

CUTTING EDGE

Cutting Edge: Atopy Promotes Th2 Responses to Alloantigens and Increases the Incidence and Tempo of Corneal Allograft Rejection¹

Clay Beauregard, Christina Stevens, Elizabeth Mayhew, and Jerry Y. Niederkorn²

A large body of evidence suggests that corneal allograft rejection is mediated by a type 1 Th cell response and that deviation toward type 2 immunity favors graft survival. However, clinical observations indicate that patients with severe ocular allergies have increased risk of corneal allograft rejection. We used a mouse model of atopic conjunctivitis to evaluate the effects of Th2 immune deviation on corneal allograft survival and possible mechanisms of graft rejection. Our results reveal the following novel findings: 1) atopic conjunctivitis promotes systemic Th2 immune responses to corneal graft donor alloantigens; 2) corneal allografts in atopic host eyes have an increased incidence and swifter tempo of rejection; 3) increased rejection is associated with alterations in systemic T cell-mediated responses to donor alloantigens; and 4) corneal allograft rejection in atopic hosts does not require the direct involvement of infiltrating eosinophils. The Journal of Immunology, 2005, 174: 6577–6581.

Prevailing dogma proposes that allograft rejection is mediated by type 1 CD4⁺ Th1 immune responses, which produce IFN- γ and IL-2 and mediate delayed-type hypersensitivity (DTH) to donor's histocompatibility Ags (1). In the classical paradigm, Th2 cells secrete IL-4, IL-5, and other specific cytokines, which cross-regulate Th1 cytokines (and vice versa), thereby suppressing clonal expansion of Th1 cells (2). Thus, it has been proposed that tilting the alloimmune response toward a Th2 pathway favors allograft survival (3).

Corneal transplantation is the most common solid organ grafting procedure in the Western hemisphere, and rejection is correlated with alloantigen-specific DTH³ and production of IFN- γ (4). In both humans and rodents, rejected corneal allografts are infiltrated with CD4⁺ T cells (5, 6) and contain increased amounts of IFN- γ (7).

The Th1-Th2 paradigm has led to the hypothesis that deviation to a Th2 immune response would improve corneal allo-

graft survival (3). However, promoting Th2 immune responses by elimination of the Th1 arm has been shown to cause eosinophil-mediated rejection of cardiac and skin allografts in mice (8–10). Previous studies involving corneal transplantation in Th2-deviated hosts have produced conflicting results ranging from allograft acceptance to complete rejection (3, 11). Clinical observations indicate that patients with severe ocular allergies are at a higher risk of corneal transplant rejection (12). In this study, we examined corneal allograft rejection in a mouse model of atopic conjunctivitis involving sensitization and topical challenge with short ragweed pollen, a common allergen in humans (13). The Th cell population in this model stimulates allergen-specific increases in IgE, IL-4, IL-5, and eosinophilic inflammation (13).

Materials and Methods

Animals

All animal studies were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas. Animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, National Institutes of Health. Female BALB/c (H-2^d) mice (Taconic Farms), female B10.D2 (H-2^d) mice (The Jackson Laboratory), female C3H/HeJ (H-2^b) mice (The Jackson Laboratory), and female C57BL/6 (H-2^b) mice (The Jackson Laboratory) were 5–9 wk of age.

Induction of atopic conjunctivitis

Allergic conjunctivitis was induced using a previously described ragweed pollen model of atopy (13). BALB/c mice were given 50 μ g of short ragweed pollen (International Biologicals) in 5 mg of alum (Pierce) by footpad injection on day 0. Atopic conjunctivitis was induced by a "multihit" topical challenge method in which immunized mice were given 1.5 mg of short ragweed pollen suspended in PBS to the right eye once per day from days 10 to 16.

Orthotopic corneal transplantation

Atopic or naive BALB/c mice were given orthotopic corneal grafts onto the right eye from naive C57BL/6, B10.D2, or BALB/c donors as described previously (14). Graft opacity, edema, and neovascularization were scored as described previously (15). Grafts were considered rejected on the day when corneal opacity was scored as 3+ on a scale of 0 to 4+. Atopic host mice received topical short ragweed pollen to the right eye 3 days/wk throughout the study.

Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, TX 75390

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² Address correspondence and reprint requests to Dr. Jerry Y. Niederkorn, Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9057. E-mail address: jerry.niederkorn@utsouthwestern.edu

³ Abbreviations used in this paper: DTH, delayed-type hypersensitivity; MST, median survival time; LN, lymph node.

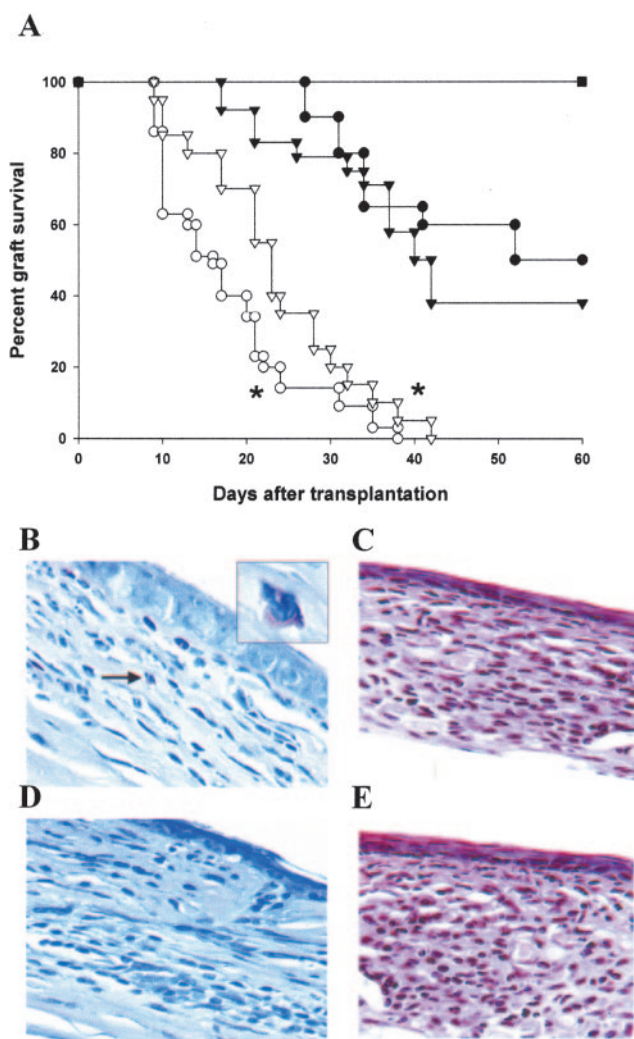
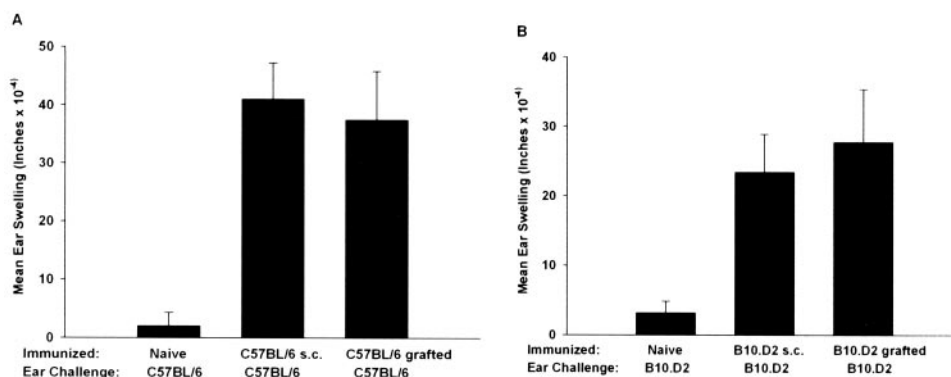


FIGURE 1. Corneal allograft survival in normal and atopic hosts. *A*, C57BL/6 corneal allografts transplanted to the eyes of normal BALB/c mice (●) ($n = 20$) or eyes of atopic BALB/c that were challenged with pollen (○) ($n = 30$). B10.D2 corneal allografts transplanted to normal BALB/c mice (▼) ($n = 19$) or to atopic BALB/c that were challenged with pollen (▽) ($n = 20$). BALB/c corneal isografts transplanted to eyes that were challenged with pollen in atopic BALB/c mice (■) ($n = 10$). *, $p < 0.001$ by Mann-Whitney U test between atopic and normal hosts. Giemsa stain and H&E stain of rejected cornea from an atopic BALB/c recipient of C57BL/6 allograft shows marked eosinophilic (B) and mononuclear inflammation (C). *Insert* shows eosinophil. Giemsa (D) and H&E (E) stains of rejected corneal allografts from a normal BALB/c recipient of C57BL/6 allograft shows no eosinophilia.

FIGURE 2. DTH to alloantigens in atopic BALB/c that have rejected a corneal allograft. Atopic BALB/c mice that rejected a C57BL/6 corneal allografts (A) or a B10.D2 corneal allografts (B). Naive BALB/c mice served as negative controls and BALB/c mice immunized s.c. with C57BL/6 spleen cells served as positive controls. Error bars indicate SEM. *, $p < 0.05$ by the Student's t test.



DTH assay

DTH in response to alloantigens was detected by an ear swelling assay as described previously (16). BALB/c mice immunized s.c. with alloantigens and complete Freund's adjuvant (Sigma-Aldrich) were used as positive controls. Net ear swelling at 24 h was recorded.

Mixed lymphocyte reactions and cytokine ELISA

We measured Th1 and Th2 cytokine production in draining cervical lymph nodes (LNs) of BALB/c corneal allograft recipients by performing MLR with irradiated C57BL/6 splenocytes at a 1:1 ratio for 48 h in 2 ml of culture medium. Supernatants were assayed for IL-4, IL-5, or IFN- γ by capture ELISA (R&D Systems).

Immunohistochemistry

We labeled 4- μ m eye cross-sections with mAbs against TIM-3 (8B.2C12; eBioscience) or T1/ST2 (DJ8; MD Biosciences) using the Vectastain Elite ABC system (Vector Laboratories). Purified rat IgG1 isotype control (BD Pharmingen) was used to determine Ab specificity.

Statistical methods

Statistical differences in median survival times (MSTs) of corneal grafts were compared using the nonparametric Mann-Whitney U test. All other groups of means were compared using the Student's t test and were considered significantly different at $p < 0.05$.

Results and Discussion

Corneal allograft survival in hosts with atopic conjunctivitis

Corneal allografts were transplanted orthotopically to normal or atopic BALB/c hosts, and graft survival was assessed for up to 60 days. Atopic conjunctivitis was induced in BALB/c hosts by footpad sensitization to short ragweed pollen followed by topical challenge to the right eye with ragweed pollen. Corneal allografts were transplanted onto the allergen-challenged eye. Atopic BALB/c hosts rejected 100% of fully mismatched (different at both MHC and minor H loci) C57BL/6 corneal allografts with a MST of 13 days and a mean rejection time of 17 ± 8 days (Fig. 1). Normal, nonatopic BALB/c hosts rejected 50% of C57BL/6 corneal allografts with a MST of 54 days and a mean rejection time of 36 ± 9 days. MSTs were significantly different ($p < 0.01$).

Atopic BALB/c hosts also rejected 100% of MHC-matched (different multiple minor H loci) B10.D2 corneal allografts with a MST of 21 days and a mean rejection time of 23 ± 9 days (Fig. 1). Normal BALB/c hosts rejected 62.5% of B10.D2 corneal allografts with a MST of 37 days and a mean rejection time of 32 ± 9 . MSTs were significantly different ($p < 0.01$). Atopic BALB/c mice did not reject syngeneic BALB/c corneal grafts (Fig. 1).

Our results demonstrate for the first time that mice with Th2-type immune deviation induced by atopy are at higher risk

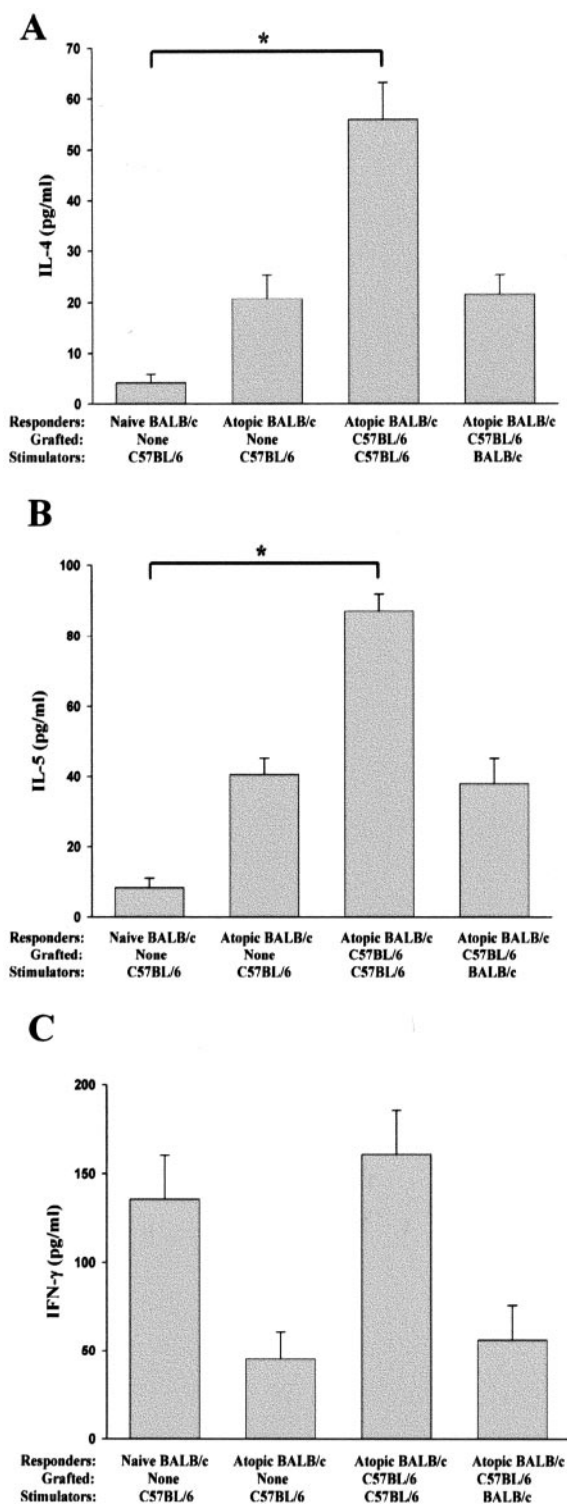


FIGURE 3. ELISA for Th1 and Th2 cytokines produced by cervical LN cells from atopic BALB/c mice that had rejected C57BL/6 corneal allografts. BALB/c LN cells were stimulated with either C57BL/6 or BALB/c LN cells in vitro, and the quantity of IL-4, IL-5, and IFN- γ was determined by ELISA. Error bars indicate SEM. *, $p < 0.05$ by the Student's t test.

to reject allografts. Rejection occurs more frequently and at a faster rate than in normal, non-Th2-polarized recipients. The increased allograft rejection in our model was not due to the direct effects of local allergic inflammation induced by topical

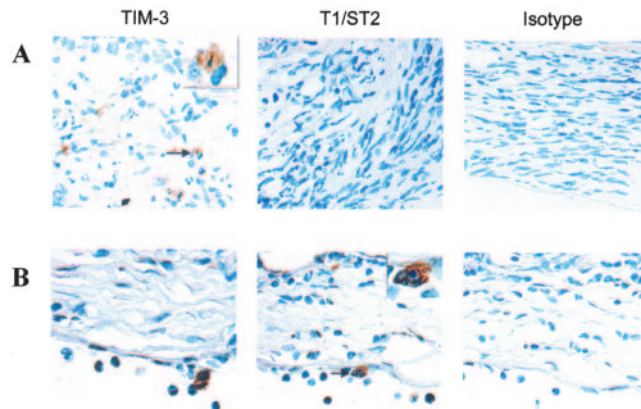


FIGURE 4. Infiltration of Th1 (TIM-3⁺) and Th2 (T1/ST2⁺) cells into rejected corneal allografts. *A*, Immunohistochemical staining of C57BL/6 corneal allografts rejected by a normal BALB/c host showed TIM-3⁺ cells (*inset*) but not T1/ST2⁺ cells. *B*, Immunohistochemical staining of C57BL/6 corneal allografts rejected by an atopic BALB/c host shows TIM-3⁺ and T1/ST2⁺ (*inset*) cells in cornea ($\times 600$).

ragweed challenge because syngeneic corneal grafts transplanted to atopic hosts did not undergo rejection.

Because corneal allograft rejection is accompanied normally by a heavy mononuclear infiltrate and a paucity of granulocytes, we assessed the presence of inflammatory cells in rejected allografts. Rejected allografts from atopic recipients had a preponderance of eosinophils, along with the typical mononuclear infiltrate (Fig. 1). Conjunctivae from atopic recipients also displayed eosinophilia, typical of atopic conjunctivitis. Rejected allografts from normal recipients did not have eosinophil infiltration but did display a heavy mononuclear infiltrate (Fig. 1). Normal conjunctivae did not contain eosinophils. Surviving syngeneic grafts from atopic recipients had neither eosinophils nor any inflammatory cell infiltrate, while these recipients displayed typical eosinophilic inflammation of the conjunctivae (data not shown).

Immune responses to donor alloantigens

Atopic mice that had rejected a corneal allograft were examined for DTH using ear challenge with donor spleen cells or third-party spleen cells. Atopic BALB/c mice that had rejected either C57BL/6 or B10.D2 corneal allografts displayed significant DTH responses to donor alloantigens compared with naive BALB/c mice challenged with donor alloantigens (Fig. 2). Atopic BALB/c mice that had rejected C57BL/6 corneal allografts did not mount DTH responses to C3H mouse spleen cells, confirming the Ag specificity of the DTH responses to C57BL/6 alloantigens (data not shown).

Draining cervical LNs from atopic BALB/c mice that had rejected C57BL/6 corneal allografts were assessed for IFN- γ , IL-4, and IL-5 production by ELISA following in vitro stimulation with C57BL/6 alloantigens. LN cells from atopic BALB/c mice that had rejected C57BL/6 corneal allografts produced significantly more IL-4 (Fig. 3A) and IL-5 (Fig. 3B) when stimulated in vitro with alloantigens than did naive BALB/c mice stimulated with alloantigens. Interestingly, LN cells from both naive BALB/c and atopic BALB/c mice that rejected C57BL/6 corneal allografts produced equally high amounts of IFN- γ compared with ungrafted atopic BALB/c mice when stimulated in vitro with alloantigens (Fig. 3C).

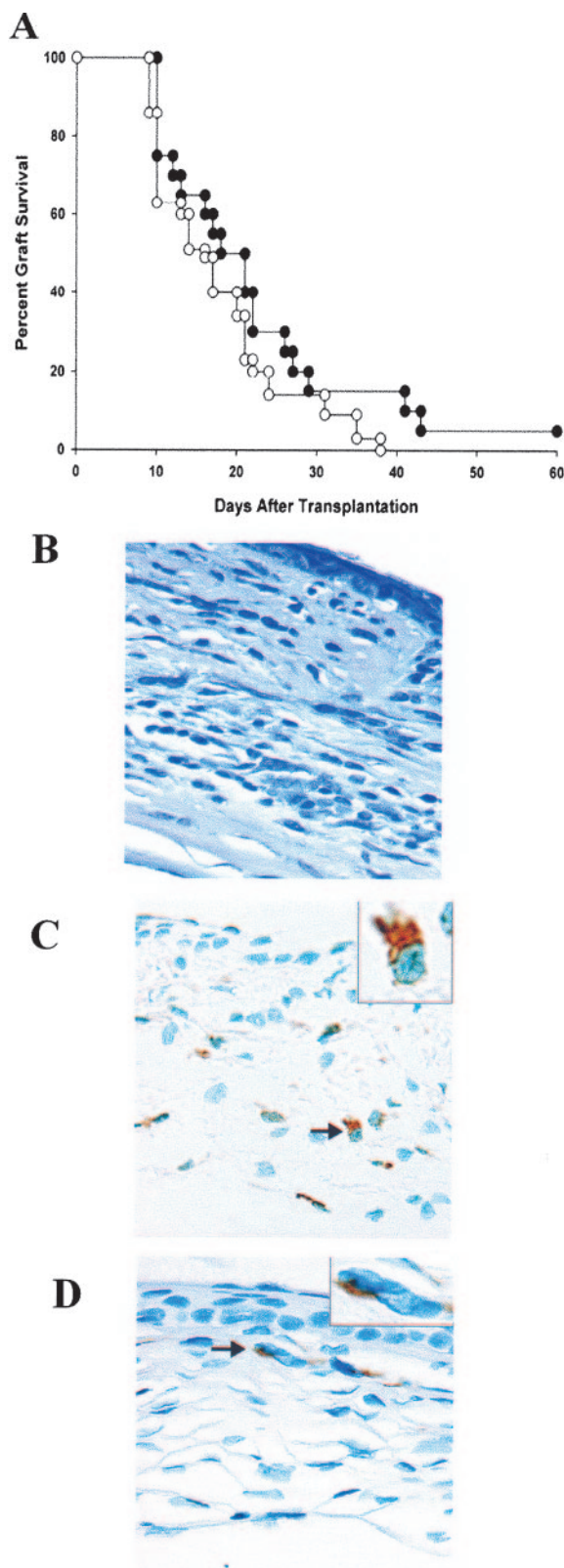


FIGURE 5. Corneal allograft rejection in nonallergen challenged eyes of atopic hosts. *A*, C57BL/6 corneal allografts transplanted to either the eye that was challenged with pollen in atopic BALB/c mice (●) or the eye that was not challenged with pollen (○). *B*, Giemsa staining of rejected corneal allografts in atopic BALB/c eyes that were not challenged with pollen. Note absence of eosinophils. Immunohistochemical staining of rejected corneal allograft in an atopic BALB/c eye that was not challenged with pollen revealed T1/ST2⁺ cells (*C*) and TIM-3⁺ cells (*D*) (arrows indicate positively stained cells in insets) (×600).

Presence of Th1 and Th2 cells in rejected corneal allografts

Because atopic BALB/c mice that had rejected corneal allografts displayed signs of both Th1 immune responses (DTH, IFN- γ production) and Th2 immune responses (eosinophilia, IL-4 and IL-5 production), we examined rejected corneal allografts for the presence of Th1 and Th2 cells immunohistochemically using mAbs to surface proteins specific for Th cell subsets. TIM-3 is expressed only on Th1 cells, whereas T1/ST2 is expressed only on Th2 cells (17, 18). As expected, C57BL/6 corneal allografts rejected by normal BALB/c mice contained TIM-3⁺ cells but no detectable T1/ST2⁺ cells (Fig. 4*A*). By contrast, C57BL/6 corneal allografts rejected by atopic BALB/c hosts contained both TIM-3⁺ and T1/ST2⁺ cells (Fig. 4*B*). In both normal and atopic BALB/c hosts, infiltrating T cells were seen in the corneal stroma, the anterior chamber adjacent to the corneal endothelial layer, and extravasating from the iris into the anterior chamber (Fig. 4).

Our findings are consistent with recent evidence that Th1 cells are important for Th2 effector elements. In a mouse model of allergen-induced airway inflammation, Th1 cells were required for trafficking of both Th2 cells and eosinophils to the lung by mediating up-regulation of VCAM-1 in the lung (19, 20). In our study, IFN- γ was produced in response to donor alloantigens and might up-regulate adhesion molecules such as VCAM, thereby allowing leukocytes to enter the cornea and effect allograft rejection. It is noteworthy that activation of allospecific Th2 immune responses did not inhibit or delay immune rejection of corneal allografts as has been reported in another model of Th2 immunity (3). Previous models of allograft rejection in Th2-polarized animal models have used IFN- γ ^{-/-} mice, CD80^{-/-} mice, or mice depleted of IL-12 with mAbs (9, 12, 21, 22), which differ from the present atopy model in that the mice are incapable of generating Th1 immune responses, a scenario that is unlikely to occur in human subjects. By contrast, the present atopy model mimics a significant category of human corneal transplant recipients. Tears from patients with allergic conjunctivitis or atopic keratoconjunctivitis contain both Th1 cytokines, including IL-12 and IFN- γ , and Th2 cytokines, including IL-4, IL-5, and IL-13 (23). Therefore, it is likely that atopic patients will mount an immune response that embodies both Th1 and Th2 responses to a corneal allograft.

Corneal allograft rejection in eyes without allergic conjunctivitis

To determine whether the increased incidence and accelerated rejection rate of corneal allografts in atopic hosts were due to a local inflammatory effect or a systemic alloimmune effect, we transplanted C57BL/6 corneas onto the unchallenged left eye of atopic BALB/c mice, while continuing to challenge the right eye with ragweed pollen as before. Only the eye that was not exposed to ragweed pollen received a corneal allograft in this experiment. A separate group of atopic BALB/c mice received a C57BL/6 corneal allograft to the right eye, which was challenged with ragweed pollen as before. We observed that 95% of C57BL/6 corneal allografts transplanted to eyes not challenged with ragweed pollen in atopic BALB/c mice were rejected (Fig. 5*A*). As expected, 100% of C57BL/6 corneal allografts transplanted to the ragweed pollen challenged eyes were rejected (Fig. 5*A*). We found no significant differences in MST or mean rejection times between allografts in nonallergic eyes or allergic eyes of atopic hosts. Unlike rejected corneal allografts in allergic

eyes of atopic hosts, rejected corneal allografts placed in nonallergic eyes contained no detectable eosinophils (Fig. 5B). Moreover, no eosinophils were detectable in conjunctivae from nonallergic eyes of atopic mice (data not shown). Eosinophils produce cationic proteins that are toxic to corneal cells in vitro. However, the absence of eosinophils in corneal allografts transplanted to nonallergen-challenged eyes of atopic hosts indicates that eosinophils are not the cause of elevated rejection in atopic hosts. As was seen in rejected allografts from allergic eyes, both T1/ST2⁺ cells (Fig. 5C) and TIM-3⁺ cells (Fig. 5D) were identified in rejected allografts from nonallergic eyes of atopic hosts. We found no apparent quantitative or qualitative differences in the overall level of corneal inflammation associated with rejection of corneal allografts in ragweed pollen challenged or unchallenged eyes of atopic hosts.

In conclusion, we have demonstrated that a pre-existing chronic atopic condition causes the host to have an increased risk of rejection of allogeneic tissue grafts compared with nonatopic hosts. This increased susceptibility to allograft rejection is a systemic condition and is not limited to the organ in which allergic inflammation is present. Moreover, corneal allograft rejection in atopic hosts involves both Th1 and Th2 immune elements that not only fail to cross-regulate each other but may in fact act synergistically in the destruction of the allograft.

Disclosures

The authors have no financial conflict of interest.

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