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A Role for Lymphotoxin in Primary Sjögren's Disease

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The etiology of salivary gland injury in primary Sjögren's disease is not well understood. We have previously described a mouse model of Sjögren's disease, IL-14α transgenic (IL14αTG) mice, which reproduces many of the features of the human disease. We now demonstrate a critical role for lymphotoxin α (LTA) in the pathogenesis of Sjögren's disease in IL14αTG mice. IL14αTG mice express LTA mRNA in their salivary glands and spleen and produce soluble LTA protein in their salivary secretions. When IL14αTG mice were crossed with LTA$^{-/-}$ mice, the IL14αTG.LTA$^{-/-}$ mice retained normal salivary gland secretions and did not develop either lymphocytic infiltration of their salivary glands or secondary lymphomas. However, both IL14αTG and IL14αTG.LTA$^{-/-}$ mice produced similar amounts of IFN-α and had similar deposition of autoantibodies in their salivary glands. Both IL14α and IL14α/LTA$^{-/-}$ mice had similar B cell responses to T-dependent and T-independent Ags, L-selectin expression, and expression of RelA, RelB, and NF-κB2 in their spleens. These studies suggest that LTA plays a critical role in the local rather than systemic inflammatory process of Sjögren's disease. Furthermore, local production of soluble LTA in the salivary glands of IL14αTG mice is necessary for the development of overt Sjögren's disease. Autoantibody deposition alone is not sufficient to produce salivary gland dysfunction. We also demonstrate that LTA is increased in the salivary gland secretions and sera of patients with Sjögren's disease, further strengthening the biological relevance of the IL14αTG model to understanding the pathogenesis of human disease. *The Journal of Immunology*, **2010, 185: 6355–6363.**

Sjögren's syndrome is a common autoimmune disorder characterized by xerostomia (dry mouth) and xerophthalmia (dry eyes) due to progressive loss of secretory function in the salivary and lacrimal glands, respectively (1, 2). In previous studies, we demonstrated that transgenic mice expressing the B cell growth factor IL-14α develop a disease that reproduces the clinical and immunological changes characteristic of Sjögren's disease. Moreover, these pathological changes occur in IL-14α transgenic (IL14αTG) mice in the same temporal sequence as observed in patients with Sjögren's disease (3–6). Confirming previous observations in human disease, we demonstrated that in IL-14α mice, the loss of salivary gland secretion precedes lymphocytic infiltration of the salivary glands (4, 7, 8). Several possible mechanisms may underlie salivary gland injury independent of cell-mediated destruction including direct cytokine toxicity from IL-12, IFN-α, IFN-γ, lymphotoxin α (LTA), or TNF-α (9–11). Alternatively, Ab-mediated injury either alone or in combination with cytokines may cause gland damage (12). Lymphocytic infiltration may also contribute to salivary gland injury by direct cell-mediated destructive processes.

Lymphotoxins (LTs) are cytokines closely related to TNF. LTA has both systemic and local actions that allow for productive interactions between lymphocytes and surrounding parenchymal and stromal cells (13–17). Membrane-bound LTA associated with lymphotoxin β (LTB) is required for the formation of normal lymph node and germinal center architecture (17). LTA/LTB is capable of inducing ectopic germinal center formation (13–17). LTA also exists in a soluble form as a trimer that has direct cytotoxic effects (18). Soluble LTA also promotes production of IFNs as well as chemokines that are important activating and migration signals to immune cells (19–21). Due to its direct cytotoxic effects, LTA has been directly implicated in the pathogenesis of human polymyositis (22). In animal models, blocking LTA action inhibited both induced and spontaneous forms of autoimmune diseases including collagen-induced arthritis, inflammatory bowel disease, myasthenia gravis, insulin-dependent diabetes, and multiple sclerosis (23, 24). In this paper, we demonstrate that in the IL14αTG animal model, LTA is a critical factor in the pathogenesis of Sjögren's disease. Furthermore, because soluble LTA is increased in the salivary gland secretions and sera of patients with Sjögren's disease, we speculate that LTA may play an important role in human illness.

Materials and Methods

Mice

LTA$^{-/-}$ mice (strain name: B6.129S2-Ltatm1Dch/J) and C57/BL6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the Laboratory Animal Facility at the State University of New York (SUNY) at Buffalo in accordance with institutional guidelines. IL14αTG mice were made by our laboratory and maintained in the Laboratory Animal Facility at SUNY Buffalo (3). To generate IL14αTG mice that did not express LTA, IL14αTG mice were crossbred with LTA$^{-/-}$ mice. These double transgenic (IL14αTG.LTA$^{-/-}$) mice were screened by PCR to ensure the presence of the IL-14α gene and lack of LTA gene by following instructions from The Jackson Laboratories.

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Abbreviations used in this paper: IL-14α TG, IL-14α transgenic; LT, lymphotoxin; LTA, lymphotoxin α; LTB, lymphotoxin β; LTBR, LTB receptor; MZB, marginal zone B cells; NP, nitrophenol; PG, parotid gland; qPCR, quantitative PCR; SMG, submandibular gland; SUNY, State University of New York.

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Quantitative PCR for the evaluation of LTA, RelA, RelB, and NF-κB2

Quantitative PCR (qPCR) reaction was set up using SYBR GreenER qPCR SuperMix for ABI Prism instrument purchased from Invitrogen (Carlsbad, CA) following the manufacturer's instructions. qPCR was run on an ABI 7900 instrument (Applied Biosystems, Carlsbad, CA) with the following program: 50°C for 2 min hold, 95°C for 10 min hold, 40 cycles of 95°C, 15 s and 60°C, 60 s. Melting curve analysis was performed as previously described (4). The primers for LTA were forward primer 5′-CACGAGGTCCAGCTCTTTTC-3′ and reverse primer 5′-AGTGCAAAGGCTCCAAAGAA-3′; for RelA were forward primer 5′-GGCCTCATCCACATGAACTT-3′ and reverse primer 5′-CACTGTCACCTGGAAGCAGA-3′; for RelB were forward primer 5′- GCGGATTTGCCGAATCAACAAGGA-3′ and reverse primer 5′-AGCTGCTCAACTCTCCAAGGACAT-3′; for NF-κB2 were forward primer 5′-TGACTGTGGAGCTGAAGTGG-3′ and reverse primer 5′-GGTGTGTTTCCAGCAAAGGT-3′; and for 18S rRNA control were forward primer 5′-CGCGGTTCTATTTTGTTGGT-3′ and reverse primer 5′-AGTCGGCATCGTTTATGGTC-3′.

Stimulation of purified B cells with LPS and rIL-14α

B lymphocytes were purified from the spleens of C57/BL6 mice using Dynabeads Mouse Pan B (Invitrogen) following the manufacturer's instructions. Cells were then cultured in RPMI 1640 medium with 10% FBS (Invitrogen) and stimulated with LPS (Sigma, St. Louis, MO) and custom-made rIL-14α. rIL-14α was made by cloning human IL-14α into PQE-30 vector from Qiagen (Valencia, CA) and expressed in *Escherichia coli* Host Strains M-15 from Qiagen. LPS from rIL-14α was removed with polymyxin beads.

Western blot analysis of LT in salivary gland secretions

Salivary gland secretions from mice were evaluated by a Western blot assay utilizing an anti-LT mAb obtained from R&D Systems (Minneapolis, MN) as previously described (4). Salivary gland secretions from patients with Sjögren's disease and from normal controls were evaluated by a Western blot assay utilizing an anti-LT mAb obtained from R&D Systems. Total protein concentration in the saliva samples was measured by the bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Equal amounts of protein were loaded in each sample well.

Analysis of serum IFN-α and serum and salivary LTA by ELISA

The levels of IFN-α in the sera of mice were determined by an ELISA kit purchased from Invitrogen and according to the manufacturer's instructions. The levels of soluble LTA in the sera of human subjects were determined by an ELISA kit purchased from R&D Systems and according to the manufacturer's instructions.

Histological and immunofluorescence evaluation of salivary gland tissues

The structure of salivary gland tissues was evaluated by standard histological techniques using H&E staining as previously described (3, 4). Immunofluorescence studies were performed as previously described using Abs to mouse IgA, IgG, and IgM from Sigma (St. Louis, MO) (4).

Determination of the volume of salivary gland secretions

The volume of salivary gland secretions was determined as previously described (4). In short, mice were anesthetized with i.p. injection of 80 mg ketamine and 10 mg xylazine per kg body weight. Body weight was measured, and then they were given 0.05 mg pilocarpine per 100 g body weight i.p. The total saliva was collected by gentle suction for a 15-min period and measured.

Flow cytometry

Flow cytometry on lymphocytes obtained from the spleen and peritoneal cavity was performed as previously described using Abs to murine CD3, CD5, CD19, CD21, CD38, CD62L, CD138, IgM, and IgD (BD Biosciences, San Jose, CA) (3).

Patients with Sjögren's disease

Patients with Sjögren's disease were recruited from the immunology clinics of the SUNY at Buffalo School of Medicine. Normal donors were age- and sex-matched friends of the patients. The diagnosis of Sjögren's disease was based on the criteria proposed by the American-European Consensus Group on Sjögren's Syndrome (25). The Institutional Review Board of the University at Buffalo for Health Sciences approved these studies.

Results

Because previous microarray studies suggested that the mRNA for LTA was expressed at high levels in the spleens of IL14αTG mice, we examined the expression of LTA mRNA in the spleens and salivary glands of IL14αTG and C57BL/6 mice by quantitative RT-PCR. Fig. 1*A* demonstrates that the mRNA for LTA was expressed at higher levels in the salivary glands and spleens of IL14αTG mice than in the corresponding tissues of C57BL/6 mice. In both cases, significantly more LTA mRNA was expressed in the salivary glands than in the spleens, suggesting that local production of LTA is important. No LTA mRNA was observed in either of these organs in $LTA^{-/-}$ mice. Western blot studies demonstrated that the LTA found in the salivary gland secretions of IL14αTG mice was in a soluble form. Furthermore, LTA was detected in the saliva of IL14αTG mice but not in the saliva of littermate control mice, $LTA^{-/-}$ mice, or IL14αTG.$LTA^{-/-}$ mice (Fig. 1B). Because the production of LTA was increased in IL14αTG mice, we examined whether rIL-14α could induce the production of LTA mRNA in purified B cells. The addition of IL-14α to LPS-stimulated B cells resulted in a significantly increased amount of LTA production (Fig. 1*C*). Next, because B1 cells are a potential source of soluble LTA, which has been associated with the induction of systemic inflammatory states (26, 27), we also evaluated the level of LTA transcripts in peritoneal B1 cells in C57BL/6 and IL14αTG mice. Fig. 1*D* demonstrates that expression of LTA mRNA in the peritoneal B1 cells is similar in both strains of mice.

To evaluate the significance of LTA in Sjögren's disease, we generated IL14αTG mice that lacked LTA (IL14αTG.$LTA^{-/-}$) (28). Fig. 2 demonstrates that IL14αTG.$LTA^{-/-}$ mice maintained normal salivary gland secretion up to 10 mo of age, whereas IL14αTG mice exhibit diminished salivary gland function (4). All strains of mice, including the control C57BL/6 and $LTA^{-/-}$ mice, demonstrated some mild diminution in salivary gland secretion between 6 and 10 mo, suggesting that there must be some age-dependent changes in salivary gland function not dependent upon LTA. Furthermore, IL14αTG.$LTA^{-/-}$ mice do not have lymphocytic infiltration within salivary glands even at 14 mo of age despite the presence of pulmonary inflammation (Fig. 3). In contrast, by 14 mo of age, IL14αTG mice have significant lymphocytic infiltration of both the submandibular and parotid glands (4).

We next explored whether LTA-induced salivary gland inflammation was mediated by IFN-α. LTA has been shown to induce the production of IFN-α under various circumstances (29), and IFN-α has been associated with Sjögren's disease (10, 30). We previously demonstrated increasing levels of IFN-α in the sera of IL14αTG mice as they aged (3). Fig. 4 demonstrates that at 8 mo of age, there is no difference in the levels of IFN-α in the sera of disease-prone IL14αTG and disease-resistant IL14αTG.$LTA^{-/-}$ mice. In contrast, IFN-α is undetectable in the sera of control C57BL/6 and $LTA^{-/-}$ mice at the same age.

We next examined whether particular B cell subpopulations were altered in IL14αTG.$LTA^{-/-}$ mice. Normally, IL14αTG mice have increased numbers of peritoneal B1 cells compared with that in normal mice (Fig. 5) (3). However in the absence of LTA (IL14αTG.$LTA^{-/-}$), the number of B1 cells in the peritoneum was the same as that in the C57BL/6 controls. The number of B2 cells and marginal zone B cells were similar in IL14αTG mice and IL14αTG.$LTA^{-/-}$ mice (Fig. 5 and data not shown).

To test whether the differences in numbers of peritoneal B1 cells had functional consequences, we immunized both IL14αTG and

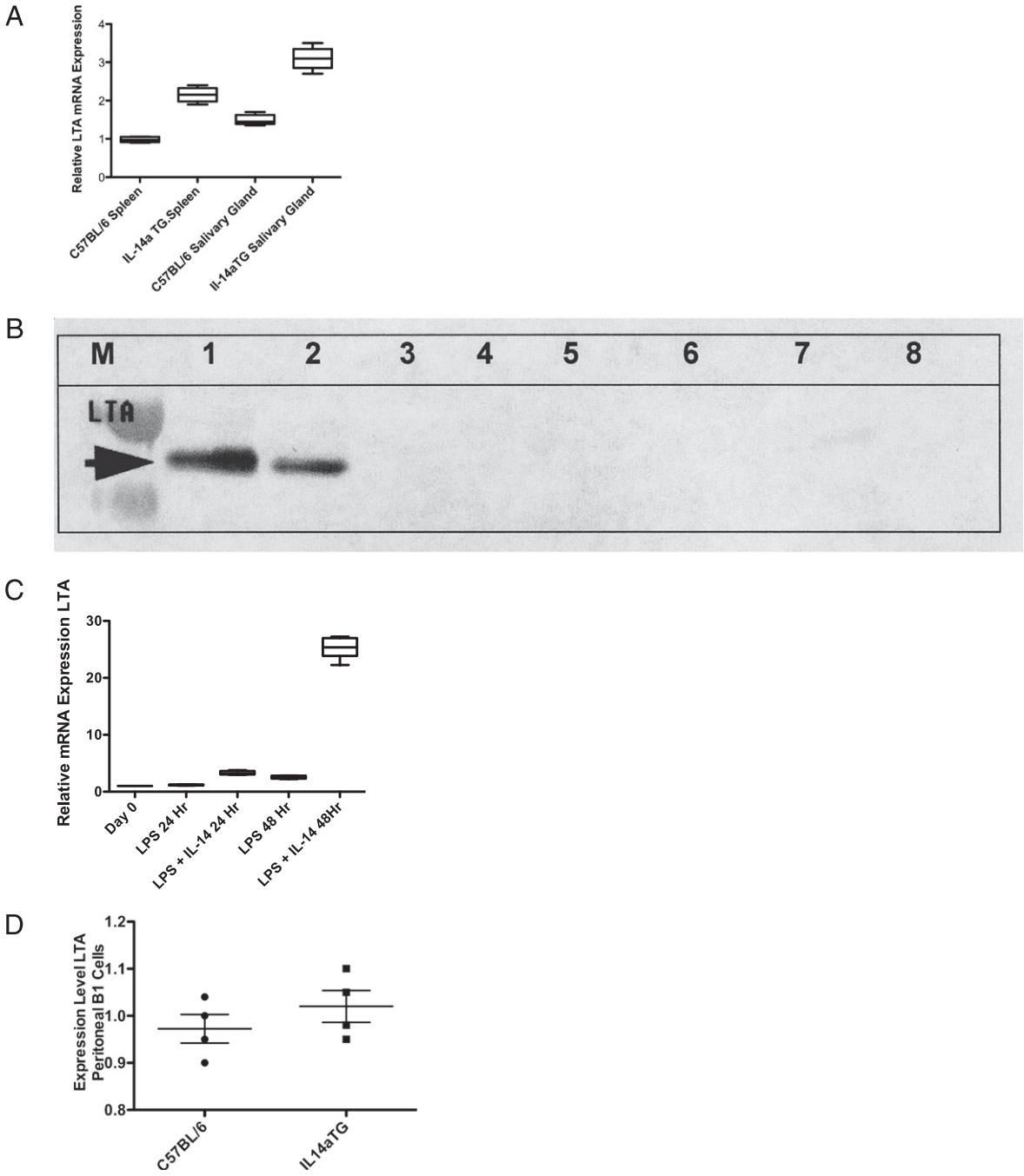

FIGURE 1. *A*, Data shown are the mean ± SEM of the relative levels of LTA mRNA in the spleens and salivary glands of six C57BL/6 and six IL14αTG mice at 10 mo of age. The differences between C57BL/6 spleen and C57BL/6 salivary gland, C57BL/6 spleen and IL14αTG spleen, C57BL/6 salivary gland and IL14αTG salivary gland, and IL14αTG spleen and IL14αTG salivary gland are all highly significant ($p < 0.001$). Statistical analysis was performed using unmatched Student *t* test. *B*, Salivary gland secretions were collected from IL14αTG mice and various control mice at 12 mo of age. Western blot assays were performed on the undiluted specimens. *Lane M*, M.w. markers. *Lane 1* and *lane 2* are from IL14αTG mice, *lane 3* and *lane 4* are from IL14αTG.LTA$^{-/-}$ mice, *lane 5* and *lane 6* are from LTA$^{-/-}$ mice, and *lane 7* and *lane 8* are from C57BL/6 mice. *C*, B lymphocytes were purified from the spleens of C57BL/6 mice and stimulated with media alone, LPS (10 μg/ml), or LPS + IL-14α (10 ng/ml) for 24 or 48 h as shown. Cells were harvested after culture and the levels of LTA mRNA determined by qPCR. The difference in LTA mRNA levels in LPS + IL-14α stimulated cells compared with that of media-stimulated cells was significant ($p < 0.001$) as was the difference in these levels between LPS and LPS + IL-14α stimulated cells (24 h, $p < 0.01$; 48 h, $p < 0.001$) as determined by Student *t* test. *D*, Peritoneal B1 cells were isolated from four C57BL/6 and four IL14αTG mice at 10 mo of age. Levels of LTA mRNA were determined by qPCR, and the relative levels of LTA mRNA expression for each mouse are shown. The difference between the levels of LTA mRNA in C57BL/6 and IL14αTG mice is not statistically significant ($p = 0.337$).

IL14αTG.LTA$^{-/-}$ mice with a T-independent Ag, nitrophenol (NP)-Ficoll. As shown in Fig. 6, the IgM responses in both IL14αTG mice and IL14αTG.LTA$^{-/-}$ mice were equivalent. Thus, although there were fewer peritoneal B1 cells in IL14αTG.LTA$^{-/-}$ mice compared with that in IL14αTG mice, B1 function was equivalent in these two strains of mice. To evaluate B2 function, we immunized IL14αTG and IL14αTG.LTA$^{-/-}$ mice with the T-dependent Ag NP-OVA and determined the IgG anti-NP response. There were no differences in anti-NP Ab responses in these two strains of mice (Fig. 6*B*).

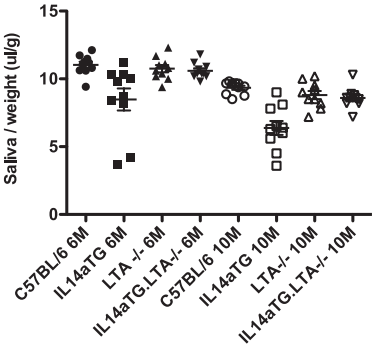

FIGURE 2. Data shown are the amount of saliva collected in 15 min after pilocarpine stimulation, as described, for 10 mice in each group, normalized to the body weight of the animals. The mean ± SEM is designated by bars. Statistical significance is reached for C57BL/6 at 6 mo versus IL14αTG at 6 mo ($p < 0.01$), C57BL/6 at 10 mo versus IL14αTG at 10 mo ($p < 0.001$), IL14αTG at 6 mo versus IL14αTG.LTA$^{-/-}$ at 6 mo ($p < 0.01$), and IL14αTG at 10 mo versus IL14αTG.LTA$^{-/-}$ at 10 mo ($p < 0.01$). Analysis was done by ANOVA using the Tukey–Kramer multiple comparison test. Using a paired Student t test, mice from each group were compared at 6 and 10 mo. Statistically significant decreases in salivary gland function were noted between the 6-mo and 10-mo time points in all groups: C57BL/6 ($p < 0.0001$), IL14αTG ($p = 0.04$), LTA$^{-/-}$ ($p = 0.001$), and IL14αTG.LTA$^{-/-}$ ($p < 0.0001$).

We assessed the expression of L-selectin (CD62L) on B and T lymphocytes to determine whether the trafficking of lymphocytes to the salivary glands might be altered. Fig. 7 demonstrates that B cells from IL14αTG.LTA$^{-/-}$ mice expressed higher levels of CD62L than that of B cells from IL14αTG mice. This result suggests that at least the initial step of B cell trafficking should not be inhibited in these mice. The expression of CD62L on T lymphocytes was only minimally depressed in IL14αTG.LTA$^{-/-}$ mice compared with that in IL14αTG mice and was not likely to have functional consequence.

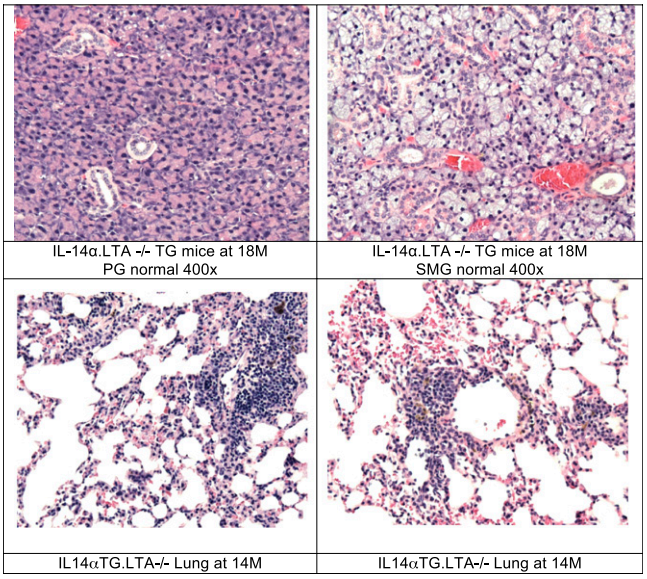

FIGURE 3. The *top panels* show histological samples of the parotid gland (PG) and the submandibular gland (SMG) of a representative IL14αTG.LTA$^{-/-}$ mouse from 24 mice studied. The *bottom panels* show histological samples of the lungs of two representative IL14αTG.LTA$^{-/-}$ mice from 24 studied. All mice were 18 mo of age. IL14αTG mice have lymphocytic infiltration of their PGs, SMGs, and lungs at this age. H&E, original magnification ×400.

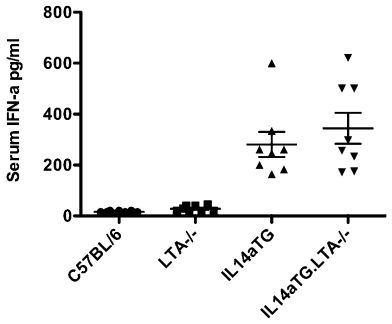

FIGURE 4. Data shown are the serum levels of IFN-α determined by commercially available ELISA (R&D Systems) at 8 mo of age for eight mice in each group: C57BL/6, LTA$^{-/-}$, IL14αTG, and IL14αTG.LTA$^{-/-}$. The difference between the IFN-α levels of C57BL/6 and IL14αTG mice were highly significant ($p < 0.001$) using Student t test. The difference between the IFN-α levels of IL14αTG and IL14αTG.LTA$^{-/-}$ mice were not significant ($p = 0.453$).

Next, we considered the possibility that LTA might be necessary for the production and deposition of anti-salivary gland autoantibodies. Previous studies have shown that IgM autoantibodies are found in the submandibular glands of IL14αTG mice prior to diminished salivary secretion and lymphocytic infiltration (4). However, disease-resistant IL14αTG.LTA$^{-/-}$ mice, which do not get salivary gland injury, had similar IgM autoantibody deposition in their submandibular glands as that of disease-prone IL14αTG mice (Fig. 8). Furthermore, both IL14αTG mouse and IL14αTG.LTA$^{-/-}$ mouse serum produced a speckled anti-nuclear fluorescence pattern similar to that of patients with Sjögren's syndrome that was not seen with normal mouse serum. These results suggest that both transgenic strains produce equivalent autoantibodies both to cytoplasmic as well as salivary gland Ags.

Because we could not identify any clear differences in lymphocyte function in IL14αTG and IL14αTG.LTA$^{-/-}$ mice, we sought to determine whether LTA was critical for induction of systemic inflammation, possibly involving other cell types. We therefore examined the levels of mRNA for several genes involved with systemic inflammation, RelA, RelB, and NF-κB2, in the spleens of

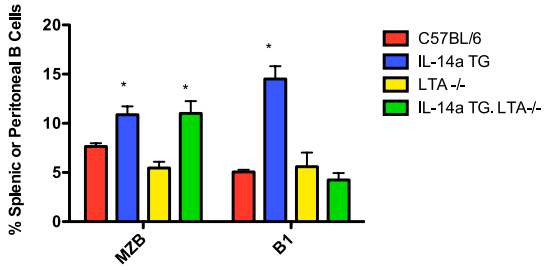

FIGURE 5. Data shown are the mean + SEM of five mice studied in each group. Flow cytometry was used to determine the percentage of marginal zone B cells (MZB) (CD19$^+$, CD21$^+$, sIgM$^+$) in the spleen and B1 cells (CD5$^+$, CD19$^+$, sIgM$^+$) in the peritoneal lavages of C57BL/6 mice, IL14αTG mice, LTA$^{-/-}$ mice, and IL14αTG.LTA$^{-/-}$ mice. For MZB, the difference between C57BL/6 and IL14αTG spleens was statistically significant ($p = 0.0201$) as was the difference between that of LTA$^{-/-}$ and IL14αTG.LTA$^{-/-}$ ($p = 0.0151$) using Student t test. The difference between IL14αTG and IL14αTG.LTA$^{-/-}$ was not statistically significant ($p = 0.725$). For peritoneal B1 cells, the differences between IL14αTG mice and both C57BL/6 ($p = 0.0002$) and IL14αTG.LTA$^{-/-}$ ($p < 0.0001$) mice were statistically significant. Values that are significantly different compared with those of the controls are noted with an asterisk.

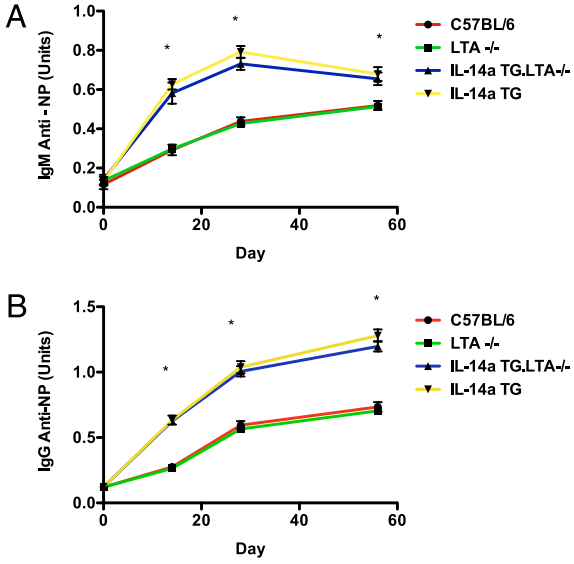

FIGURE 6. *A*, Six mice in each group were immunized with NP-Ficoll as described in *Materials and Methods* on days 0 and 14. Sera were collected on days 0,14, 28, and 56. Data shown are the mean ± SEM of the levels of IgM anti-NP Abs determined by ELISA. At time 0 there is no statistically significant difference among any of the groups as determined by Student *t* test. The difference between C57BL/6 and LTA$^{-/-}$ was not different at any time point. The difference between IL14αTG and IL14αTG.LTA$^{-/-}$ was not significant at any time point. The difference between C57BL/6 and IL14αTG was significant at times 14 ($p < 0.001$), 28 ($p < 0.001$), and 56 ($p < 0.01$) as was the difference between LTA$^{-/-}$ and IL14αTG.LTA$^{-/-}$ at times 14 ($p < 0.001$), 28 ($p < 0.001$), and 56 ($p < 0.01$). Values that are significantly different compared with those of the controls are noted with an asterisk. *B*, Six mice in each group were immunized with NP-OVA as described in *Materials and Methods* on days 0 and 14. Sera were collected on days 0, 14, 28, and 56. Data shown are the mean ± SEM of the levels of IgG anti-NP Abs determined by ELISA. At time 0 there is no statistically significant difference among any of the groups as determined by Student *t* test. The difference between C57BL/6 and LTA$^{-/-}$ was not different at any time point. The difference between IL14αTG and IL14αTG.LTA$^{-/-}$ was not significant at any time point. The difference between C57BL/6 and IL14αTG was significant at times 14 ($p < 0.001$), 28 ($p < 0.001$), and 56 ($p < 0.001$) as was the difference between LTA$^{-/-}$ and IL14αTG.LTA$^{-/-}$ at times 14 ($p < 0.001$), 28 ($p < 0.001$), and 56 ($p < 0.001$). Values that are significantly different compared with those of the controls are noted with an asterisk.

IL14αTG and IL14αTG.LTA$^{-/-}$ mice. We chose to evaluate these particular genes because they are expressed at high levels in various types of inflammatory states (31), and each of these genes is expressed at high levels in the B cell lymphomas of IL14αTG mice (32). Fig. 9 demonstrates that at 10 mo of age, the expression of RelA, RelB, and NF-κB2 are similar in IL14αTG and IL14αTG.LTA$^{-/-}$ mice. Only small but statistically significant differences were noted. These studies suggested that local production of LTA in the salivary glands is critical for development of salivary gland injury in IL14αTG mice.

The local production of LTA mRNA has previously been described in the salivary glands of patients with Sjögren's disease (33). We examined the presence of soluble LTA protein in the salivary gland secretions and sera of patients with documented early Sjögren's disease. Fig. 10*A* demonstrates that patients with Sjögren's disease, but not normal controls, have detectable soluble LTA in their salivary gland secretions. Fig. 10*B* reveals the presence of soluble LTA in the sera of these patients. The amount of LTA in the salivary gland secretions was statistically greater than the amount of LTA in the sera ($p = 0.0019$).

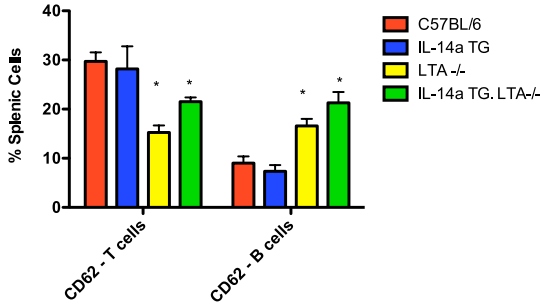

FIGURE 7. Data shown are the mean + SEM of five mice studied in each group. Flow cytometry was used to determine the percentage of L-selectin–positive B cells (CD19$^+$, CD62L$^+$) and T cells (CD3$^+$, CD62L$^+$) in the spleens of C57BL/6, IL14αTG, IL14αTG.LTA$^{-/-}$, and LTA$^{-/-}$ mice. Among the B cells, the differences between IL14αTG mice and both LTA$^{-/-}$ mice ($p = 0.0006$) and IL14αTG.LTA$^{-/-}$ mice ($p = 0.0012$) were statistically significant as determined by Student *t* test. There were no statistically significant differences among the T cells of any of the groups. Values that are significantly different compared with those of the controls are noted with an asterisk.

Discussion

These studies demonstrate that soluble LTA is produced locally in the salivary glands of IL14αTG mice and selected patients with Sjögren's disease. LTA deficiency prevents salivary gland injury in IL14αTG mice without altering the production of IFN-α, B cell function, the deposition of IgM autoantibodies in the salivary glands, or changes in the expression of several NF-κB proteins associated with systemic inflammation. Thus, in IL14αTG mice and perhaps human Sjögren's disease, LTA acts locally to directly and/or indirectly induce salivary gland injury. LTA is not involved with the pulmonary injury that occurs in IL14αTG mice, suggesting that organ-specific injury in Sjögren's disease may involve distinct mechanisms.

Various cytokines have been implicated in the pathophysiology of salivary gland injury in Sjögren's disease based on their local expression (33, 34). In some cases, the relevance of these cytokines to Sjögren's disease has been confirmed in animal models. For example, IFN-γ has been found in the serum and saliva of patients (34, 35), and deletion of IFN-γ or the IFN-γ receptor genes from NOD mice prevented the abnormal expression of salivary gland proteins as well as the immune response to them (35). With other cytokines, such as IL-10, animal models have provided conflicting results (11, 36, 37). We focused on LTA and IFN-α in these studies because microarray studies identified them and their signatures in both IL14αTG mice and patients with Sjögren's disease (33). Previous studies had implicated IFN-α in Sjögren's disease, and we identified IFN-α in the sera of IL14αTG mice (3, 10, 30).

The analysis of LTA is complicated by the fact that it exists in two very distinct forms, which likely have very different physiological roles. Membrane-bound LTA is found together with LTB dimers (or rarely) monomers and binds a specific LTB receptor (LTBR) (17). Membrane-bound LTA is critical for the development of lymph nodes and Peyer's patches through its actions on stromal and follicular dendritic cells, without direct activity on lymphocytes (17). In contrast, soluble LTA binds the TNF-α receptors TNF-RI and TNF-RII, which are present on lymphocytes (38). The effects of LTA binding TNF-RI and/or TNF-RII results in cell activation or apoptosis, depending upon other factors influencing the cells involved (39, 40). Lymphocyte function has generally been normal in LTA$^{-/-}$ mice, although the forma-

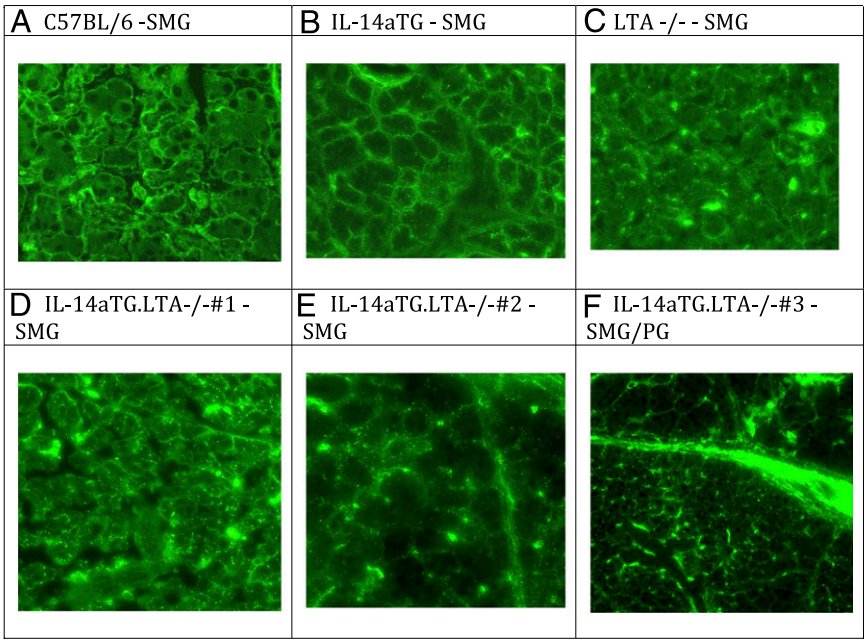

FIGURE 8. Immunofluorescence studies were performed as described in *Materials and Methods* using IgM, IgG, and IgA Abs. Only the studies with anti-IgM Abs were positive, and representative examples are shown. Examples from C57BL/6, IL14αTG, LTA$^{-/-}$, and IL14αTG.LTA$^{-/-}$ mice are shown as labeled. Fluorescein-labeled goat anti-murine IgM, original magnification ×400. PG, parotid gland; SMG, submandibular gland. *A*, Submandibular gland of a C57BL/6 mouse. *B*, Submandibular gland of an IL14αTG mouse. *C*, Submandibular gland of an LTA$^{-/-}$ mouse. *D* and *E*, Submandibular glands of IL14αTG.LTA$^{-/-}$ mice. *F*, Submandibular gland and parotid gland of an IL14αTG.LTA$^{-/-}$ mouse.

tion of IgG memory responses has been shown to be less efficient (16, 41).

LTA has been implicated as having diverse effects on the pathogenesis of autoimmune disorders. One potential role for membrane-bound LTA would be to increase the production of ectopic lymphoid tissues in particular organs leading to the development of more efficient local autoreactive responses (24, 42). A soluble LTBR-Ig was produced to investigate this hypothesis. LTBR-Ig blocked the development of collagen-induced arthritis, diabetes, and experimental allergic encephalomyelitis in certain animal models (43–45). A recent study examining LTBR-Ig in NOD mice, an animal model for both diabetes and Sjögren's syndrome, confirmed that it blocked both the development of diabetes and sialitis. However, LTBR-Ig provided incomplete protection in maintaining normal salivary gland secretions (46). In contrast, a more recent study examining collagen-induced arthritis found that soluble LTBR-Ig had no influence on the disease, whereas Abs to LTA blocked disease induction and the aberrant production of cytokines associated with it (47). The recent elucidation of the role of LT in the normal development of thymic stromal cells argues that LTBR-Ig might make autoimmunity actually worse by preventing the AIRE-induced deletion of high-affinity autoreactive T cells by thymic stromal cells (48). Finally, LT itself has been

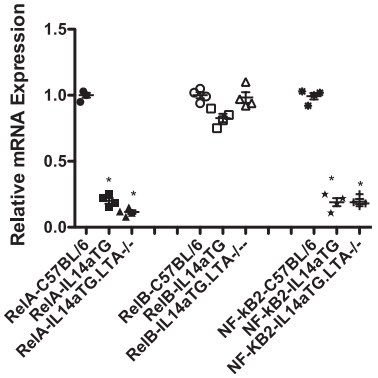

FIGURE 9. Splenic mRNA was obtained from four mice per group for C57BL/6, IL14αTG, and IL14αTG.LTA$^{-/-}$ mice at 10 mo of age. Relative levels of mRNA expression for RelA, RelB, and NF-κB2 were determined by RT-PCR and are shown as individual values. There were small, statistically significant differences between the expression of RelA ($p = 0.018$) and RelB ($p = 0.02$) in IL14αTG and IL14αTG.LTA$^{-/-}$ mice but not in the expression of NF-κB2 ($p = 0.14$) as determined by Student *t* test. The levels of mRNA for RelA and NF-κb when comparing splenic mRNA for C57BL/6 mice versus either IL14αTG or IL14αTG.LTA$^{-/-}$ splenic mRNA was significant ($p < 0.001$). Values that are significantly different compared with those of the controls are noted with an asterisk.

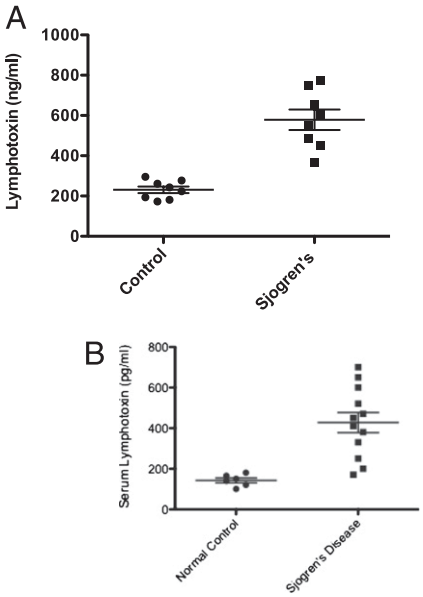

FIGURE 10. *A*, Saliva was obtained over a 5-min period from patients with Sjögren's disease and from age- and sex-matched controls after stimulation with sour candy. A commercially available ELISA (R&D Systems) was used to determine LTA levels. The differences between the levels of LTA in the saliva of patients with Sjögren's disease and in that of normal controls was statistically significant using Student *t* test ($p < 0.0001$). *B*, Sera were obtained from six normal donors (age- and sex-matched to six of the Sjögren's disease patients) and from 12 patients with Sjögren's disease. A commercially available ELISA (R&D Systems) was used to determine LTA levels. The differences between the serum levels of soluble LT in normal controls and in patients with Sjögren's disease was statistically significant ($p = 0.0011$) using Student *t* test.

shown to protect against diabetes in two studies utilizing NOD mice (49, 50). In the IL14αTG model of Sjögren's disease, we demonstrated that salivary gland injury occurs before the infiltration of the glands with lymphocytes (4). Therefore, early injury to the salivary glands is unlikely to be due to membrane-bound LTA/LTB effects. However, membrane LTA/LTB may contribute to the later stages of lymphocytic inflammation observed in the salivary glands of IL14αTG mice.

Another potential mechanism by which LTA could participate in autoimmunity is by the direct activation of B cells participating in the disease process. LTA is an autocrine growth factor for B lymphocytes and has been shown to enhance normal memory B cell responses (27, 51–55). These functions have been attributed partially to signaling through TNF-RI, which is activated by soluble LTA (56). TNF-RII activation by either TNF-α or LT also leads to B cell activation (57). Furthermore, LTA is critical for the production of chemokines, especially CXCL13, necessary for normal B cell homing (21).

In our studies, the only B cell abnormality noted was a normalization of the number of peritoneal B1 cells in IL14αTG.LTA$^{-/-}$ mice compared with that in IL14αTG mice (Fig. 5). This was likely due to differences in B1 compartmentalization, as the production of IgM Abs to NP-Ficoll, which is dependent upon B1 cells, was completely normal (Fig. 6) (58). Other B cell functions in IL14αTG.LTA$^{-/-}$ mice including the IgG response to NP-OVA (Fig. 6) and the production of autoantibodies were normal (Fig. 8). Although LTA can certainly influence the development of other cell types, including NK cells and NKT cells (59, 60), we did not perform more extensive studies on these cell populations because they are not found in the salivary gland lesions of the IL14αTG mice (4).

Another potential role for LTA in autoimmunity is to promote the production of disease-inducing cytokines. LTA has been shown to induce the production of type 1 IFNs (29, 61). We have observed production of IFN-α in IL14αTG mice (3). Increased levels of IFN-α have also been observed in the sera of patients with both systemic lupus erythematosus and Sjögren's disease (62). Our current findings suggest that IFN-α does not determine salivary gland injury in IL14αTG mice, as the levels of IFN-α were similar in the sera of IL14αTG and IL14αTG.LTA$^{-/-}$ mice (Fig. 4). Preliminary studies examining IL14αTG mice lacking the type 1 IFNR (IL14αTG.IFNα/βR$^{-/-}$) support this conclusion. Five of seven IL14αTG.IFNα/βR$^{-/-}$ mice studied at 12 mo of age have lymphocytic infiltration of their submandibular glands, and two of seven have lymphocytic infiltration of their lacrimal glands.

LTA could contribute to autoimmunity by directly killing vulnerable tissues. Soluble LT has been shown to be directly cytolytic (18). This activity has been invoked for its role in experimental colitis and human polymyositis (22, 63). Increased amounts of soluble LTA have been identified in the cerebrospinal fluids of patients with multiple sclerosis, which could directly have toxic effects (64). The fact that soluble LTA as well as LTA transcripts were identified in the saliva and salivary tissues respectively in IL14αTG mice (Figs. 1*B*, 2, 3) would support a role for LTA-induced local cytotoxicity in promoting Sjögren's disease. The lack of salivary LTA in disease-resistant LTαTG.LTA$^{-/-}$ mice further supports this hypothesis. The fact that the spleens of IL14αTG and IL14αTG.LTA$^{-/-}$ mice express similar levels of mRNA transcripts for inflammatory proteins (Fig. 9) further supports the fact that LTA has a more local than systemic effect. The fact that soluble LTA is found at high levels in the salivary gland secretions (Fig. 10*A*) and sera of patients with Sjögren's disease (Fig. 10*B*) suggests that LTA-induced cytotoxicity may also be an important factor in human disease.

Several additional interesting observations resulting from these studies are worthy of mention. First, discordance was noted in the injury to the salivary glands and the lungs in IL14αTG mice. Salivary gland injury is dependent upon LTA, and lung injury is not (Fig. 3). Similar discordance has been noted in the NOD mouse when analyzing diabetes and salivary gland injury (46, 65). Although this suggests that the mechanism of injury in these two organs may be different, it must also be considered that the mechanism of lung injury in IL14αTG mice is different from the mechanism of lung injury in IL14αTG.LTA$^{-/-}$ mice. Previous studies have demonstrated that LTB$^{-/-}$ and LTA$^{-/-}$ mice develop increased numbers of lymphocytes in the lungs because of abnormal cellular trafficking and lymph node formation (66).

It is noteworthy that salivary gland injury did not occur in IL14αTG.LTA$^{-/-}$ mice, despite the deposition of Abs in the salivary glands (Figs. 2, 3, 8). This discrepancy suggests that autoantibodies in and of themselves are not pathogenic but must work in conjunction with other mediators such as LTA to induce salivary gland injury. Alternatively, autoantibody production and deposition may be an epiphenomenon reflecting the existence of salivary gland injury. It is also possible that different types of autoantibodies are deposited in the salivary glands at various stages of the disease having different pathogenic potential. Future studies will examine these different possibilities.

Also of interest is the lack of lymphoma development in IL14αTG.LTA$^{-/-}$ mice. The incidence of lymphoma is markedly increased in Sjögren's disease (67–69). More than 95% of IL14αTG mice develop B cell lymphomas by 18 mo of age (3). None of 24 IL14αTG.LTA$^{-/-}$ mice followed to 22 mo of age developed lymphoma. Little is known regarding the evolution of the chronic inflammation of Sjögren's disease into lymphoma, but one hypothesis is that the rapid proliferation of autoreactive cells leads to accumulation of mutations that ultimately allow the malignant state to prevail (70–72). Another hypothesis is that there are intrinsic abnormalities in the B cells of Sjögren's patients that allow malignancy to develop after further environmental insults (73). The absence of LTA in IL14αTG.LTA$^{-/-}$ mice somehow prevents malignant B cell transformation. The mechanisms by which this occurs will be the topic of future studies.

In summary, the data presented in this study support the hypothesis that early Sjögren's disease may be caused by local LTA production leading to salivary gland injury. These findings may explain the observation made in both patients and animal models that injury to the salivary glands occurs prior to and independent of parenchymal lymphocytic infiltration (74, 75). As the current definition of Sjögren's disease in humans includes the identification of lymphocytic infiltration of salivary glands, most patients are likely being missed during the early stage of disease when the potential for salivary gland repair may still be present (25, 76). Future work must identify early markers for disease to develop effective treatment strategies.

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Disclosures

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