VISTA Is Crucial for Corneal Allograft Survival and Maintenance of Immune Privilege

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Submitted: April 16, 2019
Accepted: October 27, 2019

Citation: Kunishige T, Taniguchi H, Ohno T, Azuma M, Hori J. VISTA is crucial for corneal allograft survival and maintenance of immune privilege. *Invest Ophthalmol Vis Sci*. 2019;60:4958–4965. https://doi.org/10.1167/iovs.19-27322

Purpose. V-domain immunoglobulin suppressor of T cell activation (VISTA) is a novel immune checkpoint receptor and ligand for regulating T cell proliferation and cytokine production. The purpose of the present study was to determine the role of VISTA in the immune privilege of corneal allografts.

Methods. Expression of VISTA mRNA in mouse eyes was assessed with reverse-transcription PCR. Corneas of C57BL/6 mice were orthotopically transplanted into the eyes of BALB/c wild-type recipients treated with anti-VISTA mAb, and graft survival was assessed. A separate set of BALB/c mice treated with anti-VISTA mAb or rat IgG received injection of C57BL/6 splenocytes into the anterior chamber, and induction of allospecific anterior chamber-associated immune deviation (ACAID) was assessed. $CD4^+$ and $CD8^+$ T cells in the spleen were assessed with flow cytometry.

Results. VISTA mRNA was constitutively expressed in the cornea, and the expression of VISTA was localized to $CD11b^+$ cells on the corneal stroma. Survival of allografts treated with anti-VISTA mAb was less than that of the control. ACAID was induced less efficiently in BALB/c mice treated with VISTA mAb. The proportions of $CD8^+$ T cells and $CD8^+$ $CD103^+$ T cells ($CD8^+$ T regulatory cells) in the spleen of BALB/c mice treated with anti-VISTA mAb were significantly lower than those of the control.

Conclusions. VISTA may play an essential role in the acceptance of corneal allografts via involvement with allospecific ACAID, which suppresses T cell infiltration into the cornea.

Keywords: corneal allograft, immune privilege, ACAID, VISTA, immune checkpoint

The eye is provided with immune protection against pathogens in a manner that greatly reduces the threat of inflammation-induced vision loss.[1] Immune-mediated inflammation and allograft rejection are greatly reduced in the eye, a phenomenon called "immune privilege."[2] Corneal transplants are the least rejected among all organ transplants, a characteristic that is also attributable to immune privilege.[1–3] However, if corneal transplantation is performed on eyes that have been deprived of immune privilege, the rejection rate becomes as high as heart and skin transplantation.[1,2,4] Thus, immune privilege must be present to prevent rejection after corneal transplantation.[1–3]

Anterior chamber-associated immune deviation (ACAID) and the immune suppressive microenvironment in the eye are needed to maintain immune privilege after corneal transplantation.[1–3,5,6] ACAID is a well-known phenomenon in which antigen-specific peripheral tolerance is induced after antigen introduction into the anterior chamber (AC).[7] In response to threats to vision, the eye expresses soluble and cell surface immunomodulatory factors to suppress cells and molecules that mediate innate and adaptive immune inflammation.[3,6,8] This intraocular milieu is called the "immune suppressive microenvironment."[6]

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,[8] glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand (GITR-L),[5,9] galectin-9,[10,11] and inducible costimulatory molecule (ICOS) and ICOS ligand[12,13] are involved in immune suppression in the cornea and are essential for immune privilege for corneal transplantation. The molecular mechanisms of immune privilege in the eye are not fully understood. To provide the rationale for development of new therapeutic strategies in the eye, further investigations of the mechanisms underlying immune privilege are needed.

V-domain Ig suppressor of T cell activation (VISTA)/PD-1H is a novel co-inhibitory immune checkpoint receptor and ligand whose extracellular domain bears homology to theB7 family ligand PD-L1.[14–16] VISTA is primarily expressed on hematopoietic cells, and VISTA expression is highly regulated on myeloid antigen-presenting cells (APCs) and T cells.[14] VISTA can function as a receptor as well as a ligand.[17] Indeed, structural modeling suggests homology to PD-1[15] and PD-L1.[14] Soluble VISTA-Ig fusion protein or VISTA expression on APCs inhibits T cell proliferation and cytokine production in vitro.[14]

Various studies have investigated that that VISTA regulates T-cell responses in various murine models of carcinoma, autoimmune and allergic disease.[14,18–23] Anti-VISTA treatment exacerbates the development of the T cell–mediated autoimmune disease experimental autoimmune encephalomyelitis in mice.[14] $VISTA^{-/-}$ T cells induce exacerbated graft-versus-host

disease compared to wild-type T cells.[18] VISTA is an immune checkpoint regulator important in the maintenance of skin homeostasis and inflammation in murine model allergic skin inflammation.[20] VISTA deficiency or blockade of VISTA also accelerated asthmatic responses.[21,23]

Antibodies targeting the immune-checkpoint proteins CTLA-4, PD-1, and PD-L have become new therapies for cancer.[24–29] VISTA expression also plays a critical role in shaping anti-tumor immunity.[19,22] VISTA monoclonal antibody (mAb) treatment increases the number of tumor-specific T cells in the periphery and enhances the infiltration, proliferation, and effector function of tumor-reactive T cells.[22] VISTA mAb administration as a monotherapy significantly suppresses the growth of both transplantable and inducible melanoma.[22]

The involvement of VISTA in immune privilege of the eye has never been investigated. Here, we examined for the first time the role of VISTA in immune privilege of the eye using a well-established murine model of corneal transplantation and ACAID.

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 8–10 weeks of age. They were treated according to the Association for Research in Vision and Ophthalmology 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.

Abs and Flow Cytometry

Anti-mouse VISTA (MIH63, rat IgG2a mAb) was generated as previously described.[19–21] For fluorescence immunohistochemistry or flow cytometry, mAbs against CD3 (145-2C11, Armenian hamster IgG), CD4 (GK1.5, rat IgG2b), CD8 (53.6.72, rat IgG2a), CD11b (M1/70, rat IgG2b), CD25 (PC61.5, rat IgG1), Foxp3 (FJK-16s, rat IgG2a), CD103 (M290, rat IgG2a), and anti-VISTA mAb were used. All fluorescein isothiocyanate (FITC)-, Phycoerythrin (PE)-, allophycocyanin (ACP)-, and biotin-conjugated mAbs and isotype control Igs were obtained from eBioscience (San Diego, CA, USA). Culture supernatant from the 2.4G2 hybridoma (anti-CD16/CD32 mAb) was used to block non-specific binding. Stained cells were then analyzed (BD FACSverse, BD Biosciences, San Jose, CA, USA; Flow Jo software, Tomy Digital Biology, Tokyo, Japan).

Reverse-Transcription PCR (RT-PCR)

Corneas were isolated from a total of 19 normal BALB/c and 10 C57BL/6 (B6) mouse eyes. Total RNA was extracted using NucleoSpin RNA (TaKaRa). First-strand cDNA was prepared using PrimeScript RT Master Mix (TaKaRa, Shiga, Japan). Standardization of cDNA samples was based on the content of GAPDH cDNA. PCR was performed using mouse VISTA or GAPDH primers. The sequences of sense and antisense primers were as follows: VISTA, 5′-GAAGTCCAGGTCTCTGAA GAC-3′ and 5′-CTGGTAGCTAGAGCACGTGAT-3′. GAPDH, 5′-GCATGGCCTTCCGTGTTCCT-3′ and 5′-GGTCCTCAGTGTA GCCCAAGATGC-3′.

PCR 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 of thermostable DNA polymerase (TaKaRa Ex Taq). After 45 cycles of amplification, the PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Orthotopic Corneal Transplantation and Treatment

Penetrating keratoplasty was performed as previously described.[30] C57BL/6 mice were used as donors, and wild-type (WT) 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 in diameter) were placed in the same-sized recipient bed with eight interrupted 11-0 nylon (Mani, Tochigi, Japan) sutures, which were removed 7 days post-grafting. After the grafting procedure, recipients were administered 0.2 mg anti-VISTA monoclonal antibody (MIH63) or control rat IgG intraperitoneally three times a week for 8 weeks. Syngeneic transplantation using WT BALB/c donor corneas into a WT 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 (J.H.), who evaluated each graft for survival according to a previously reported scoring system that defines graft survival as follows: 0, clear graft; 1+, minimal superficial nonstromal opacity; 2+, minimal deep stromal opacity with pupil margin and iris vessels visible; 3+, moderate deep stromal opacity with only the pupil margin visible; 4+, intense deep stromal opacity with the AC visible; and 5+, maximum stromal opacity with total obscuration of the AC.[30] Grafts with score 2+ (mild stromal opacity with iris structure visible) or greater after 3 weeks were considered rejected.

Histology and Immunohistochemistry

For immunohistochemistry, graft-bearing eyes were removed and frozen in OCT 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 VISTA, CD4, CD8, CD11b, and rat IgG (eBioscience). Other sections were stained with hematoxylin and eosin. Briefly, after blocking with 2% bovine serum albumin, sections were incubated with PE-, FITC-, or biotin-conjugated primary Ab diluted to 4 μg/ml for 2 hours. This was followed by staining with streptavidin-PE (eBioscience) that was diluted to 4 μg/ml for 1 hour at room temperature. 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.[8] BALB/c mice received AC injection of 5×10^5 C57BL/6 splenocytes 2 weeks prior to 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 1 week after immunization. Twenty-four hours after the ear challenge, ear thickness was measured using a low-pressure micrometer (Mitsutoyo; MTI, Kanagawa, Japan).

Ear swelling was determined as follows: specific ear swelling = (measurement of right ear at 24 hours − measurement of right ear at 0 hours) − (measurement of left ear at 24 hours − measurement of left ear at 0 hours) $\times 10^{-3}$ mm. Ear swelling responses 24 hours after injection are presented as individual values ($\times 10^{-3}$ mm) for each tested animal and as a group mean ± standard error of the mean. For 3 weeks, three times a week, treatments with 0.2 mg anti-VISTA mAb or control rat IgG mAb were performed starting from the day of AC injection and continuing until the day of ear injection. As a positive control, a similar number of irradiated spleen cells were injected into the right ear pinnae of BALB/c mice that had been immunized 1 week previously by subcutaneous injection of 1×10^7 C57BL/6 spleen cells. As a negative control, 1×10^6 irradiated C57BL/6 spleen cells were injected into the right ear pinnae of naïve mice that had not been previously injected into the AC or immunized.

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

In vitro experimental models of the efferent phase of corneal rejection in culture dishes were used as described previously.[8] $CD4^+$ T cells (90%–94% pure as estimated by flow cytometry) were purified from the spleens of 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, C57BL/6, or C3H/He mice, using the MACS magnetic cell sorting and separation system and $CD4^+$ T-Cell Isolation Kit 2 (Miltenyi Biotec, Bergisch Gladbach, Germany). Corneas pretreated with anti-VISTA mAb (MIH63) or control rat IgG from C57BL/6 mice 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.[8] As a positive control for corneal cell death, normal C57BL/6 corneas were incubated with Triton X-100 without Ab treatment or incubation with T cells. As negative controls, normal C57BL/6 corneas without Ab treatment or incubation with T cells were used.

Statistical Analyses

Corneal graft survival rates were compared using Kaplan-Meier survival curves and the Logrank test. Ear-swelling measurements, corneal endothelial cell death, infiltrating $CD4^+$ and $CD8^+$ cells, and percentage of gated spleen cells were analyzed using the two-tailed Student's t test. Probability (P) values < 0.05 were considered statistically significant.

Results

VISTA Is Expressed in Normal Corneas

RT-PCR revealed that VISTA mRNA was expressed in freshly isolated spleen and cornea of normal mouse (Fig. 1A). Immunofluorescent staining indicated that the expression of VISTA was localized to $CD11b^+$ cells on the corneal stroma (Figs. 1B, 1C). VISTA was not expressed on the epithelium or in endothelial cells (Figs. 1B, 1C).

The numbers of $VISTA^+$ $CD11b^+$ cells and whole $CD11b^+$ cells were counted in the confocal microscopy images of the areas randomly selected which is same as the size of 212.55 µm × 212.55 µm square (magnification, ×40) in the corneal sections of naïve BALB/c mice ($n = 7$). The numbers of $VISTA^+$ $CD11b^+$ cells and whole $CD11b^+$ cells were 1.0 ± 0.6 and 2.3 ± 1.0 cells per image, respectively. Thus, the proportion of $VISTA^+$ $CD11b^+$ cells was 43.75% of whole $CD11b^+$ cells in the corneal sections of normal mouse eyes.

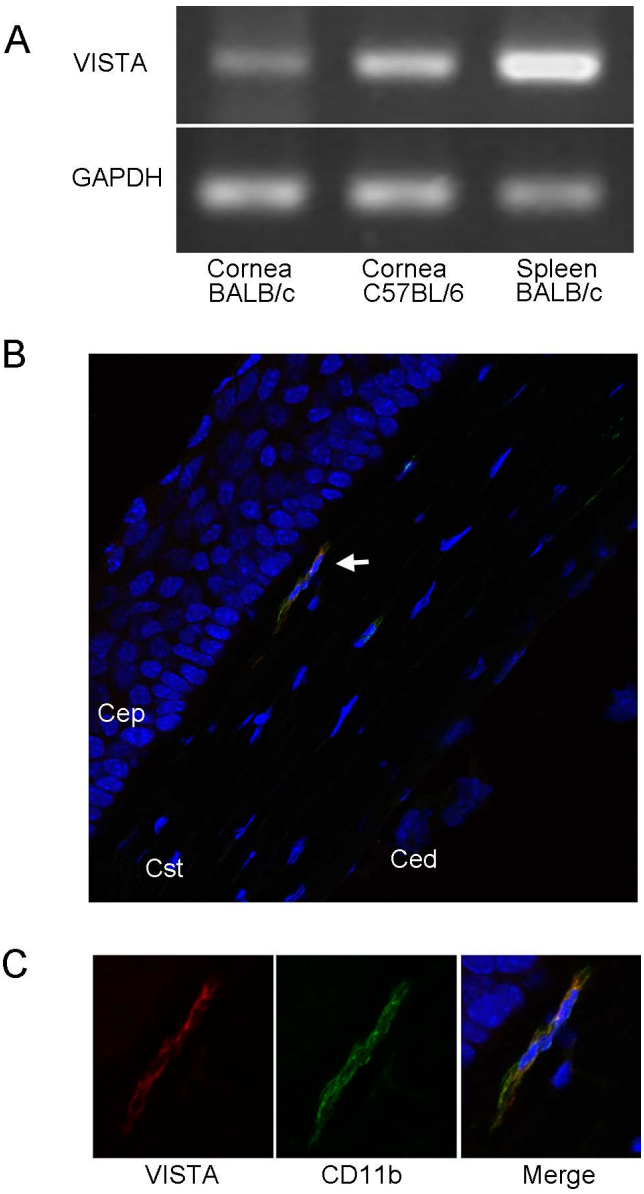

Figure 1. VISTA is expressed in the normal cornea. Total mRNAs were extracted from freshly isolated corneas of normal eyes from naïve BALB/c and C57BL/6 mice, and then reverse transcribed and amplified by PCR. PCR products were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining (**A**). Cryostat sections of normal eyes from naïve BALB/c mice were examined following immunofluorescent staining (**B**). Cryostat sections were stained with biotinylated anti-VISTA mAb followed by streptavidin-PE (*red*) and FITC-conjugated anti-CD11b mAb (*green*). Nuclei were stained with DAPI (*blue*). "Ced," "Cst," and "Cep" denote corneal endothelium, stroma, and epithelium, respectively. Original magnification, ×40. High-magnification images (**C**) shows a cell that is double positive for VISTA and CD11b at higher magnification.

Accelerated Corneal Allograft Rejection in Recipients Treated With Anti-VISTA mAb

Normal corneas of C57BL/6 mice were transplanted orthotopically into normal eyes of BALB/c mice. In all recipients, 0.2 mg

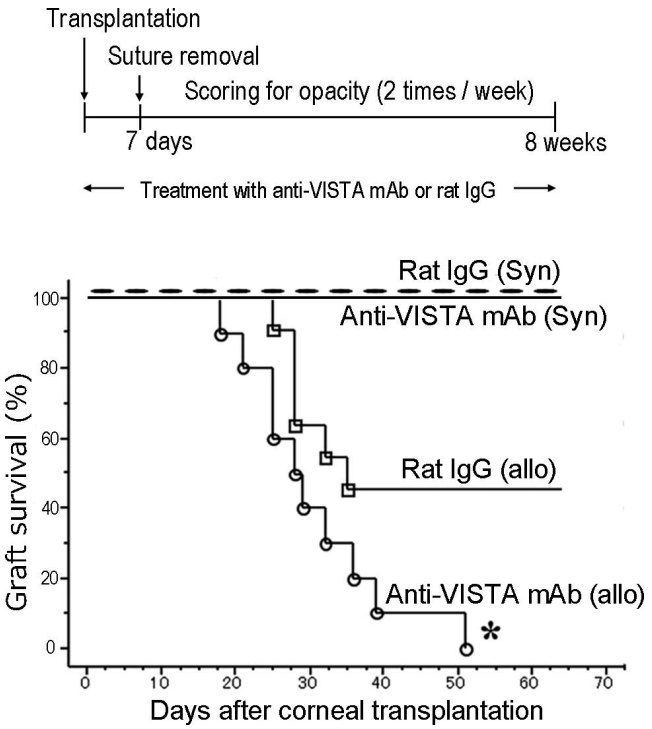

Figure 2. Blockade of VISTA accelerates corneal allograft rejection. Normal corneas from C57BL/6 mice were transplanted orthotopically into normal eyes of WT BALB/c mice ($n = 10$–11 per group). Normal corneas from WT BALB/c mice were also transplanted orthotopically into WT BALB/c mice as a syngeneic transplantation model ($n = 6$ per group). In the recipients, 0.2 mg anti-VISTA mAb or control rat IgG was administered intraperitoneally three times/week after grafting. Graft survival was clinically assessed and compared. Corneal allograft survival rates were compared using Kaplan-Meier survival curves and the Long test ($n = 10$–11 per group, $^*P < 0.05$).

anti-VISTA mAb or control rat IgG was administered intraperitoneally 3 times/week for 8 weeks after grafting. Graft survival was clinically assessed and compared. Approximately 50% of allografts survived 8 weeks in 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.[8] Administration of control IgG thus does not affect corneal allograft survival. Conversely, all allografts were rejected when recipients were treated with anti-VISTA mAb. Allograft survival in the recipients treated with anti-VISTA mAb was significantly shorter than that in the control ($P < 0.05$; Fig. 2).

As syngeneic corneal grafts, normal corneas from BALB/c mice were transplanted orthotopically into BALB/c mice, and the recipients were treated with anti-VISTA mAb or rat IgG. All syngeneic grafts survived in the anti-VISTA mAb- or control rat IgG-treated WT BALB/c mice. This indicated that treatment with anti-VISTA antibody does not affect the survival of syngeneic BALB/c corneal allografts.

Treatment With Anti-VISTA mAb Increases T Cell Infiltration in Allografts

All recipients were treated with anti-VISTA mAb or control rat IgG. Graft-bearing eyes were isolated at 3–5 weeks. Hematoxylin and eosin staining showed that the number of infiltrating cells in the corneas was increased following anti-VISTA treatment (Fig. 3A) compared to control recipients (Fig. 3B). Cryostat sections of graft-bearing eyes were examined following immunofluorescent staining with PE-conjugated anti-CD4 mAb (red) and anti-CD8 mAb (red). Nuclei were stained with DAPI (blue). The number of $CD4^+$ and $CD8^+$ cells in corneal sections were counted. The number of $CD4^+$ and $CD8^+$ cells in the corneas of the recipients treated with anti-VISTA mAb was greater than that in the control recipients ($P < 0.01$, Fig. 3C–F).

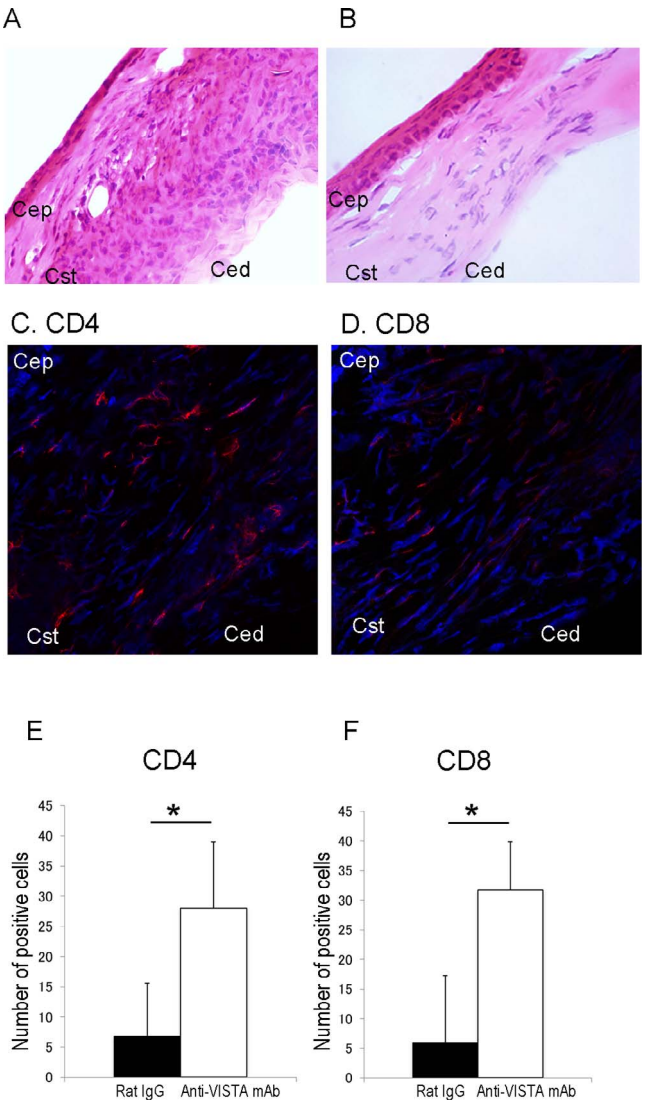

Figure 3. Infiltration of $CD4^+$ and $CD8^+$ T cells into anti-VISTA mAb-treated grafts. Cryostat sections of graft-bearing eyes were examined following hematoxylin and eosin staining (**A, B**) and immunofluorescent staining (**C, D**). Cryostat sections of anti-VISTA mAb-treated grafts (**A**) and anti-rat mAb-treated grafts (**B**) at 3–5 weeks were stained with hematoxylin and eosin. Cryostat sections of surviving allografts at 3–5 weeks were stained with PE-conjugated anti-CD4 (**C**) or CD8 (**D**) mAb (*red*), and nuclei were stained with DAPI (*blue*). Original magnification, ×40 (**A**–**D**). "Ced," "Cst," and "Cep" denote corneal endothelium, stroma, and epithelium, respectively. The number of $CD4^+$ and $CD8^+$ cells in corneal sections were counted (**E, F**). The masked cell count assessment was performed by a single observer (T.K.). Data are the mean ± standard deviation of 3–4 corneas in each group and were analyzed using the two-tailed Student's t test ($^*P < 0.01$).

VISTA Signaling Is Partially Involved in the Induction of ACAID

ACAID is an essential mechanism for immune privilege of the eyes and maintains acceptance of corneal allografts.[1,2] We

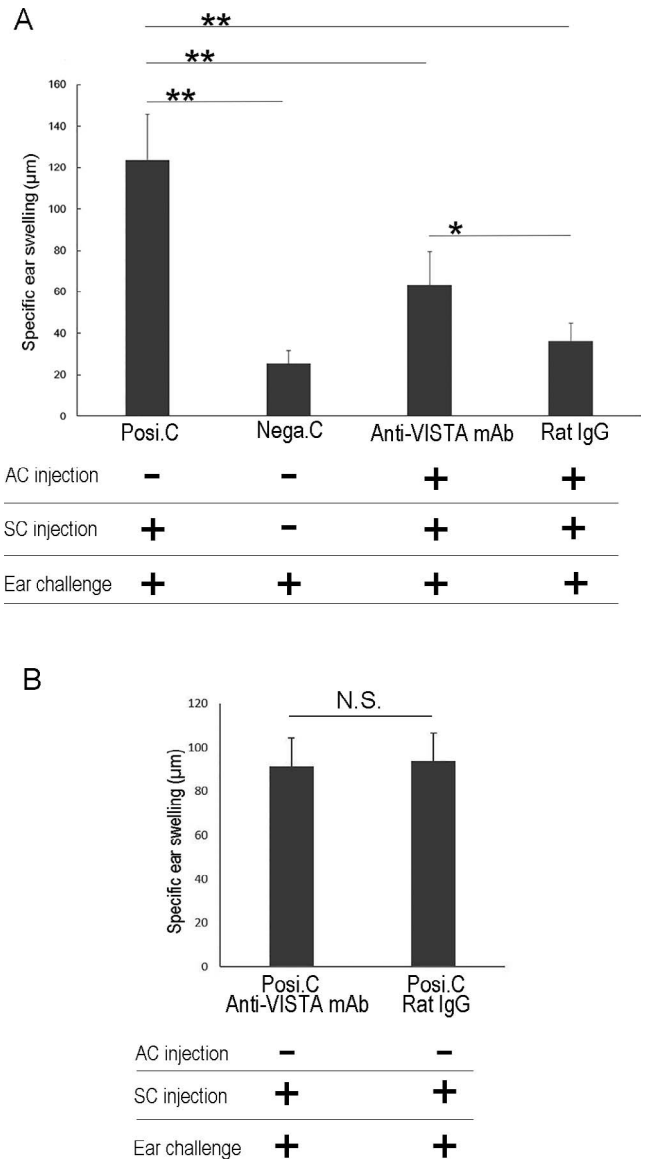

Figure 4. VISTA is partially involved in the induction of ACAID. C57BL/6 spleen cells were used as alloantigens and injected into the right anterior chamber (AC) of normal eyes of mice treated with anti-VISTA mAb and anti-rat mAb. Two weeks later, C57BL/6 spleen cells were injected subcutaneously to 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 delayed-type hypersensitivity. Positive control mice (Posi.C) received subcutaneous immunization and ear challenge without previous AC injection. Negative control mice (Nega.C) received only the ear challenge without AC injection or immunization. (**A**) shows the results of ACAID induction. (**B**) shows the results of an experiment with mice treated with anti-VISTA or control rat IgG as a positive control ($n = 6$ per group, $^{*}P < 0.05$, $^{**}P < 0.005$, N.S.: not significant). Data were analyzed using the two-tailed Student's t test. "SC" denotes subcutaneous.

hypothesized that the vulnerability of corneal allografts noted after treatment with anti-VISTA mAb may result from failure of ACAID-inducing Tregs. To investigate this, we used in vivo experimental models including ACAID. B6 spleen cells were used as alloantigens and were injected into the right AC of normal BALB/c eyes. After 2 weeks, B6 spleen cells were injected subcutaneously to sensitize the mice. After one more week, B6 spleen cells were injected into the ear pinnae to determine the delayed hypersensitivity (DH) response 24 hours later. Treatments with anti-VISTA mAb were administered for 3 weeks starting from the day of AC injection until the day of ear challenge. The DH response was induced in sensitized mice without prior AC injection (positive controls) compared with unsensitized naïve mice (negative controls).

DH responses were suppressed in control IgG-treated mice and anti-VISTA-treated mice, indicating that ACAID was induced in control IgG-treated mice and anti-VISTA-treated mice ($P < 0.01$; Fig. 4A). However, DH responses were significantly higher in anti-VISTA-treated mice compared with control IgG-treated mice ($P < 0.05$; Fig. 4A), suggesting that the partial abrogation of ACAID response was observed in anti-VISTA-treated mice. Control rat IgG treatment did not induce significant ear swelling and is indeed comparable to the negative control (Supplementary Fig. S1).

Next, we analyzed whether anti-VISTA treatment alone enhances the expression of positive delayed hypersensitivity. B6 spleen cells were injected subcutaneously to sensitize the mice. One week later, B6 spleen cells were injected into the ear pinnae and the DH response was determined 24 hours later. Anti-VISTA mAb or control rat IgG as a positive control was administered for 1 week. The DH responses were not significantly different between the anti-VISTA-treated mice and the control IgG-treated mice, suggesting that anti-VISTA treatment does not enhance the expression of positive delayed hypersensitivity (Fig. 4B).

These results indicate that VISTA is at least partly involved in the induction of ACAID.

Treatment With Anti-VISTA mAb Decreases the Proportion of Splenic $CD8^+$ $CD103^+$ T Cells in the ACAID Model

A separate set of ACAID model BALB/c mice treated with anti-VISTA mAb or rat IgG was assessed. Seventy-two hours after induction of ACAID, expression of CD4, CD8, and CD103 in the spleen cells of ACAID model mice was examined with flow cytometry. CD103 was used to identify alloreactive regulatory $CD8^+$ T cells.[31]

No significant differences were observed between the proportions of $CD4^+$ T cells among splenic T cells of ACAID model mice treated with anti-VISTA mAb and that of the control (Fig. 5A). On the other hand, the proportions of $CD8^+$ and $CD8^+$ $CD103^+$ T cells among splenic T cells of BALB/c mice treated with anti-VISTA mAb were significantly lower than those in the control recipients (Fig. 5B–D). Cryostat sections of surviving graft-bearing eyes at 3 weeks were examined following immunofluorescent staining for CD8 and CD103. $CD8^+$ $CD103^+$ T cells were present in the corneal graft (Fig. 5E).

VISTA Does Not Mediate a Local Protective Effect From Allospecific Killing by $CD4^+$ T Cells in the Cornea

The above results led us to hypothesize that the expression of VISTA in the cornea and/or on T cells protects corneal allografts from alloreactive infiltrating T cells by involving ACAID and $CD8^+$ Tregs. To further investigate another possible mechanism of VISTA-mediated corneal allograft survival, we examined destruction of corneal endothelial cells (CECs) by alloreactive T cells in vitro.[8] As a model of the effector phase of corneal rejection, the corneas from C57BL/6 (B6) eyes pretreated with anti-VISTA mAb or control rat IgG were incubated with $CD4^+$ T cells. Dead CECs stained with PI were counted and compared. No significant differences were

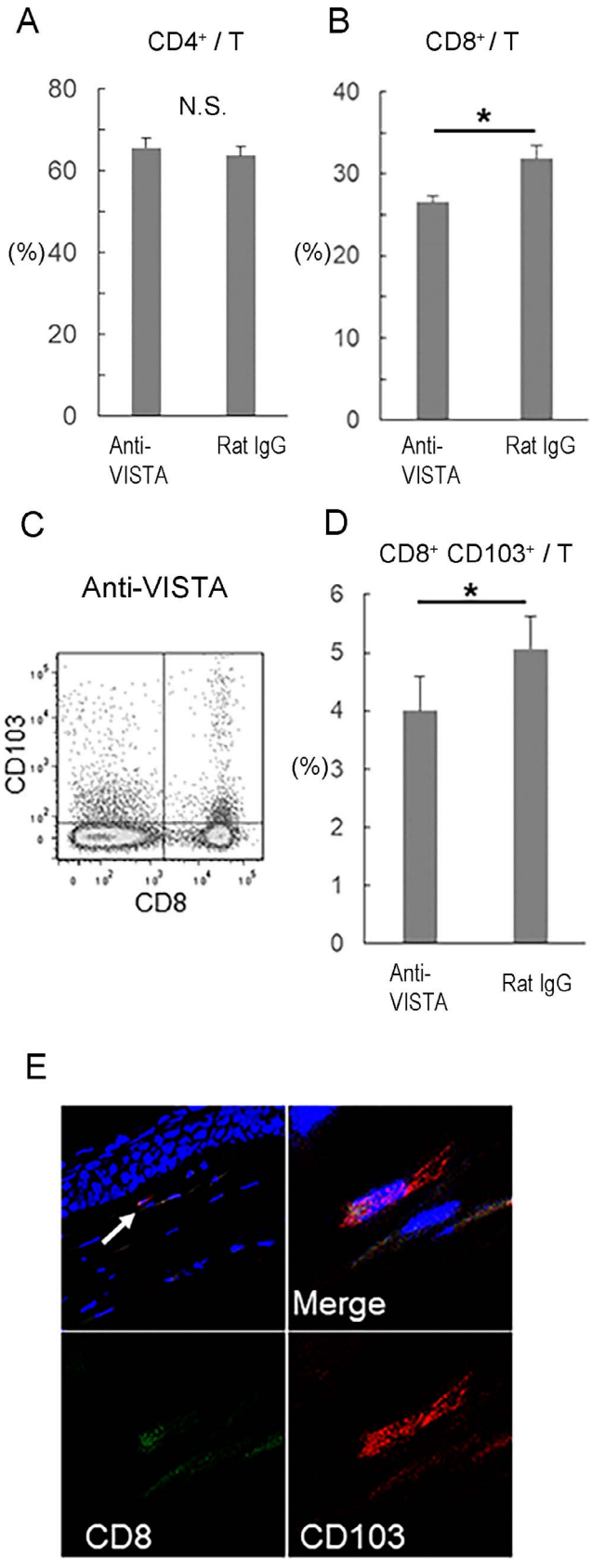

Figure 5. $CD8^+$ $CD103^+$ T cells in the spleen and the cornea of mice treated with anti-VISTA mAb. Seventy-two hours after induction of ACAID, expression of CD4, CD8, and CD8/CD103 in T cells from the spleen of ACAID model mice treated with anti-VISTA mAb or rat IgG was examined with flow cytometry. (**A**) shows the proportion of $CD4^+$ T cells among T cells. (**B**) shows the proportion of $CD8^+$ T cells among T cells. (**C**) shows representative flow cytometry data of $CD8^+$ $CD103^+$ T cells, and (**D**) shows the proportion of $CD8^+$ $CD103^+$ T cells among T cells. Cryostat sections of surviving allografts at 3 weeks (**E**) were stained with FITC-conjugated anti-CD8 mAb (*green*) and PE-conjugated anti-CD103 mAb (*red*). Nuclei were stained with DAPI (*blue*). Arrow shows the $CD8^+$ $CD103^+$ cells, and a double-positive cell at higher magnification is shown. Data are the mean ± SD of three experiments per group. $^*P < 0.05$, N.S.: not significant. Data were analyzed using the two-tailed Student's *t* test.

observed between the number of dead CECs treated with anti-VISTA monoclonal antibodies (mAb) and control IgG after incubation with alloreactive T cells (Fig. 6). Thus, VISTA does not mediate a local protective effect from allospecific killing by $CD4^+$ T cells within the cornea.

Discussion

This experiment was performed to determine whether VISTA is involved in the immune-privileged status of corneal allotransplantation and to explore the underlying mechanisms. We demonstrated with immunohistochemistry that VISTA is expressed in $CD11b^+$ cells in the corneal stroma. With RT-PCR, we showed that VISTA mRNA is also expressed in the cornea. The number of $CD4^+$ and $CD8^+$ T cells in the corneas of the recipients treated with anti-VISTA mAb was greater than that in the control recipients. The allografts became more vulnerable to rejection. Similar to our observations, VISTA blockade using a neutralizing anti-VISTA mAb (MIH 63) preferentially activated T cells in murine models of squamous cell carcinoma, allergic skin inflammation, and allergic asthma.[19–21] These results indicate that VISTA plays an essential role in protecting corneal allografts to control T cells from rejection.

We explored two possible mechanisms for the VISTA-mediated corneal allograft protection from rejection. One possibility was that VISTA is involved in induction of antigen-specific systemic immune tolerance to eye-derived antigens, a phenomenon known as ACAID. Induction of donor-specific ACAID is associated with long-term graft acceptance and promotes the survival of corneal allografts.[1] The present study revealed that ACAID was induced in the presence of control rat IgG, but that ACAID induction was suppressed by administration of anti-VISTA mAb. Thus, VISTA is involved in the induction of ACAID. In ACAID models, we also demonstrated that the proportion of $CD8^+$ $CD103^+$ T cells among splenic T cells of recipients treated with anti-VISTA mAb was significantly lower than that in control recipients. When ACAID is induced, $CD4^+$ and/or $CD8^+$ T cells differentiate into ACAID-inducing regulatory T cells (ACAID-Tregs) in the spleen. $CD4^+$ ACAID-Tregs inhibit the differentiation of Th1 cells in secondary lymph tissues such as LNs, whereas $CD8^+$ ACAID-Tregs inhibit the function of effector T cells (Th1 and Th2) at the local site.[32,33] The present study demonstrated that $CD8^+$ $CD103^+$ regulatory T cells were present in surviving allografts. Regulatory $CD8^+$ $CD103^+$ alloreactive T cells are induced by allogeneic myeloid dendritic cells, and they inhibit the proliferative capacity of alloreactive T cells.[31] Keino et al. have reported that CD103 expression is necessary on $CD8^+$ T cells in vivo for induction of ACAID.[34] $VISTA^+$ $CD11b^+$ cells may capture antigens as resident APCs, and in the spleen, they activate $CD8^+$ $CD103^+$ cells, which differentiate into antigen-specific regulatory T cells that migrate and inhibit induction of DH. Thus, $VISTA^+$ $CD11b^+$ cells may affect the function of $CD8^+$ $CD103^+$ T cells to induce ACAID.

The other possible mechanism of VISTA-mediated ocular immune privilege is that corneal VISTA mediates the immune

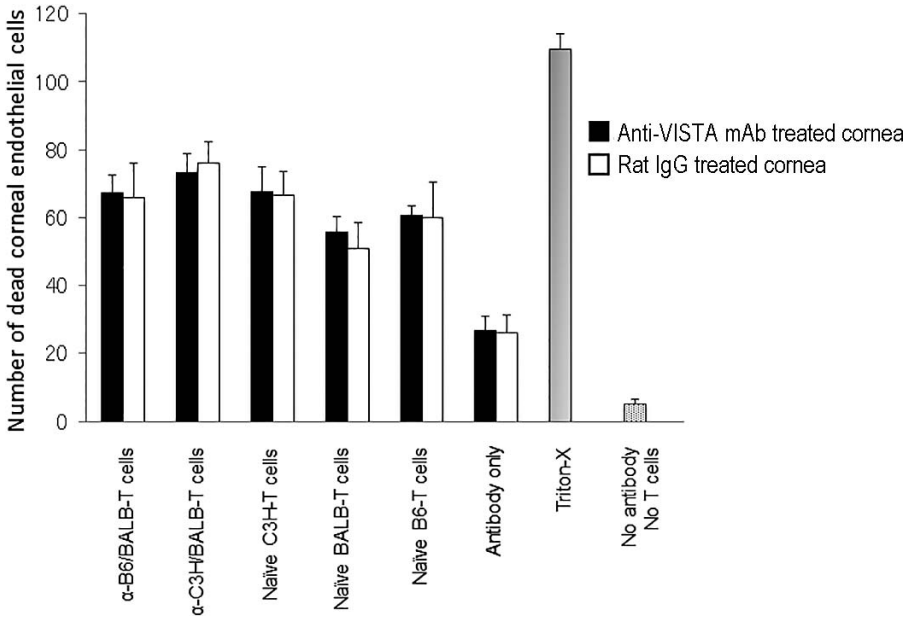

FIGURE 6. VISTA is not involved in alloreactive $CD4^+$ T cell cytotoxicity against corneal endothelial cells. C57BL/6 corneas pretreated with anti-VISTA mAb or anti-rat mAb were incubated with purified $CD4^+$ T cells from the spleens of BALB/c mice presensitized with C57BL/6 splenocytes (α-B6/BALB-T cells) or third-party C3H/He splenocytes (α-C3H/BALB-T cells), or from the spleens of naïve C3H/He mice (naïve C3H-T cells) or naïve BALB/c mice (naïve BALB-T cells) or C57BL/6 mice (naïve B6-T cells). After a 6-hour incubation, corneal endothelial cell (CEC) death was detected by staining unfixed tissue with propidium iodide (PI) followed by confocal microscopic examination. Positive control corneas were incubated with Triton X-100. As a negative control, corneas were incubated without T cells. Data are the mean ± SD of PI+ CECs from five corneas in each group. N.S.: not significant. Data were analyzed using the two-tailed Student's *t* test.

suppressive intraocular microenvironment, in which inflammatory cells within the eye are eliminated or suppressed. To examine whether VISTA mediates a local protective effect from killing by effector T cells in corneal allografts, we evaluated CEC destruction by alloreactive T cells in vitro. No significant differences were observed between the number of dead CECs treated with anti-VISTA mAb and control IgG after incubation with alloreactive T cells. Thus, VISTA does not mediate a local protective effect from the allospecific killing by CD4 T cells within the cornea.

As described above, the present study showed that a mechanism of VISTA-mediated immune privilege in corneal transplantation is tolerance (ACAID) via splenic $CD8^+$ $CD103^+$ T regulatory cells, but not local immune suppression in the cornea. We have previously reported that some of other immune checkpoints such as ICOS ligand[12] and GITR-L[5] have a role on induction of $FoxP3^+$ $CD25^+$ $CD4^+$ T regulatory cells, but we found that anti-VISTA treatment did not affect the proportion of $FoxP3^+$ $CD25^+$ $CD4^+$ T cells in LNs in the recipients of corneal allografts (data not shown).

We demonstrated that the proportion of $VISTA^+$ $CD11b^+$ cells is less than half of whole $CD11b^+$ cells in the areas randomly selected in the corneal sections of normal mouse eyes. To understand functional role of $VISTA^+$ $CD11b^+$ cells, further studies including the distribution and frequency of $VISTA^+$ $CD11b^+$ cells are needed.

To further elucidate the mechanism of immune privilege for corneal transplantation, further immunologic analysis will be necessary. The cornea constitutively expresses various immune-regulating molecules, such as B7-H1,[8] CD95L,[35,36] GITR-L,[5] galectin-9,[10] and ICOS-ICOS ligand[12] 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.

In summary, our present results indicate that VISTA plays a critical role in the maintenance of the immune privileged status of corneal allografts. VISTA is constitutively expressed in the cornea and is involved in allospecific ACAID, which suppress T cell infiltration into the cornea. Forced expression of VISTA may be a new strategy for conferring an immune privileged status on other organs to suppress allograft rejection.

Acknowledgments

The authors thank Hiroshi Takahashi, MD, PhD, at Nippon Medical School for his support on this project.

Supported by grants from Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) (17K11493, 26462700, 15K20290).

Commercial relationships: none.

Disclosure: **T. Kunishige**, None; **H. Taniguchi**, None; **T. Ohno**, None; **M. Azuma**, None; **J. Hori**, None

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