Role of Tumor Necrosis Factor Receptor Expression in Anterior Chamber-Associated Immune Deviation (ACAID) and Corneal Allograft Survival

Jerry Y. Niederkorn, Elizabeth Mayhew, Jessamee Mellon, and Sushma Hegde

PURPOSE. To determine the role of tumor necrosis factor receptors (TNFRs) in corneal allograft rejection.

METHODS. Corneal epithelial and endothelial cells were examined by flow cytometry for the expression of TNFRI and TNFRII and their susceptibility to TNF-α-induced apoptosis. Corneal allografts from normal and TNFRI and TNFRII knockout (KO) C57BL/6 mice were transplanted to BALB/c hosts, and the fate of the allografts was monitored. C57BL/6 spleen cells were injected into the anterior chamber (AC) of BALB/c mice to induce anterior chamber-associated immune deviation (ACAID) and promote corneal allograft survival. The presence of ACAID suppressor cells in corneal allograft recipients was tested using a local adoptive transfer (LAT) assay.

RESULTS. Murine corneal epithelial and endothelial cells expressed TNFRI and TNFRII and were susceptible to TNF-α-induced apoptosis, yet corneal allografts from either TNFRI or TNFRII donors did not enjoy a lower incidence of rejection or a prolongation in survival time compared to corneal allografts from normal C57BL/6 donors. Moreover, all 31 of the TNFRI KO corneal grafts were rejected by naive BALB/c hosts. Rejection of TNFRII KO corneal grafts occurred even though suppressor cells developed in the hosts and inhibited the expression of delayed-type hypersensitivity to donor alloantigens.

CONCLUSIONS. Expression of TNFRII on corneal cells conveys a degree of protection against immune rejection of corneal allografts by a mechanism that is independent of ACAID. Moreover, disruption of ACAID before the application of TNFRII to corneal allografts fails to improve survival and does not replace the TNFRI-dependent protective mechanism. (Invest Ophthalmol Vis Sci. 2004;45:2674–2681) DOI:10.1167/iovs.04-0144

Corneal transplantation is the oldest, most common, and usually most successful form of solid tissue allografting. Even though HLA matching is not normally performed, and systemic immunosuppressive drugs are not used, up to 90% of first-time corneal allografts succeed. These features have led to the conclusion that corneal allografts enjoy an immune privilege that is not found in other forms of organ transplantation. Several factors contribute to the immune privilege of corneal allografts including (1) the absence of patent blood and lymphatic vessels in the corneal graft bed; (2) the low expression of major histocompatibility (MHC) class I and II antigens on corneal cells; (3) the expression of complement regulatory proteins in the aqueous humor and on corneal cells; (4) the expression of FasL on corneal cells; and (5) anterior chamber-associated immune deviation (ACAID). ACAID is a unique form of immune regulation that is induced when antigens are introduced into the anterior chamber of the eye. Th1 immune responses, such as delayed-type hypersensitivity (DTH), are actively suppressed in an antigen-specific manner within 7 days of anterior chamber priming. Corneal allografts are in direct contact with the anterior chamber, and it has been demonstrated that corneal allograft survival correlates closely with the graft’s capacity to induce ACAID to donor alloantigens. The appearance of corneal allograft rejection coincides with the loss of ACAID and the emergence of donor-specific DTH. Moreover, maneuvers that prevent the induction of ACAID, such as splenectomy, produce a sharp increase in the incidence of corneal graft rejection.

Despite ocular immune privilege, a significant number of corneal allografts undergo immune rejection. Although Maumenee demonstrated the immunologic basis of corneal graft rejection more than 50 years ago, the exact immune effector mechanisms that mediate corneal allograft destruction still remain a mystery. A large body of evidence suggests that corneal allograft rejection is a cell-mediated, T-cell–dependent process. The best evidence to date points to CD4+ T cells as pivotal contributors to the immune rejection of corneal allografts. Rodents deficient in CD4+ T cells—either by gene disruption or systemic treatment with anti-CD4 depleting antibody—display sharply reduced corneal allograft rejection.

The most obvious pathway for a CD4+ T cell’s contribution to corneal allograft rejection is through their well-established role in DTH. During the DTH response, CD4+ Th1 cells elaborate a variety of cytokines that may influence corneal allograft rejection. Chief among these are interferon (IFN)-γ and tumor necrosis factor (TNF-α). However, IFN-γ does not appear to be a key player in this process, as corneal graft rejection occurs in IFN-γ knockout (KO) mice. By contrast, TNF-α has been implicated in corneal allograft rejection. TNF-α mRNA has been detected in the beds of rejecting corneal allografts, and TNF-α protein levels are significantly elevated in the aqueous humor and the serum of hosts that reject corneal allografts.

The present study considered the hypothesis that TNF-α can induce apoptosis in corneal cells and plays a key role in the immune rejection of corneal allografts. Studies were also performed to confirm the hypothesis that corneal allograft rejection correlates with the loss of ACAID suppressor cells.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2b), TNFRI (p55) knockout (KO), TNFRII (p75) KO, BALB/c (H-2d), and BALB/c IFN-γ KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed and cared for in accordance with the guidelines of the University Commit-
Corneal Cell Lines

C57BL/6 corneal cell cultures were established as described previously. 26 Cell lines were maintained in complete MEM-(CMEM) at 37°C and 5% CO₂. CMEM consisted of MEM supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 2 mM MEM vitamins, and 1% penicillin-streptomycin-fungizone solution (all from BioWhittaker, Walkersville, MD).

Flow Cytometry

Expression of murine TNFRI and TNFRII was assessed by flow cytometry, as previously described. 27 Corneal cells (1 × 10⁶) were incubated with 1 μg/mL goat antimurine TNFRI or antimurine TNFRII antibody (R&D Systems, Minneapolis, MN) for 30 minutes on ice, washed three times, and incubated with FITC-labeled rat anti-goat IgG secondary antibody for 20 minutes at 4°C, washed three additional times in PBS, fixed in 1% paraformaldehyde, and assessed for fluorescence by flow cytometry (FACScan; BD Biosciences, Lincoln Park, NJ). All events were analyzed with the accompanying software (CellQuest; BD Biosciences).

Annexin V Staining to Evaluate In Vitro Apoptosis

Apoptosis in the corneal cells was also measured by detection of annexin V binding to phosphatidylserine expressed on the cell membranes of apoptotic cells. 28 Corneal cells were incubated with recombinant murine TNF-α (50–200 ng/mL; BD Biosciences, San Diego, CA) or in CMEM alone for 24, 48, and 72 hours at 37°C. Apoptosis was then assessed using a commercially available detection kit (TACS Annexin-V FITC Apoptosis Detection Kit; R&D Systems). In this assay, annexin V-FITC’s binding to phosphatidylserine (PS) was used as an indicator for apoptotic cells. Although PS is normally confined to the inner leaflet of the plasma membrane (PM), it appears on the external leaflet of the PM during apoptosis, preceding even the nuclear changes that typically characterize apoptosis. Propidium iodide (PI) staining was used to identify cells that had lost membrane integrity and were thus classified as being necrotic rather than apoptotic. During flow cytometry, cells that were positive for annexin V-FITC fluorescence (FL1) only were identified as being apoptotic, whereas cells that were positive for both PI fluorescence (FL1) and annexin V-FITC fluorescence (FL3) were identified as being necrotic.

Statistical Analysis

Median survival times (MSTs) and mean rejection times (MRTs) were calculated for the various corneal allografts. The Mann-Whitney test determined the statistical significance in MST. The differences in the incidences of rejection were evaluated by χ² analysis. Results for DTH assays were evaluated by Student’s t-test. Differences in all experiments were considered to be statistically significant at P < 0.05.

RESULTS

Corneal Epithelial and Endothelial Cell Expression of TNFRI and TNFRII and Susceptibility to TNF-Induced Apoptosis

Although TNF receptors are expressed on most cells in the body, it was important to determine their expression on corneal cells. C57BL/6 corneal epithelial and endothelial cells were examined by flow cytometry for the expression of TNFRI and TNFRII. The results of a typical analysis are shown in Figure 1 and demonstrate that C57BL/6 corneal epithelial and endothelial cells expressed significant amounts of TNFRII. By contrast, corneal endothelial cells expressed a markedly lower percentage of TNFRI-positive cells.

In vitro annexin V staining was performed to determine whether corneal epithelial and endothelial cells were susceptible to TNF-induced apoptosis. Corneal cells were incubated...
with recombinant murine TNF-α (50–200 ng/mL) for 24, 48, and 72 hours and assessed for apoptosis. The results indicated that both corneal epithelial and endothelial cells underwent TNF-induced apoptosis, which was detectable as early as 24 hours after initial exposure and persisted for 72 hours (Fig. 2). However, corneal endothelial cells were significantly more susceptible to apoptosis than epithelial cells. The results demonstrate that corneal epithelial and endothelial cells express significant quantities of both TNFRI and TNFRII and are susceptible to TNF-induced apoptosis.

Fate of Corneal Allografts from TNFRI and TNFRII KO Donors

Previous studies have suggested that TNF-α may play a significant role in corneal allograft rejection. TNF-α mRNA expression is elevated in the graft beds of hosts that receive corneal allografts, and there is a sharp increase in TNF-α levels in the serum and aqueous humor in hosts that reject corneal allografts. The expression of TNFRI and TNFRII receptors on corneal epithelial and endothelial cells and the susceptibility of these cells to TNF-induced apoptosis prompted us to consider the hypothesis that corneal allografts lacking TNFRI or TNFRII would experience a reduced incidence or delayed onset of immune rejection. Accordingly, corneal grafts from TNFRI KO, TNFRII KO, and normal C57BL/6 donors were transplanted orthotopically to BALB/c hosts. As in previous experiments, 50% of the normal C57BL/6 corneal grafts underwent rejection in BALB/c hosts, with a mean rejection time of 29 ± 7.4 days (Fig. 3). Corneal allografts from C57BL/6 TNFRI KO donors were rejected at the same tempo (25.38 ± 8.85 days) and with the same incidence (8/15; 53%) as allografts from normal C57BL/6 donors (4/8; 50%). By contrast, all the TNFRII KO corneal grafts (31/31; 100%) underwent rejection (Fig. 3). The high incidence of TNFRII KO graft rejection was not due to graft failure or the inability of the TNFRII KO corneal grafts to survive the surgical procedure, as TNFRII KO corneal homografts transplanted to TNFRII KO recipients survived indefinitely (data not shown).

Effect of Viable TNFRII KO Cells on the Induction of ACAID

Previous studies have shown that mice bearing long-term-surviving orthotopic corneal allografts also express antigen-specific downregulation of DTH that is reminiscent of ACAID, whereas the appearance of corneal allograft rejection coincides with the loss of ACAID and the emergence of donor-specific DTH. Maneuvers that prevent the induction of ACAID, such as splenectomy, result in a steep increase in the immune rejection of corneal allografts. With this in mind, we considered the hypothesis that the 50% survival rate for normal C57BL/6 corneal allografts was attributable to the capacity of normal C57BL6 corneal allografts to induce ACAID and that the immune rejection of 100% of the TNFRII KO corneal allografts was due to the inability of TNFRII KO cells to induce ACAID. This hypothesis is consistent with findings of Elzey et al., who found that TNFRI KO spleen cells derivatized with trini-
trophenol (TNP) were capable of inducing ACAID in syngeneic mice, whereas TNFRII KO cells were not. We explored the hypothesis that TNFRI KO cells were capable of inducing ACAID in response to C57BL/6 alloantigens, but TNFRII KO cells could not. A conventional footpad-swelling assay was used to examine the capacity of TNFRI KO and TNFRII KO cells to induce ACAID. Briefly, $1 \times 10^6$ plastic nonadherent spleen cells from TNFRI KO, TNFRII KO, or normal C57BL/6 donors were injected into the AC of BALB/c mice. Seven days later the mice were immunized with an SC injection of $1 \times 10^6$ C57BL/6 cells. DTH responses to C57BL/6 alloantigens were evaluated 14 days after the SC injection. AC injection of either normal or TNFRI KO spleen cells induced ACAID (Fig. 4). By contrast, AC injection of TNFRII KO cells failed to induce

![Graph A](image1.png)

**FIGURE 2.** Susceptibility of murine corneal cells to TNF-α-induced apoptosis. C57BL/6 corneal epithelial (A) and endothelial (B) cells were incubated with various concentrations of recombinant murine TNF-α for 24, 48, and 72 hours. Apoptosis was determined by flow cytometry, using annexin V staining as an indicator of apoptosis. PI-positive cells were removed by gating before determining annexin V-positive cells. The results shown are typical of three independent assays. Cells treated with staurosporine (5 μg/mL) served as the positive control and cells incubated with normal medium were used as the negative control. Probabilities were determined by Student's $t$-test. Experiments using corneal epithelial cells: $P = 0.0001$ for all TNF-α treatment groups compared with the MEM-treated control, except for 50 ng/mL TNF-α 24-hour ($P = 0.0002$), TNF-α 72-hour ($P = 0.0047$), and 100 ng/mL TNF-α 72 hour ($P = 0.0006$). Experiments using corneal endothelial cells: $P = 0.0001$ compared with MEM controls in all groups except 100 ng/mL TNF-α 24-hour ($P = 0.0029$) and 200 ng/mL TNF-α ($P = 0.0059$).

![Graph B](image2.png)

**FIGURE 3.** Fate of corneal allografts from TNFRI KO donors and TNFRII KO donors. BALB/c mice were grafted with C57BL/6 corneal allografts prepared from normal donors, TNFRI KO donors, or TNFRII KO donors. (A) TNFRI KO corneal grafts ($n = 15$) were compared with corneal allografts from normal C57BL/6 donors ($n = 10; P > 0.05$). (B) Corneal allografts from TNFRII KO donors ($n = 31$) were compared with corneal allografts form normal C57BL/6 donors ($n = 8; P < 0.001$).
cells. Although signaling through the TNFRI receptor upregulates Fas expression on the AC-injected, TNP-derivatized cells, the TNFRII receptor does not support TNF-mediated upregulation of Fas. Therefore, experiments were performed to confirm that the survival of C57BL/6 corneal allografts in an effort to induce ACAID and promote the acceptance of corneal allografts.

DTH Responses in Corneal Allograft Recipients

The results to this point supported the hypothesis that the rejection of TNFRII KO corneal allografts was due to the inability of the allogeneic corneal cells to induce ACAID. Therefore, experiments were performed to confirm that the survival of C57BL/6 corneal allografts correlates with the presence of suppressor cells that inhibit the expression of DTH, whereas the rejection of TNFRII KO corneal allografts coincides with the presence of DTH to C57BL/6 alloantigens. A LAT assay was used to reveal the presence of regulatory T cells in hosts that had received either normal C57BL/6 or TNFRII KO C57BL/6 corneal allografts. Briefly, spleen cells were collected from three categories of BALB/c mice: hosts that had rejected C57BL/6 corneal allografts; hosts bearing clear C57BL/6 corneal allografts on day 30; and hosts that had rejected TNFRII KO corneal allografts. Five hundred thousand spleen cells from each of the corneal allografted groups were mixed with 5 x 10^5 spleen cells from BALB/c mice that had been SC immunized with 1 x 10^6 normal C57BL/6 spleen cells 14 days earlier. The BALB/c spleen cell mixture was combined with 1 x 10^5 C57BL/6 spleen cells (alloantigen) and injected into the pinnae of naïve BALB/c mice. The negative control consisted of BALB/c spleen cells from naïve mice mixed with 1 x 10^5 C57BL/6 spleen cells (alloantigen) and injected into the pinnae of naïve BALB/c mice. The positive control was a mixture of 5 x 10^5 naïve BALB/c spleen cells mixed with 5 x 10^5 spleen cells from BALB/c mice previously immunized against C57BL/6 alloantigens and 1 x 10^5 C57BL/6 cells. The results of the LAT assay confirmed the previous DTH findings from corneal allografted hosts and revealed the presence of regulatory T cells that suppressed DTH responses (Fig. 6). Unexpectedly, spleen cells from BALB/c mice that had rejected TNFRII KO corneal allografts suppressed the expression of DTH by spleen cells from mice SC immunized with C57BL/6 alloantigens. By contrast, spleen cells from hosts that had rejected C57BL/6 corneal allografts did not suppress DTH in the LAT assay. As expected, in hosts bearing clear C57BL/6 corneal allografts, regulatory T cells developed that suppressed DTH responses to C57BL/6 alloantigens. This experiment was performed three times with similar results.

These results suggest that TNFRII KO corneal allografts underwent rejection in the face of suppressor cells. Additional experiments were performed to determine whether induction of ACAID before the application of TNFRII KO corneal allografts would promote corneal graft survival. Plastic nonadherent spleen cells or corneal endothelial cells from C57BL/6 mice were injected into the AC of normal BALB/c mice 7 to 10 days before transplantation of TNFRII corneal allografts in an effort to induce ACAID and promote the acceptance of corneal allografts.
The fate of the TNFRII KO corneal allografts was followed in the AC-injected BALB/c mice and compared with C57BL/6 corneal allograft survival in untreated BALB/c hosts. Although this protocol is known to induce ACAID and promote corneal allograft survival in other donor–host combinations, no beneficial effects were observed. In three separate experiments, 93% (14/15) of the TNFRII corneal grafts transplanted to AC-primed BALB/c mice underwent rejection at a tempo equal to and in some cases, faster than that observed with normal C57BL/6 corneal grafts (data not shown).

**DISCUSSION**

The results reported herein indicate that corneal epithelial and endothelial cells express TNFRI and TNFRII and are susceptible to TNF-α-induced apoptosis. This observation, combined with previous reports linking TNF-α to corneal graft rejection, prompted us to explore the fate of corneal allografts lacking either TNFRI or TNFRII. The biological activity of TNF-α is regulated by two receptors, TNFRI (p55) and TNFRII (p75), which share homologous extracellular domains. Depending on the cell type, TNF-α can induce apoptosis through various mechanisms, including receptor homo- or heterodimerization and activation of downstream signaling pathways such as the NF-κB or JNK pathways.
either TNFR1 or TNFR2. Both corneal endothelial and epithelial cells were found to express TNFR1 and TNFR2 and were susceptible to TNF-α-induced apoptosis. Accordingly, we expected to observe either a delay in graft rejection or a reduced incidence of rejection of corneal allografts lacking either TNFR1 or TNFR2. The results of three separate experiments involving 31 recipients of TNFR1 KO corneal allografts indicated that the opposite occurred; that is, the absence of TNFR1 on the grafted corneas was associated with a 100% incidence of graft rejection. Thus, the absence of TNFR1 increased the risk of corneal graft rejection. This further implies that signaling through TNFR1 on donor cells promotes an immunoregulatory event that either prevents the induction or the expression of alloimmunity. ACAID is the most likely candidate for such an immunoregulatory mechanism.

It has been reported that TNF-α plays a pivotal role in the induction of ACAID. AC injections have been shown to upregulate the expression of TNF-α in the AC.27,35 TNF-α enhances Fas-mediated apoptosis of lymphoid cells by promoting a decrease in intracellular levels of FADD-like IL-1β-converting enzyme inhibitory protein (FLIP) and an in increase in the production of the proapoptotic protein Bax.27 However, this effect necessitates signaling through TNFR1. Normal and TNFR1 KO cells undergo apoptosis after AC injection, whereas TNFR1 KO cells resist Fas-induced apoptosis in the eye. More important, hapten-derivatized T cells from TNFR1 and normal mice induce ACAID, but cells from TNFR1 KO mice are ineffective. Collectively, these results suggest that signaling through TNFR1 sensitizes lymphoid cells for Fas-induced apoptosis, which is necessary for the induction of ACAID by a variety of antigens, including allografts.54 It is noteworthy that TNF-α is a principal inducer of IL-10 biosynthesis,35 and it has been shown that IL-10 is essential for the induction of ACAID.36-37 Thus, the exceptionally high incidence of rejection of the TNFR1 KO corneal allografts may be attributable to the resistance of the corneal cells to Fas-mediated apoptosis within the AC. This is supported by results indicating that cells from TNFR1 KO mice were incapable of inducing ACAID. However, when apoptosis of the TNFR1 KO cells was induced by x-irradiation before AC injection, ACAID was induced. The failure of TNFR1 KO cells to induce ACAID to C57BL/6 allografts suggests that the high incidence of rejection of TNFR1 KO corneal grafts may be offset if ACAID were induced in hosts destined to receive TNFR1 KO corneal allografts. However, AC injection of either plastic-nonadherent, normal C57BL/6 spleen cells or C57BL/6 corneal endothelial cells before the application of TNFR1 KO corneal allografts failed to improve graft survival using a protocol that is known to induce ACAID and promote the survival of normal corneal allografts.29 These results suggest that inducing ACAID with donor lymphoid cells does not promote the survival of TNFR1 KO corneal allografts, which implies that the high incidence of TNFR1 KO corneal allograft rejection is unrelated to the grafts’ inability to induce ACAID. Furthermore, suppressor cells that inhibit the expression of DTH to C57BL/6 alloantigens develop in BALB/c mice that have rejected TNFR1 KO corneal allografts. Of note, such suppressor cells are not detected in BALB/c hosts that have rejected C57BL/6 corneal allografts. Suppressor cells are, however, detectable in BALB/c hosts bearing long-term corneal allografts from normal C57BL/6 donors. These results indicate that the presence of ACAID does not guarantee corneal allograft survival and that presence of TNFR1 on corneal cells conveys some form of protection that reduces the likelihood of immune rejection. We are unaware of any reports suggesting that TNFR1 has a similar protective effect in other forms of organ transplantation. Thus, the nature of this protection remains to be identified. Understanding this mechanism could have important clinical applications in promoting corneal allograft survival.

References