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$\alpha\beta$ TCR$^+$ T Cells, but Not B Cells, Promote Autoimmune Keratitis in B10 Mice Lacking $\gamma\delta$ T Cells

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Purpose. To investigate additional factors in the spontaneous development of keratitis previously reported in B10.TCR$\delta^{-/-}$ female mice.

Methods. The study tested whether susceptible B10.TCR$\delta^{-/-}$ mice have dry eyes compared with resistant B6.TCR$\delta^{-/-}$ females and also rederived the B10.TCR$\delta^{-/-}$ strain to test for the role of an infectious agent. Also assessed was whether adoptive transfer of $\alpha\beta$ T cells from autoimmune mice induced keratitis in resistant mice. In addition, a potential role was examined for B cells or autoantibodies by B-cell inactivation, and the role of female hormones was tested by ovariectomy. Finally, the study investigated whether adoptive transfer of Vγ1$^+$ $\gamma\delta$ T cells confers protection.

Results. Tear production in B10.TCR$\delta^{-/-}$ females was actually higher than in B6.TCR$\delta^{-/-}$ controls. Rederived B10.TCR$\delta^{-/-}$ mice still developed keratitis. Keratitis was induced in resistant mice after adoptive transfer of $\alpha\beta$ T cells from keratitic donors. Inactivation of B cells from susceptible mice had no effect on the development of keratitis. Ovariectomy did not significantly reduce disease in B10.TCR$\delta^{-/-}$ females. Adoptive transfer of Vγ1$^+$ cells from wild-type donors reduced keratitis in B10.TCR$\delta^{-/-}$ females.

Conclusions. Neither low tear levels nor ovarian hormones contribute to spontaneous keratitis in B10.TCR$\delta^{-/-}$ female mice, nor does it appear to depend on an infectious agent carried vertically in this strain. However, $\alpha\beta$ T cells from keratitic hosts are sufficient to induce disease in the resistant B10.TCR$\beta^{-/-}\delta^{-/-}$ strain. Autoaggressive $\alpha\beta$ T cells in the absence of Vγ1$^+$ T cells in B10.TCR$\delta^{-/-}$ mice may be insufficiently checked to prevent disease. (*Invest Ophthalmol Vis Sci.* 2012;53:301–308) DOI:10.1167/iovs.11-8855

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The role of $\gamma\delta$ T cells as immunoregulatory cells has been documented in many different settings, but in the eye, these cells appear to be of particular importance. Mice that lack or have been depleted of $\gamma\delta$ T cells do not develop anterior chamber-associated immune deviation (ACAID)[1–3] and also reject allogeneic corneal grafts much more readily than do $\gamma\delta$ T-cell-sufficient mice,[2] pointing to the role of these cells in maintaining tolerance to antigens normally present in the eye. Mice with herpes stromal keratitis, an infectious disease that eventually progresses to autoimmune keratitis after the virus (HSV) has been cleared (reviewed in Ref. 4), have been shown to be particularly susceptible to progression to HSV infection of the brain if they lack $\gamma\delta$ T cells,[5] consistent with the idea that $\gamma\delta$ T cells normally downregulate immune responses that are evoked in the cornea and thus prevent inflammatory damage that leads to this complication. Increases in $\gamma\delta$ T cells during autoimmune disorders of the human eye have also been noted, including Behçet's disease[6] and ocular cicatricial pemphigoid,[7,8] as well as in chronic corneal graft rejection,[8] which suggests that $\gamma\delta$ T cells play a similar regulatory role in the human eye. We recently reported that, in female mice of the C57BL/10 background, which lack $\gamma\delta$ T cells because of genetic disruption of the TCR-δ constant region (B10.TCR$\delta^{-/-}$ mice), keratitis develops spontaneously, such that by 18 weeks of age, 70% to 80% of adult females show evidence of disease.[9] The development of keratitis is dependent on the B10 background, because mice with the same genetic defect but having instead the closely related C57BL/6 background do not develop keratitis. The disease is also much more prevalent in females than in males. Our previous study additionally indicated that male hormones do not protect against keratitis, because orchiectomized males show no increase in disease incidence, but that $\alpha\beta$ T cells appear to play a role in the development of keratitis, because mice depleted of $\alpha\beta$ T cells with a monoclonal antibody or treated with the immunosuppressive drug cyclosporine, developed keratitis at a reduced level.

In this article, we investigate additional factors that could play a role in this spontaneous eye disease, including dry eye, ovarian hormones, an insidious infectious component, and autoimmune $\alpha\beta$ T cells and B cells. Of these, our results indicate that only autoaggressive $\alpha\beta$ T cells play a role in inducing keratitis. Moreover, Vγ1$^+$ $\gamma\delta$ T cells provide some resistance against development of the disease.

Materials and Methods

Mice

C57Bl/10J (B10) mice, C57BL/6J (B6) mice, and B6.TCR$\delta^{-/-}$ mice[10,11] were either newly obtained from The Jackson Laboratory (Bar Harbor, ME) or maintained in our colony from Jackson Laboratory stock. The B10.TCR$\delta^{-/-}$ and B10.TCR$\beta^{-/-}\delta^{-/-}$ strains were backcrossed in our

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facility, as previously described.[9] The work described in this article was reviewed and approved by the National Jewish Institutional Animal Care and Use Committee and adhered to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Keratitis Scoring

The keratitis was scored once every 2 weeks by gross observation of the mice, and a severity score was assigned for each side, as previously described.[9] The maximum possible score for an individual mouse was 10.

Tear Measurements

To approximate the Schirmer test used clinically, we used phenol red–impregnated cotton thread (Zone-Quick thread tear test; Oasis, Glendora, CA). Mice to be tested were anesthetized briefly with isoflurane vapor, and the thread was applied to the outer canthus of each eye for 60 seconds. The area wetted was then recorded (in millimeters) for each, according to the scale present on the thread. The mice were tested at 6 to 10 weeks of age, a time when B10.TCR$\delta^{-/-}$ mice are not likely to have developed keratitis. Any mice with obvious keratitis at the time of testing were excluded from the analysis.

Rederivation of the B10.TCR$\delta^{-/-}$ Strain

The rederivation was performed in the barrier of the National Jewish Health animal facility, which is designed to prevent transfer of any potential infectious agents that could be accidentally introduced from the regular (SPF) animal facility. Personnel must shower before entering the barrier from another National Jewish animal room, and all equipment and supplies are sterilized before transfer into the barrier. Personnel working in the barrier wear a uniform, shoe covers, hair cover, face mask, and gloves. The mice are housed in ventilated units, and the cages are opened and manipulated in laminar flow hoods. All mice housed or admitted to the barrier are rederived into the facility, except for certified mice from The Jackson Laboratory.

To rederive the strain, female B10.TCR$\delta^{-/-}$ mice of 3 to 4 weeks of age were superovulated by giving each an IP injection of pregnant mare serum gonadotropin (0.1 mL of 50 IU/mL in PBS) and, 46 to 48 hours later, an IP injection of human chorionic gonadotropin (0.1 mL of 50 IU/mL in PBS). Four to 7 hours later, each female was added to a cage containing either an experienced male B10.TCR$\delta^{-/-}$ breeder mouse or a male 7 weeks of age or older that had been exposed to females for approximately 24 hours, followed by a 2- to 3-day rest. After 15 to 18 hours, the females were checked for the presence of a vaginal plug. Positive females were euthanized 2 days later. The embryos were removed from the oviducts, placed in cyropreservation medium in straws, and slow frozen in a cryomed, controlled-rate freezer (Biocool; SP Scientific, Warminster, PA). The embryos remained frozen for approximately 10 months, and the strain was then rederived in the barrier facility into SPF foster mothers newly obtained from The Jackson Laboratory. The embryos were thawed and injected, as described previously,[12] into the oviduct of an outbred ICR female made pseudopregnant by copulation with a vasectomized male, confirmed 3 days previously by the presence of a vaginal plug.

Rederived pups were maintained in the barrier facility. After reaching 8 weeks of age, two rederived females were bred with C57BL/10 wild-type males newly obtained from The Jackson Laboratory to produce F1 offspring that were +/− at the TCR-δ locus and then interbred to produce F2 pups. All breeding and maintenance were performed in the barrier facility for the duration of the experiment.

To identify F2 offspring that were TCR$\delta^{-/-}$, the F2 mice were bled from the tail vein within 1 month of weaning, collecting up to 6 drops of blood into 2.5 mL of balanced salt solution containing 0.0038 USP units of heparin (Sigma-Aldrich, St. Louis, MO). After RBC lysis in 9 mL of Gey's solution, the suspension was underlaid with 0.5 mL FBS and spun at 1200 rpm for 8 minutes. Blood T cells in the pellet were then enriched by nylon wool passage,[13] using a 0.25-g nylon wool column in a 3-mL syringe. The presence of $\gamma\delta$ T cells was confirmed by two-color flow cytometry with an anti-mouse CD3 monoclonal antibody (KT3[14]) and an anti-mouse TCR-δ monoclonal antibody (GL3[15]). Female F2 mice having no detectable $\gamma\delta$ T cells were deemed TCR$\delta^{-/-}$. All rederived B10.TCR$\delta^{-/-}$ females were scored for keratitis after reaching 18 weeks of age.

Adoptive T-Cell Transfers

For keratitic $\alpha\beta$ T-cell adoptive transfers, splenic T cells from keratitic B10.TCR$\delta^{-/-}$ females having an overall score of at least 4 were used as donors. Spleens were homogenized in cell culture medium containing antibiotics[16] plus 5% FBS, and the RBCs were lysed with Gey's solution. The cells were then passed over nylon wool to enrich for T cells[13] and resuspended in sterile balanced saline plus 5% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA). Then, 1 to 2 $\times$ 10^6 were injected into the tail vein of 6- to 12-week-old B10.TCR$\beta^{-/-}\delta^{-/-}$ female hosts. Keratitis develops spontaneously in B10.TCR$\beta^{-/-}\delta^{-/-}$ mice at a low rate (~20% of females by 18 weeks of age[9]). Any that had discernible keratitis at the time of adoptive transfer were excluded from the experiment.

For Vγ1$^+$ T cell transfers, spleen cells from normal B10 donors were similarly prepared as described above. Spleen cell suspensions were treated with 40 μg/mL 2.4G2 monoclonal antibody[17] plus 20 μg/mL mouse gamma globulin (Jackson ImmunoResearch) for approximately 60 minutes at 4°C to block Fc-receptors, washed once, and then incubated with a biotinylated monoclonal antibody specific for Vγ1 (2.11[18]) for 15 minutes at 4°C. The Vγ1$^+$ cells were next purified using streptavidin magnetic beads with MS minicolumns (MACS; Miltenyi Biotec, Auburn, CA), in accordance with the manufacturer's instructions. In some experiments, the eluted cells were passed a second time over another MS column, to improve purity. An aliquot was then stained with an FITC-labeled anti-$\gamma\delta$ TCR monoclonal antibody (GL3[19]) plus streptavidin-PE and analyzed by flow cytometry to assess the purity of the Vγ1$^+$ cells. Among the live lymphocytes in various preparations, Vγ1$^+$ cells ranged from 41% to 79%. In all cases, less than 1% represented Vγ1-negative $\gamma\delta$ TCR$^+$ cells. Purified Vγ1$^+$ cells were then adoptively transferred into 5- to 9-week-old B10.TCR$\delta^{-/-}$ female hosts with no evident keratitis at the time of injection by tail vein inoculation, injecting 1.7 to 3.25 $\times$ 10^5 Vγ1$^+$ cells per mouse.

For CD4$^+$ $\alpha\beta$ T-cell transfers, spleen cells from normal B10 mice were purified over nylon wool columns as described above, and the eluted cells were incubated in a cocktail of biotinylated monoclonal antibodies recognizing mouse CD8α,[20] CD19 (eBioscience, San Diego, CA), NK1.1,[21] and TCR-$\gamma\delta$,[19] to label CD8$^+$ $\alpha\beta$ T cells, B cells, NK cells, and $\gamma\delta$ T cells, respectively. The labeled cells were then incubated with streptavidin beads (MACS; Miltenyi Biotec) and passed over LD columns (MACS; Miltenyi Biotec) to remove all labeled cells together. An aliquot of unbound negatively selected cells was then taken for flow cytometric analysis and stained with an FITC-labeled anti-$\alpha\beta$ TCR monoclonal antibody plus anti-CD4-PE (eBioscience). Of the live lymphocytes, 96% were $\alpha\beta$ TCR$^+$ and CD4$^+$. Purified CD4$^+$ cells were then adoptively transferred into 7- to 12-week-old B10.TCR$\beta^{-/-}\delta^{-/-}$ female hosts with no evident keratitis at the time of injection, by tail vein inoculations, injecting 0.3 to 1.0 $\times$ 10^6 purified CD4$^+$ cells per mouse.

B-Cell Inactivation

B10.TCR$\delta^{-/-}$ females without evident keratitis at 7 weeks of age were treated with a monoclonal antibody that inactivates B cells by serial injection of the hamster anti-mouse CD79β (Igβ) monoclonal antibody HM79. This antibody, diluted in balanced saline, was injected at a dose of 200 μg per mouse into the tail vein at 7, 8, 9, 11, 13, 15, and 17 weeks of age. This antibody has been shown to efficiently inactivate and, with repeated doses, to deplete B cells in vivo.[22,23] Two weeks after the final antibody injection, the keratitis was scored. The sham-

treated controls were instead injected on the same schedule with normal Syrian hamster gamma globulin (Jackson ImmunoResearch, West Grove, PA) diluted to the same concentration. In one experiment, the mice were euthanized after the treatment and their spleens removed for flow cytometric analysis. B cells from these animals were stained using a monoclonal antibody against CD19 (biotinylated; eBioscience) plus streptavidin-PE (InVitrogen, Carlsbad, CA). T cells were also stained as a positive control with an FITC-labeled anti-CD3ε monoclonal antibody (KT3[14]).

Ovariectomy

Six- to 8-week-old B10.TCR$\delta^{-/-}$ females were weighed and anesthetized with isoflurane. The hair in the midback region was shaved, and the skin was disinfected with 70% ethanol. Ointment (DuoLube; Bausch & Lomb, Rochester, NY) was applied to the eyes with a cotton swab (Q-tip; Unilever, New York, NY), to ensure that they did not dry out excessively during surgery, and a steady flow of isoflurane in air and oxygen was supplied via a vaporizer by placing the mouse's nose in a nose cone. A horizontal dorsal incision approximately 1 cm long was made on the side through the skin about halfway between the ribs and the base of the tail and another through the underlying muscle layer into the peritoneal cavity. The ovary and surrounding fatty tissue were withdrawn, the ovary exposed, the oviduct tied off with a resorbable suture (4-0 self-absorbing, braided Vicryl), and the ovary excised. Two to three sutures were used to close the internal incision, and 10-mm wound clips were applied to close the skin. The second ovary was then exposed on the other side and removed similarly. For sham-ovariectomized controls, the ovaries were briefly exposed, then reinserted into the opening and the mouse closed up. The mice were treated with buprenorphine at 1 mg/kg by subcutaneous injection just before surgery and every 12 to 16 hours for the first 48 hours after surgery. Wound clips were removed 10 to 14 days after surgery. The keratitis in the mice was scored until 18 to 19 weeks of age. Any mice that had discernible keratitis before the time of surgery were excluded from the analysis.

Statistics

Differences between tear levels in the different groups were analyzed with a one-tailed Student's *t*-test. Differences in the incidence of keratitis between two groups were analyzed with a two-tailed Fisher's exact test. Because keratitis scores were assessed by an assigned rank, the differences in average keratitis score were analyzed with a nonparametric one-tailed Wilcoxon rank sum or Mann-Whitney U test (all analyses: Prism; GraphPad, San Diego, CA).

Results

The High Incidence of Keratitis in B10.TCR$\delta^{-/-}$ versus B6.TCR$\delta^{-/-}$ Female Mice Is Not the Result of Reduced Tear Production

In humans, keratitis is often associated with keratoconjunctivitis sicca, or dry eye.[24] We therefore hypothesized that a failure to produce an adequate amount of tears could contribute to the susceptibility of B10.TCR$\delta^{-/-}$ females to keratitis, compared with B6.TCR$\delta^{-/-}$ females. We used a method described previously to approximate the Schirmer test used clinically in humans [25] for comparing tear production in the two strains. However, we found that in fact the B10-background mice produced higher tear levels. This was also true of wild-type B10 versus B6 mice, and the difference was significant for both comparisons (Fig. 1). Thus, low tear production does not predispose the B10.TRC$\delta^{-/-}$ strain to the development of keratitis.

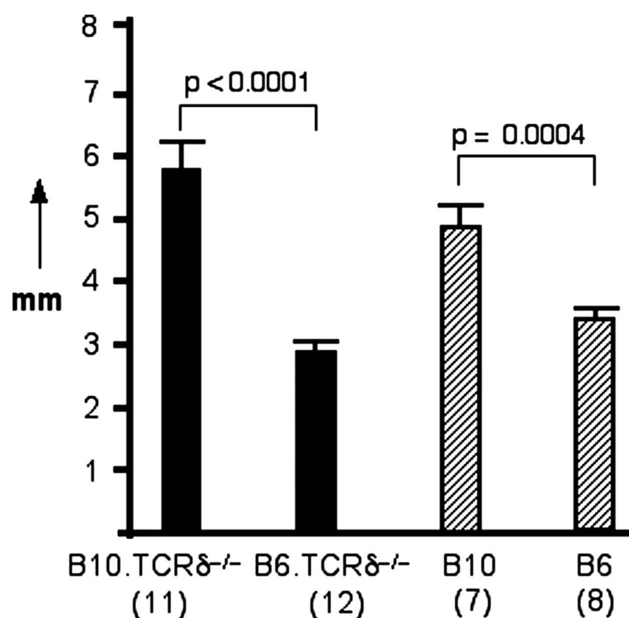

Figure 1. Keratitis-susceptible B10.TCR$\delta^{-/-}$ mice had higher tear levels than did keratitis-resistant B6.TCR$\delta^{-/-}$ mice. Tear levels were measured in both eyes of individual mice, and the average for each group is shown. Errors bars, SEM. The total number examined in each group is indicated in parentheses.

Rederived B10.TCR$\delta^{-/-}$ Mice Still Show a High Incidence of Spontaneous Keratitis

To test whether an infectious agent transmitted to the offspring at birth could cause keratitis in B10.TCR$\delta^{-/-}$ mice, we rederived this strain by removing embryos at the eight-cell stage from pregnant mothers and implanting them into uteri of pseudopregnant mothers of an unrelated strain. The rederivation was performed in a barrier facility in the National Jewish Health animal facility, which is designed to prevent potential transfer of infectious agents from the regular SPF facility, and the rederived strain was maintained in the barrier. Two females were obtained from the rederivation, both of which developed keratitis after 18 weeks of age, suggesting that rederived B10.TCR$\delta^{-/-}$ females are similarly apt to develop keratitis. To obtain more for this analysis, these two females were crossbred with C57BL/10 males obtained from The Jackson Laboratory, and the offspring were then intercrossed to produce F2 progeny. The F2 mice having two defective TCRδ loci were then identified by staining peripheral blood T cells with an anti-TCR-γδ monoclonal antibody. To prevent any potential reinfection, the B10.TCR$\delta^{-/-}$ F2 progeny were maintained in the barrier facility until they reached 18 weeks of age, and then the keratitis was scored. As can be seen (Fig. 2), the rederived B10.TCR$\delta^{-/-}$ females developed keratitis at a rate that was not significantly different from that of the original B10.TCR$\delta^{-/-}$ females housed in the regular facility during the same period. Thus, we conclude that the disease probably does not depend on the transmission of an unknown infectious agent. This conclusion adds support to our previous hypothesis that the keratitis stems instead from an autoimmune attack that develops against the normally immune-privileged cornea.

Adoptive Transfer of αβ T Cells from Keratitic Donors Increases the Frequency of Keratitis in B10.TCR$\beta^{-/-}\delta^{-/-}$ Hosts

In many autoimmune diseases, there is evidence that T cells, particularly CD4^{+} αβ T cells, induce the development of dis-

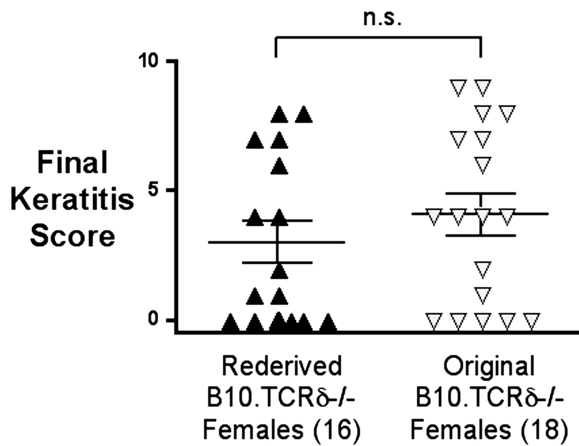

Figure 2. Rederivation of the B10.TCR$\delta^{-/-}$ strain did not significantly reduce the incidence of keratitis. Individual keratitis scores by 18 weeks are shown for the rederived B10.TCR$\delta^{-/-}$ females versus females of the original B10.TCR$\delta^{-/-}$ strain tested over approximately the same time period. The number of individual mice screened in each group is shown in parentheses. The small difference between means was not significant (NS).

ease (reviewed in Ref. 26), and our previous results also suggest a role for $\alpha\beta$ T cells in the keratitis that develops in B10.TCR$\delta^{-/-}$ mice.[9] To test this notion more directly, we examined whether keratitis could be induced in resistant B10.TCR$\beta^{-/-}\delta^{-/-}$ mice by adoptive transfer of $\alpha\beta$ T cells from keratitic B10.TCR$\delta^{-/-}$ donors. As shown in Figure 3A, we found that these $\alpha\beta$ T cells were indeed sufficient to induce keratitis. Unexpectedly, we also found that the mice that received these $\alpha\beta$ T cells underwent a dramatic weight loss (not shown). A similar weight loss was also noted when enriched normal CD4$^+$ $\alpha\beta$ T cells from B10 donors were adoptively transferred into B10.TCR$\beta^{-/-}\delta^{-/-}$ hosts, although these mice did not develop keratitis (Fig. 3B). Thus, the weight loss in both cases may be mediated by CD4$^+$ $\alpha\beta$ T cells, but does not appear to be caused by an autoimmune attack by keratitis-inducing $\alpha\beta$ T cells.

Inactivation of B Cells from B10.TCR$\delta^{-/-}$ Females Has No Effect on the Development of Keratitis

Autoantibodies play a pathologic role in several autoimmune diseases, and the elimination of B cells during the course of the disease can therefore ameliorate the symptoms.[27] Our finding that adoptively transferred $\alpha\beta$ T cells from keratitic hosts can induce disease (Fig. 3) does not eliminate a possible role for B cells in B10.TCR$\delta^{-/-}$ keratitis, because the transferred T cells could potentially act as helper cells for B cells producing anticorneal autoantibodies. Alternatively, the T-cell preparation could contain contaminating B cells at sufficient levels to induce keratitis, if the B cells are potentially pathogenic. To test whether B cells could play a role in the spontaneous keratitis of B10.TCR$\delta^{-/-}$ mice, we treated B10.TCR$\delta^{-/-}$ females periodically with an antibody against a pan-B-cell antigen to keep the peripheral B cells depleted or in an inactive state, beginning at a young age before keratitis developed. As shown in Figures 4A and 4B, there was no effect of this treatment on keratitis, since mice treated with normal hamster IgG developed disease at a similar rate and severity as those treated with the B-cell-depleting antibody. We confirmed the efficacy of the antibody treatment by staining spleen cells from B-cell-inactivated versus sham-treated mice with an anti-CD19 monoclonal antibody, 2 weeks after the final injection, which showed that the overall B-cell counts were still reduced by an average of more than 25-fold (Fig. 4C). We therefore conclude that B cells do not play a role in the development of spontaneous keratitis in B10.TCR$\delta^{-/-}$ mice.

The Incidence of Keratitis Is Not Significantly Decreased after Ovariectomy

In both humans and mice, many autoimmune diseases are more prevalent in females than in males.[28] Our previous results indicated that a lack of male hormones cannot explain the higher prevalence of keratitis in B10.TCR$\delta^{-/-}$ females compared to males.[9] However, female hormones could instead promote the development of autoaggressive lymphocytes, or in some other way act to increase susceptibility to keratitis. Therefore, we tested whether ovariectomized B10.TCR$\delta^{-/-}$ females are less likely to develop keratitis than sham-ovariectomized controls. As shown in Figure 5, we found only a slight, nonsignificant difference between ovariectomized mice and sham-ovariectomized controls. Thus, the presence of ovarian hormones cannot explain why keratitis is six to seven times more prevalent in female than in male B10.TCR$\delta^{-/-}$ mice.

Adoptive Transfer of Vγ1$^+$ Cells from Wild-Type Donors Reduced the Incidence of Keratitis in B10.TCR$\delta^{-/-}$ Females

We had shown that adoptive transfer of $\gamma\delta$ T cells from wild-type B10 donors could reduce the incidence of keratitis

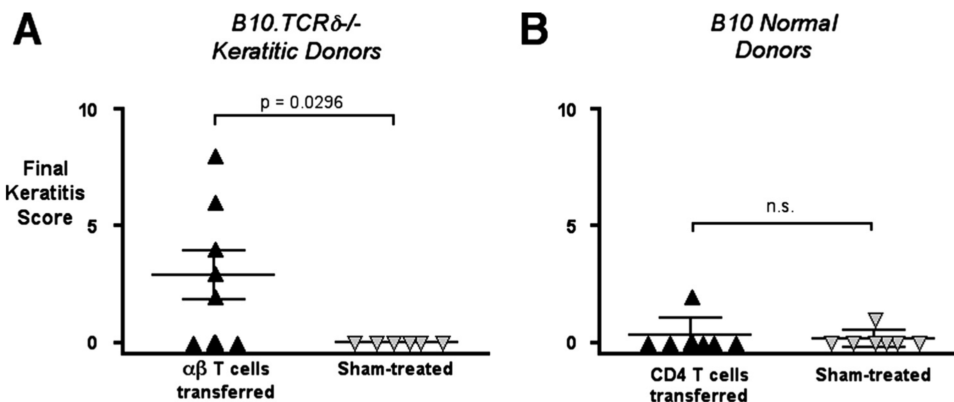

Figure 3. (**A**) Adoptively transferred $\alpha\beta$ T cells from keratitic B10.TCR$\delta^{-/-}$ donors induce keratitis in B10.TCR$\beta^{-/-}\delta^{-/-}$ hosts. Average final keratitis scores of mice at 18 to 19 weeks of age are shown for 8 B10.TCR$\beta^{-/-}\delta^{-/-}$ hosts into which $\alpha\beta$ T cells from keratitic B10.TCR$\delta^{-/-}$ donors were transferred, and for 6 B10.TCR$\beta^{-/-}\delta^{-/-}$ controls that were sham treated at the same time with the cell diluent only. Scores obtained for individual mice are indicated by each symbol with the mean and SD bars superimposed as horizontal lines. $P > 0.05$ was regarded as nonsignificant (NS). (**B**) Adoptively transferred CD4$^+$ $\alpha\beta$ T cells from normal B10 donors do not induce keratitis in B10.TCR$\beta^{-/-}$ $\delta^{-/-}$ hosts. Average keratitis scores are indicated as in (**A**), for seven B10.TCR$\beta^{-/-}\delta^{-/-}$ hosts into which $\alpha\beta$ T cells from normal B10 donors were transferred and for seven B10.TCR$\beta^{-/-}\delta^{-/-}$ controls that were sham-treated at the same time with the cell diluent only.

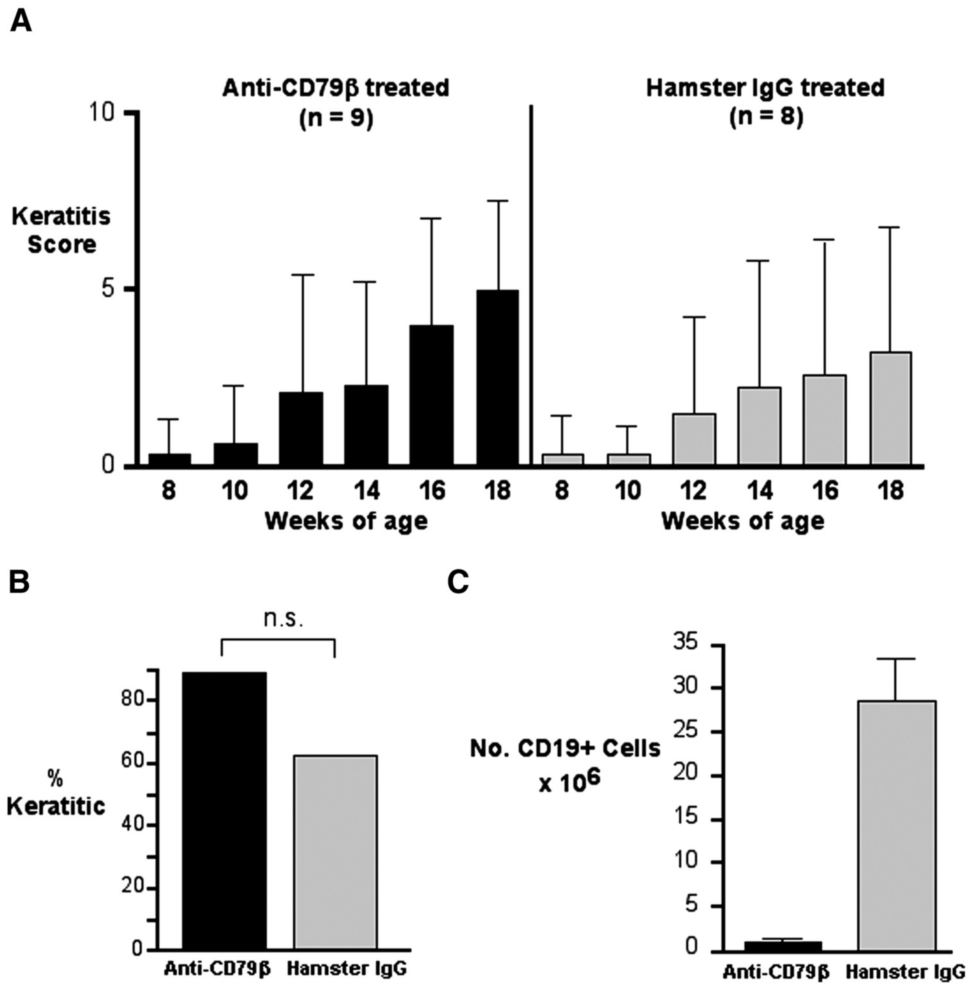

FIGURE 4. B-cell inactivation does not affect the incidence or severity of keratitis in B10.TCR$\delta^{-/-}$ females. (**A**) The average keratitis scores for mice treated with anti-CD79β antibody or sham-treated with hamster IgG as a control are shown. Each column indicates the average at a given age. Error bars, SD. The total number of mice tested in each group is shown in parentheses. (**B**) The incidence of keratitis of the mice in (**A**) at 18 to 19 weeks, shown as the percentage of mice in the group that developed keratitis compared with the total number in the group. The difference was not significant (NS). (**C**) The average number of CD19^{+} cells obtained from spleens of mice shown in (**A**) was calculated from the percentage determined by flow cytometry, as determined for individual mice. Error bars, SE.

in B10.TCR$\delta^{-/-}$ females. However, different $\gamma\delta$ T cell subsets have distinct functional properties (reviewed in Ref. 29). In other disease models, we and others have shown that Vγ1^{+} $\gamma\delta$ T cells have an anti-inflammatory effect.[30–33] We therefore tested whether purified Vγ1^{+} $\gamma\delta$ T cells from B10 donors are sufficient to reduce the incidence of keratitis when adoptively transferred to B10.TCR$\delta^{-/-}$ female hosts. As shown in Figure 6, reconstitution of these mice with Vγ1^{+} cells indeed reduced the average disease incidence by approximately twofold. This transfer did not result in any weight loss in the hosts, unlike the transfer of $\alpha\beta$ T cells into B10.TCR$\beta^{-/-}\delta^{-/-}$ hosts (Fig. 3).

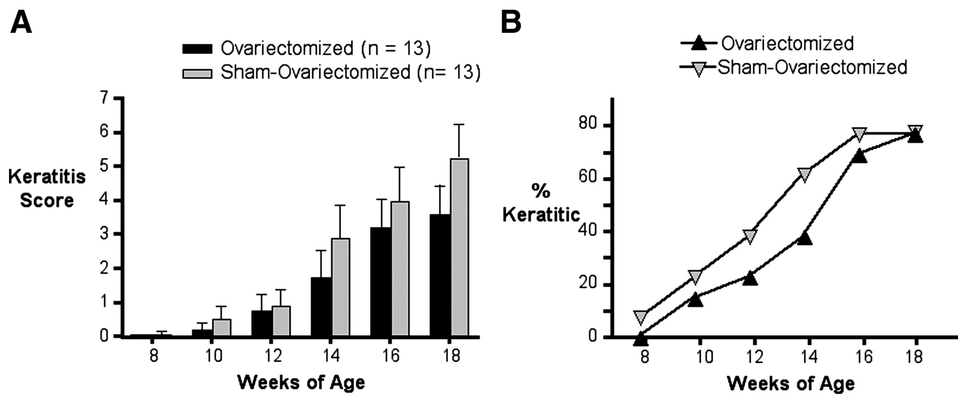

FIGURE 5. Ovariectomy does not significantly affect the incidence of keratitis in B10.TCR$\delta^{-/-}$ females. (**A**) The average keratitis scores for ovariectomized B10.TCR$\delta^{-/-}$ mice and for sham-ovariectomized B10.TCR$\delta^{-/-}$ mice are shown. Each column indicates the average score at a given age. Error bars, SE. The total number of mice tested in each group is shown in parentheses. The small difference was not significant at any age. (**B**) The disease incidence for each group shown in (**A**) at various ages, calculated as the percentage of mice with keratitis divided by the total number in the group. Again, the small difference was not significant at any age.

Figure 6. Vγ1$^+$ $\gamma\delta$ T cells from the spleens of normal B10 donors can suppress the incidence of keratitis in B10.TCR$\delta^{-/-}$ hosts. Average final keratitis scores at 18 to 19 weeks of age are shown for 12 B10.TCR$\delta^{-/-}$ mice, into which Vγ1$^+$ cells from normal B10 donors were transferred, and for 12 sham-treated controls that received only cell diluent. Scores obtained for individual mice are indicated by each symbol, with the mean and SE bars superimposed as horizontal lines.

Discussion

The B10.TCR$\delta^{-/-}$ mice develop what appears to be autoimmune keratitis spontaneously, rather than after infection or immunization, and as such they represent an intriguing model for investigating immune dysregulation in the eye. In this study, we attempted to determine additional factors that contribute to the susceptibility of these mice to keratitis.

Our observation that B10.TCR$\delta^{-/-}$ and B10 mice on average have higher tear levels than do B6.TCR$\delta^{-/-}$ and B6 mice (Fig. 1) was the opposite of what we had expected to find, since dry eye is often associated with keratitis. Conceivably, the opposite could be true, that excess tear levels are in some way pathologic, if the mice tend to build up a pool of stagnant tears due to an obstruction of the nasolacrimal duct, which allows bacteria to grow abnormally, as sometimes occurs congenitally in young children.[34] Another possibility is that the tears produced by B10 mice, though more copious, may lack something in their chemical composition that sets the stage for keratitis. Finally, the increased tear level in B10-background mice could be symptomatic of a preexisting corneal irritation due to unknown causes, which often goes on to develop into overt keratitis when $\gamma\delta$ T cells are absent. We plan to further investigate these possibilities, because the difference between the two strains was quite striking. That B10.TCR$\delta^{-/-}$ mice with keratitis go on to develop dry eye seems unlikely, because we found no evidence of lacrimal gland inflammation or degeneration in histologic sections.[9]

Keratitis can result from infection of the eye with either a viral or bacterial agent, and in particular herpes simplex virus[35,36] and *Pseudomonas aeruginosa*[37] have been studied in this regard. Both types 1 and 2 herpes simplex virus are known to be transmissible during birth in rare instances in humans.[38] Therefore, it seemed possible that a congenital eye infection may in part be responsible for the high frequency with which keratitis develops in B10.TCR$\delta^{-/-}$ females. Our finding that rederived B10.TCR$\delta^{-/-}$ females kept in a barrier facility and shielded from direct or indirect contact with the original strain still developed keratitis at approximately the same frequency as did the original strain (Fig. 2) indicates that congenital infection is unlikely to play a role in bringing about the disease.

Indirect evidence that spontaneous keratitis in the B10.TCR$\delta^{-/-}$ strain is promoted by $\alpha\beta$ T cells, based on the results of four different experiments, was presented in our prior study.[9] First, depleting B10.TCR$\delta^{-/-}$ females of $\alpha\beta$ T cells by injection of a TCRβ-specific monoclonal antibody reduced the incidence of disease. Second, when we crossed B10.TCR$\delta^{-/-}$ mice with B10.TCR$\beta^{-/-}$ mice to generate B10.TCR$\beta^{-/-}\delta^{-/-}$ mice, which are able to produce neither $\alpha\beta$ nor $\gamma\delta$ T cells, this double-knockout strain had a substantially reduced tendency to develop keratitis. Third, treatment of B10.TCR$\delta^{-/-}$ females with cyclosporine, which inhibits the activation of T cells, also reduced the incidence of disease. Fourth, we were able to detect both CD4$^+$ and CD8$^+$ $\alpha\beta$ T cells infiltrating the corneas of B10.TCR$\delta^{-/-}$ mice with keratitis. Our finding that the adoptive transfer of enriched $\alpha\beta$ T cells derived from keratitic donors promotes the development of keratitis in B10.TCR$\beta^{-/-}\delta^{-/-}$ hosts (Fig. 3) provides direct evidence that $\alpha\beta$ T cells mediate the disease. Therefore, the prevalent mechanism by which keratitis develops in B10.TCR$\beta^{-/-}\delta^{-/-}$ mice is most likely T-dependent autoimmunity. However, since not only wild-type B10 mice but also B10.TCR$\beta^{-/-}\delta^{-/-}$ mice show a low incidence of spontaneous keratitis, there must be another mechanism that can sometimes lead to this disease. The $\alpha\beta$ T cells do not appear to promote keratitis by helping autoreactive B cells to differentiate and secrete anticorneal autoantibodies, because B-cell inactivation by monoclonal antibody treatment did not affect the rate or severity of disease in B10.TCR$\delta^{-/-}$ mice (Fig. 4). An unexpected observation from the adoptive transfer experiments with $\alpha\beta$ T cells was that the host mice suffered a profound weight loss. This was evident even when the mice were given $\alpha\beta$ T cells from normal B10 mice, rather than from keratitic B10.TCR$\delta^{-/-}$ donors (Fig. 3B). However, the B10 strain has been noted to carry genes that promote susceptibility to autoimmunity, in contrast to B6 strain,[39] so it is possible that the weight loss reflects other autoimmunities which develop after the transfer of $\alpha\beta$ T cells.

The greatly increased susceptibility of B10.TCR$\delta^{-/-}$ females compared with males is reminiscent of many other autoimmune diseases, in which females are also more often affected. Our previous results suggested that this difference in susceptibility is not due to a protective effect stemming from male hormones, as was found previously in experimental autoimmune encephalomyelitis,[40] because orchiectomizing B10.TCR$\delta^{-/-}$ males did not result in an increase in keratitis. The preponderance of autoimmune disease in females has alternatively been attributed to the ability of estrogens to stimulate immune cells, influencing processes such antigen presentation, lymphocyte activation, and cytokine gene expression.[28] However, ovariectomy of B10.TCR$\delta^{-/-}$ mice, which greatly diminishes female hormone levels, had no significant effect on the rate or incidence of keratitis (Fig. 5). Another possible explanation is that the susceptibility of females versus males stems from immune-relevant genes that are encoded on the X chromosome, which tend to be overexpressed in females and can lead to the expansion of autoimmune lymphocytes.[41]

$\gamma\delta$ T cells normally appear to prevent keratitis from developing in B10 mice, and our results from this study suggest that the suppressive $\gamma\delta$ T cells may be a Vγ1$^+$ subset (Fig. 6). Certain Vγ1$^+$ cells have been shown to be potent producers of IL-4,[42–45] and increased levels of IL-4 could mediate the effect reported here after the adoptive transfer of Vγ1$^+$ cells. Whether Vγ1$^+$ cells are the $\gamma\delta$ T cells that normally mediate protection against the development of keratitis remains to be

seen, because the artificially introduced cells could prevent the development of keratitis in another way. Indeed, a subset of $\gamma\delta$ T cells has been described that normally resides in the limbus of the murine eye and plays a role in corneal wound healing.[46–48] Virtually all the T cells in the limbus express the $\gamma\delta$ TCR. This restricted distribution implies that they have a very specific role to play, and it seems likely that the limbal $\gamma\delta$ T cells protect the cornea against autoimmune attack. Whether the limbal $\gamma\delta$ T cells are in fact Vγ1$^+$ is currently unknown, however.

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