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Long-Term Survival of Corneal Allografts Is Dependent on Intact CD1d-Reactive NKT Cells

Koh-Hei Sonoda, Masaru Taniguchi, and Joan Stein-Streilein

BALB/c mice that tolerate the allogeneic grafts develop allogeneic-specific anterior chamber-associated immune deviation. Because CD1d-reactive NKT cells are essential for anterior chamber-associated immune deviation, we postulated that the survival of C57BL/6 (B6) corneal graft in BALB/c mice was also dependent on CD1d-reactive NKT cells. The B6 corneal graft rejection rate in BALB/c vs Jα281 knockout (KO) mice, which lack NKT cells, was measured. While there were no difference in the early phase of rejection, the survival rates at 12 wk after grafting for BALB/c and Jα281 KO mice were 50 and 0%, respectively. Because anti-CD1d mAb abrogated the corneal graft survival in the wild-type mice we concluded that CD1d-reactive NKT cells were essential for graft survival. Moreover, allospecific T regulatory (Tr) cells correlated with acceptance of B6 grafts in BALB/c mice, and the adoptive transfer of these allospecific Tr cells to Jα281 KO mice allowed a 50% survival rate of B6 corneal grafts. In conclusion, CD1d-reactive NKT cells are required for induction of allospecific Tr cells and are essential for survival of corneal allografts. Mechanisms that contribute to cornea graft acceptance may lead to new therapies for improvement in graft survival in high-risk corneas and other transplanted tissues and grafts.

Corneal transplantation is the most successful type of solid tissue transplantation in humans (1). The success of corneal transplantation is usually explained by the fact that the cornea is an immune-privileged tissue, capable of resisting immune-mediated destruction (2–4), as well as the fact that the cornea is grafted into an immune-privileged site, the eye (5, 6). However, immune privilege is a dynamic rather than a passive state, and not all orthotopic allografts succeed in humans or experimental animals (7–9). Immune rejection remains a significant clinical problem and ∼10% of patients lose their sight because of corneal graft failure (8).

Inoculation of allogeneic tissue into the anterior chamber (a.c.) of the eye suppresses allospecific systemic delayed-type hypersensitivity (DTH) (10, 11). The model of peripheral tolerance termed a.c.-associated immune deviation (ACAID) is a systemic mechanism that contributes to the active maintenance of immune privilege of the eye. Hosts bearing a long-term clear corneal allograft display an Ag-specific down-regulation of DTH response to donor alloantigens that is reminiscent of ACAID (12). In contrast, hosts that developed donor-specific DTH following keratoplasty invariably rejected their corneal allografts (13, 14). Because orthotopically grafted corneae form the anterior wall of the a.c., grafted endothelial cells express histocompatibility Ags within this immune-privileged site and induce ACAID (14, 15). It was noted that during ACAID induction donor Ag-specific T regulatory (Tr) cells were induced in the spleen (12, 13, 16) and contributed to long-term allograft survival.

Recently we reported that CD1d-reactive NKT cells are central to the development of the Ag-specific Tr cells in ACAID (17). NKT cells belong to a specialized population of lymphocytes that coexpress the TCRβ chain and NK markers (18, 19). A major subpopulation of murine NKT cells expresses a unique invariant Vα14Jα281 Ag receptor not expressed by conventional T cells (18, 20–23). Similarly, NKT cells exist in the human and express the invariant Vα24Jβ18 TCRβ chain (24, 25). NKT cells are restricted by MHC class I-like CD1d molecules (26–28). The CD1d molecule is also required for the development of NKT cells, because CD1d knockout (KO) mice selectively lack NKT cells (29–31). Moreover, the NKT cell must interact with the CD1d molecule for the induction of tolerance, because blocking the CD1d interaction with a CD1d-specific Ab, either in vivo (17) or in vitro (32), blocks the development of Ag-specific Tr cells.

In this report, we show that when a C57BL/6 (B6) donor graft was transplanted to a BALB/c recipient, removal of the CD1-reactive invariant NKT cells from the recipient mice resulted in 0% survival of donor corneas, whereas there was 50% survival if NKT cells were intact. Because NKT cell deficiency prevented the induction of allogeneic Tr cells that facilitate graft survival, the NKT cells are required for ACAID induction to alloantigens as well as soluble Ags (17). Moreover, our data suggest that methods that induce CD1d-reactive NKT cell-dependent tolerance before grafting corneas may lead to prevention of graft rejections in high-risk corneas and suggest novel therapies for patients.

Materials and Methods

Mice

Female 8- to 10-wk-old mice were used in all experiments. BALB/c mice were obtained from Taconic Farms (Taconic, NY). Jα281 KO mice (NKT KO mice) were generated at Chiba University (Chiba, Japan) and backcrossed eight times to BALB/c mice (N8) (23). All mice were maintained...
on food and water ad libitum until they reached the desired weight (20–24 g). All animals were treated humanely and in accordance with the Schepens Animal Care and Use Committee and National Institutes of Health guidelines.

Orthotopic corneal allograft
As described previously (32), before all surgical procedures, recipient mice were anesthetized with an i.p. injection of a mixture of 3 mg of ketamine and 0.0075 mg of xylazine. The central 2 mm of the donor cornea was excised and secured in recipient graft beds with eight interrupted 11-0 nylon sutures (Sharpoint; Vanguard, Houston, TX). Antibiotic ointment was applied to the corneal surface, and the eyelids were closed for 72 h with an 8-0 nylon tarsorrhaphy. All grafted eyes were examined after 72 h. At 1–2 weeks after surgery, technical difficulties (hyphema, infection, or loss of a.c.) were excluded from further consideration. The transplant sutures were removed in all cases on day 7.

Evaluation and scoring of orthotopic corneal allograft
At weekly intervals, grafts were evaluated by slit lamp microscopy and scored for opacification and neovascularization. As previously described (33), the scoring system from 0 to 5+ included the following characteristics: 1) degree of opacification: 0, clear and compact graft; 1+, minimal superficial opacity; 2+, mild deep (stromal) opacity with pupil margin undisturbed (iris structure) visible; 3+, moderate stromal opacity with only pupil margin visible; 4+, intense stromal opacity with a.c. visible; 5+, maximal corneal opacity with total obscuration of the a.c. (grafs with an opacity score of 2+ or greater after 3 wk were considered rejected (immunological failure)) (33); and 2) degree of neovascularization: 0, no vessels; 1+, ves- sels in one or two quadrants of recipient bed only; 2+, vessels in three or four quadrants of recipient bed only; 3+, vessels at recipient-graft border in one or two quadrants; 4+, vessels at recipient-graft border in three or four quadrants; 5+, vessels in peripheral stroma of graft, one or two quadrants; 6+, vessels in peripheral stroma of graft, three or four quadrants; 7+, vessels in central stroma of graft, one or two quadrants; 8+, vessels in central stroma of graft, three or four quadrants.

Direct assessment of DTH
Mice were sensitized to the alloantigen with an inoculation i.v. of 10⁷ allogeneic splenocytes. On the seventh day after sensitization, mice received 2 × 10⁵ irradiated (2000 rad) allogeneic donor splenocytes injected into the right pinnace and ear thickness was measured with an engineer’s micrometer (Mitutoyo, Paramus, NJ) 24 h post ear pinnace inoculation (12).

LAT assay for DTH
Allogeneic-specific regulatory Tr cells were detected by local adoptive transfer (LAT) assay as described before (12, 16). BALB/c mice were sensitized with an inoculation (i.v.) of 10⁷ allogeneic (B6) splenocytes. Fourteen days postsensitization, the T cells that were primed to the allogeneic Ags were enriched from the dissociated spleen cells by removing B cells and macrophages using IMMULAN columns (Biotex Laboratories, Houston, TX) and were used as DTH effector cells in a LAT assay. Irradiated (2000 rad) B6 spleen cells were used as stimulator cells. Regulator cells were column-enriched splenic T cells harvested from BALB/c mice 7 days post a.c. inoculation of 5 × 10⁶ irradiated B6 spleen cells. Effector, stimulator, and regulator cells (5 × 10⁵ of each) were mixed and resuspended in 10 μl of HBSS for inoculation into the right ear pinnae of naive mice. Ear swelling was measured with an engineer’s micrometer 24 h post ear pinnace inoculation. Naive T cells from unmanipulated BALB/c mice were used as effector cells and regulator cells for the negative control. T cells primed to alloagric Ags were used as effector cells and naive T cells from unmanipulated mice were used as regulator cells for positive control.

Antibodies
The Abs used for flow cytometry analysis were as follows: Fc block (anti-mouse FcγRIII/IIb mAb, 2.4G2), biotin-conjugated anti-Ly49c (5E6), bi- otin-conjugated anti-CD1 mAb (1B1), FITC-conjugated anti-CD3 mAb (145-2C11), and CyChrome 5-conjugated anti-TCRβ mAb (H57-597) were all purchased from BD PharMingen (San Diego, CA). PE-conjugated anti-B220 mAb (RA3-6B2) and PE-conjugated Mac-1 (M1/70.15) were purchased from Caltag Laboratories (South San Francisco, CA). Streptavidin-PE was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

The Abs used for in vivo treatment were as follows: anti-CD1 mAb (3C11, rat IgM) was also purified from mouse ascites using protein A columns. Purified rat Ig was purchased from Sigma-Aldrich (St. Louis, MO) and used as control for anti-CD1 mAb. Abs were inoculated i.p. (50 μg each) every 4 days after grafting until the grafted corneas were rejected.

Flow cytometry
Splenic NK and NKT cells were analyzed by flow cytometry. RBC were lysed by adding Tris-buffered ammonium chloride to a cell pellet of spleen cells and incubated for 2 min at room temperature. After washing, staining was performed in the presence of saturating concentrations of Fc block (blocks FcγRII/III). Cells were stained with the following three reagents and colors (using concentrations recommended by the manufacturer): biotin-conjugated anti-Ly49c mAb counterstained with streptavidin-PE, CyChrome 5-conjugated anti-TCRβ chain mAb, and FITC-conjugated anti-CD3 mAb. Stained cells were analyzed on an EPICS XL flow cytometer (Beckman Coulter, Miami, FL).

Blocking of NKT/CD1 cell interaction in vivo
Purified anti-CD1 mAb (3C11: 50 μg in 100 μl of PBS) or control rat Ig (50 μg in 100 μl of PBS) was injected i.p. into recipient BALB/c mice to block the subsequent interaction of CD1d with NKT cells (17). Flow cytometry studies of spleen cells harvested from the 3C11-treated mice confirmed that the CD1-positive cells (biotin-conjugated anti-CD1 mAb (B1) counterstained by streptavidin-PE) neither were depleted nor showed changes in the populations of T cells (FITC-conjugated anti-CD3 mAb), B cells (PE-conjugated anti-B220 mAb), NK/NKT cells (triple staining: FITC-conjugated anti-CD3 mAb, CyChrome 5-conjugated anti-TCRβ chain mAb, and biotin-conjugated anti-Ly49c mAb counterstained by streptavidin-PE), and macrophages (PE-conjugated Mac-1) (data not shown).

Adaptive transfer of Tr cells into grafted J281 KO mice
After RBC lysis, T cells were enriched from dissociated spleen cells from naive or B6 graft-accepted mice by removing B cells and macrophages using IMMULAN columns (Biotex Laboratories, Houston, TX). Enriched T cells were treated with biotin-conjugated anti-Ly49c, MicroBeads-con- jugated anti-mouse pan-NK cells (DX5) (Miltenyi Biotec, Auburn, CA) and anti-NKT cell (U5A2-13, rat IgG2a) (34, 35) (kindly provided by Dr. H. Wakasugi (National Cancer Center Research Institute, Tokyo, Japan)), washed twice in PBS (pH 7.2) containing 0.5% BSA and 2 μM EDTA. Ab-labeled cells were treated with anti-rat Ig MicroBeads and streptavidin MicroBeads (Miltenyi Biotec) for 15 min and washed twice. To harvest NK/NKT cell-depleted population, cells were applied to type MS⁺ positive selection column with MiniMACS (Miltenyi Biotec) and collected eluted population. Cells were stained with CyChrome 5-conjugated anti-TCRβ chain mAb and streptavidin-PE. T cell enrichment and NKT cell depletion were confirmed by flow cytometry.

Recipient J281 KO mice grafted with allogeneic corneas were inoculated (i.v.) with 10³ mouse enriched T cells (NKT/NK-depleted cells) harvested from either naive BALB/c mice or BALB/c mice with accepted B6 corneas of 8-wk duration, 4 wk after grafting. Following this treatment, grafts were evaluated by slit lamp microscopy at weekly intervals.

Statistics
Data were analyzed for significant differences in ear swelling among experimental groups by ANOVA and Scheffe’s test. A value of p ≤ 0.05 was considered significant. Kaplan-Meier survival curves were constructed and the Mantel-Cox test was used to compare the probability of corneal graft survival. A value of p ≤ 0.05 was considered significant.

Results
Role of Vα14⁺ NKT cells in early inflammation and graft rejection
To investigate the role of NKT cells in the corneal allograft, we compared the allograft (B6) survival between BALB/c and J281KO (BALB/c background). During the natural time course of corneal allograft in BALB/c mice, the maximum inflammation was observed 7 days after grafting (Fig. 1A). The early inflammation was thought to be caused mainly by interrupted sutures or surgical treatment, and not mediated by allo-specific immune response, because the same level of inflammation was observed in syngeneic graft controls (33). Most of the allografts that did not suffer from technical problems became clear (a score of <2 in opacity) by day 14 or 7 days after removal of sutures on day 7.
Role of NKT cells in survival of cornea grafts

Three weeks after grafting, grafted corneas began to show signs of allospecific immune rejection (33). As documented before, 50% of B6 cornea grafts survive in BALB/c mice (33), and the 50% of grafted corneas that were rejected occurred between the third and seventh week after transplantation. The surviving grafted corneas were accepted by 12 wk in BALB/c mice (Fig. 2A). Although there were no differences in opacity until the sixth week, all B6 grafted corneas in Ja281 KO mice were rejected by 8 wk after grafting (Fig. 2A). After the third week the persistently opaque corneas were ultimately rejected (Fig. 2B). Thus the long-term graft survival was dependent on the NKT cells expressing the invariant TCR.

Role of CD1d in allograft survival

Invariant (Vo14Ja281) NKT cells are restricted by CD1d. To confirm that the NKT cells must interact with CD1d to achieve tolerance of the allografts, we blocked the NKT cell CD1d interaction with specific Ab in vivo. Previous studies showed that anti-CD1d mAb, 3C11, indeed blocks the interactions between NKT cells and CD1d molecule and abrogated generation of ACAID induced by a protein Ag (OVA) (17). Again, there were no critical differences between control Ig-treated and anti-CD1d-treated mice in the inflammation for the first 14 days (data not shown). Nor were there remarkable changes in the rejection rate between these two groups before 6 wk after grafting. However, between 6 and 8 wk post-grafting all BALB/c mice treated with the anti-CD1d Ab rejected their B6 corneas. Mice treated with control Ab accepted the corneas (Fig. 3). Therefore, CD1d-reactive NKT cells are essential for long-term allograft survival in B6 donor cornea grafts to BALB/c recipients.

Ja281 KO mice receiving allografts failed to induce DTH suppression

To explore the potential mechanism of late-phase graft rejection in Ja281 KO mice, we compared the allospecific DTH response in grafted BALB/c mice and Ja281 KO mice. Twelve weeks after corneas were grafted, mice received a s.c. inoculation of B6 splenocytes (10⁷). After 7 days, mice were challenged intradermally in the ear pinnae with 2 × 10⁶ irradiated B6 splenocytes and ear swelling was measured 24 h later. It is well documented that suppression of allospecific DTH response is correlated with graft acceptance (8, 12). BALB/c mice that rejected their allografts (50%) responded to the Ag challenge with ear swelling equal to the positive control (Fig. 4); however, BALB/c mice that accepted the allografts (50%) exhibited reduced ear swelling (Fig. 4). It is known that BALB/c mice with accepted corneal allografts induce inflammation in corneal allografts in BALB/c and Ja281 KO mice. A, Clinical evaluation of the opacity and neovascularization in grafted cornea. BALB/c (n = 20) or Ja281 KO (n = 16) mice received orthotopic corneal grafts from B6 donors. The central 2 mm of the donor cornea was excised and secured in recipient graft beds with eight interrupted 11-0 nylon. Transplant sutures were removed on day 7. The graft opacity and neovascularization on the graft were evaluated by clinical score on days 3, 7, and 14. The ordinate shows the score and the abscissa shows the days from grafting. Data are the combined results of three independent experiments. B, Typical pictures of grafted cornea (~day 14). BALB/c or Ja281 KO mice received orthotopic corneal grafts from B6 donors. Typical pictures of the anterior segment of the grafted eye are shown at each time point. The clinical scores (opacity and neovascularization) are indicated below the pictures.

FIGURE 1. Early inflammation in corneal allografts in BALB/c and Ja281 KO mice. A, Clinical evaluation of the opacity and neovascularization in grafted cornea. BALB/c (n = 20) or Ja281 KO (n = 16) mice received orthotopic corneal grafts from B6 donors. The central 2 mm of the donor cornea was excised and secured in recipient graft beds with eight interrupted 11-0 nylon. Transplant sutures were removed on day 7. The graft opacity and neovascularization on the graft were evaluated by clinical score on days 3, 7, and 14. The ordinate shows the score and the abscissa shows the days from grafting. Data are the combined results of three independent experiments. B, Typical pictures of grafted cornea (~day 14). BALB/c or Ja281 KO mice received orthotopic corneal grafts from B6 donors. Typical pictures of the anterior segment of the grafted eye are shown at each time point. The clinical scores (opacity and neovascularization) are indicated below the pictures.
Mice were inoculated s.c. with \(10^7\) donor (B6) splenocytes (nonirradiated) to receive donor cell inoculations (a.c.). Twelve weeks after corneal grafting, mice were inoculated s.c. with \(10^7\) irradiated (2000 rad) donor splenocytes without allogeneic priming. The positive DTH control, age-matched naive BALB/c, or Jax281 KO mice that received allogeneic grafts (or control nontreated mice) and were treated with biotinylated anti-Ly49C mAb and streptavidin-PE and then analyzed by flow cytometry (Fig. 5A). In these studies NKT cell KO mice that received the adoptive transfer of Tr cells accepted their corneal allografts (Fig. 5B). Although NKT cells are known to produce immunosuppressive cytokines (30, 31, 36, 37), our data indicate that NKT cells themselves are not the regulatory cells functioning in corneal allograft survival but that the Tr cells are conventional Ag-specific T cells. CD1d-reactive NKT cells are essential for the generation of Tr cells, but once the Ag-specific Tr cells are generated the NKT cell is no longer necessary for graft survival.

Adoptive transfer of Tr cells from WT mice with allografts abrogated the graft rejection in Jax281 KO mice

The precise role and mechanism of the involvement of NKT cells in corneal graft survival were further investigated by adoptively transferring splenic Tr cells harvested from BALB/c mice that displayed long-term accepted corneal grafts into Jax281 KO mice 4 wk after receiving B6 corneal grafts. At this time, NKT cells were depleted from enriched T cells by treatment with a combination of biotinylated anti-Ly49C mAb (5E6) and anti-NK1.1 cell mAb (US52A-13) before passing them over a mixture of streptavidin and goat anti-rat conjugation magnetic beads. The quality of depletion technique was confirmed by flow cytometry (Fig. 5A). In these studies NKT cell KO mice that received the adoptive transfer of Tr cells accepted their corneal allografts (Fig. 5B). Although NKT cells are known to produce immunosuppressive cytokines (30, 31, 36, 37), our data indicate that NKT cells themselves are not the regulatory cells functioning in corneal allograft survival but that the Tr cells are conventional Ag-specific T cells. CD1d-reactive NKT cells are essential for the generation of Tr cells, but once the Ag-specific Tr cells are generated the NKT cell is no longer necessary for graft survival.

FIGURE 3. Effect of anti-CD1 mAb treatment in grafted WT mice. Survival rates of orthotopic corneal grafts from B6 donors in BALB/c mice treated with i.p. inoculation of either control Ig or anti-CD1d mAb every 4 days (50 μg per mouse in each inoculation) after corneal grafting were compared. The ordinate shows the percentage of grafts accepted and the abscissa shows the weeks post grafting. *, Significant differences (p ≤ 0.05). Data are the combined results of two independent experiments.

FIGURE 4. Allospecific DTH response in mice bearing cornea grafts or receiving donor cell inoculations (a.c.). Twelve weeks after corneal grafting, mice were inoculated s.c. with \(10^7\) donor (B6) splenocytes (nonirradiated). Seven days later mice received \(2 \times 10^6\) irradiated (2000 rad) donor splenocytes into the right ear pinna. Ear thickness was measured after 24 h. The negative DTH control, age-matched naive BALB/c, or Jax281 KO mice received \(2 \times 10^6\) irradiated (2000 rad) donor splenocytes without allogeneic priming. The positive DTH control mice were sensitized with age-matched B6 splenocytes s.c. and were not grafted. Changes (Δ) in ear swelling measurements (24 h post ear challenge) are shown on the ordinate.

Individual mice that rejected their allografts; *, significant differences (p ≤ 0.05).

FIGURE 5. Adoptive transfer of Tr cells into grafted Jax281 KO mice that received allogeneic grafts. A. Confirmation of T cell enrichment and NKT cell depletion. Column-enriched splenic T cells were harvested from BALB/c mice that accepted the cornea grafts (or control nontreated mice) and were treated with biotin-conjugated anti-Ly49c, MicroBeads-conjugated anti-mouse pan-NK cells (DX5), and anti-NKT cell Ab (US52A-13, rat IgG2a). Ab-labeled cells were treated with anti-rat Ig MicroBeads and streptavidin MicroBeads. To deplete NK/NKT cell population, cells were applied to type MS positive selection column with MiniMACS (Miltenyi Biotec) and the eluate was collected. The negatively selected cells and the similarly treated whole spleen cell population not exposed to the magnetic field were stained with CyChrome 5-conjugated anti-TCRβ chain mAb and streptavidin-PE and then analyzed by flow cytometry.

The fluorescence intensity for Cy5-TCRβ chain and PE-Ly49c are shown on the ordinate and abscissa, respectively. The percentage of cells within the T cell (large square), NKT cell (small square), and NK cell (rectangle) quadrants before (whole splenocytes) and after (cells for transfer) selection are listed in the blocks. B. Figure 5. Effect of regulatory T cell transfer on grafts in Jax281 KO mice. Splenic T cells from BALB/c mice that had accepted their corneal grafts for 8 wk were adoptively transferred into Jax281 KO (BALB/c background) mice that received orthotopic corneal grafts from B6 donors. For control, Jax281 KO mice with grafts received splenic T cells from naive BALB/c mice. The ordinate shows the percentage of grafts accepted and the abscissa shows the weeks from grafting. *, Significant differences (p ≤ 0.05). Data are the combined results of two independent experiments.
needed for the effector phase of suppression. Thus, CD1d-reactive NKT cells are essential for the generation of allogeneic Tr cells that promote corneal acceptance.

Ja281 KO mice failed to induce allogeneic-specific ACAID and Tr cells

Previously, we showed a role for NKT cells in OVA-induced ACAID. To further examine the role of NKT cells in the generation of Tr cells (ACAID) to alloantigens, we injected B6 spleen cells into the a.c. of Ja281 KO mice. The successful grafting of an allogeneic cornea is equal to putting the alloantigens into the non-inflamed a.c. for induction of ACAID against allogeneic Ags (15). In fact, individual animals develop allospecific Tr cells in the spleen within a week of inoculation of allogeneic cells into non-inflamed eyes by the same mechanism that leads to acceptance of grafts (11, 38).

One panel each of BALB/c or Ja281 KO mice was inoculated (a.c.) with $5 \times 10^5$ irradiated B6 spleenocytes 7 days before receiving an s.c. inoculation of $10^7$ B6 spleenocytes. Seven days after allogeneic cell immunization, mice were challenged in the ear pinnae with $2 \times 10^6$ irradiated B6 spleenocytes and ear swelling was measured 24 h later. We found that DTH responses were suppressed in a.c. inoculated BALB/c but not in Ja281 KO mice that received the a.c. inoculation of B6 spleenocytes (Fig. 6A). A direct measure of Tr cell induction was assessed in a LAT assay. Inoculation (a.c.) induced allospecific Tr cells in BALB but not Ja281 KO mice (Fig. 6B). Thus, in the absence of NKT cells, a.c. inoculation does not induce the generation of efferent allogeneic Tr cells.

Discussion

This report shows that CD1d-reactive NKT cells are required for the long-term survival of corneal allografts. Fifty percent of recipient mice acquire allogeneic-specific ACAID, develop allogeneic-specific Tr cells, and accept corneal grafts indefinitely. Previously, we reported that CD1d-reactive NKT cells were required for the induction of Tr cells to protein Ags inoculated into the a.c. (17). This report shows that the NKT cell-dependent tolerance concept applies to allogeneic Ags as well.

Compared with other organ transplantation (skin, heart, liver, kidney, etc.), corneal transplantation has some unique features. Corneal transplantation is the most successful type of solid tissue transplantation in humans (1) and long-term acceptance is achieved without immunosuppressive therapy. The success of corneal transplantation was originally explained by the lack of lymphatic drainage in the cornea. Aqueous humor contains the nutrition for donor cornea instead of blood vessels. Thus, the surgeon does not need to do angiostomy for corneal transplantation.

The success of corneal transplantation is also correlated with the immune-privileged status of the eye. In addition to the immune-privileged quality of the corneal tissue itself (4, 39, 40), the orthotopic cornea graft becomes part of the a.c. of the eye, which is an immune-privileged site. It is well documented that corneal allograft acceptance is closely related to allospecific Tr cell induction, which imparts allogeneic ACAID (5, 12, 41). However, not all orthotopic allografts actually succeed in humans (42). Grafts are rejected in so-called “high-risk” recipients. The high-risk condition appears to occur when the immune privilege state is compromised. Grafts that follow corneal herpes infection, burning, etc., are unable to induce allospecific Tr cells and therefore are rejected (8, 9, 43). To achieve graft acceptance in a high-risk patient, the precise mechanism of allospecific Tr cell induction in corneal transplantation needs to be elucidated.

![Image]

**FIGURE 6.** Generation of Tr cells in WT and Ja281 KO mice inoculated a.c. with allogeneic cells. A. Allogeneic-specific ACAID induction in Ja281 KO mice. One panel (five mice per group) of age-matched BALB/c or Ja281 KO mice was inoculated (a.c.) with $5 \times 10^5$ irradiated B6 spleenocytes 7 days before receiving a s.c. inoculation of $10^7$ B6 spleenocytes. After 7 days, mice were challenged intradermally in the ear pinna with $2 \times 10^6$ irradiated B6 spleenocytes and ear swelling was measured 24 h later. For negative DTH control, age-matched naive BALB/c or Ja281 KO mice received $2 \times 10^6$ irradiated (2000 rad) donor spleenocytes without allogeneic priming. For positive DTH control, mice were inoculated with age-matched B6 spleenocytes s.c. but did not receive a previous a.c. inoculation of cells. Changes ($\Delta$) in ear swelling measurements (24 h post ear challenge) are shown on the ordinate. *, A significant difference ($p < 0.05$). B. LAT. Fourteen days after $10^6$ allogeneic (B6) spleenocytes were inoculated s.c. into naive BALB/c mice, T cells were enriched from recipient spleens and used as allogeneic sensitized T effector cells. Irradiated (2000 rad) B6 spleenocytes, T cells enriched from spleens of BALB/c, or Ja281 KO mice that were previously (7 days) inoculated (a.c.) with $5 \times 10^5$ irradiated B6 spleenocytes were tested as regulator cells. Effector, stimulator, and regulator cells (5 x $10^5$ of each) were resuspended in 10 $\mu$L of HBSS and inoculated into the ear pinnae of naive mice. Ear swelling was measured at 24 h. As a negative control, naive T cells from unmanipulated BALB/c mice were used as effector cells and regulator cells. Alloprimed T cells were used as effector cells, and naive T cells from unmanipulated mice were used as regulator cells for positive control. Mixtures of cells injected into the ear pinnae are indicated along the abscissa below each bar. Changes ($\Delta$) in ear swelling measurements (24 h post ear challenge) are shown on the ordinate. *, A significant difference ($p < 0.05$).
Recently, several reports showed that CD1d-reactive Vα14 NKT cells were involved in transplantation tolerance induced by systemic administration of Ab to the donor organ. Such Ab treatment apparently induced tolerance and promoted graft survival even in nonimmune-privileged sites (44, 45). Ikeharra et al. (46) demonstrated that NKT cells were crucial for acceptance of islet xenografts in mice treated with anti-CD4 Ab. Also, Seino et al. (47) showed that NKT cells played a crucial role in the induction of cardiac allograft tolerance in an experimental model of tolerance that follows anti-LFA-1/ICAM-1 or anti-B7-1/B7-2 Ab treatment, and Terabe et al. (48) reported that NKT mediated repression of tumor immunosurveillance. These studies support our notion that Vα14 NKT cells are required for the induction of allospecific Tr cells and perhaps in a variety of tolerance models that may share the commonality of effector Tr cells (6).

The timing of corneal graft rejection in Jα281 KO mice and anti-CD1d mAb-treated mice is a point for discussion. Mice rejected their allograft between 6 and 8 wk (Figs. 1–3). There may be at least two different mechanisms involved in the host immune reaction against an allograft. One mechanism is clinically effective during the first 6–8 wk after grafting and independent of CD1d-reactive NKT cells. In both NKT cell KO-deficient mice and anti-CD1d mAb-treated mice corneal grafts were rejected at the same rate as grafts in the control group (Figs. 1 and 2). The other mechanism appears to be effective after this initial rejection phase and conspires to maintain clarity of the allograft. This relatively late-phase immune response was closely correlated to CD1d activation of invariant NKT cells. In human corneal transplantation, most of the immunological graft rejection suddenly occurs between 2 and 7 wk postoperation. The relationship between the time course of corneal graft rejection in human and mouse is not clear. However, CD1d-reactive invariant NKT cells do exist in humans (24, 25) and may contribute to corneal graft survival as well.

Another point to consider is why Tr cells appear to be generated more slowly in the allograft system compared with when OVA is inoculated into the eye. It is thought that CD1d-restricted NKT cell-dependent tolerance is induced more slowly in mice after cornea grafting than after direct injection (a.c.) of Ag because the release of the eye-derived APCs carrying the alloantigens from the graft is delayed. It is reported that the innervation (3) (and it is postulated that the cellular traffic patterns) are disrupted by the surgical manipulation needed for placement of the graft. Moreover, we show in this manuscript that direct injection of allospleen cells into the a.c. induces Tr cells at a time course similar to OVA inoculation (Fig. 6).

The elucidation of mechanisms for tolerance that use CD1d-reactive NKT cells to induce allogeneic Tr cells in grafted mice or allogeneic cell inoculated (a.c.) mice can be guided by studies in a similar model of CD1d/NKT cell-dependent tolerance induced to a.c. inoculation of soluble protein Ags (OVA) (17). In this model, we found that NKT cells produced IL-10 in response to CD1d stimulation and that NKT cell-derived IL-10 was critical to the development of the Ag-specific Tr cells as well. By extrapolation, we predict that IL-10-mediated mechanisms are involved with the generation of allogeneic-specific Tr cells. Further investigations are needed to clarify this point.

Our data do not deny the possible contribution of other type of cells in the induction of allogeneic tolerance. Above all, several CD1d-reactive cells other than Vα14 NKT cells (49–51) may contribute to the acceptance of corneal allograft. Also, multiple cell types express CD1d and could provide essential signals to Vα14 NKT cells for Tr cell induction. However, the inability to show survival of grafts in Jα281 KO mice is strong evidence that corneal graft survival in this mouse strain combination is dependent on CD1d-restricted NKT cells.

These data have fomented ideas for developing therapeutic maneuvers based upon those that promote the generation of Ag-specific, CD1d-reactive, NKT cell-dependent Tr cells to prevent and reverse allogeneic sensitization in mice and humans. Thus, the molecular studies will be extended into humans with the goal of therapeutic applications for rejecting corneal graft leading to tolerance involving CD1d/NKT cell interactions.

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