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B7-H1-Induced Apoptosis as a Mechanism of Immune Privilege of Corneal Allografts

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The programmed death-1 (PD-1) costimulatory pathway has been demonstrated to play a role in the regulation of immune responses and peripheral tolerance. We investigated the role of this pathway in establishing an immune privilege status of corneal allografts in mice. B7-H1, but not B7-DC or PD-1, was expressed constitutively in the eye, i.e., cornea, iris-ciliary body, and retina.

Corneal transplantation is so far the most successful of solid organ transplants that are performed in humans (1–3). In the vast majority of uncomplicated cases, only topical, rather than systemic, immunosuppression is required to secure the graft’s survival. This positive clinical experience is matched by the results obtained in experimental models where orthotopic corneal transplants have been performed in immunocompetent mice and rats. Under these conditions, considerable success has been observed for the corneal allografts (4, 5). The usual explanation for the extraordinary success of orthotopic corneal allografts, either in humans or in experimental animals, is related to the phenomenon termed immune privilege (4, 6). In orthotopic grafting, the corneal graft is sutured into an avascular (both blood and lymph) rim of recipient cornea. Moreover, the graft necessarily forms the anterior surface of the anterior chamber. The anterior chamber is a well-described immune-privileged site where grafts of a variety of foreign tissues are accepted for prolonged and often indefinite intervals of time (7, 8). Acceptance of corneal allografts at this site is no exception. Anterior chamber-associated immune deviation (ACAUD) is a well-known phenomenon in which Ag-specific peripheral tolerance is induced after Ag injection into the anterior chamber (9, 10). The anterior chamber contains biologically relevant concentrations of various immunomodulatory neuropeptides, growth factors, cytokines, and soluble cell surface receptors, such as α-melanocyte-stimulating hormone (11), vasoactive intestinal peptide (12), calcitonin gene-related peptide (13), TGF-β (14), thrombospondin (15), macrophage migration inhibitory factor (16), IL-1 receptor antagonist (17), CD46 (18), CD55 (18), CD59 (18), and CD95L (19). These factors suppress innate and adaptive immunity and maintain the immune suppressive microenvironment within the eye (11–19).

Although the site of engraftment is immune privileged, the cornea, when used as an allograft, also has been considered to be an immune-privileged tissue. Early experiments by Medawar and by Barker and Billingham (20, 21) indicated that the cornea had the capacity to avoid or escape destruction by the alloimmune rejection process. Normal cornea lacks blood vessels and lymphatic vessels (22). The central part of cornea, which is used as donor tissue, contains only a small population of MHC class II-expressing APCs (23). Although it has recently been reported that bone marrow-derived cells are present within normal cornea, most of these cells have an immature phenotype lacking MHC class II expression (24). Moreover, normal corneal cells, i.e., epithelial, stromal, and endothelial cells, express no MHC class II and only weak MHC class I Ags (25–27). In addition, normal corneal endothelial cells constitutively express immune-modulating factors such as CD95L (28). Thus, corneal endothelium is considered to play a central role in the protection of corneal allografts from immunologic rejection when transplanted orthotopically in the eyes (29) and heterotopically beneath the kidney capsule (30, 31). Molecular mechanisms of corneal invulnerability are not perfectly understood. Further investigations of the mechanisms of immune privilege are necessary to develop new therapeutic approaches to prevent blinding inflammation within the eye, and also the destructive inflammation observed in other tissues and organs.

Recently, a novel negative regulatory molecule, which is a new member of the B7-CD28 superfamily, has been described, and it is referred to as programmed death-1 (PD-1) (32). This molecule is...
a type I transmembrane protein that was originally identified in a T cell line undergoing programmed cell death. It has been found to be expressed on activated T and B cells and on a subset of thymocytes (32). PD-1 contains an ITIM in its cytoplasmic tail (33, 34) that negatively regulates T cell Ag receptor signaling through interactions with specific ligands. B7-H1 (PD-1) and B7-den-drritic cell (DC) (PD-L2), which belong to the B7 family, were cloned (35, 36) and identified as potential ligands for PD-1 (37, 38). The PD-1 ligands show tissue distribution profiles that are distinct from those of the other B7 family members. The expres-
sion of B7-DC is restricted and mainly found only in lymphoid tissues and on DC (36, 38, 39). Although the expression of B7-H1 has been detected in lymphoid tissues, including activated APCs, monocytes, and B cells (35, 37), B7-H1 is also expressed in non-
lymphoid organs, such as the heart, lung, placenta, kidney, and liver (35, 37). B7-H1 expression has also been found in the majority of human cancers and has been shown to lead to increased apoptosis of activated T cells (40). It has been postulated that this may be a potential mechanism for immune evasion. Although it is well known that the eye is an immune-privileged tissue, there have been no reports on the expression or role of these molecules within the eye.

In this study, we demonstrate for the first time that B7-H1 is constitutively expressed in eye tissue. We used blocking Abs to PD-1, B7-H1, and B7-DC to investigate whether these molecules play a role in establishing the immune-privileged status of corneal allografts. Our data indicate that the interaction between B7-H1 and PD-1 plays an important role in the survival of corneal allografts. Constitutive expression of B7-H1 on the corneal endothel-
ium and stroma induces apoptosis of effector T cells at the graft site. This action of B7-H1 contributes to the immune-privileged status of corneal allografts.

Materials and Methods

Mice and anestheisa

Male BALB/c, C57BL/6, and C3H/He mice were purchased from Sankyo Lab Service. All mice were used at 8–10 wk of age and 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. Each mouse was anesthetized by i.m. injection of a mixture of 3.75 mg of ketamine and 0.75 mg of xylazine before all surgical procedures.

Abs and reagents

Anti-mouse B7-H1 (MIH5, rat IgG2a mAb), anti-mouse B7-DC (TY25, rat IgG2a mAb), and anti-mouse PD-1 (RMP1-14, rat IgG2a mAb) were generated as previously described (39, 41, 42).

For flow cytometry and immunohistochemistry, FITC-, PE-, or biotin-conjugated anti-mouse B7-H1 (MIH5, rat IgG2a), anti-mouse B7-DC (TY25, rat IgG2a), anti-mouse PD-1 (J43, hamster IgG), anti-mouse CD4 (RM4-5, rat IgG2a), anti-mouse CD8 (53-6.7, rat IgG2a), anti-mouse CD11b (M1/70, rat IgG2a), anti-mouse CD11c (N418, Armenian hamster IgG2a), and isotype matches for use as control IgGs were purchased from eBioscience.

Orthotopic corneal transplantation and treatment

Penetrating keratoplasty was performed as previously described (43). Briefly, 2-mm diameter donor corneas were placed in the same sized re-
cipient bed with eight interrupted sutures (11-0 nylon; Mani). Sutures were removed at 8 days postgrafting. C57BL/6 mice were used as donors and BALB/c mice were used as recipients. Three times a week for 8 wk, 0.2 mg of anti-mouse PD-1 mAb (RPM1-14), anti-mouse B7-H1 mAb (MIH5), anti-mouse B7-DC mAb (TY25), or control rat IgG was administered i.p.

Evaluation of corneal allograft

Orthotopic grafts were observed by operative microscopy at least twice a week. A masked assessment of orthotopic corneal grafts was performed by a single observer (M. Wang) who examined each graft for survival accord-
ing 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 anterior chamber visible; and 5+, maximum stromal opacity with total obscuration of the anterior chamber (43). Grafts with opacity scores of 2+ or greater after 3 wk were consid-
ered to have been rejected.

RT-PCR

Cornea, iris-ciliary body, and neural retina were isolated from a total of 10 normal mouse eyes. Total RNA was extracted from each tissue using Isogen (Nippongene). First-strand cDNA was prepared using a SuperScript First Strand Synthesis System (Invitrogen Life Technologies) from 5 μg of total RNA. Standardization of cDNA samples was based on the content of β-actin cDNA. Primers for mouse β-actin were 5′-GTGGGCGCGCTCTAGGCCACCCAA-3′ and 5′-CTCTTTGGATGTCACGACGGATCT-3′. Primers for mouse B7-H1 were 5′-ATGAGGATATTGGCTGCGATATATT-3′ and 5′-TACGCTCCTCCTGAAATTGTGT-3′. PCR was performed in a total volume of 20 μl in PCR buffer in the presence of 0.2 mM dNTP, 1 μM of each primer, and 1 U of TaqDNA polymerase (Advanced Biotechnologies). After 35 cycles of amplification, the PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Histology and immunohistochemistry

Eyes bearing corneal allografts were removed for histological assessment at 2 and 4 wk after transplantation, fixed with 10% formalin, embedded in paraffin, sectioned, and stained with H&E. Approximately 20 sections were prepared from each graft-bearing eye. For immunohistochemistry, normal eyes and graft-bearing eyes were removed and frozen in OCT compound (Sakura Finetech) in acetone-dry ice and stored at −80°C. Cryostat sections (5 μm) were fixed in cold acetone, followed by immunofluorescent staining for the detection of mouse PD-1, B7-H1, B7-DC, CD4, CD8, CD11c, and CD11b. Briefly, after blocking with 2% BSA, the sections were incubated with FITC-, PE-, Cy-3-, or biotin-conjugated primary Ab diluted to 0.5 μg/ml for 2 h. This was followed by staining with streptavidin-
alkaline phosphocyanin (eBioscience) or streptavidin-PD (Jackson Immunore-
search Laboratories) that was diluted to 4 μg/ml for 1 h at room temper-
atur. After washing with PBS, sections were mounted with 4',6-
diamidino-2-phenylindole (DAPI)-containing mounting medium and observed under a confocal microscope.

TUNEL

For the TUNEL assay, grafted eyes were removed and frozen in OCT compound. Cryostat sections (5 μm) were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in 0.1% so-
dium carbonate for 2 min on ice. After washing twice with PBS, TUNEL

Assessment of donor-specific ACAID

ACAID induction was tested as previously described (44). Briefly, recipient BALB/c mice received an anterior chamber (AC) injection of 5 × 105 donor C57BL/6 spleen cells. One week after AC injection, recipients were immunized by s.c. injection of 1 × 107 C57BL/6 spleen cells. Seven days after the immunization, 1 × 105 irradiated (2000 rad) C57BL/6 spleen cells were injected into the right ear pinnae. Twenty-four hours after the ear challenge, ear thickness was measured with a low-pressure micrometer (Mitsuoyo, MTT). Ear swelling was determined as follows: specific ear swelling = ((24-h measurement of right ear − 0-h measurement of right ear) − (24-h measurement of left ear − 0-h measurement of left ear)) × 10−5 mm. Ear swelling responses 24 h after injection are presented as individual values (10−3 mm) for each tested animal and as a group mean ± SEM. For 3 wk, treatments with anti-PD-1, anti-B7-H1, and/or anti-B7-DC mAb were performed starting from the day of the AC injection and con-
tinuing until the day of the 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 were immunized 1 wk previously by a s.c. injection of 10 × 106 C57BL/6 spleen cells. As a negative control, 1 × 106 irradiated
C57BL/6 spleen cells were injected into the right ear pinnae of naïve mice that were not previously AC injected or immunized.

**In vitro assay of corneal endothelial cell destruction by alloreactive T cells**

To examine corneal endothelial destruction by alloreactive T cells in vitro, we constructed a model of the effenter phase of corneal rejection in culture dishes. Fresh normal corneas from C57BL/6 eyes were incubated with 10 μg of anti-B7-H1 mAb (MHI5) or control rat IgG for 2 h in 5% CO2 at 37°C, and then washed twice with PBS. T cells were purified from the spleens of BALB/c mice that were presensitized by s.c. immunization with C57BL/6 spleen cells or with 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 (Miltenyi Biotec) with the Pan T cell Isolation kit (anti-CD45R/B220, anti-CD49b/DX5, anti-CD11b/Mac-1, and anti-Ter119 mAbs; Miltenyi Biotec), according to the manufacturer’s instructions. Purified CD3+ T cells (96–98% pure as estimated by FACSCalibur (BD Biosciences)) were suspended in RPMI 1640. The cornea pretreated with anti-B7-H1 mAb or control rat IgG was incubated with 2.5 × 10^7 T cells for 6 h in 5% CO2 at 37°C, and then washed twice with PBS. The unfixed corneal samples were incubated with 50 μg/ml propidium iodide (PI) for 30 min to stain the nuclei of dead endothelial cells. Using confocal microscopy (×40 magnification), PI-positive cells were counted at three randomly selected areas in the corneal endothelium of each corneal sample, as previously described (45). As a positive control for corneal cell death, normal C57BL/6 cornea was incubated with Triton X-100 without Ab treatment or incubation with T cells. As negative controls, normal C57BL/6 cornea with Ab treatment and incubation without T cells, and cornea without Ab treatment or incubation with T cells were used.

**Statistical analyses**

The corneal graft survival rates were compared using the Kaplan-Meier survival curves and the Breslow-Gehan Wilcoxon test. Ear-swelling measurements and corneal endothelial cell death were analyzed using the two-tailed Student t test. Probability (p) values <0.05 were considered statistically significant.

**Results**

**B7-H1 expression in normal mouse eyes**

RT-PCR revealed that B7-H1 mRNA was strongly expressed in freshly isolated cornea and iris-ciliary body, and weakly expressed in the neural retina of normal mouse eyes (Fig. 1). Immunofluorescent staining indicated that the expression of B7-H1 was localized to corneal endothelial cells and deep stromal cells but was not expressed on the corneal epithelium. B7-H1 protein was also strongly expressed in the iris-ciliary body of normal mouse eyes. Neither PD-1 nor B7-DC was expressed in normal cornea or the iris-ciliary body (Fig. 1).

**Expression of B7-H1 and PD-1 in corneal allografts**

When normal corneas of C57BL/6 were transplanted orthotopically into normal eyes of BALB/c mice, B7-H1 was strongly expressed on corneal endothelial cells and stromal cells in the corneal allografts (Fig. 2, B–D). PD-1-expressing CD4+ T cells infiltrated the allografts and accumulated in the corneal endothelium when the allografts were being rejected (Fig. 2, H–L). There was no expression of B7-DC in the allografts (Fig. 2, E–G).

**Blockade of B7-H1 or PD-1 accelerates corneal allograft rejection**

Normal corneas of C57BL/6 mice were transplanted orthotopically into normal eyes of BALB/c mice. In all recipients, 0.2 mg of anti-PD-1 mAb, anti-B7-H1 mAb, anti-B7-DC mAb, or control rat IgG was administered i.p. three times a week for 8 wk after the grafting. Graft survival was clinically assessed and compared. As shown in Fig. 3, ~50% of allografts survived >8 wk in the control IgG-treated recipients. We have previously reported that ~50% of corneal allografts from C57BL/6 donors survive in untreated BALB/c recipients (43). Thus, the administration of the control IgG does not affect the corneal allograft survival. In contrast, all allografts were rejected within 50 days when the recipients were treated with anti-PD-1 or anti-B7-H1 mAb. Survival of allografts in the anti-PD-1 or anti-B7-H1 mAb-treated mice was significantly shorter than that in the control IgG-treated mice (anti-PD-1, p < 0.05; anti-B7-H1, p < 0.01). In contrast, survival of allografts in the anti-B7-DC mAb-treated mice was statistically indistinguishable from that seen in the control IgG-treated mice (Fig. 3).

**Blockade of PD-1, B7-H1, and/or B7-DC does not abolish ACAID**

Eye-associated tolerance, termed ACAID, is one of the major mechanisms for immune privilege of the eyes and maintains acceptance of corneal allografts (10). We first hypothesized that the vulnerability of corneal allografts that was noted after blockade of PD-1/B7-H1 might result from failure to induce ACAID. To examine this, we tested the effect of PD-1/B7-H1 blockade on allograft-specific ACAID induction using a simple model. B6 spleen cells were used as alloantigens and injected into the right anterior chamber of normal BALB/c eyes. Two weeks later, B6 spleen cells were injected s.c. to sensitize the mice. One more week later, B6 spleen cells were challenged into the ear pinnae to determine the delayed hypersensitivity (DH) response 24 h later.
were stained with DAPI (C, followed by streptavidin-PE and FITC-conjugated anti-CD4 mAb (E) mAb, or control IgG (F), antB7-DC (H), anti-PD-1 (B7-H1 (Bmulticolor immunofluorescence staining with biotin-conjugated anti-
molecules expressed in the cornea interact with the effector PD-1

tion led us to postulate a new hypothesis in which the B7-H1
The failure of the PD-1/B7-H1 blockade to abolish ACAID induc-
the induction of ACAID (Fig. 4). These results indicated that PD-
or anti-B7-DC and/or anti-B7-H1 mAb did not significantly affect
pressed the DH response in the control IgG-treated mice, indicat-
ing the induction of ACAID. The treatments with either anti-PD-1
and/or anti-B7-H1 mAb or control IgG (L), followed by streptavidin-PE and FITC-conjugated anti-CD4 mAb (I). Nuclei were stained with DAPI (C, F, and J). Merged color images are shown in
D, G, and K. Graft center (A–G) and corneal endothelium of the graft
junction (H–L) are shown. Representative sections are shown. “Ced” de-
notes corneal endothelium. Original magnification, ×40.

For 3 wk, treatments with anti-PD-1, anti-B7-H1 and/or anti-B7-DC mAb were applied starting from the day of AC injection and until the day of ear challenge. As shown in Fig. 4, the DH response was induced in the sensitized mice without a prior AC injection (positive control) as compared with unsensitized naive mice (negative control). The prior AC injection significantly suppressed the DH response in the control IgG-treated mice, indicating the induction of ACAID. The treatments with either anti-PD-1 or anti-B7-DC and/or anti-B7-H1 mAb did not significantly affect the induction of ACAID (Fig. 4). These results indicated that PD-1/B7-H1 interaction was not involved in the induction of ACAID.

B7-H1 blockade suppresses apoptosis of infiltrating T cells in corneal allografts

The failure of the PD-1/B7-H1 blockade to abolish ACAID induction led us to postulate a new hypothesis in which the B7-H1 molecules expressed in the cornea interact with the effector PD-1+T cells at the local site. To test this new hypothesis, we performed histological and immunohistological analyses and TUNEL assays in attempt to detect apoptosis of infiltrating T cells in corneal allografts. At 2 wk after grafting, allografts in either control IgG or anti-B7-H1 mAb-treated mice still survived clinically as represented by H&E staining of the graft centers (Fig. 5A, a and d). However, cellular infiltration was found at the graft junction in both types of grafts, with a more severe infiltration in the allografts treated with anti-B7-H1 mAb (Fig. 5A, b and e). In the control IgG-treated grafts, massive apoptosis was observed in the infiltrating cells, which included CD4+ T cells (Fig. 5B, a–e). In contrast, apoptotic cells were hardly detectable among the infiltrating cells, which included PD-1-expressing CD4+ cells, in the anti-B7-H1 mAb-treated grafts (Fig. 5B, f–j). At 4 wk, clinical survival was observed in the control IgG-treated allografts, which exhibited little inflammation (Fig. 5A). There were no PD-1-expressing cells in the allografts (Fig. 5C, a and b). In contrast, the anti-B7-
H1 mAb-treated allografts were undergoing rejection with severe

FIGURE 4. Blockade of PD-1, B7-H1, and/or B7-DC does not abolish ACAID. B6 spleen cells were used as alloantigens and injected into the right AC of BALB/c normal eyes. Two weeks later, B6 spleen cells were injected s.c. to sensitize the mice. After one more week, a challenge was conducted by injecting B6 spleen cells into right ear pinnas of each mouse, and 24 h later, specific ear swelling was measured as an indication of DH. Treatments with anti-PD-1, anti-B7-H1, and/or anti-B7-DC mAb were performed starting from the day of the AC injection and continuing until the day of the ear challenge. DH responses were similarly suppressed in anti-PD-1, anti-B7-H1, anti-B7-DC, or control IgG (C.IgG)-treated groups, and no significant differences were observed among them. Positive control mice (Posi.C) received the s.c. immunization and the ear challenge without previous AC injection. Negative control mice (Neg.C) received only the ear challenge without AC injection or immunization.

FIGURE 3. Blockade of B7-H1 or PD-1 accelerates corneal allograft rejection. Normal corneas of C57BL/6 were transplanted orthotopically into normal eyes of BALB/c mice. After the grafting procedure, recipients were injected with 0.2 mg of anti-PD-1 mAb, anti-B7-H1 mAb, anti-B7-DC mAb, or control rat IgG i.p. three times a week for 8 wk. Graft survival was clinically assessed and compared. Survival of allografts treated with anti-PD-1 or anti-B7-H1 mAb was significantly less than that in the control allografts. (Anti-PD-1, *, p < 0.05; anti-B7-H1, **, p < 0.01; n = 9–10 in each group.)
infiltration of PD-1-expressing CD4+ and CD8+ T cells (Fig. 5, Af and C, c–g).

**B7-H1 protects corneal endothelial cells from killing by alloreactive T cells in vitro**

The above results led us to hypothesize that constitutive expression of B7-H1 in the cornea has the capacity to protect corneal allografts from alloreactive infiltrating T cells by inducing apoptosis of these effector cells. To further substantiate this possibility, we established a model of corneal endothelial cell destruction by alloreactive T cells in vitro. In previous studies by us and others, corneal endothelial cells have been documented to be the target of alloreactive T cells in both human and rodent corneal transplantations (45). As a model of the effector phase of the corneal rejection, normal C57BL/6 corneas were incubated with purified T cells from the spleens of BALB/c mice presensitized against C57BL/6 Ags. Purified T cells from the spleens of BALB/c mice presensitized against third-party C3H/He Ags were used as non-allospecific activated T cells. Splenic T cells from naive BALB/c, C57BL/6, or C3H/He mice were used as allogeneic, syngeneic, or third-party naive T cells, respectively. As shown in Fig. 6, the number of dead corneal endothelial cells in the anti-B7-H1 mAb-treated corneas was significantly larger than that in the control IgG-treated corneas after incubation with activated T cells against third-party alloantigens too (p = 0.0248). Moreover, the number of dead corneal endothelial cells in the anti-B7-H1 mAb-treated corneas was significantly larger than that in the control IgG-treated corneas after incubation with T cells from naive C3H/He mice (p = 0.0051). In contrast, no significant difference was observed between the anti-B7-H1 mAb and control IgG-treated corneas when syngeneic B6 T cells were used as the effector cells. These results suggest that B7-H1 protects corneal endothelial cells from killing by alloreactive T cells irrespectively of a prior sensitization.

**Discussion**

The present study was designed to investigate whether PD-1 and its ligands (B7-H1 and B7-DC) are involved in the immune-privileged status of the eye using the corneal allotransplantation model and to explore the underlying mechanism.

We demonstrated for the first time that normal eye tissue, such as cornea, iris-ciliary body, and neural retina, constitutively express B7-H1. However, the present study revealed that the PD-1/B7-H1 interaction plays an important role in
protecting corneal allografts from rejection. Therefore, the constitutive expression of B7-H1 on corneal endothelial cells and stromal cells may be in part responsible for the immune-privileged status of corneal allografts.

We explored two possible mechanisms for the B7-H1-mediated corneal allograft protection from rejection. One possibility was that the PD-1/B7-H1 interaction might be involved in the induction of Ag-specific systemic immune tolerance to eye-derived Ags, known as ACAID. Ags placed in the anterior chamber are captured by resident APCs, which then migrate through the trabecular meshwork out of the eye and into the blood. Once these cells reach the marginal zone of the spleen, active TGF-β, IL-10, and CCL5 attract and activate Ag-specific CD4+ and CD8+ T cells, which differentiate into Ag-specific regulatory T cells that inhibit induction and expression of delayed hypersensitivity (10). It has been confirmed that induction of donor-specific ACAID is associated with long-term graft acceptance and promotes the survival of corneal allografts (10).

Our results demonstrate that ACAID was induced in the recipients treated with control IgG, because Ag-specific DH was suppressed. DH was also similarly suppressed in the recipients treated with anti-PD-1, anti-B7-H1, and anti-B7-DC mAbs. These results indicate that the induction of ACAID is independent of the PD-1/PD ligand interaction and that the acceptance of corneal grafts can be abrogated even though ACAID remains intact. The B7-H1-mediated protection of corneal allografts from immune rejection is due to a mechanism other than ACAID.

The other possible mechanism is that the corneal B7-H1 supports the immunosuppressive microenvironment, in which inflammatory cells within the eye are deleted or suppressed. The TUNEL staining of corneal allografts clearly indicated that the infiltrating cells were undergoing apoptosis in the recipients treated with control IgG, thereby leading to survival of the allograft. In contrast, TUNEL staining was negative for inflammatory cells, including PD-1+ and CD4+ cells, in the recipients treated with anti-B7-H1 mAb. In these recipients, graft destruction occurred leading to accelerated rejection. These results suggest that the B7-H1 expressed in the cornea functions as an apoptosis-inducing molecule against infiltrating effector cells. B7-H1 expressed on the donor corneal stroma and endothelium may have the capacity to delete PD-1+ effector T cells by inducing apoptosis, probably when these T cells infiltrate into the grafts from peripheral recipient corneal beds through stromal neovessels carrying lymph and blood, and when T cells adhere onto the graft endothelium from the anterior chamber. Consistent with this notion, Dong et al. (40) have reported that B7-H1 expressed in tumors promotes apoptosis of infiltrating cells and that intrahepatic apoptosis of T cells decreases in B7-H1 knockout mice (46). Yu et al. (47) have also demonstrated that liver stellate cells inhibit T cell responses via B7-H1-mediated T cell apoptosis.

To further substantiate the B7-H1-mediated protection of corneal allografts from effector T cells, we evaluated the corneal endothelial cell destruction by alloreactive T cells in vitro. The killing of corneal endothelial cells by alloreactive T cells in vitro was significantly enhanced in the corneas pretreated with anti-B7-H1 mAb as compared with those pretreated with control IgG. This finding demonstrates that B7-H1 expressed on corneal endothelial cells plays a substantial role in the protection of corneal endothelium from destruction by effector T cells. Interestingly, the B7-H1-mediated protection was also observed after incubation with third-party-reactive T cells, indicating that B7-H1 protects cornea from bystander injury by activated T cells. The most surprising finding was that the B7-H1-mediated protection was still observed even after incubation with allogeneic T cells from naive mice. This suggests that naive T cells were sensitized by allogeneic corneal endothelial cells to promote their injury. This manner of Ag sensitization is called “peripheral sensitization,” which was first postulated by Medawer more than 50 years ago. Our present results may indicate that peripheral sensitization exists in the cornea and that the constitutive expression of B7-H1 on corneal endothelial cells inhibits the peripheral sensitization. However, it is still unclear why B7-H1-mediated protection of C57BL/6 corneal endothelium was observed only after incubation with allogeneic T cells from naive C3H mice, but not BALB/c mice. Although we have not directly examined the differences in kinetic expression and function of PD-1 between BALB/c and C3H/He strains, it is possible that the contribution of PD-1 to peripheral sensitization may differ depending on background genetic factors. This is because PD-1-deficient mice show distinct autoimmune symptoms between BALB/c and C57BL/6 background (33, 48). Another possibility is that T cells from naive mice were not equal to naive T cells, and they contained memory T cells that cross-reacted with alloantigens and killed the corneal endothelial cells. The potential of memory T cells to cross-react with C57BL/6 Ags may be higher in naive C3H mice than that in naive BALB/c mice.

Some experiments have shown that the ligation of PD-1 by B7-H1 inhibits the proliferation and cytokine production by activated T cells (37). PD-1-deficient mice have been shown to spontaneously develop systemic autoimmune diseases (48). Indeed, in several animal models, which include experimental autoimmune encephalomyelitis (49), autoimmune diabetes in prediabetic NOD mice (50), and hapten-induced contact hypersensitivity (41), blockade of the PD-1/B7-H1 pathway caused acceleration and exacerbation of the disease. These data support a role for B7-H1 as a negative regulator of T cell responses via PD-1. However, some investigators have reported that B7-H1 costimulates T cell proliferation and cytokine production in vitro (51). In the experimental
colitis model (52), blockade of B7-H1 suppressed expansion of T cells and Th1 cytokine production in vivo. Subudhi et al. (53) have demonstrated that B7-H1 can provide positive costimulation for T cells to promote pancreatic islet allograft rejection.

The role of the PD-1/B7-H1 pathway remains controversial. It is unclear whether B7-H1 costimulation is PD-1-dependent or whether it is mediated by an alternative receptor for B7-H1 (54). Moreover, the cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based switch motif, in addition to an ITIM (34, 35), which may be responsible for transmission of a positive co-stimulatory signal via PD-1.

Although our present results support a role for B7-H1 as a negative regulator, we propose that B7-H1-mediated protection is mediated by inducing apoptosis in PD-1-expressing T cells, at least in the cornea. Although the mechanism of B7-H1-induced apoptosis remains unclear, it is noteworthy that B7-H1-induced apoptosis has been only observed in immune-privileged tissues or sites such as tumors (40), liver (46, 47), and cornea so far. This suggests that B7-H1 can induce apoptosis only when B7-H1-expressing tissues also express other immune-regulating factors such as CD95L (29). The cornea constitutively expresses various immune-regulating factors, such as B7-H1, CD95L (28, 29), MHC class Ib (55), and CD59 (56), in distinct localization patterns within the tissue. The fact that the immune-privileged status of the cornea can be abolished by dysfunction of even just one of these molecules suggests that each of these molecules plays a nonredundant or cooperative role to maintain immune privilege. Further studies are needed to address this possibility.

In summary, the present results indicate that B7-H1 plays a critical role in the maintenance of the immune-privileged status of corneal allografts. B7-H1 is constitutively expressed on corneal endothelial and stromal cells and induces apoptosis of T cells via PD-1 within the cornea. Therefore, the cornea can be an immune-privileged tissue due in part to its constitutive expression of B7-H1. Forced expression of B7-H1 could provide a new strategy for conferring an immune-privileged status on other organs to suppress allograft rejection.

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References


