher in the accepted allografts and syngeneic grafts than in the rejected allografts. On the other hand, expression of ICOS was significantly higher in the rejected allografts than in the accepted allografts and syngeneic grafts (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Fig. 3).

Hematoxylin-eosin staining revealed that the rejected allografts were infiltrated with several inflammatory cells (Fig. 4D), whereas the accepted allografts had few inflammatory cells (Fig. 4A). Immunofluorescent staining showed that ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells were present in the accepted allografts (Figs. 4B, 4C). On the other hand, the rejected allografts were infiltrated with many ICOS$^+$ CD4$^+$ T cells that were Foxp3$^-$ (Figs. 4E, 4F). No significant differences were observed between the numbers of ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells and ICOS$^+$ Foxp3$^-$ CD4$^+$ T cells in the accepted graft junction (Fig. 4G). Numbers of ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells were significantly lower than that of ICOS$^+$ Foxp3$^-$ CD4$^+$ T cells at the rejected graft junction (*$P < 0.05$; Fig. 4H).

Inducible Costimulator–ICOSL Interactions Are Partially Involved in the Induction of ACAID

Eye-associated tolerance, termed ACAID, is a major mechanism of immune privilege in the eyes and suppresses rejection of corneal allografts.[1] We first hypothesized that the vulnerability of corneal allografts that was observed after blockade of ICOS-ICOSL may have resulted from a failure to induce ACAID. To address this possibility, we examined the effect of ICOS-ICOSL blockade on alloantigen-specific ACAID induction by using an established model. C57BL/6 spleen cells were used as alloantigens and injected into the right anterior chamber of WT or ICOS$^{-/-}$ BALB/c eyes. After 2 weeks, C57BL/6 spleen cells were injected subcutaneously to sensitize the mice. After one more week, C57BL/6 spleen cells were injected into the ear pinnae to determine the delayed-type hypersensitivity (DTH) response 24 hours later. The DTH response was induced in positive controls (sensitized mice without prior anterior chamber [AC] injection) and compared with negative controls (unsensitized naïve mice). Delayed-type hypersensitivity responses were suppressed in WT and ICOS$^{-/-}$ mice, indicating that ACAID was induced in WT and ICOS$^{-/-}$ mice (**$P < 0.01$; Fig. 5). However, DTH responses were significantly higher in ICOS$^{-/-}$ mice compared with WT mice, suggesting that the suppressive function of ACAID in ICOS$^{-/-}$ mice was lower than that in WT mice (*$P < 0.05$; Fig. 5). These results indicate that the ICOS-ICOSL interaction is at least partly involved in the induction of ACAID.

Inducible Costimulator–ICOSL Has a Protective Effect From Allospecific Killing by CD4$^+$ T Cells and Induces Tregs

The above results led us to hypothesize that the expression of ICOSL in the cornea protects corneal allografts from alloreactive infiltrating T cells by inducing Tregs. To further investigate the mechanism of ICOS-ICOSL–mediated corneal allograft survival, we examined destruction of CEC by alloreactive T cells in vitro.[5] As a model of the effector phase of corneal rejection, the corneas from C57BL/6 eyes were incubated with CD4$^+$ T cells from WT or ICOS$^{-/-}$ BALB/c mice that had been presensitized with C57BL/6 splenocytes. Dead CECs stained with PI were counted. In post coculture of corneal tissue and T cells immunized with C57BL/6 spleen cells, CEC damage due to alloreactive T-cell cytotoxicity was significantly greater with ICOS$^{-/-}$ T cells than with WT T cells (*$P < 0.01$; Fig. 6A). This indicates that ICOS-ICOSL has a protective effect from allospecific killing by CD4$^+$ T cells in the cornea.

After a 6-hour incubation, the proportion of Foxp3$^+$ CD25$^+$ Tregs among CD4$^+$ T cells was assessed with flow cytometry. The preincubation proportion of Tregs in ICOS$^{-/-}$ T cells was not significantly different from that of WT T cells. The proportion of Tregs in ICOS$^{-/-}$ T cells remained unchanged after incubation with and without corneal tissue. However, after incubation without and with corneal tissue, the proportion of Tregs in WT T cells increased significantly (*$P < 0.05$; Figs. 6B, 6C). The proportion of Tregs in WT T cells was significantly larger than that observed in ICOS$^{-/-}$ T cells after incubation with and without corneal tissue (*$P < 0.05$; Fig. 6C). This suggests that ICOS signaling plays a significant role in local induction of Tregs in the cornea.

DISCUSSION

The present study was designed to investigate whether ICOS and ICOSL are involved in the immune-privileged status of the eye using the corneal allotransplantation model and to explore the underlying mechanism. We demonstrated with RT-PCR that ICOSL mRNA was constitutively expressed in the cornea, iris-CB, and retina of naïve mice. We also showed that blockade of ICOSL and ICOS deficiency in the recipients accelerated rejection of corneal allografts. These results indicate that the ICOS-ICOSL interaction plays an essential role in protecting corneal allografts from rejection.

We explored two possible mechanisms for the ICOS-mediated corneal allograft protection from rejection. One possibility was that the ICOS-ICOSL interaction is involved in the induction of antigen-specific systemic immune tolerance to eye-derived antigens, known as ACAID. Antigens placed in the AC are captured by resident antigen-presenting cells, which then migrate through the trabecular meshwork and into the blood. Once these cells reach the marginal zone of the spleen,

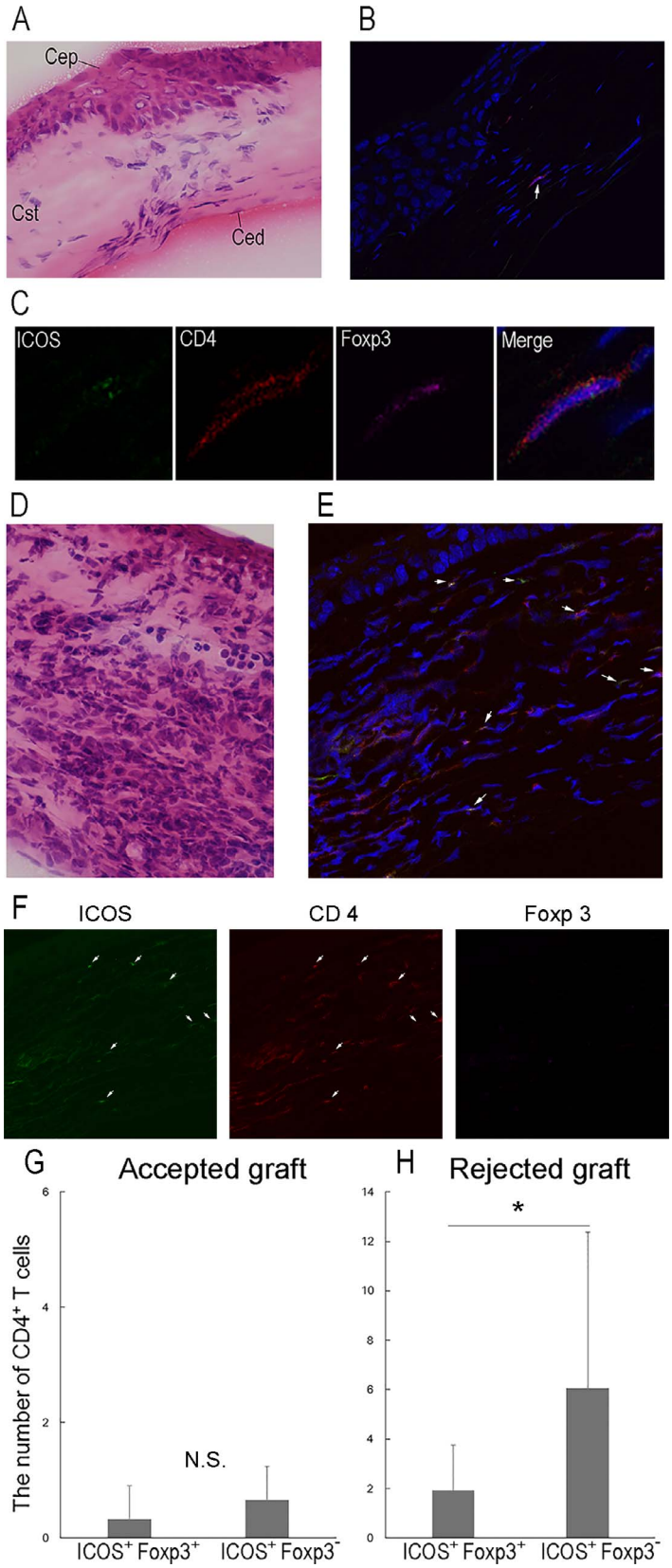

FIGURE 4. Large numbers of ICOS$^+$ Foxp3$^-$ CD4$^+$ T cells infiltrate into rejected grafts but not accepted grafts. Cryostat sections of graft-bearing eyes were examined by H&E staining (**A**, **D**) and immunofluorescent staining (**B**, **C**, **E**, **F**). Cryostat sections of surviving allografts at 3 to 4 weeks (**A**, **B**, **C**) were stained with biotinylated anti-ICOS mAb followed by streptavidin-FITC (*green*), PE-conjugated anti-CD4 mAb (*red*), and APC-conjugated anti-Foxp3 mAb (*pink*). Nuclei were stained with DAPI (*blue*). *Arrows* in (**B**) show the ICOS$^+$ CD4$^+$ Foxp3$^+$ cells, and (**C**) shows a triple-positive cell at higher magnification. Cryostat sections of rejected allografts at 3 to 4 weeks (**D**, **E**, **F**) were also examined by immunofluorescent staining for the detection of ICOS, CD4, and Foxp3. Nuclei were stained with DAPI. *Arrows* in (**E**) and (**F**) show the ICOS$^+$ CD4$^+$ Foxp3$^-$ cells. "Ced," "Cst," and

"Cep" denote corneal endothelium, stroma, and epithelium, respectively. Original magnification, ×40. No significant differences were observed between numbers of ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells and ICOS$^+$ Foxp3$^-$ CD4$^+$ T cells in the accepted graft junction (**G**). Numbers of ICOS$^+$ Foxp3$^+$ CD4$^+$ T cells were significantly lower than that of ICOS$^+$ Foxp3$^-$ CD4$^+$ T cells at the rejected graft junction (*$P < 0.05$; [**H**]). Data are presented as the mean ± SD of three to four corneas in each group and analyzed using the 2-tailed Student's *t*-test.

active transforming growth factor-β, IL-10, and chemokine (C-C motif) ligand 5 (CCL5) attract and activate antigen-specific CD4$^+$ and CD8$^+$ T cells to differentiate into antigen-specific Tregs that inhibit induction and expression of DTH.[1] Induction of donor-specific ACAID is associated with long-term graft acceptance and promotes the survival of corneal allografts.[1]

Our results demonstrated that ACAID was induced in the WT recipients, because antigen-specific DTH was suppressed. Delayed-type hypersensitivity was also suppressed in the ICOS$^{-/-}$ recipients, although DTH was higher in ICOS$^{-/-}$ mice than in WT mice. These results suggest that ICOS-ICOSL is at least partially involved in ACAID; however, ICOS-ICOSL–mediated protection of corneal allografts from immune rejection appears to involve another mechanism in addition to ACAID.

The other possible mechanism is that corneal ICOSL supports the immunosuppressive microenvironment, in which inflammatory cells within the eye are deleted or suppressed. To assess the expression of ICOS and ICOSL in whole graft cornea, quantitative real-time PCR and immunofluorescent staining were performed. Quantitative real-time PCR and immunofluorescent staining of allografts revealed that the expression of ICOSL was maintained, and ICOS$^+$ Foxp3$^+$ Tregs were present in the accepted allografts. In contrast, ICOSL expression was reduced, and many infiltrating ICOS$^+$ Foxp3$^-$ CD4$^+$ T cells infiltrated in rejected allografts. Inducible costimulator is expressed on the surface of both effector T cells and Tregs. Inducible costimulator signaling may have a different function in these cells, depending on the expression of ICOSL and its ligation. The expression of ICOSL in the cornea may mediate the suppressive function of ICOS$^+$ Tregs, leading to allograft acceptance.

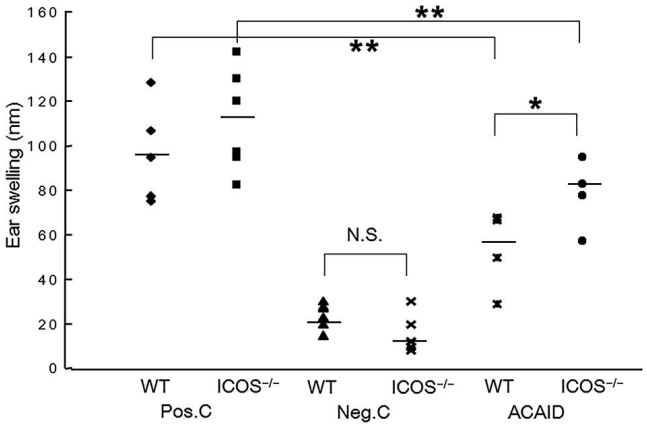

FIGURE 5. Inducible costimulator–ICOSL interactions are partially involved in the induction of ACAID. C57BL/6 spleen cells were used as alloantigens and injected into the right AC of normal eyes of ICOS$^{-/-}$ or WT BALB/c mice. Two weeks later, C57BL/6 spleen cells were injected subcutaneously to sensitize the mice. After one more week, a challenge was conducted by injecting C57BL/6 spleen cells into the right ear pinna of each mouse, and specific ear swelling was measured 24 hours later as an indication of DTH. Positive control mice (Pos.C) received subcutaneous immunization and ear challenge without previous AC injection. Negative control mice (Neg.C) received only the ear challenge without AC injection or immunization. *$P < 0.05$; **$P < 0.01$; N.S., not significant. Data are analyzed using the 2-tailed Student's *t*-test.

To further substantiate the ICOS-ICOSL–mediated protection of corneal allografts from effector T cells, we evaluated CEC destruction by alloreactive T cells in vitro. After coculture of normal corneal tissue with alloantigen-primed CD4$^+$ T cells, CEC damage by alloreactive T cells was significantly greater with ICOS$^{-/-}$ T cells than with WT T cells. After the interaction between T cells and corneal tissue, the proportion of Foxp3$^+$ Tregs was increased in WT, but not ICOS$^{-/-}$, T cells. This suggests that the local expansion of ICOS-expressing Tregs is mediated by the interaction between ICOS on T cells and ICOSL expressed in corneal tissue and leads to the protection of corneal tissue from damage by alloreactive T cells. Thus, ICOSL on corneal tissue and ICOS-expressing Tregs in the allografts may play an essential role in protecting the corneal allografts from rejection.

The importance of Tregs has been demonstrated in establishing tolerance when orthotopic corneal allografts are transplanted into the eye.[6,24,25] Inducible costimulator plays an essential role in controlling the immune regulatory system, as demonstrated by previous studies showing that ICOS-deficient mice have defects in tolerance induction.[26,27] Recent studies showing a reduction in CD4$^+$ Foxp3$^+$ Tregs in ICOS-deficient mice indicate an important role for ICOS in maintaining Treg homeostasis.[28,29] Chen et al.[30] also demonstrated that the surviving and proliferative Tregs are ICOS$^+$, whereas the death-prone Tregs are ICOS$^-$. Regulatory T cells that are ICOS$^+$ exhibit much stronger suppressive activity than ICOS$^-$ Tregs.[30] From our data, using corneal transplantation in vivo and in vitro, the ICOS-ICOSL pathway contributed to ICOS$^+$ Treg-mediated protection of corneal tissue and was involved in ACAID. Therefore, the ICOS-ICOSL pathway likely contributes to immune privilege for corneal transplantation using at least these two mechanisms.

Although other laboratories have reported that ICOS fusion protein (ICOSIg) gene therapy does not prevent allograft rejection following corneal transplantation[31] and that allograft survival in BALB/c recipients treated with anti-ICOS antibody is not prolonged compared with the isotype control antibody,[32] ICOS blockade has the opposite effects on immune responses depending on the timing of the blockade. Some studies have reported a role for ICOS and ICOSL as inhibitory costimulatory molecules. Inducible costimulator–ICOSL signal blockade during antigen priming exacerbates experimental autoimmune encephalomyelitis and acute graft-versus-host disease.[13,14] In a cardiac allograft model, early ICOS blockade accelerates allograft rejection.[16,17] We showed that blockade of ICOSL in the induction phase and ICOS deficiency accelerated the rejection of corneal allografts. Although our present results support a role for ICOS-ICOSL in inhibitory costimulation, we propose that ICOSL-mediated protection of corneal allografts is mediated by induction of ICOS-expressing Tregs. We propose that the ICOS-ICOSL pathway is essential for the immune privilege for corneal transplantation. To further clarify the mechanism of immune privilege for corneal transplantation, further immunologic analysis will be important. The cornea constitutively expresses various immune-regulating molecules, such as B7-H1,[5] CD95L,[33,34] GITR-L,[6] and galectin-9,[7] in distinct localization patterns within the tissue. The fact that the immune-privileged status of the cornea can be abolished by dysfunction of just one of these molecules suggests that each molecule plays a nonredundant or cooperative role in maintenance of immune privilege.

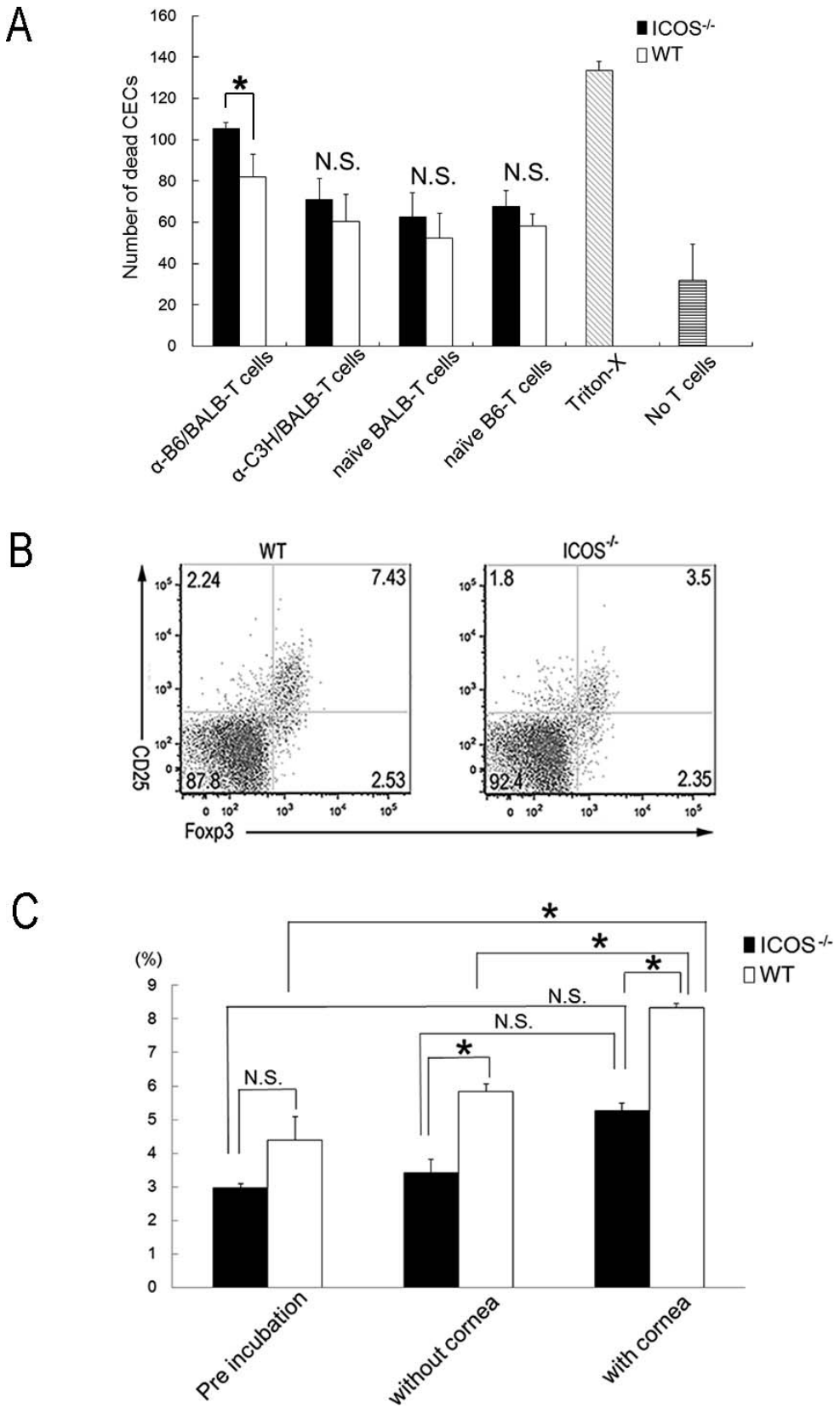

Figure 6. Inducible costimulator attenuates alloreactive $CD4^+$ T-cell cytotoxicity against CECs and is required for induction of Tregs. Corneas from C57BL/6 mice were incubated with purified $CD4^+$ T cells from the spleens of WT or $ICOS^{-/-}$ BALB/c mice presensitized with C57BL/6 splenocytes (α-B6/BALB-T cells) or third-party C3H/He splenocytes (α-C3H/BALB-T cells), or from the spleens of naïve BALB/c mice (naïve BALB-T cells) or C57BL/6 mice (naïve B6-T cells). After a 6-hour incubation, CEC death was detected by staining unfixed tissue with PI followed by confocal microscopic examination (**A**). Positive control corneas were incubated with Triton X-100. As a negative control, corneas were incubated without T cells. Data are presented as the mean ± SD of PI^+ CECs from five corneas in each group. $^*P < 0.01$; N.S., not significant. Data are analyzed using the

2-tailed Student's *t*-test. Alloreactive T cells from ICOS$^{-/-}$ or WT mice following coincubation with B6 corneas were examined by flow cytometry (**B**, **C**). (**B**) shows representative flow cytometry data of CD4^{+}-gated CD25^{+} Foxp3^{+} T cells after coculture with corneal tissue. (**C**) shows the proportion of Foxp3^{+}CD25^{+} cells among CD4^{+} T cells presented as the mean ± SD of three experiments in each group. $^{*}P < 0.05$; N.S., not significant. Data are analyzed using the 2-tailed Student's *t*-test.

In summary, our present results indicate that ICOS-ICOSL plays a critical role in the maintenance of the immune-privileged status of corneal allografts. Inducible costimulatory ligand is constitutively expressed in the cornea and induces ICOS^{+} Tregs within the cornea. Forced expression of ICOSL may be a new strategy for conferring an immune-privileged status on other organs to suppress allograft rejection.

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