Absence of 4–1BB Gene Function Exacerbates Lacrimal Gland Inflammation in Autoimmune-Prone MRL-*Fas*^{lpr} Mice

Dass S. Vinay,[1] *Jung D. Kim,*[1,2] *Tatsuhiko Asai,*[1,3] *Beom K. Choi,*[2] *and Byoung S. Kwon*[1,2]

Purpose. To define the role of endogenous 4-1BB (an important T-cell costimulatory molecule) in the regulation of ocular disease, MRL-*Fas*^{lpr} mice deficient in 4-1BB were generated, and their lacrimal gland function was studied.

Methods. 4-1BB$^{-/-}$MRL/MpJ-*Tnfrs*^{lpr}/*Tnfrs*^{lpr} (*lpr*/4-1BB$^{-/-}$) mice were generated and used at the ninth backcross. Mice were killed at various times, and lacrimal gland cellularity was analyzed by flow cytometry. Tear and tissue samples were analyzed by Western blotting for the presence of aquaporin 5 (AQP5) and 120-kDa fragments of α-fodrin. Cytokine expression of lacrimal glands was assessed by flow cytometry and RT-PCR analysis.

Results. Absence of the 4-1BB gene function in *lpr* mice resulted in early and increased infiltration of mononuclear cells into lacrimal glands compared with 4-1BB intact *lpr* mice. The severity of lesions in *lpr*/4-1BB$^{-/-}$ mice was closely associated with enhanced accumulation of primarily CD4$^+$ T cells within the lacrimal glands and with increased expression of IL-4. Elevated levels of AQP5 and cleaved 120-kDa fragments of α-fodrin were found in tears and lacrimal gland lysates, respectively, of *lpr*/4-1BB$^{-/-}$ but not *lpr*/4-1BB$^{+/+}$ mice.

Conclusions. Deletion of 4-1BB in *lpr* mice accelerates lacrimal gland lesions through increased CD4$^+$ T-cell infiltration and their production of immune modulators. (*Invest Ophthalmol Vis Sci.* 2007;48:4608–4615) DOI:10.1167/iovs.07-0153

MRL/MpJ-*Tnfrs*^{lpr}/*Tnfrs*^{lpr} (*lpr*) mice develop, besides lupus, a severe lacrimal gland disease closely resembling human Sjögren syndrome,[1] which affects 1% to 3% of the world population, making it one of the most prevalent autoimmune disorders. Four million of these patients live in the United States, and more than 90% of that number are women.[1] The pathophysiology of lacrimal gland disease is thought to encompass two distinct phases: an initiation phase, in which priming of autoreactive lymphocytes occurs, and an effector phase, in which pathogenic lymphocytic infiltrates begin to accumulate in the lacrimal glands (dacryoadenitis), resulting in decreased production of tears (dry eye).[1] The secretory dysfunction has been shown to result in part from damage to glandular epithelial cells, which are replaced by fibrosis caused by infiltrating pathogenic lymphocytes.[2,3]

4-1BB, a 50- to 55-kDa tumor necrosis factor receptor (TNFR) family member, is an important T-cell costimulatory molecule.[4–8] Although 4-1BB signaling preferentially promotes the proliferation and survival of CD8$^+$ T cells,[9] it also supports IL-2 production by CD4$^+$ T cells and prevents activation-induced cell death.[10] In vivo administration of agonistic anti-4-1BB mAb prevents the development of various autoimmune and nonautoimmune conditions.[11] Mice deficient in 4-1BB mice develop normally and are viable and fertile. They have normal humoral responses to vesicular stomatitis virus, display diminished virus-specific cytokine production and CTL activity, and experience increased turnover of myeloid precursor cells in the peripheral blood, bone marrow, and spleen.[12] The 4-1BB-null mice also have suboptimal NK/NKT cells and associated functions,[13] are highly susceptible to hapten-induced inflammatory bowel disease (data not shown), and display resistance to herpes simplex virus–induced stromal keratitis.[14]

Given the important role CD4$^+$ T cells play, especially in the regulation of ocular disease,[15–18] and the evidence that the 4-1BB$^{-/-}$ mice display enhanced CD4$^+$ T-cell division,[12,19] we hypothesized that the inherently hyperproliferative and dysregulated[20] 4-1BB$^{-/-}$ CD4$^+$ T-cell phenotype, when developed on an MRL *Fas*^{lpr} background, should further enhance CD4$^+$ T-cell responses and disease severity.

In the present study, we report that this is the case. As expected, we noticed early and massive CD4$^+$, but not CD8$^+$, T-cell accumulation in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice over *lpr*/4-1BB$^{+/+}$ mice and acute lacrimal gland inflammation. Taken together, these results indicate a potential role of endogenous 4-1BB in the modulation of lacrimal gland disease in the model described.

Methods

Mice

MRL/MpJ-*Tnfrs6*^{lpr}/*Tnfrs*^{lpr} (*lpr*) and control Balb/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The 4-1BB–deficient mice (Balb/c) have been described previously.[12] To generate the *lpr* mice deficient in 4-1BB, we mated the homozygous parents from individual single knockout backgrounds. Independent colonies were established and backcrossed among siblings over nine generations so that MRL background genes were greater than 90%. The genotype of the mutant mice was assessed by Southern blot analysis or PCR of tail DNA. All mice were housed in the LSU Health Sciences Center specific pathogen-free animal facility. All experiments were performed using strain-, age-, and sex-matched mice. Animal experimentation protocols were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.

From the [1]LSU Eye Center, LSU Health Sciences Center School of Medicine, New Orleans, Louisiana; and the [2]Immunomodulation Research Center, Department of Biological Sciences, University of Ulsan, Ulsan, Republic of Korea.

[3]Current affiliation: Department of Ophthalmology, Hamamatsu University School of Medicine, Handayama Hamamatsu-City, Shizuoka Perfecture, Japan.

Supported in part by the United States Public Health Service (Grant R01EY013325 [BSK]); Innovative Research Award from the Arthritis Foundation, Atlanta, GA; Grants KRF-2005-201-E00008 and KRF-2005-084-E00001; the Stem Cell Project (Grant 2006-04212); Science Research Center funds to the Immunomodulation Research Center, from the KOSEF and the Korean Science and Engineering Foundation.

Submitted for publication February 7, 2007; revised April 27 and June 25, 2007; accepted August 20, 2007.

Disclosure: **D.S. Vinay**, None; **J.D. Kim**, None; **T. Asai**, None; **B.K. Choi**, None; **B.S. Kwon**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Byoung S. Kwon, Immunomodulation Research Center, University of Ulsan, Ulsan, Korea 749-680; bkwon@lsuhsc.edu.

Investigative Ophthalmology & Visual Science, October 2007, Vol. 48, No. 10

Measurement of Aqueous Tear Production

Tear production was measured with phenol red–impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA).[21]

Evaluation of Lacrimal Gland Histology

Lacrimal gland sections (7 μm) were cut, stained with hematoxylin and eosin (H&E), and graded as described by Jabs et al.[22] Briefly, H&E-stained histologic sections were scored from 0 to 4 based on the presence or absence of foci of 50 or more mononuclear inflammatory cells: grade 0, no inflammatory cells; grade 1, scattered inflammatory cells without any focus; grade 2, at least one focus; grade 3, multiple foci; and grade 4, multiple foci plus presence of lacrimal gland damage (e.g., replacement of lobular architecture by mononuclear inflammatory cells, fibrosis).

Immunohistochemistry

Frozen tissue sections (7 μm) were thaw-mounted onto glass slides, fixed in ice-cold ethanol, and rehydrated in PBS (pH 7.6) for 20 minutes, followed by immersion in 10% rabbit serum for an additional 30 minutes Staining for CD4, CD8, IL-4, and IFN-γ was performed with appropriate unlabeled antibodies (1/50) and visualized with HRP-conjugated anti-rat IgG and avidin-biotinylated enzyme complex reagents (Vector Laboratories Inc., Burlingame, CA). Diaminobenzidine reagent (Vector) was used to visualize the immunostaining; this step was followed by counterstaining with hematoxylin (Sigma-Aldrich, St. Louis, MO). Microphotographs were taken with a microscope (Eclipse E600; Nikon, Melville, NY) and camera (FDX35; Nikon, Tokyo, Japan).

Detection of Immunoglobulin Deposits

Lacrimal glands were removed from mice and snap frozen in 1 to 2 mL optimum cutting temperature (OCT) compound (Miles, Elkhart, IN). Cryostat sections (7 μm) were prepared with microtome and fixed in ice-cold ethanol for 20 minutes. They were then soaked in PBS for 20 minutes and blocked with PBS containing 10% rabbit serum (Sigma-Aldrich) in a moist chamber. After rinsing, they were incubated with dilutions of FITC-conjugated anti-IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL). The titers were enumerated as the reciprocal value of the highest anti–IgG1- and anti–IgG2a-FITC dilution that gave a clear positive reaction.

Immunoblotting

For immunoblot detection of aquaporin 5 (AQP5) in the tear fluid, mice were anesthetized and tears were collected with a cotton thread, as described. Cotton threads from three to five mice were pooled in microcentrifuge tubes containing 100 μL PBS and were boiled for 5 minutes. Protein released from the cotton threads was determined by BCA assay (Pierce Chemical, Rockford, IL). Lysates of lacrimal glands were prepared and cleared by centrifugation, and supernatants were stored at −80°C until analysis. Samples (20 μg/lane) were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed overnight with anti-AQP5 (1/200 dilution; C-19; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit polyclonal anti-fodrin antibody (1/25,000; a gift of Yoshio Hayashi, Department of Pathology, Tokushima University School of Dentistry, Tokushima, Japan). Bound immune complexes were detected with appropriate HRP-conjugated secondary antibody in conjunction with ECL reagent (Pierce).

Flow Cytometry

Phenotypes of lymphocytes (1×10^6 cells in 100 μL) were analyzed at 4°C after an initial blocking step with 1 μg unlabeled anti-FcγR Ab (clone 2.4G2; prepared in house) with the use of (FACSCalibur; Becton-Dickinson, Mountain View, CA). Unless mentioned, all fluorochrome-labeled mAbs used were purchased from ebioscience (San Diego, CA).

Isolation of T Cells and Analysis of IL-4 and IFN-γ Expression

Single-cell suspensions of lacrimal glands were prepared by treatment of minced tissue fragments with collagenase D (100 U/mL) and DNAse I (15 μg/mL; 40 minutes at 37°C). T-cell subsets were purified using T-cell enrichment columns (R&D Systems, Minneapolis, MN), washed, and incubated in complete medium (RPMI 1640 supplemented with 10% FBS, antibiotics, sodium pyruvate, L-glutamine, nonessential amino acids, and 2-mercaptoethanol; cRPMI) containing antibiotic (Brefeldin A; Sigma-Aldrich; 5 μg/mL) for 6 hours at 37°C and 5% CO_2. The cells (purity greater than 96%) were washed, surface stained with the indicated PE-conjugated antibodies, fixed, permeabilized, incubated with FITC-labeled anti–IL-4 or anti–IFN-γ, and analyzed by flow cytometry. Total RNA was isolated from purified T cells using TRI reagent (Sigma-Aldrich). Levels of specific cytokine mRNA were determined by RT-PCR. In brief, 2 μg RNA was reverse transcribed using random hexamer primers specific for IL-4 (forward, 5′-GGTCTCAACCCCCAGCTAGT; reverse, 3′-TGATGCTCTTTAGGCTTCCA), IFN-γ (forward, 5′-TGAACGCTACACACTGCATCTTGG; reverse, 3′-CGACTCCTTTTCCGCTTCCTGAG) and GAPDH (forward, 5′-ATCATCTCCGCCCCTTCTGC; reverse, 3′-CCACCACCCTGTTGCTGTAG). The PCR products were stained with ethidium bromide after electrophoresis on 1% agarose gels.

TUNEL Assay

Lacrimal gland tissue was fixed in 10% buffered formalin. Sections (7 μm) were cut, fixed, deparaffinized, and processed using a commercial kit (Oncogene Research Products, Boston, MA) according to the manufacturer's protocol. Immunostaining was analyzed by counting for the presence of apoptotic cells (dark spots) in 10 randomly selected high-power fields. Numbers were tabulated, and mean ± SD was obtained. Sections were analyzed with the use of a microscope (Eclipse E600; Nikon).

Statistical Analysis

Each experiment contained four to eight mice, used both lacrimal glands, and was repeated at least three times with similar results. All statistical analyses were conducted with Student's *t*-test. Results were considered statistically significant at $P \leq 0.05$.

Results

Increased Infiltration of Mononuclear Cells in the Lacrimal Glands of *lpr*/4–1BB$^{-/-}$ Mice

In the *lpr* mouse strain, lupus and Sjögren syndrome (lacrimal and salivary gland malfunction) coexist with a host of other clinical disorders, including arthritis.[1,23–25] To test the importance of the endogenous 4-1BB pathway in the development of autoimmune lesions, especially those of the lacrimal glands, we enumerated the rates of mononuclear cell infiltration into these organs. The *lpr*/4-1BB$^{-/-}$ mice showed a slight but not significant increase in organ mass (Figs. 1A, 1B) compared with the *lpr*/4-1BB$^{+/+}$ mice. Despite this, extensive and early lacrimal gland inflammation was observed in *lpr*/4-1BB$^{-/-}$ mice compared with *lpr*/4-1BB$^{+/+}$ mice, as verified by H&E staining (Fig. 1C). By 3 months of age, the *lpr*/4-1BB$^{-/-}$ mice showed extensive mononuclear cell infiltration (grade 3; Fig. 1C, inset) that became intense by 5 months of age (grade 4; Fig. 1C, inset). On the other hand, the *lpr*/4-1BB$^{+/+}$ mice showed no infiltrates at 1 month of age (data not shown), minimal inflammatory lesions by 3 months of age (grade 1; Fig. 1C, inset), and extensive infiltrates (grade 3; Fig. 1C, inset)—though comparatively less than seen in *lpr*/4-1BB$^{-/-}$ mice—by 5 months of age (Fig. 1C). Calculations of mononuclear inflammatory cells suggested significantly increased infiltrating cells in *lpr*/4-1BB$^{-/-}$ mice at 3 months and 5

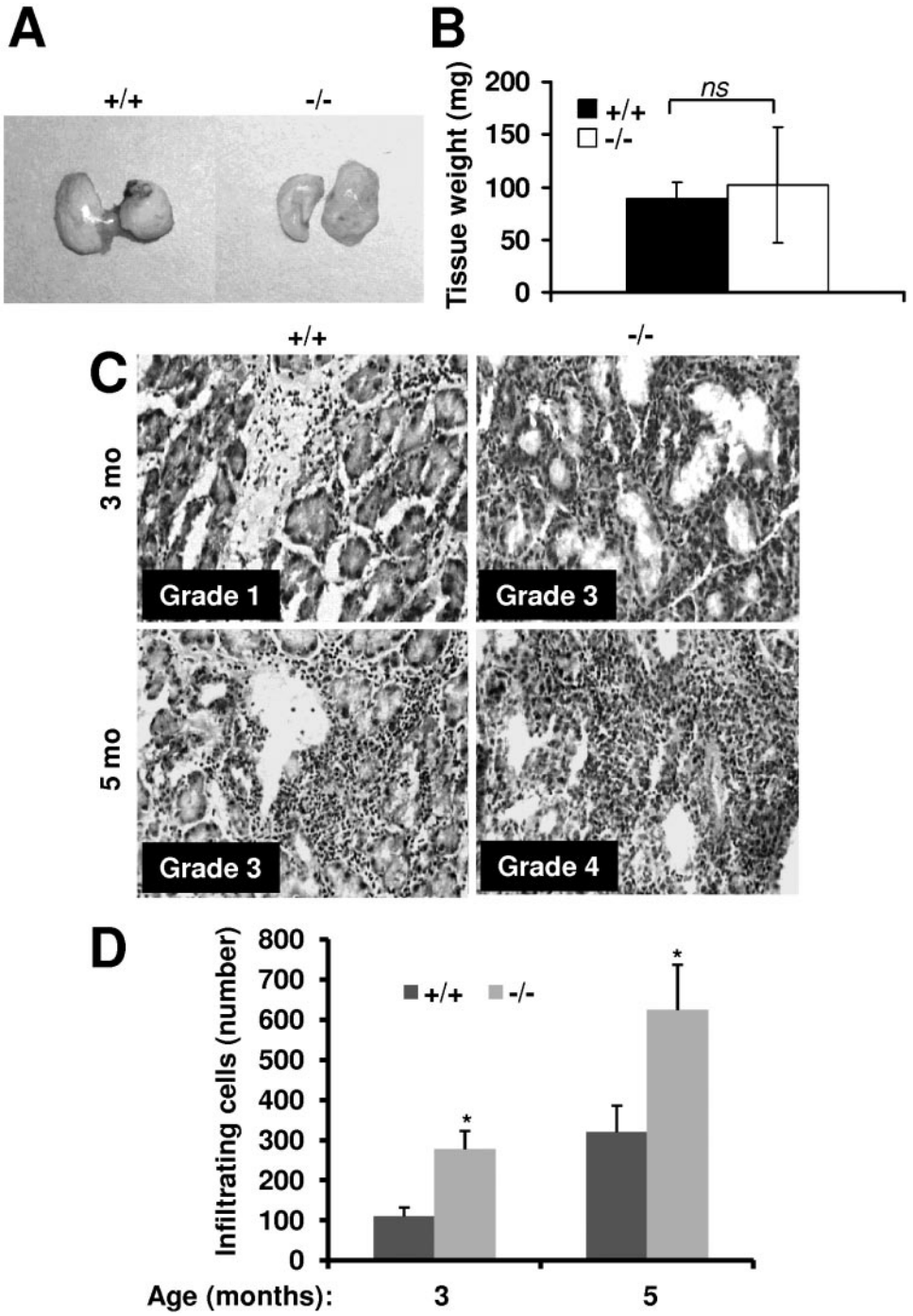

FIGURE 1. 4-1BB deficiency causes enhanced lacrimal gland infiltration in *lpr* mice. The *lpr*/4-1BB$^{+/+}$ and *lpr*/4-1BB$^{-/-}$ mice were killed at 5 months of age. Size (**A**) and weight (**B**) of lacrimal glands. (**C**) H&E staining of 3- and 5-month-old *lpr*/4-1BB$^{+/+}$ and *lpr*/4-1BB$^{-/-}$ mice (n = 7). Original magnification, ×20. Histology scoring of lacrimal gland sections was performed in 10 randomly selected high-power fields, represented as Arabic numerals (*inset*). (**D**) The extent of mononuclear inflammatory cells was analyzed by counting (dark spots) in 10 randomly selected high-power fields. At least three histologic sections per lacrimal gland of individual mouse were evaluated, and pooled data from four independent experiments were considered for analysis. Numbers were tabulated, and mean ± SD was obtained. Similar results were seen in two other experiments. $P < 0.05$.

months of age (Fig. 1D). All control animals (age- and sex-matched Balb/c and Balb/c 4-1BB$^{-/-}$ mice) had entirely normal histologic integrity of lacrimal glands (data not shown).

Augmented CD4$^+$ T-Cell Numbers in *lpr*/4–1BB$^{-/-}$ Mice

Most studies agree that disease severity in *lpr* mice closely correlates with the levels of leukocyte infiltration.[26] Previous studies demonstrate a predominance of CD4$^+$ T cells in the infiltrates of lacrimal glands of the *lpr* mice,[15–18] and treatment with anti-CD4 antibody suppresses the inflammation.[27] Immunohistochemical analysis of lacrimal glands of 3- and 5-month-old mice revealed increased CD4$^+$ T cells in *lpr*/4-1BB$^{-/-}$ mice compared with *lpr*/4-1BB$^{+/+}$ mice, which showed increases smaller than those seen in *lpr*/4-1BB$^{-/-}$ mice (Fig. 2A; n = 7). Quantitative analysis of infiltrates revealed significant increases in CD4$^+$ T-cell numbers beginning at 3 and 5 months of age in *lpr*/4-1BB$^{-/-}$ mice compared with *lpr*/4-1BB$^{+/+}$ mice (Fig. 2B; n = 4). CD8β^+ T-cell numbers of lacrimal glands, however, did not show much variation among the groups (Fig. 2C; n = 4). These findings suggest that the absence of 4-1BB gene function in *lpr* mice alters leukocyte trafficking, especially in CD4$^+$ T cells, in and out of lacrimal glands, affecting the severity of disease resolution.

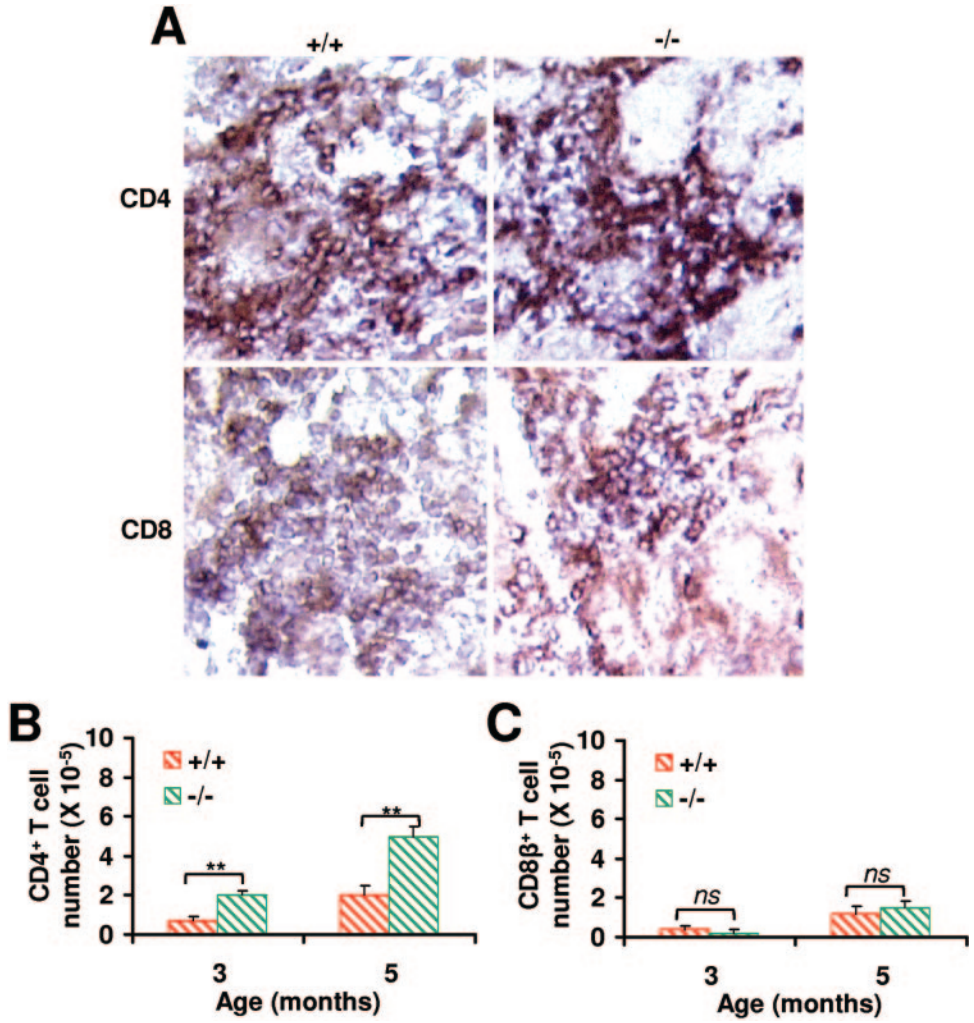

FIGURE 2. Increased $CD4^+$ T-cell numbers in the lacrimal glands of *lpr*/4-$1BB^{-/-}$ mice. (**A**) Lacrimal gland sections (7 μm) of 5-month-old mice were cut and processed for immunohistochemistry. Original magnification, ×20. Representative findings of four independent experiments are shown ($n = 7$). (**B**, **C**) Lacrimal glands were collected and digested with collagenase/DNAse I. Cells (1×10^6/sample) were stained with fluorochrome-labeled anti-CD4 (**B**) and anti-CD8β (**C**) Abs and analyzed by flow cytometry. Absolute numbers of individual cell type were calculated from the total cell number. Data, pooled from four independent flow cytometry experiments, are calculated and presented as mean ± SD. Each experiment used both lacrimal glands ($n = 4$).

4–1BB Deficiency Demonstrates Symptoms of Lacrimal Gland Dysfunction in *lpr* Mice

Different animal models of ocular disease are characterized by distinct pathologic features.[1] For example, tear production is reduced with advanced Sjögren syndrome in nonobese diabetic (NOD) mice but not in *lpr* mice,[28] despite the development of full-fledged disease in both models.[1,23–25] Our findings agree with those of previous reports[1] in that tear production was not significantly different between *lpr*/4-$1BB^{+/+}$ and *lpr*/4-$1BB^{-/-}$ mice at any time during the course of the study (Fig. 3A; $n = 20$).

Despite increased infiltration of pathogenic leukocytes and normal tear production in the lacrimal glands of *lpr* mice, secretion of AQP5 into tears has been suggested as a diagnostic marker for lacrimal gland dysfunction in *lpr* mice.[29] Therefore, we analyzed the expression pattern of AQP5 in tears collected from 5-month-old *lpr*/4-$1BB^{+/+}$ and *lpr*/4-$1BB^{-/-}$ mice. Larger amounts of AQP5 were seen in tears of *lpr*/4-$1BB^{+/+}$ mice than of *lpr*/4-$1BB^{+/+}$ mice (Fig. 3B; $n = 6$). We further confirmed the severity of lacrimal gland malfunction in *lpr*/4-$1BB^{-/-}$ mice by studying the cleavage pattern of α-fodrin. The 120-kDa α-fodrin (a cleaved byproduct of 240-kDa native protein) is an autoantigen against which autoantibodies are generated in experimental animals and human patients with Sjögren syndrome[30] and is generated as result of intense apoptosis in the inflamed lacrimal glands.[31,32] Our results demonstrate enhanced expression of the 120-kDa cleaved product of the α-fodrin protein in the lysates of lacrimal glands from *lpr*/4-$1BB^{-/-}$ mice compared with *lpr*/4-$1BB^{+/+}$ mice (Fig. 3C; $n = 6$). We also examined the possibility of differences between the extent of apoptosis in lacrimal glands of *lpr*/4-$1BB^{+/+}$ mice and *lpr*/4-$1BB^{-/-}$ mice. Results of TUNEL assay clearly demonstrated significant increases in the number of apoptotic cells in the lacrimal glands of *lpr*/4-$1BB^{-/-}$ mice compared with *lpr*/4-$1BB^{+/+}$ mice (Figs. 3D, 3E; $n = 6$). No apoptosis was detected in age-matched control Balb/c and Balb/c 4-$1BB^{-/-}$ mice (data not shown).

Increased Double-Negative T-Cell Accumulation and Immunoglobulin Deposition

Growing evidence suggests that the development of autoimmunity in *lpr* mice is closely correlated with the appearance of cells that coexpress T-cell and B-cell phenotypes ($CD4^-CD8^-B220^+CD3^+$; double-negative T cells [DN T cells]). Despite their well-described role in lupus nephritis,[33] the importance of DN T cells in the development of lacrimal gland inflammation is not completely understood. Prompted by the increased lacrimal gland infiltration of $CD4^+$ T cells in *lpr*/4-$1BB^{-/-}$ mice, we analyzed whether the proportions of DN T cells also increased in these organs. Our results demonstrate nearly double the DN T-cell percentages in lacrimal glands of *lpr*/4-$1BB^{-/-}$ mice compared with *lpr*/4-$1BB^{+/+}$ mice (Fig. 4A; $n = 7$). Similar increases were noted in the spleens and salivary glands of *lpr*/4-$1BB^{-/-}$ mice (data not

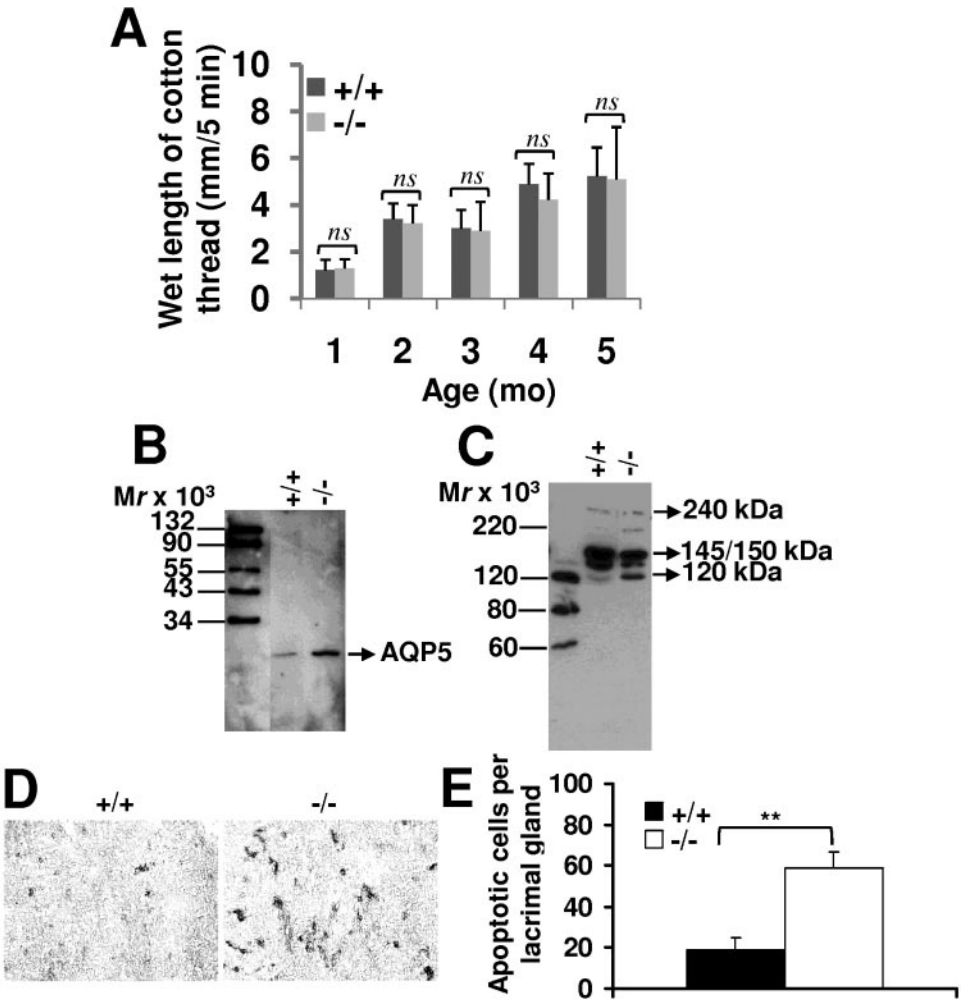

FIGURE 3. 4-1BB-deficient *lpr* mice show increased lacrimal gland disease. (**A**) Tear production was measured in 1- to 5-month-old *lpr*/4-1BB$^{+/+}$ and *lpr*/4-1BB$^{-/-}$ mice, in terms of wet length of cotton thread (mm). Pooled data from three independent experiments (each experiment contained six or seven mice) were calculated, and mean ± SD is shown ($n = 20$). (**B**, **C**) Proteins from tear samples (**B**) and lacrimal gland lysates (**C**) from 5-month-old mice were resolved at 20 μg/lane under reducing conditions, transferred to polyvinylidene difluoride membranes, and probed with antibodies to AQP5 (**B**) or α-fodrin (**C**) Abs. Biotinylated molecular weight markers were run as standards to assess the relative migration of AQP5 and α-fodrin. (**D**) TUNEL assay on deparaffinized lacrimal gland biopsy specimens performed on 5-month-old mice. Immunostaining was analyzed by counting for the presence of apoptotic cells (dark spots) in 10 randomly selected high-power fields. Numbers were tabulated, and mean ± SD was obtained. Note the abundant apoptotic cells, represented by dark spots, in *lpr*/4-1BB$^{-/-}$ mice. Sections shown are representative of all tissue samples analyzed ($n = 6$). Original magnification, ×20. (**E**) Apoptotic cells (**D**) were visually counted under a microscope. At least 10 randomly selected fields were counted and expressed as mean ± SD.

shown). The significance of such increased proportions of DN T cells in lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice is unclear and remains a challenging task for future research. One of the hallmarks of autoimmune disease in *lpr* mice is the accumulation of immunoglobulin deposition in target organs.[24] Consistent with this, we found increased immunoglobulin deposition in the kidneys (data not shown) and lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice compared with *lpr*/4-1BB$^{+/+}$ mice (Fig. 4B; $n = 7$). Interestingly, we noticed dominant IgG1 (Fig. 4B; left panels) but not IgG2a (Fig. 4B; right panels) deposits in the lacrimal glands. Titration experiments confirmed that lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice showed high levels of IgG1 deposits compared with *lpr*/4-1BB$^{+/+}$ mice at 3 and 5 months of age (Fig. 4C; $n = 7$). Levels of IgG2a, on the other hand, were comparatively low and insignificant between the age and mouse groups studied (Fig. 4B, right panels; Fig. 4C). Splenic B-cell numbers (especially B1 B cells) and function, germinal center formation, serum IgG2a/2b titers, and serum anti-dsDNA antibodies were significantly produced in *lpr*/4-1BB$^{-/-}$ mice compared with their *lpr*/4-1BB$^{+/+}$ counterparts (Vinay DS et al., manuscript in preparation). Taken together, these data suggest that increased production of CD4$^+$ T cells (present study) and enhanced B-cell function (data not shown) in *lpr*/4-1BB$^{-/-}$ mice appear to be responsible for the increased immunoglobulin deposition in *lpr*/4-1BB$^{-/-}$ mice.

Increased IL-4 Expression in Lacrimal Glands of *lpr*/4–1BB$^{-/-}$ Mice

We tested whether 4-1BB deficiency altered cytokine production in lacrimal gland infiltrates of *lpr* mice and accounted for the severity of inflammation seen in these mice. 4-1BB-deficient *lpr* mice showed increase in IL-4 expression compared with 4-1BB-sufficient *lpr* in the lacrimal glands (Fig. 5A, upper panels; $n = 4$). In contrast, the IFN-γ expression was comparatively low in *lpr*/4-1BB$^{+/+}$ and *lpr*/4-1BB$^{-/-}$ mice (Fig. 5A, lower panels; $n = 4$), a finding we verified by RT-PCR. As seen by the immunohistochemical findings, RT-PCR data also suggested significantly increased IL-4 in *lpr*/4-1BB$^{-/-}$ compared with *lpr*/4-1BB$^{+/+}$ mice at 3 and 5 months of age (Fig. 5B; $n = 4$). Levels of IFN-γ were low compared with IL-4 but increased in *lpr*/4-1BB$^{-/-}$ mice (Fig. 5C; $n = 4$). Because we found increased CD4$^+$ T-cell accumulation in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice and because these cells play important roles in the development of lacrimal gland inflammation in *lpr* mice,[15–18] we wanted to test whether these cells were responsible for the observed cytokine increase in *lpr*/4-1BB$^{-/-}$ mice. To do so, we negatively purified T-cell subsets (CD4$^+$ and CD8$^+$) from lacrimal glands and evaluated their intracellular expression of IL-4 and IFN-γ by flow cytometry. When freshly purified T-cell subsets were cultured in vitro for 5 hours in the presence of antibiotic (Brefeldin A; to cause an accumulation of intracellular cytokines), the CD4$^+$ T cells from *lpr*/4-1BB$^{-/-}$ mice showed more than a twofold increase in IL-4 but

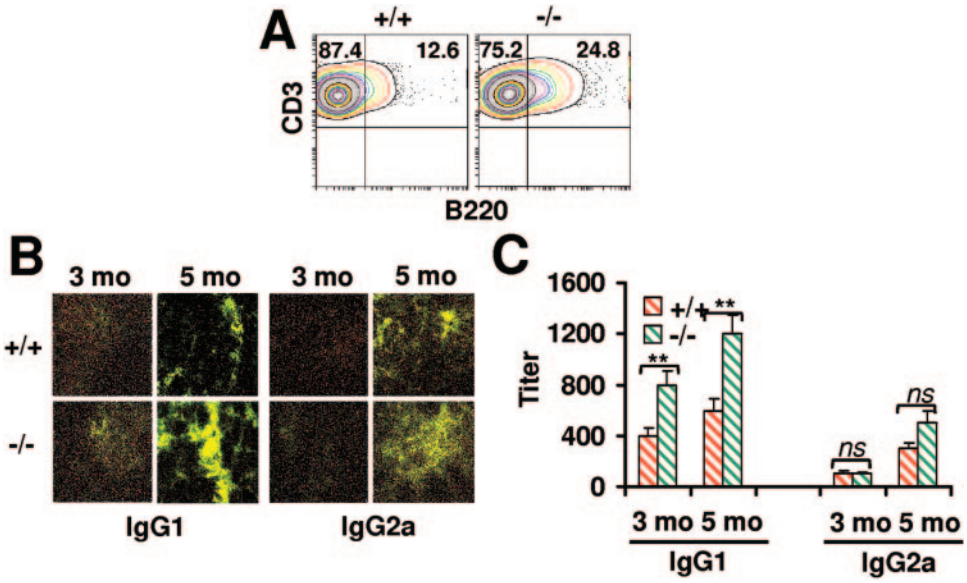

FIGURE 4. Increased DN T-cell and immunoglobulin deposition in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice. The *lpr*/4-1BB$^{+/+}$ and *lpr*/4-1BB$^{-/-}$ mice were killed at 5 months of age. (**A**) Lacrimal glands were collected and digested with collagenase/DNAse I. Single-cell suspensions were processed for two-color flow cytometry after an initial blocking step in anti-FcγR antibody. Numbers in each panel represent the percentage of positive cells for the indicated cell populations ($n = 7$). (**B**) Lacrimal glands were excised, embedded (Tissue-Tek), and snap frozen. Sections (7 μm) were cut and stained with dilutions of FITC-conjugated anti-mouse IgG1 and IgG2a. A representative result obtained with 1/100 dilution of FITC-anti-IgG1 and IgG2a is shown. (**C**) Staining of lacrimal gland sections was achieved (**B**), and titers of IgG1 and IgG2a deposits in the lacrimal glands were assessed. Magnification, ×40 ($n = 7$).

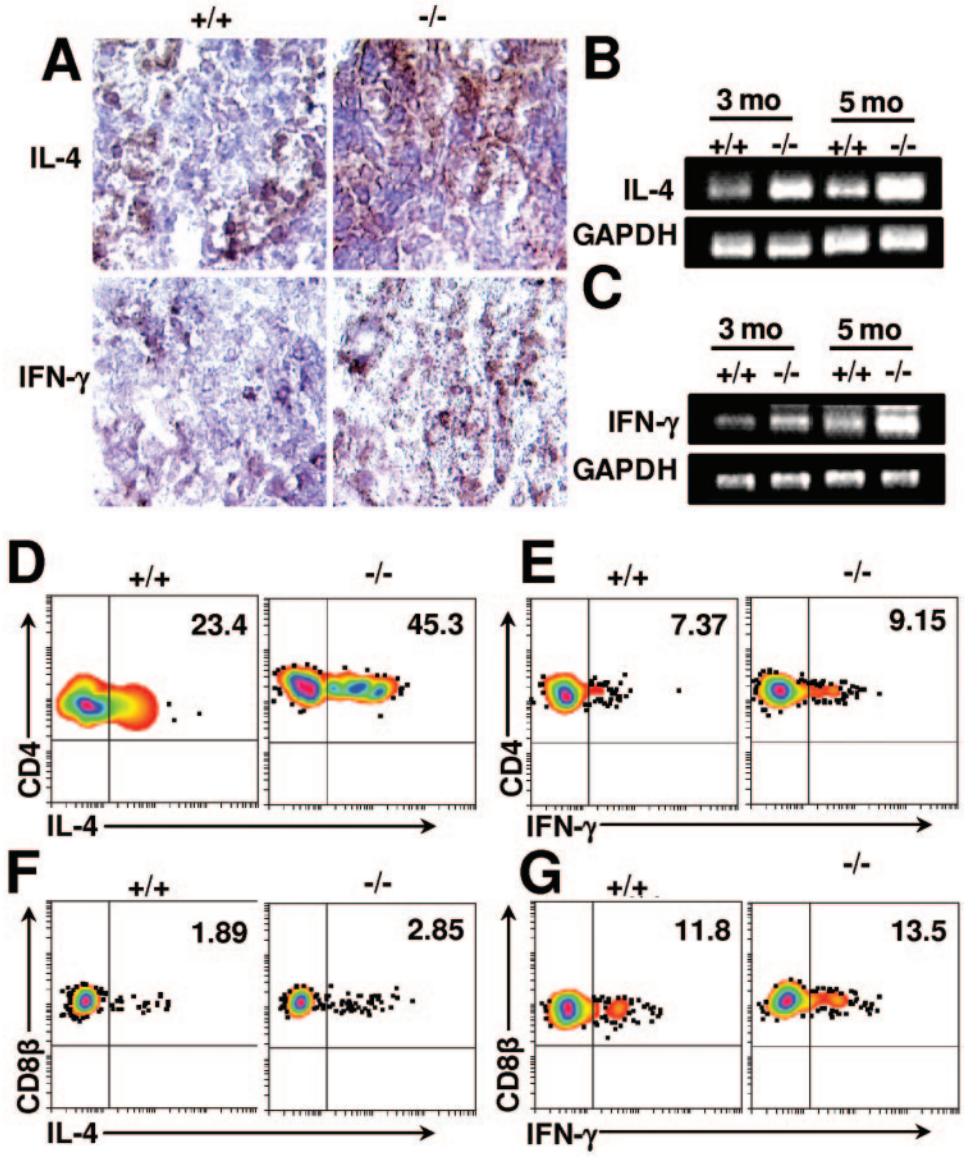

FIGURE 5. Increased IL-4 expression in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice. (**A**) Lacrimal glands of 5-month-old mice were collected, sections (7 μm) were cut, and IL-4 and IFN-γ were detected by immunohistochemistry ($n = 4$). Representative findings of three independent experiments are shown. Original magnification, ×20. (**B–E**) Purified T-cell subsets isolated from the lacrimal glands of 3- and 5-month-old mice were used for RT-PCR analysis of IL-4 (**B**) and IFN-γ (**C**). Pooled samples from three independent experiments were used for the experiment ($n = 4$). T-cell subsets purified from the lacrimal glands of 5-month-old mice were cultured in cRPMI and antibiotic (Brefeldin A; 5 μg/mL) for 6 hours. Cells were washed, surface stained with indicated antibodies, fixed, and permeabilized, and intracellular IL-4 (**D**, **F**) and IFN-γ (**E**, **G**) were detected by flow cytometry. Representative findings of three independent experiments are shown ($n = 4$).

not IFN-γ over *lpr*/4-1BB$^{+/+}$ mice (Figs. 5D–G; $n = 4$). RT-PCR analysis of purified T cells isolated from the lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice showed increased expression of IFN-γ (albeit lower than IL-4) as opposed to the flow cytometry data (compare Figs. 5C, 5E). Repeat experiments showed similar patterns. This variability might have occurred because of the high sensitivity of RT-PCR analysis compared with flow cytometric evaluation. Although direct evidence is lacking, it is possible that in RT-PCR analysis—in which we used whole T cells and not T-cell subsets—minor contamination of NKT and DN T cells was responsible for the observed discrepancy in IFN-γ levels in RT-PCR (Fig. 5C), in contrast to flow cytometry analyses (Figs. 5E, 5G; $n = 4$). Future studies will be devoted to understanding the role and source of IFN-γ and the implications in the development of lacrimal gland lesions in the model described. Thus, increased CD4$^+$ T-cell accumulation and production of immune modulators appear responsible for the severity of lacrimal gland inflammation seen in *lpr*/4-1BB$^{-/-}$ mice.

Discussion

One of the key findings emerging from this study was that deletion of 4-1BB in *lpr* mice compared with 4-1BB intact mice resulted in increased lacrimal gland disease. The basis for the relationship between 4-1BB deficiency and the severity of disease phenotype in *lpr* mice was sought. Based on our present findings, we conclude that 4-1BB deficiency in *lpr* mice results in increased infiltration of pathogenic CD4$^+$ T cells. Lacrimal gland inflammation in *lpr* mice is similar to that seen in humans with Sjögren syndrome because most (approximately 63%–74%)[34] infiltrating cells are CD4$^+$ T cells; fewer are CD8β$^+$ cells, B cells, and macrophages.[16] In support of this, eliminating CD4$^+$ T cells eliminates disease in *lpr* mice.[27] Thus, the increase in CD4$^+$ T-cell numbers in lacrimal glands (present study), coupled with their enhanced proliferative abilities[12,19] and biological function,[20] partly explains the differences seen in the severity of lacrimal gland disease in both mouse groups.

Reports indicate that even at the height of disease severity, *lpr* mice maintain unaltered tear production[1] despite the heavy infiltration of lacrimal glands with pathogenic lymphocytes.[16,35–38] Accordingly, our results also revealed comparable tear production in both groups and at all stages of life studied (Fig. 3A; $n = 20$). Despite normal tear production, our results clearly show that lacrimal gland function is greatly affected in *lpr*/4-1BB$^{-/-}$ compared with *lpr*/4-1BB$^{+/+}$ mice. This conclusion is based on our present finding that a 120-kDa fragment of α-fodrin is expressed more definitely in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ than of *lpr*/4-1BB$^{+/+}$ mice (Fig. 3C). The 120-kDa α-fodrin subunit is an important organ-specific autoantigen in the pathogenesis of Sjögren syndrome in animal models and humans.[30] The 120-kDa fragment is cleaved from mature 240-kDa α-fodrin, in association with apoptosis,[31,32] and is frequently detected in biopsy specimens and sera of affected patients and in mouse models.[30] Why the apoptosis-induced 120-kDa α-fodrin cleaved product developed in mouse lacrimal glands in our study is difficult to explain, especially in our Fas-deficient model. Although Fas–FasL interactions are absent because of the *lpr* mutation in the MRL-*Fas*^{lpr} mice, apoptosis is known to occur in these mice in a Fas-independent manner.[39] Our present study indicates that increased apoptosis in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ compared with *lpr*/4-1BB$^{+/+}$ mice might have led to the cleavage of α-fodrin into a 120-kDa fragment. The cause of the accumulation of the120-kDa cleaved α-fodrin fragment in the lacrimal glands of *lpr*/4-1BB$^{-/-}$ mice is unclear; this protein has not been reported in mouse strains other than NFS/*sld*.[30] It may be that the increase in pathogenic leukocytes by their actions converted the *lpr*/4-1BB$^{-/-}$ mice into a more susceptible phenotype. To our knowledge, the presence of the 120-kDa cleaved α-fodrin fragment has not been reported previously in the Sjögren syndrome model in MRL-*Fas*^{lpr} mice. That lacrimal gland damage is more severe in *lpr*/4-1BB$^{-/-}$ mice is further evidenced by the secretion of significant amounts of AQP5 in the tears. AQP5, which is specifically localized in the apical membrane of acinar duct cells in mouse[40] and human[41] lacrimal gland, is known to leak into the tears of *lpr* mice on glandular damage.[42]

Several reports demonstrate that the autoimmune mechanism in *lpr* mouse spleens and lymph nodes is mediated predominantly by Th1-type [43,44] and Th2-type[45,46] responses in lacrimal glands. Our results demonstrated strong IL-4 expression in the lacrimal glands of *lpr* mice and comparatively low IFN-γ. Repeat experiments yielded similar results, ruling out the possibility of any artifact. We performed additional experiments to confirm whether IL-4 predominated in lacrimal gland lesions. RT-PCR analysis performed on purified lacrimal gland infiltrating T cells (Figs. 5B, 5C) and flow cytometric enumeration of cytokines at the single-cell level (Figs. 5D–G) and RNase protection assay (data not shown) suggested that IL-4 rather than IFN-γ is the dominant cytokine expressed in the lacrimal glands of this mouse strain.

The reasons for Th2-type responses, especially those mediated by IL-4 (we did not observe IL-10 overexpression in any group of mice; data not shown), in the lacrimal glands of *lpr* mice (present study) and Th1 responses in the spleens and lymph nodes[43,44] are unclear to us. Reports show increased levels of IL-4 but not IFN-γ in the lacrimal glands of *lpr* mice by immunohistochemical analysis and by the more sensitive RT-PCR analysis.[45] Evidence also demonstrates that the observed increases in IL-4 were derived from the infiltrating CD4$^+$ T cells.[46] In addition, evaluation of patients with human autoimmune lymphoproliferative syndrome revealed a prominent bias toward the Th2 phenotype,[47] a result similar to that seen in the lacrimal glands of *lpr* mice shown in the present study. Thus, lacrimal gland disease in *lpr* mice appears to be mediated differently than is systemic autoimmune disease in humans.[48]

In summary, this study provides strong evidence that deletion of the endogenous 4-1BB pathway accelerates lacrimal gland lesions through the upregulation of pathogenic CD4$^+$ T cells and their production of immune modulators. Finally, the present findings, coupled with future studies, may offer the possibility of testing 4-1BB pathway manipulation as an effective treatment for autoimmune lesions associated with Sjögren syndrome, which remains one of the most prevalent autoimmune disorders for which we have found no complete cure.

References

1. van Blokland SCA, Versnel MA. Pathogenesis of Sjögren's syndrome: characteristics of different mouse models for autoimmune exocrinopathy. *Clin Immunol.* 2002;103:111–124.
2. Fox RI. Sjögren's syndrome. *Curr Opin Rheumatol.* 1995;7:409–416.
3. Talal N. Sjögren's syndrome. *Curr Opin Immunol.* 1989–1990;2:622–624.
4. Vinay DS, Kwon BS. Role of 4-1BB in immune responses. *Semin Immunol.* 1998;10:481–489.
5. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity. *Nat Rev Immunol.* 2003;3:609–620.

6. Mittler RS, Foell J, McCausland M, et al. Anti-CD137 antibodies in the treatment of autoimmune disease and cancer. *Immunol Res*. 2004;29:197–208.
7. McHugh RS, Whitters MJ, Piccirillo CA, et al. $CD4^+CD25^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity*. 2002;16:311–323.
8. Wilcox RA, Chapoval AI, Gorski KS, et al. Expression of functional CD137 receptor by dendritic cells. *J Immunol*. 2002;168:4262–4267.
9. Takahashi C, Mittler RS, Vella AT. 4–1BB is a bona-fide CD8 T cell survival signal. *J Immunol*. 1999;162:5037–5040.
10. Hurtado JC, Kim YJ, Kwon BS. Signals through 4–1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J Immunol*. 1997;158:2600–2609.
11. Vinay DS, Cha K, Kwon BS. Dual immunoregulatory pathways of 4-1BB signaling. *J Mol Med.* 2006;84:726–736.
12. Kwon BS, Hurtado JC, Lee ZH, et al. Immune responses in 4–1BB (CD137)-deficient mice. *J Immunol*. 2002;168:5483–5490.
13. Vinay DS, Choi BK, Bae JS, Kim WY, Gebhardt BM, Kwon BS. CD137-deficient mice have reduced NK/NKT cell numbers and function, are resistant to lipopolysaccharide-induced shock syndromes, and have lower IL-4 responses. *J Immunol*. 2004;173:4218–4229.
14. Seo SK, Park YH, Choi JH, et al. Blocking 4–1BB/4–1BB ligand interactions prevents herpetic stromal keratitis. *J Immunol*. 2003;171:576–583.
15. Adamson TC, Fox RI, Frisman DM, Howell FV. Immunohistological analysis of lymphoid infiltrates in primary Sjögren's syndrome using monoclonal antibodies. *J Immunol*. 1983;130:203–208.
16. Jabs DA, Prenderghast RA. Reactive lymphocytes in lacrimal gland and renal vasculatic lesions of autoimmune MRL/lpr mice express L3T4. *J Exp Med*. 1987;166:1198–1203.
17. Ohno K, Takahashi T, Maki K, Ueda M, Taguchi O. Successful transfer of localized autoimmunity with positively selected $CD4^+$ cell to SCID mice lacking functional B cell. *Autoimmunity*. 1999;29:103–110.
18. Tabbara K, Sharara N. Sjögren's syndrome: pathogenesis. *Eur J Ophthalmol*. 1999;9:1–7.
19. Lee SW, Vella AT, Kwon BS, Croft M. Enhanced CD4 T cell responsiveness in the absence of 4–1BB. *J Immunol*. 2005;174:6803–6808.
20. Maerten P, Kwon BS, Shen C, et al. Involvement of 4–1BB (CD137)-4–1BB ligand interaction in the modulation of CD4 T cell-mediated inflammatory colitis. *Clin Exp Immunol*. 2006;143:228–236.
21. Sakamoto R, Bennett ES, Henry VA, et al. The phenol red thread tear test: a cross-cultural study. *Invest Ophthalmol Vis Sci*. 1993;34:3510–3514.
22. Jabs DA, Lee B, Burek CL, Saboory AM, Prenderghast RA. Cyclosporine therapy suppresses ocular and lacrimal gland disease in MRL/Mp-*lpr/lpr* mice. *Invest Ophthalmol Vis Sci*. 1996;37:377–383.
23. Sjögren H. Zur Kenntnis Keratocunjuctivitis sicca. *Acta Ophthalmol*. 1933;2:1–151.
24. Theofilopoulos AN, Dixon FJ. Etiopathogenesis of murine SLE. *Immunol Rev*. 1981;55:179–216.
25. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol*. 1985;37:269–390.
26. Talal N, Moutsopoulos HM, Kassan SS. *Sjögren's Syndrome: Clinical and Immunological Aspects*. Berlin: Springer-Verlag; 1987;193–199.
27. Jabs DA, Prenderghast RA. Autoimmune ocular disease in MRL/Mp-lpr/lpr mice is suppressed by anti-CD4 antibody. *Invest Ophthalmol Vis Sci*. 1991;32:2718–2722.
28. Barabino S, Dana MR. Animal models of dry eye: a critical assessment of opportunities and limitations. *Invest Ophthalmol Vis Sci*. 2004;45:1641–1646.
29. Hirai S, Ishida N, Watanabe K, Mita S. Leakage of aquaporin 5 in the tear of dacryoadenitis mice. *Invest Ophthalmol Vis Sci*. 2000;41:2432–2437.
30. Haneji N, Nakamura T, Takio K, et al. Identification of alpha-fodrin as a candidate autoantigen in primary Sjögren's syndrome. *Science*. 1997;276:604–607.
31. Saegusa K, Ishimaru N, Yanagi K, et al. Prevention and induction of autoimmune exocrinopathy is dependent on pathogenic autoantigen cleavage in murine Sjögren's syndrome. *J Immunol*. 2002;169:1050–1057.
32. Vanags DM, Pörn-Ares MI, Coppola S, Burgess DH, Orrenius S. Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J Biol Chem*. 1996;271:31075–31085.
33. Cohen PL, Eisenberg RA. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu Rev Immunol*. 1991;9:243–269.
34. Jabs DA, Prenderghast RA. Murine models of Sjögren's syndrome: immunohistological analysis of different strains. *Invest Ophthalmol Vis Sci*. 1988;29:1437–1443.
35. Skarstein K, Johannessen AC, Holmdahl R, Johnson R. Effects of sialadenitis after cellular transfer in autoimmune MRL/*lpr* mice. *Clin Immunol Immunopathol*. 1997;84:177–184.
36. Johnson R, Tarkowski A, Backman K, Holmdahl R, Klareskog L. Sialadenitis in the MRL-*lpr* mouse: morphological and immunohistochemical characterization of resident and infiltrating cells. *Immunology*. 1987;60:611–616.
37. Johnson R, Holmdahl R. Infiltrating mononuclear cells in salivary glands and kidneys in autoimmune MRL/Mp-*lpr/lpr* mice express IL-2 receptor and produce interferon-gamma. *J Oral Pathol Med*. 1990;19:330–334.
38. Hayashi Y, Haneji N, Hamano H, Yanag, K. Transfer of Sjögren's syndrome-like autoimmune lesions into SCID mice and prevention of lesions by anti-CD4 and anti-T cell receptor antibody treatment. *Eur J Immunol*. 1994;24:2826–2831.
39. Jabs DA, Lee B, Whittum-Hudson J, Prendergast RA. The role of Fas-Fas ligand-mediated apoptosis in autoimmune lacrimal gland disease in MRL/MpJ mice. *Invest Ophthalmol Vis Sci*. 2001;42:399–401.
40. Ishida N, Hirai SI, Mita S. Immunolocalization of aquaporin homologs in mouse lacrimal glands. *Biochem Biophys Res Commun*. 1997;238:891–895.
41. Tsubota K, Hirai S, King LS, Agre P, Ishida N. Defective cellular trafficking of lacrimal gland aquaporin-5 in Sjögren's syndrome. *Lancet*. 2001;357:688–689.
42. Zoukhri D, Kublin CL. Impaired neurotransmitter release from lacrimal and salivary gland nerves of a murine model of Sjögren's syndrome. *Invest Ophthalmol Vis Sci*. 2001;42:925–932.
43. Murray LJ, Lee R, Martens C. In vivo cytokine gene expression in T cell subsets of the autoimmune MRL/Mp-*lpr/lpr* mouse. *Eur J Immunol*. 1990;20:163–170.
44. Takahashi S, Fossati L, Iwamoto M, et al. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J Clin Invest*. 1996;42:561–568.
45. Jabs DA, Prenderghast RA, Rorer EM, Hudson AP, Whittum-Hudson A. Cytokines in autoimmune lacrimal gland disease in MRL/MpJ mice. *Invest Ophthalmol Vis Sci*. 2001;42:2567–2571.
46. Jabs DA, Lee B, Whitum-Hudson JA, Prenderghast RA. Th1 versus Th2 immune responses in autoimmune lacrimal gland disease in MRL/Mp mice. *Invest Ophthalmol Vis Sci*. 2000;41:826–831.
47. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann Intern Med*. 1999;130:591–601.
48. Jabs DA, Burns WH, Prenderghast RA. Paradoxic effect of anti-CD4 therapy on lacrimal gland disease in MRL/Mp-*lpr/lpr* mice. *Invest Ophthalmol Vis Sci*. 1996;37:246–250.