Lacrimal Gland Involvement in Graft-Versus-Host Disease: A Murine Model

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PURPOSE. To describe lacrimal gland involvement in a murine model of acute graft-versus-host disease (GVHD).

METHODS. Histopathologic examination was performed on lacrimal glands of mice affected by GVHD at 1, 2, 4, and 6 weeks after allogeneic bone marrow transplantation (BMT). Histopathologic scoring, based on characteristic GVHD findings in human disease involved evaluation of periductal inflammation, apoptosis, ductal stasis, ductal debris, and fibrosis. CD3, CD4, CD8, CD20, and CD68 antibodies were used to stain leukocyte subsets in GVHD lacrimal gland infiltrates. Lacrimal glands from syngeneic BMT mice were used in control experiments.

RESULTS. Patchy periductal inflammation and focal fibrosis were significantly elevated as early as 2 weeks after allogeneic BMT. Histopathologic scoring of lacrimal glands after allogeneic BMT was significantly different at 4 ($P = 0.005$) and 6 ($P < 0.0001$) weeks when compared with scores in syngeneic control mice. The leukocytes in lacrimal gland GVHD infiltrates were predominately CD3$^+$ T lymphocytes, most of which were CD8$^+$, with fewer CD4$^+$ cells present.

CONCLUSIONS. This study describes the first murine model of lacrimal gland GVHD with features that closely mimic those described in human disease and indicates that lacrimal involvement occurs in acute GVHD. (*Invest Ophthalmol Vis Sci.* 2005; 46:2692–2697) DOI:10.1167/iovs.05-0040

Bone marrow transplantation (BMT) is a successful and common treatment for a variety of hematologic malignancies, solid tumors, and acquired or congenital benign stem cell disorders. The most frequently performed type of BMT is from an allogeneic donor who is human leukocyte antigen (HLA) compatible. Although HLA typing of several major antigens is performed, there are other antigens that remain mismatched. Therefore, allogeneic BMT grafts are predisposed to graft-versus-host disease (GVHD) in which the donor graft mounts an immunologic response against host tissues.

GVHD is a major cause of morbidity and mortality occurring in up to 80% of allogeneic BMT survivors.[1] It is classified as acute or chronic based on the time of disease onset after BMT. Acute GVHD occurs within the first 3 months after transplantation, whereas chronic GVHD occurs more than 100 days after transplantation. Acute GVHD is typically characterized by gastrointestinal ulceration, hepatic dysfunction, and erythematous skin lesions. The chronic form includes skin lesions, oral and esophageal mucositis, pulmonary insufficiency, chronic liver disease, and ocular disease.

Ocular involvement is rare in acute GVHD, presenting as pseudomembranous conjunctivitis, corneal ulceration, and scleritis.[2–6] The prevalence of ocular disease is 60% to 90% in chronic GVHD with external disease as the primary manifestation.[7–9] The clinical findings include lacrimal insufficiency, conjunctivitis, superficial punctate keratopathy, corneal epithelial erosion, sterile corneal ulceration, and corneal perforation.

Insufficient aqueous tear production occurs in 19% to 50% of patients after BMT and in 70% with chronic GVHD.[8,10] Clinical symptoms include blurring of vision, photophobia, ocular irritation, redness and mucous discharge that are accompanied by common signs of superficial punctate keratopathy and reduced tear production. The ocular dryness in these patients is generally severe—especially if present in conjunction with conjunctivitis and conjunctival scarring—and may result in corneal scarring, ulceration, and perforation that may culminate in loss of the eye. Current treatment includes aggressive ocular lubrication, punctal occlusion, tarsorrhaphy, and topical cyclosporine.

In human chronic GVHD leukocytic infiltration of the lacrimal gland results in parenchymal destruction and aqueous tear deficiency. Histopathologic studies of lacrimal glands from humans with GVHD revealed stasis of secretions and epithelial cell debris in ductal lumina as well as periductal inflammation and fibrosis.[11–13] This study is the first to characterize lacrimal gland involvement in a murine model of GVHD. Our results indicate that this model closely correlates with the known histopathologic findings in lacrimal glands of humans with GVHD.

METHODS

Bone Marrow Transplantation

The procedures used in this study have been described previously.[14,15] Briefly, as described by Cooke et al.,[15] female B6D2F1 (F1) mice were purchased from the Frederick Cancer Research and Development Center (National Cancer Institute [NCI], Frederick, MD) or Jackson Laboratories (Jax; Bar Harbor, ME) and underwent transplantation at 12 weeks of age with bone marrow and T cells from 8- to 10-week-old syngeneic F1 mice or allogeneic C57BL/6 Ly 5.2$^+$ (B6) mice also obtained from NCI or Jax. Bone marrow was harvested from the femurs and tibias of donor mice. Mixtures of

5.0×10^6 BM cells and 2.0×10^6 nylon wool purified nonadherent splenic T cells were resuspended in Leibovitz L-15 serum-free medium (Invitrogen-Gibco, Grand Island, NY) and transplanted into recipients by tail vein injection (0.25 mL/injection). On the day of transplantation, host mice received 1100 or 1300 cGy total-body irradiation (TBI; ^{137}Cs source Gammacell 40 Model C-161 Type 8 irradiator; MDS Nordion International, Ottawa, Ontario, Canada) delivered in two equal doses (at 92–94 cGy/min) separated by 3 hours, to reduce gastrointestinal toxicity. Under these conditions, mice succumb within 5 to 6 days after TBI, unless given bone marrow cells. After transplantation, mice were housed in sterile, microisolator cages and fed normal mouse chow and acidified water for 3 weeks after BMT and filtered water thereafter until analysis. Successful donor cell engraftment in this model was confirmed by examining the percentage of Ly-5.2^+ (CD45.1^+) cells in peripheral blood and spleen at day 28 or 42 after transplantation, as previously described.[15] Mice were killed and lacrimal glands harvested at 1, 2, 4, and 6 weeks after BMT.

All experimentation involving live mice was conducted in accordance with standard operating procedures approved by the University Committee on the Use and Care of Animals at the University of Michigan and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

GVHD Assessment

Survival was monitored daily, and clinical GVHD scores were measured weekly. GVHD severity was graded with a previously described clinical scoring system that incorporates five parameters: weight loss, activity, posture, fur texture, and skin condition.[16] Individual weights were obtained and recorded on day 1 and weekly thereafter. Changes were graded from 0 to 2 for each parameter to generate a clinical index with a maximum score of 10. In this study, GVHD, often leading to death, was evident when clinical scores reached 6 or higher.

Tissue Harvest and Histopathological Examination and Scoring

At 1, 2, 4, and 6 weeks after BMT, 70 mice were killed by CO_2 asphyxiation followed by cervical dislocation. Right and left lacrimal glands were removed from mice and sectioned in half immediately. Portions of each lacrimal gland were fixed with 3.7% buffered formaldehyde solution for histopathologic analysis, whereas the remaining portions were placed in optimal cutting temperature (OCT) embedding medium (Sakura Finetek, Torrance, CA), snap frozen in liquid nitrogen, and stored at $-80°C$ for immunohistochemical studies. For histopathology, lacrimal gland tissue was embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin. Slides were coded and examined in a masked fashion by a trained ocular pathologist (VME), using a semiquantitative scoring system modified from those previously used for grading abnormalities in other organs affected by GVHD[14,17] and analyzing parameters of GVHD present in other organs as well as those reported in human lacrimal glands.[11,12] Histopathologic parameters were established for scoring the lacrimal gland: inflammatory cell infiltrate, lacrimal secretion stasis, epithelial cell debris in lumina, fibrosis, and epithelial cell apoptosis. Apoptosis was ascertained by assessing the presence of chromatin clumping, nuclear pyknosis, or nuclear debris. The scoring system for each parameter denoted 0 as normal; 0.5 as focal and rare; 1 as focal and mild; 2 as multifocal and mild; 3 as multifocal and moderate; and 4 as multifocal and severe, similar to those published in human[18,19] and experimental[14,17] GVHD histopathology. Scores were added to provide a total score for each gland.

Immunohistochemical Analysis

CD4 (rabbit; sc-7219), CD8 (rabbit; sc-7188), CD3 (goat; sc-1127), CD20 (goat; sc-7735), and CD68 (goat; sc-7083) polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) for immunohistochemical staining of 5-μm frozen sections. Analysis was
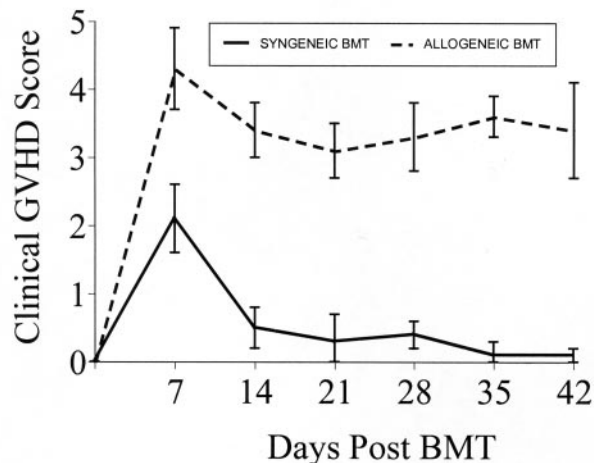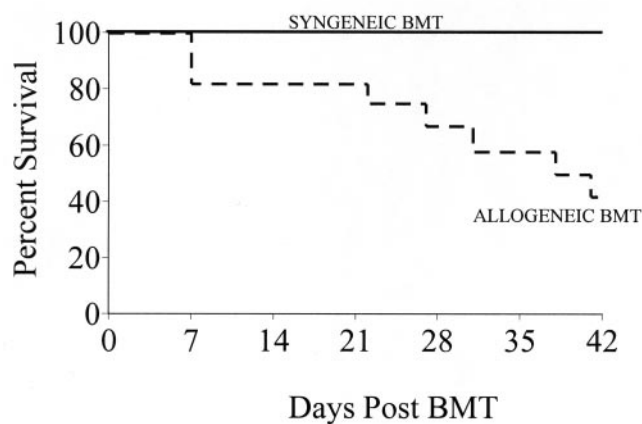

FIGURE 1. Clinical GVHD scores (**A**) and survival estimates (**B**) after syngeneic or allogeneic BMT. Recipient mice received transplants of 5×10^6 bone marrow cells and 2.0×10^6 T cells from syngeneic (B6D2F1) or allogeneic (B6 Ly 5.2^+) mice after conditioning with 1100 or 1300 cGy TBI administered in a split dose. The clinical GVHD scores (syngeneic, $n = 10$; allogeneic, $n = 12$) for all mice were determined by summing five parameters and plotted as the mean $\pm$ SD. Survival estimates are shown by Kaplan-Meier cumulative curves obtained by combining results of two similar experiments.

performed on three lacrimal glands 6 weeks after allogeneic BMT and on three glands 6 weeks after syngeneic BMT, each gland taken from a separate mouse. Complementary goat and rabbit avidin-biotin complex (ABC) staining kits (Vectastain Elite; Vector Laboratories, Burlingame, CA) were used according to the manufacturer's protocol after primary incubations with antibody dilutions of 1:400 for CD4 and CD8 and 1:100 for CD3, CD20, and CD68. Bound antibody was visualized by development with 3-amino-9-ethylcarbazole (0.5 mg/mL; Sigma-Aldrich Chemical, St. Louis, MO) in 0.1 M acetate buffer (pH 5.2), containing 0.01% H_2O_2, to yield a granular, red-brown reaction product. The sections were counterstained with hematoxylin and mounted (Gelmount; Biomeda, Foster City, CA). Slides were coded and examined in a masked fashion as noted earlier, with a semiquantitative scoring system reflecting the number of positive-staining cells: $-$, no staining; $+/-$, focal staining; $+$, multifocal staining; $++$, diffuse with mild positivity; $+++$, diffuse with moderate positivity; and $++++$ diffuse with intense positivity.
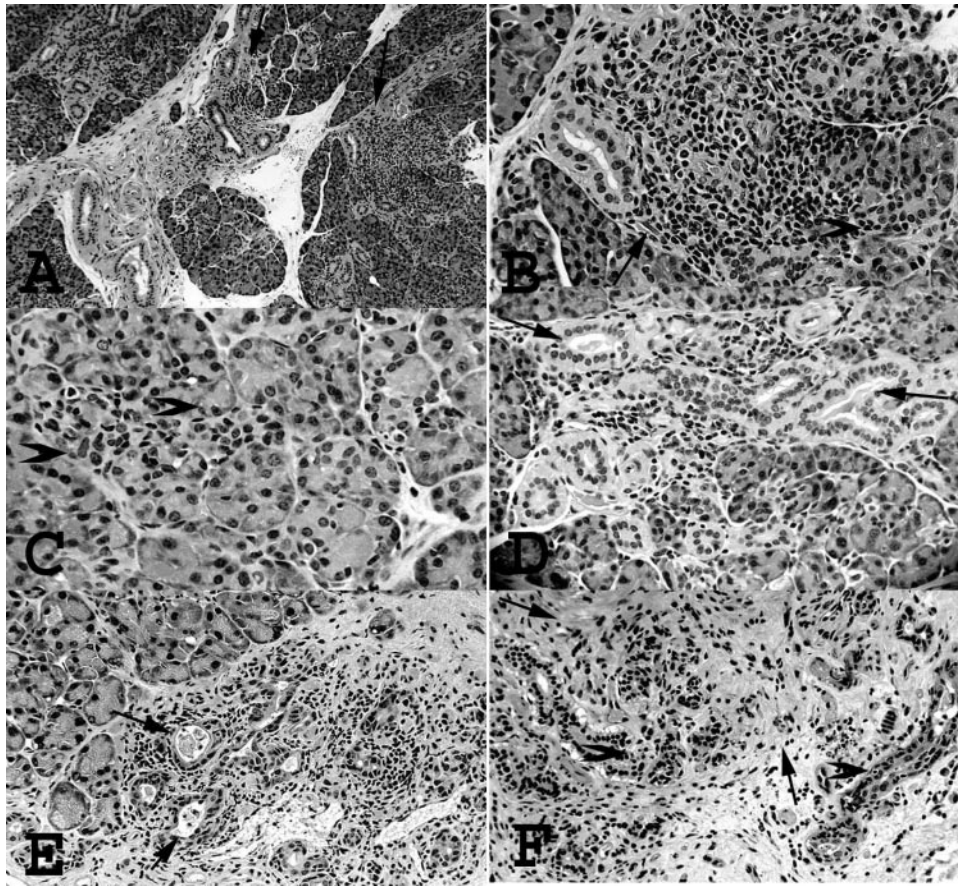
FIGURE 2. Histopathologic findings in lacrimal glands of mice 6 weeks after allogeneic BMT. Diffuse, patchy, but intense chronic inflammation centered around lacrimal ducts (*arrows*) (**A**) with lesser degrees of inflammation in nearby acini. Intense periductal (*arrow*) and acinar (*arrowhead*) infiltration by chronic inflammatory cells, principally lymphocytes (**B**). Permeation of acinar septae by chronic inflammatory cells (**C**, *arrowheads*). Periductal chronic inflammation, early fibrosis, and stasis (*arrows*) of secretions in ducts (**D**). Intense, periductal inflammation with luminal cellular debris (*arrows*) and moderate periductal fibrosis (**E**). Severe periductal fibrosis (*arrows*), residual chronic inflammation, and epithelial leukocytic infiltration with apoptosis (**F**, *arrowhead*). Hematoxylin and eosin; magnification: (**A**) ×50; (**B**, **D-F**) ×150; (**C**) ×250.

Statistical Interpretations

Numerical data are expressed as the mean $\pm$ SD. Statistical comparisons between groups were completed by analysis of variance with post hoc analysis (Scheffé multiple comparison test). $P < 0.05$ was considered significant.

RESULTS

Two weeks after allogeneic BMT, all animals began to display clinical features of GVHD including abnormal mobility, posture, fur texture, skin integrity, and weight loss. These changes were fully developed in 4- and 6-week allogeneic BMT recipients, but were not observed in any of the syngeneic BMT recipients (Fig. 1). External ocular findings observed included crusting of the lid margins, loss of periorbital fur, and blepharospasm, which were not observed in syngeneic BMT recipients.

Histopathologic Scoring

Inflammatory cell infiltrate was noted as early as 2 weeks after allogeneic BMT. The histologic scoring of the infiltrate was significantly greater in allogeneic BMT mice when compared with syngeneic control mice after 2 ($P = 0.01$), 4 ($P = 0.0004$), and 6 ($P < 0.0001$) weeks (Fig. 2A). This patchy inflammation was principally found around lacrimal gland ducts (Fig. 2B), but also permeated the septae surrounding lacrimal acini (Fig. 2C). Stasis of lacrimal gland secretions in ducts (Fig. 2D) was greater in allogeneic BMT mice than in syngeneic BMT control mice at 4 ($P = 0.06$) and 6 ($P = 0.06$) weeks after transplantation. Ductal luminal cellular debris (Fig. 2E) was greater in allogeneic BMT mice than in control mice at 4 weeks ($P = 0.06$) and significantly greater at 6 weeks ($P = 0.005$). Lacrimal

gland fibrosis replaced areas of acinar destruction and was present surrounding remaining ducts (Fig. 2F). The fibrosis was significantly greater in the mice after allogeneic BMT than in control mice at 4 ($P = 0.01$) and 6 ($P < 0.0001$) weeks. Unlike findings in other target organs of GVHD, no significant endotheliitis was present in lacrimal glands at any time point.

Using the five parameters we established to be significantly elevated in lacrimal glands of mice with GVHD, including the additional parameter of epithelial cell apoptosis, we determined a composite histopathologic score for each gland. The mean composite scores of allogeneic and syngeneic BMT mice at each time point were compared. Significant differences were found at 4 ($P = 0.005$) and 6 ($P < 0.0001$) weeks, but not at 1 and 2 weeks after BMT (Fig. 3). At 6 weeks, when peak lacrimal gland GVHD involvement was present, we found the major histopathologic parameters to be inflammation, fibrosis, and apoptosis (Table 1). These parameters contributed to a composite score for glandular tissue after allogeneic BMT that was more than three times greater than that of tissue after syngeneic BMT. Similar findings were present in lacrimal glands 4 weeks after allogeneic BMT ($n = 11$) compared with syngeneic BMT ($n = 8$), with the most significant parameters being inflammation, fibrosis, and ductal stasis (all $P < 0.05$).

Immunohistochemical Analysis

The lacrimal gland leukocytic infiltrates in mice 6 weeks after allogeneic BMT were dominated by CD3$^+$ T lymphocytes (Table 2). Immunohistochemistry also revealed that most of the T lymphocytes were CD8$^+$ (Fig. 4A), although a considerable number of CD4$^+$ T lymphocytes (Fig. 4B) were also present. Mononuclear phagocytes were also noted mul-
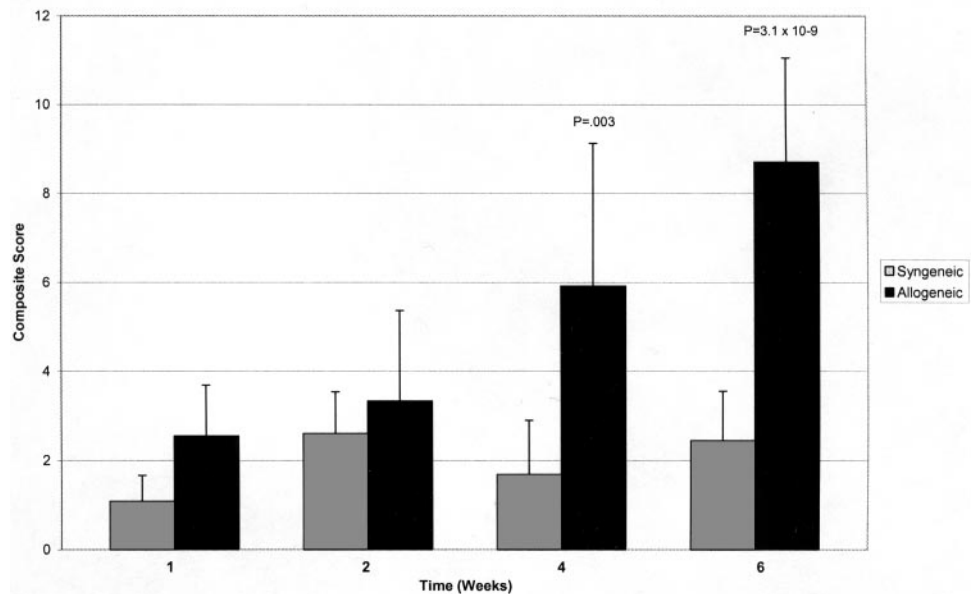
FIGURE 3. Mean composite score of five histopathologic parameters present in lacrimal glands of mice after syngeneic (▨) and allogeneic (■) BMT.

tifically when CD68 positivity was used as a marker (Fig. 4C). No significant staining occurred with CD20 antibodies for B lymphocytes, and no neutrophils were identified in any preparation.

DISCUSSION

GVHD is a complex immunologic process that involves distinct inflammatory cell populations and cytokines that interact, ultimately resulting in apoptotic injury to target tissues, including the lacrimal gland. Chemotherapy and radiotherapy of the host, which are necessary to permit the host to receive BMT and induce cytokine release from initially affected tissues, enabling them to activate donor T lymphocytes by upregulating major histocompatability complex (MHC) antigens and other adhesion molecules.[20,21] In response, activated donor T lymphocytes proliferate and secrete several cytokines, including IFN-γ which is critical for the priming of circulating monocytes and tissue macrophages.[22–24] When present exogenous endotoxin potentiates macrophage priming in various target tissues.[25,26] The activated T lymphocytes and primed macrophages recognize and attack host tissue cells in target tissues, resulting in apoptotic cell death, fibrosis, and loss of target tissue function.

Our results are the first to describe histopathologic lacrimal gland changes associated with GVHD after allogeneic BMT in a murine model. Histopathologic findings of GVHD in other organs after murine BMT are well characterized and compare favorably in the time course and histologic findings we observed in the lacrimal gland.[14–17,20,21] Similar to the lung, no histopathologic injury was present 2 weeks after BMT, but consistently intense leukocytic infiltration was seen after 6 weeks.[16] In the lung, dense mononuclear cell infiltrates predominate around bronchioles and pulmonary vessels with lesser degrees of parenchymal alveolar–interstitial infiltration,[16] corresponding to the lacrimal glandular involvement occurring principally around ducts, with less inflammation involving glandular acini. In both sites, the infiltrates are chiefly composed of lymphocytes and mononuclear phagocytes. In the lung, scattered neutrophils, frequently admixed with fibrin, are also found, presumably in response to locally derived bacterial endotoxin. We did not observe neutrophils in affected lacrimal gland tissue, a finding that is likely to be due to the lower exposure of this tissue to exogenous bacteria. Involvement of the liver is also delayed in this model, with significant involvement 4 to 6 weeks after experimental BMT.[27] Mononuclear cells aggregate around portal triads and infiltrate lobules, participating in bile duct epithelial and hepatocyte damage.[17] We consistently noted similar involvement of lacrimal ducts and, to a lesser extent, lacrimal acini. As in the lacrimal gland, the liver infiltrates did not contain neutrophils. Inasmuch as our study was limited to one model of acute murine GVHD, it is possible that the timing, degree, and character of lacrimal gland involvement may vary with other types of BMT.

In humans, lacrimal glands have been considered targets in chronic GVHD, with clinical manifestations of keratitis sicca occurring later than 100 days after BMT, the accepted clinical transition point between acute and chronic GVHD. Our model, which involves grafting from C57BL/6 donors into B6D2F1 recipients, is known to promote development of acute rather

TABLE 1. Mean Peak Histopathologic Scores for Parameters of GVHD in Lacrimal Glands of Mice 6 Weeks after BMT

	Inflammation	Lacrimal Secretion Stasis	Epithelial Cell Apoptosis	Fibrosis	Epithelial Cell Debris in Lumina	Composite
Syngeneic	0.6 ± 0.3	0.7 ± 0.6	0.8 ± 0.5	0.2 ± 0.3	0.2 ± 0.2	2.4 ± 1.1
Allogeneic	3.0 ± 0.8	1.2 ± 0.7	1.7 ± 0.9	2.0 ± 1.0	0.8 ± 0.7	8.7 ± 2.4
P	<0.0001	$=0.06$	<0.01	<0.0001	<0.01	<0.0001

Syngeneic, $n = 15$; allogeneic, $n = 11$.

than chronic GVHD. The rapid kinetics of damage noted in our model may be explained by the strong histocompatibility differences between donor and host of multiple major and minor H antigenic sites, as well as the lack of immunosuppressants. Moreover, autoantibodies that cause much of the long-term pathologic features of chronic GVHD are absent in this model.[28] The lacrimal gland involvement commencing within 4 weeks of BMT in this untreated model, strongly suggests that the lacrimal gland is a target of progressive, acute GVHD. This contention is further supported by the fact that the only other reported animal model of lacrimal gland GVHD, a hyperacute model in rats, demonstrated similar histopathologic and immunopathologic findings.[29] Taken together, these features of the animal models raise the possibility that lacrimal gland involvement in humans is initiated during acute GVHD, even though its clinical manifestations of dry eye are typically not appreciated until the patient enters the clinical phase of chronic GVHD. Thus, prevention of keratitis sicca due to GVHD in humans may require treatment soon after BMT, before signs and symptoms of dryness are present.

Stasis of lacrimal gland secretions, epithelial cell debris within lacrimal gland duct lumina, periductal inflammation, and periductal fibrosis were consistently present in the lacrimal gland lesions of our acute GVHD murine model. Each of these features has also been described in lacrimal glands of humans with chronic GVHD,[11–13] indicating the clinical relevance of this new experimental lacrimal gland model in understanding how this disease process in humans may initiate acutely and progress to a chronic phase when keratitis sicca becomes clinically manifest.

In humans with GVHD, histopathologic studies of lacrimal gland specimens have identified chronic inflammatory cell infiltrates in the periductal regions with an obliterative fibrosis. Although located mainly around medium-sized ducts, the inflammatory cells were also seen, to a lesser extent, in the acini.[1,30] In all the studies, the infiltrate comprised chiefly T lymphocytes and scattered, closely associated macrophages.[1,30] Using immunohistochemistry, Ogawa et al.[13] identified CD4+ and CD8+ T lymphocytes as the primary cell type present in the lacrimal gland inflammatory infiltrate. Only a few CD20+ B lymphocytes were observed in these infiltrates.[13,30]

Our histopathologic and immunohistochemical results on lacrimal gland GVHD in the murine model correlate closely with the leukocyte subpopulations identified in human disease. Using established leukocyte markers, we found that CD3+ T lymphocytes predominated in the murine periductal and acinar infiltrates. Similar to the human lacrimal gland, the murine GVHD lesions showed mainly CD8+ and CD4+ T lymphocytes (Table 2). Also, as in the human studies, scattered CD68+ macrophages were present while CD20+ B lymphocyte staining was negligible.

The pathogenesis of insufficient aqueous tear production in human GVHD is not completely understood, but it appears that the periductal region of the lacrimal gland is the epicenter of inflammation and fibrosis that impairs glandular function. In this region, donor T lymphocytes bind to and are activated by
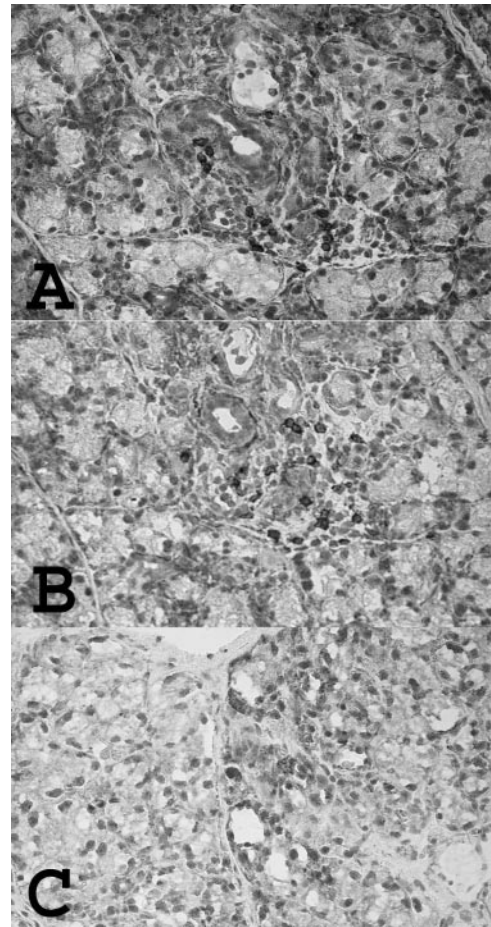

FIGURE 4. Immunohistochemistry for leukocyte markers in lacrimal glands of mice 6 weeks after allogeneic BMT. Most of the leukocytes were T lymphocytes staining for CD8 (**A**) or CD4 (**B**), whereas CD68+ mononuclear phagocytes (**C**) were present multifocally. Hematoxylin counterstain; magnification, ×150.

antigen-presenting mononuclear cells and stromal fibroblasts.[13] Activated T lymphocytes then exert various effector functions including cytotoxic effects on parenchymal cells causing apoptosis and stimulating effects on fibroblasts resulting in their proliferation and synthesis of extracellular matrix, both yielding interstitial fibrosis of advanced lacrimal gland GVHD.[13,30,31]

In conclusion, this study is the first to describe lacrimal gland GVHD in a well-characterized BMT murine model. The pathology closely parallels that observed in human disease, and the rapid kinetics suggest that lacrimal glands are targets of acute GVHD as well as chronic GVHD. Study of the mechanisms in this murine model, which closely correlates with human disease, may provide insights that will lead to amelioration or prevention of lacrimal gland GVHD dysfunction in humans.

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TABLE 2. Immunohistochemical Scoring for Lacrimal Glands in Mice 6 Weeks after BMT

	Syngeneic	Allogeneic
CD3	+/−	+++
CD4	−	++
CD8	+/−	+++
CD20	−	+/−
CD68	−	+

For both BMT groups, *n* = 3.

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