



Increased Susceptibility to Autoimmune Gastritis in Thymic Stromal Lymphopoietin Receptor-Deficient Mice

This information is current as of December 7, 2011

Hisayo Nishiura, Masahiro Kido, Nobuhiro Aoki, Satoru Iwamoto, Ryutaro Maruoka, Aki Ikeda, Tsutomu Chiba, Steven F. Ziegler and Norihiko Watanabe

J Immunol; Prepublished online 28 November 2011;
doi:10.4049/jimmunol.1003787

<http://www.jimmunol.org/content/early/2011/11/28/jimmunol.1003787>

-
- | | |
|----------------------|---|
| Subscriptions | Information about subscribing to <i>The Journal of Immunology</i> is online at http://www.jimmunol.org/subscriptions |
| Permissions | Submit copyright permission requests at http://www.aai.org/ji/copyright.html |
| Email Alerts | Receive free email-alerts when new articles cite this article. Sign up at http://www.jimmunol.org/etc/subscriptions.shtml/ |

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.



Increased Susceptibility to Autoimmune Gastritis in Thymic Stromal Lymphopoietin Receptor-Deficient Mice

Hisayo Nishiura,^{*,†} Masahiro Kido,^{*,†} Nobuhiro Aoki,^{*,†} Satoru Iwamoto,^{*,†} Ryutaro Maruoka,^{*,†} Aki Ikeda,^{*,†} Tsutomu Chiba,^{*} Steven F. Ziegler,[‡] and Norihiko Watanabe^{*,†}

Thymic stromal lymphopoietin (TSLP), mainly produced by epithelial cells, activates a variety of cell types, including dendritic cells, mast cells, T cells, and B cells. It is involved in the pathogenesis of allergic inflammation in the lung, skin, and gastrointestinal tract. In addition, TSLP promotes Th2-type intestinal immunity against helminth infection and regulates Th1-type inflammation in a mouse model of colitis, suggesting that it plays crucial roles in intestinal immune homeostasis. Although autoimmune gastritis (AIG), mediated by inflammatory Th1 responses, develops in the gastric mucosa, it is not clear whether TSLP is involved in regulating these responses in AIG. The aim of this study was to examine the roles of TSLP in the development of AIG. Because BALB/c mice thymectomized 3 d after birth (NTx mice) develop AIG, we used this model to test the role of TSLP in the development of AIG. We found that in AIG-bearing mice, TSLP was expressed in the inflamed stomach and that the serum anti-parietal cell Ab levels in neonatal thymectomized TSLPR-deficient mice (NTx-TSLPR^{-/-} mice) were significantly elevated over those in NTx-TSLPR^{+/+} mice. In addition, NTx-TSLPR^{-/-} mice exhibited an earlier onset of AIG than that observed in NTx-TSLPR^{+/+} mice. The rapid development of AIG in NTx-TSLPR^{-/-} mice resulted in more aggressive CD4⁺ T cell infiltration and more severe loss of parietal and chief cells in the progression phase of AIG, accompanied by enhanced production of IL-12/23p40 and IFN- γ . Taken together, these data suggested that TSLP negatively regulates the development of AIG. *The Journal of Immunology*, 2012, 188: 000–000.

Autoimmune gastritis (AIG) is a typical organ-specific autoimmune disease. Patients with AIG often have complications, such as gastric cancer, gastric carcinoid tumors, or pernicious anemia accompanied by achlorhydria (1–3). The histological findings of AIG are characterized by a chronic mononuclear cell infiltration affecting only or predominantly the corpus mucosa and causing loss of parietal and chief cells from the gastric gland (1). AIG's serologic hallmark is the production of characteristic circulating autoantibodies, including Ab against

H⁺K⁺-ATPase, in the parietal cells of the stomach (4, 5). Mouse models of AIG share many pathological and clinical features with human AIG and help to clarify the mechanisms involved in its development (6).

BALB/c mice thymectomized 3 d after birth (NTx mice) are one of the mouse models of AIG. NTx mice possess disease-relevant CD4⁺CD25⁺ regulatory T cells (Tregs), but these Tregs cannot fully prevent autoimmune disease development (7–9). Adult NTx mice frequently develop AIG, showing lymphocytic infiltration with selective loss of parietal and chief cells from the gastric mucosa, as well as production of autoantibodies to parietal cells (10). In addition, AIG in NTx mice is characterized by a marked infiltration of CD4⁺ T cells, which produce large amounts of IFN- γ . The development of AIG in NTx mice is severely impaired in mice with depleted CD4⁺ T cells or in those receiving blocking Abs to IFN- γ (11–14). Therefore, the inflammatory Th1 responses induced by CD4⁺ T cells are critical for the development of this animal model of AIG.

Thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, is mainly produced by epithelial cells and activates many hematopoietically derived cells, including dendritic cells (DCs), mast cells, T cells, and B cells (15, 16). TSLP is involved in the development of allergic inflammation in various organs, including the gastrointestinal tract (15–19). Interestingly, TSLP promotes Th2-type intestinal immunity against helminth infection and regulates Th1-type inflammation in mouse models of colitis (20, 21), suggesting that it plays crucial roles in intestinal immune homeostasis. Although AIG is also mediated by the inflammatory Th1 responses developed in the gastric mucosa, it is not clear whether TSLP is involved in regulating the development of AIG.

In the current study, to examine whether TSLP affects the development of AIG, we used BALB/c TSLP receptor (TSLPR)-deficient mice thymectomized 3 d after birth (NTx-TSLPR^{-/-} mice). We found that in AIG-bearing mice, TSLP was expressed

^{*}Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; [†]Center for Innovation in Immunoregulative Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; and [‡]Immunology Program, Benaroya Research Institute, Seattle, WA 98195

Received for publication November 15, 2010. Accepted for publication October 30, 2011.

This work was supported in part by Grants-in-Aid for Scientific Research (20390207, 21229009, and 23590973) from the Japan Society for the Promotion of Science; a Health and Labor Sciences Research grant on Intractable Diseases from the Ministry of Health, Labor and Welfare, Japan; Grants-in-Aid for Research from the Japanese Society of Gastroenterology, the Kato Memorial Trust for Nambyo Research, the Waksman Foundation of Japan, and National Institutes of Health Grant AI68731 (to S.F.Z.). The Center for Innovation in Immunoregulative Technology and Therapeutics is supported in part by the Special Coordination Funds for Promoting Science and Technology of the Japanese Government and in part by Astellas Pharma in the Formation of Innovation Center for Fusion of Advanced Technologies Program.

Address correspondence and reprint requests to Dr. Norihiko Watanabe, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail address: norihiko@kuhp.kyoto-u.ac.jp

Abbreviations used in this article: AIG, autoimmune gastritis; DC, dendritic cell; GLN, paragastric lymph node; MLN, mesenteric lymph node; NTx mice, BALB/c mice thymectomized 3 d after birth; NTx-TSLPR^{-/-} mice, thymic stromal lymphopoietin receptor-deficient BALB/c mice thymectomized 3 d after birth; PD-1, programmed cell death 1; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/\$16.00

in the inflamed stomach. In comparison with NTx-TSLPR^{+/+} mice, NTx-TSLPR^{-/-} mice exhibited increased production of anti-parietal cell Abs, as well as early onset and enhanced severity of inflammation of the gastric mucosa. These data suggested that TSLP regulates the development of AIG.

Materials and Methods

Mice

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), and TSLPR-deficient mice on a BALB/c background were generated, as described previously (22, 23). In this study, we used TSLPR^{-/-} mice that had been backcrossed onto a BALB/c background for 12 generations. All of these mice were bred and housed under specific pathogen-free conditions. Thymectomy of the mice 3 d after birth was performed, as described previously (24, 25). All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Histologic examination

After 24 h of starvation, mice were sacrificed, and the stomachs were immediately removed. The local pH in the corpus area of the stomach was measured with pH test paper (Advantec, Tokyo, Japan). Half of each stomach was fixed in neutral buffered formalin, embedded in paraffin wax, and cut into sections 4 μ m thick. These sections were stained with H&E. The other half of each stomach was frozen for immunohistochemistry. The degree of gastritis was determined according to a modification of a semi-quantitative scoring system, as described previously (26). Chronic inflammation, characterized by infiltration of mononuclear cells, was graded from 0 to 3, where 0 = no increase in inflammatory cells, 1 = slight infiltration of the lamina propria by lymphocytes, 2 = moderately dense infiltration, and 3 = very dense infiltration. Atrophic changes were graded from 0 to 3, according to the loss of specialized cells, chief cells, and parietal cells, (0 = no loss; 1 = mild loss of specialized cells, limited to half of the corpus glands; 2 = moderate loss of specialized cells, diffusing to more than half the corpus glands; and 3 = severe loss/almost complete loss of specialized cells throughout the gastric body). The degree of foveolar hyperplastic change of mucus neck cells of the corpus glands was scored on a scale of 0 to 3 (0 = no hyperplastic change; 1 = focal hyperplastic change of mucus neck cells of the corpus glands; 2 = moderate hyperplastic change of mucus neck cells, diffusing to the corpus glands with less than twice the height of a normal foveolar epithelial layer; and 3 = severe hyperplastic change of mucus neck cells, diffusing to the corpus glands with more than twice the height of a normal foveolar epithelial layer). Incidence of AIG was determined by greater than grade 2 chronic inflammation, characterized by moderately dense infiltration of mononuclear cells. These infiltrates were further confirmed to be CD4⁺ cells by immunohistological staining using FITC-conjugated anti-CD4 (eBioscience, San Diego, CA).

Immunohistological analysis

Fluorescence immunohistology was performed on frozen sections, as described previously (25). In brief, sections of 6 μ m were cut from tissue blocks of frozen mucosal samples onto glass slides. The sections were air dried for 30 min, fixed in acetone for 5 min, and blocked with PBS containing 1% BSA for 30 min. The sections were stained with FITC-conjugated anti-CD4 for 1 h or with biotinylated anti-TSLP (R&D Systems, Minneapolis, MN) for 1 h, followed by staining using Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA). After the final wash, the slides were mounted by Mowiol (Merck Chemicals, Darmstadt, Germany) and examined under a fluorescence microscope. Cell numbers of CD4⁺ T cells in the gastric mucosa were counted under high magnification. Briefly, 10 complete longitudinal profiles of gastric units were selected at random from the body of the stomach in each mouse, and the numbers of CD4⁺ cells/gastric unit were counted. Data were expressed as the average number of cells/section of gastric unit for each animal. For detection of autoantibodies for the gastric gland, stomachs were collected from wild-type BALB/c mice. Sections were stained with 100 $\times$ diluted sera from the mice, followed by FITC-conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL).

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed, as described previously (27). After the paragastric lymph node (GLN) was located and removed, gastric tissues were frozen in RNAlater (Qiagen, Hilden, Germany). Total

RNA was extracted using an RNeasy minikit (Qiagen), according to the manufacturer's instructions. Single-stranded cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed using SYBR Green I Master (Roche Applied Science, Basel, Switzerland). The real-time quantitative reactions were performed using a LightCycler 480 (Roche Applied Science), according to the manufacturer's instructions. Values are expressed as arbitrary units relative to GAPDH. The following primers were used: *GAPDH*: 5'-CAACTTTGT-CAAGCTCATTTCC-3' and 5'-GGTCCAGGGTTTCTTACTCC-3'; *TSLP*: 5'-CAGCTGTCTCCTGAAAATCG-3' and 5'-AAATGTTTTGTCTGGG-GAGTG-3'; *T-bet*: 5'-TCAACCAGCACCAGACAGAG-3' and 5'-AAACA-TCCGTGAATGGCTTGTG-3'; *GATA3*: 5'-TTATCAAGCCCAAGCGAAG-3' and 5'-TGTTGGTGGTCTGACAGTTC-3'; *ROR γ t*: 5'-CCGCTGAGAG-GGCTTCAC-3' and 5'-TGCAGGAGTAGGCCACATTACA-3'; *IFN- γ* : 5'-GGATGCATTCATGAGTATTGC-3' and 5'-CCTTTTCCGCTTCCTGAG-G-3'; *IL-4*: 5'-CGCCATGCACGGAGATG-3' and 5'-ACGAGCTCACTC-TCTGTGGTGTT-3'; and *IL-17A*: 5'-TTTAACCTCCCTTGGCGCAAAA-3' and 5'-CTTCCCTCCGCATTGACAC-3'.

ELISA

Serum anti-parietal cell Ab levels were measured by ELISA, as described previously (26). Briefly, duplicate wells of microtiter plates (Nunc, Roskilde, Denmark) were incubated with 10 μ g/ml Ags, extracts prepared from the normal gastric mucosa, in PBS for 16 h at 4°C. The wells were blocked with PBS containing 5% nonfat dried milk and then incubated with serial dilutions of sera for 1 h. The wells were then incubated with HRP-labeled goat anti-mouse IgG (Serotec, Oxford, U.K.) diluted at a predetermined concentration for 1 h at room temperature. After rigorous washing, each well was reacted with substrate solution (R&D Systems) for 10 min. The reaction was terminated with 50 μ l 2 mol/l H₂SO₄, and absorbency at 490 nm was determined with a microplate reader.

Isolation of mononuclear cells

Single-cell suspensions from the tissues were prepared, as described previously (25, 28). In brief, mice were sacrificed, and the stomach, GLN, mesenteric lymph node (MLN), and spleen were immediately removed. After the removal of GLN, the stomach was opened, and stomach contents were removed by rinsing several times in PBS. The stomach tissue was repeatedly injected with a total of 10 ml PBS with 5% FBS using a 5-ml syringe attached to a 26-g syringe needle. Following the injections, the mucosa was gently massaged with the needle and was cut to help release any trapped cells. The cell suspension was filtered sequentially through 70- μ m nylon mesh. Collected cells were placed on a 40/75% discontinuous Percoll gradient (GE Healthcare, Little Chalfont, U.K.) and centrifuged at 20°C for 20 min.

Flow cytometry

Flow cytometric analysis was performed, as described previously (25, 27). Cells were stained with allophycocyanin-Cy7-conjugated anti-CD4 (BD Biosciences) and PE-conjugated anti-CD3 (eBioscience). Stained cells were analyzed with FACSCanto II (BD Biosciences). Data were analyzed using Cell Quest Pro (BD Biosciences). Dead cells were excluded based on side- and forward-scatter characteristics. The number of viable indicated cells was calculated as follows: (percentage of cells in the cell type) $\times$ (number of viable cells).

Intracellular cytokine staining

Intracellular cytokine staining was performed, as described previously (25, 27). For T cell cytokine production, isolated cells were restimulated with 50 ng/ml PMA (Sigma, St. Louis, MO) + 2 μ g/ml ionomycin (Sigma) at a concentration of 1×10^6 cells/ml in RPMI Medium 1640 (Invitrogen), supplemented with 10% (v/v) heat-inactivated FCS (Sigma), penicillin G, streptomycin (both from Invitrogen), and 2-ME (Nacalai Tesque, Kyoto, Japan). After 2 h, brefeldin A (Sigma) was added at 10 μ g/ml. After 2 h, cells were collected and stained for cell surface molecules using allophycocyanin-Cy7-conjugated anti-CD4 and PE-conjugated anti-CD3. Cells were fixed and permeabilized using a Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, An Der Grub, Austria) and stained with FITC-conjugated anti-IFN- γ (eBioscience). For DC cytokine production, isolated cells were cultured at a concentration of 1×10^6 cells/ml with brefeldin A. After 2 h, cells were collected and stained for cell surface molecules using allophycocyanin-conjugated anti-CD11b (eBioscience) and FITC-conjugated anti-CD11c (BD Biosciences). Cells were fixed, permeabilized, and stained with PE-conjugated anti-IL-12/23p40 (clone C17.8; eBioscience).

Statistical analysis

Statistical analysis was performed using the Student *t* test for paired or unpaired data to compare the values between two groups and using the Wilcoxon *t* test for nonparametric paired data. The incidence of AIG was compared with the Fisher exact test. The *p* values < 0.05 were considered significant.

Results

Inflamed stomach in AIG-bearing mice exhibits enhanced expression of TSLP

Increased TSLP expression in epithelial cells is induced through exposure to viral, bacterial, and parasitic pathogens, as well as to various cytokines (15, 16). First, we examined whether expression of TSLP was affected in the inflamed stomachs of mice with experimental AIG. We examined TSLP gene expression in AIG-bearing NTx mice, finding that these mice exhibited significantly elevated levels of mRNA expression of TSLP in the inflamed stomach compared with those in the normal stomach of BALB/c mice of the same age (Fig. 1A). In addition, to confirm this finding, frozen stomach sections from AIG-bearing NTx mice were stained with anti-TSLP Abs. Ig isotype-control Abs did not produce any positive staining. In contrast to immunostaining for

TSLP in normal gastric mucosa, we found obviously detectable anti-TSLP staining of epithelial cells in the inflamed gastric mucosa from AIG-bearing NTx mice (Fig. 1B).

TSLPR deficiency exaggerates autoantibody production in the mouse model of AIG

A previous study reported low-level TSLP gene expression throughout the small and large intestine, as well as that TSLP regulated a mouse model of colitis (20, 21). To test the possibility that TSLP is involved in the regulation of AIG, we performed thymectomy 3 d after birth in TSLPR^{-/-} or TSLPR^{+/+} mice in BALB/c background. We found that parietal cells of the stomach section in normal BALB/c mouse were stained with 100× diluted sera from 14-wk-old NTx-TSLPR^{-/-} and NTx-TSLPR^{+/+} mice but not normal BALB/c mice, indicating that both NTx-TSLPR^{-/-} and NTx-TSLPR^{+/+} mice developed AIG and that sera from these mice contained specific anti-parietal cell Abs (Fig. 2A). Using an ELISA

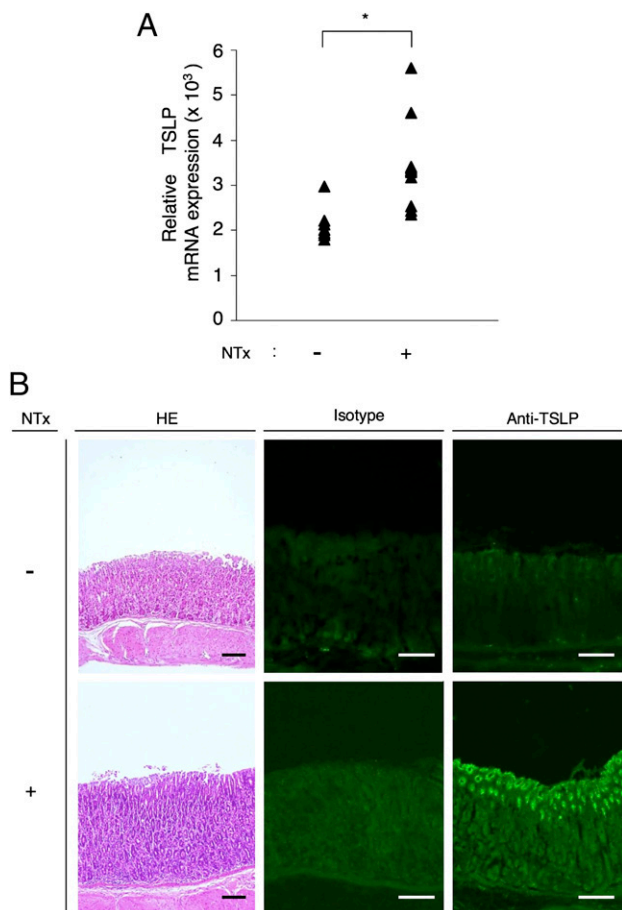


FIGURE 1. Inflamed stomach in AIG-bearing mice exhibits enhanced expression of TSLP. *A*, TSLP mRNA expression in inflamed gastric tissues from AIG-bearing NTx mice and normal controls from BALB/c mice at 12 wk of age. Expression levels of mRNA encoding TSLP were measured using real-time quantitative RT-PCR. Each symbol represents relative TSLP expression in an individual mouse. **p* < 0.05, Student *t* test for unpaired data. *B*, Immunohistological staining of the gastric mucosa. Inflamed gastric mucosa from AIG-bearing mice (lower panels) and normal mucosa from BALB/c mice (upper panels) were stained with H&E (HE), Ig isotype control Abs (Isotype), or anti-TSLP. Scale bars, 100 μ m.

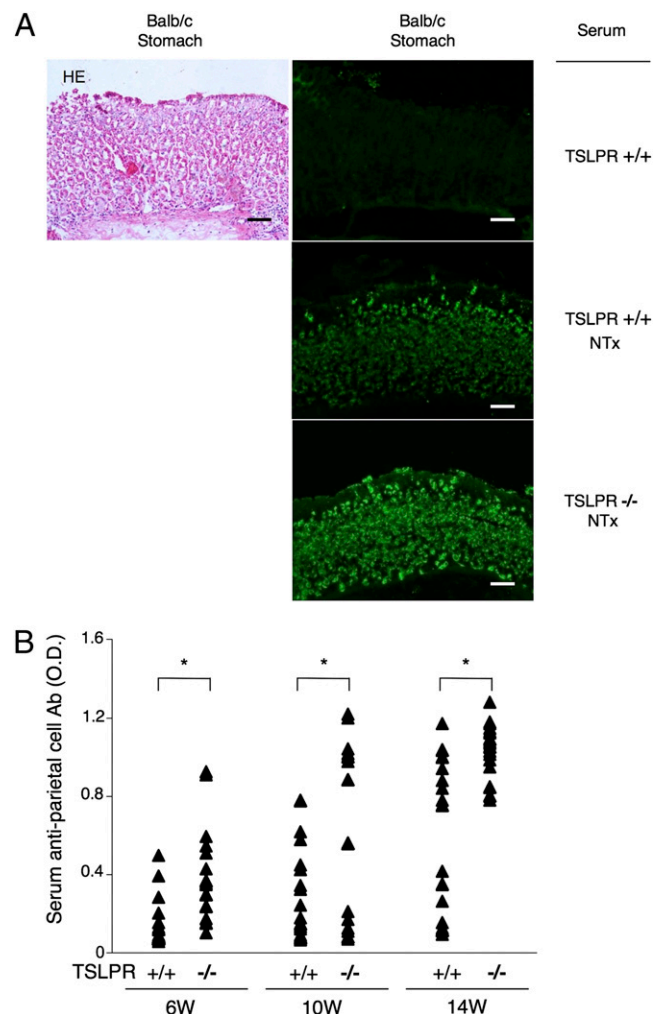


FIGURE 2. TSLPR deficiency exaggerates autoantibody production in the mouse model of AIG. TSLPR^{+/+} and TSLPR^{-/-} mice in BALB/c background were performed NTx. Sera were collected from indicated mice. *A*, Autoantibodies detected by fluorescence immunohistology. Gastric tissues of normal BALB/c mice at 12 wk of age were stained with H&E (left panel) or with 100× diluted sera from indicated 14-wk-old mice, followed by FITC-conjugated anti-mouse IgG (right panels). Scale bars, 100 μ m. *B*, Serum anti-parietal cell Ab levels were measured by ELISA. Each symbol represents OD in serum from individual NTx-TSLPR^{+/+} (*n* = 18) and NTx-TSLPR^{-/-} (*n* = 18) mice at 6, 10, and 14 wk. **p* < 0.05, Wilcoxon *t* test.

to examine the serum levels of anti-parietal cell Abs, we compared the levels of production of anti-parietal cell Abs. In 6-wk-old NTx mice, serum levels of anti-parietal cell Abs in NTx-TSLPR^{-/-} mice were significantly higher than those in NTx-TSLPR^{+/+} mice (Fig. 2B). In addition, although anti-parietal cell Ab titers gradually increased in NTx-TSLPR^{+/+} mice in an age-dependent manner, serum levels of anti-parietal cell Abs in NTx-TSLPR^{-/-} mice were significantly greater than those in NTx-TSLPR^{+/+} mice at 10 and 14 wk of age (Fig. 2B). These results suggested that TSLPR deficiency exaggerates autoimmunity in this mouse model of AIG.

TSLPR deficiency induces elevated histopathology of AIG in mice

Next, we examined whether TSLPR deficiency exacerbated gastric inflammation in our mouse model of AIG. Histological examination revealed that the gastric mucosa in 12-wk-old NTx-TSLPR^{+/+} mice had chronic gastritis with mononuclear cell infiltration, loss of parietal and chief cells, and hyperplasia of the foveolar mucus neck cells. Compared with the findings in NTx-TSLPR^{+/+} mice, NTx-TSLPR^{-/-} mice showed more severe mononuclear cell infiltration and complete loss of parietal and chief cells, accompanied by enhanced hyperplasia of the foveolar mucus neck cells (Fig. 3A). These findings were further confirmed by the gastric pH and gastritis scoring system that evaluates chronic inflammation, characterized by the infiltration of mononuclear cells; atrophic changes, based on the loss of parietal and chief cells; and hyperplastic changes of foveolar mucus neck cells. In contrast to NTx-TSLPR^{+/+} mice, gastric pH and total gastritis score were significantly elevated in NTx-TSLPR^{-/-} mice (Fig. 3B). These data

suggested that TSLPR deficiency exacerbates inflammation and atrophy of gastric mucosa in AIG.

To further investigate infiltrating cells in the gastric mucosa, these cells were examined by immunohistology. Although AIG in NTx mice is characterized by a marked infiltration of CD4⁺ T cells, the cell numbers of CD4⁺ T cells in the gastric mucosa increased in NTx-TSLPR^{-/-} mice (Fig. 4A, 4B). To confirm this finding, isolated mononuclear cells from the stomach were analyzed by flow cytometry, and CD4⁺ T cell numbers were counted (Fig. 4C, 4D). We found significantly increased numbers of CD4⁺ T cells in the stomachs of NTx-TSLPR^{-/-} mice. Taken together, these data suggested that TSLPR deficiency exacerbates CD4⁺ T cell infiltration in the gastric mucosa in AIG.

TSLPR deficiency induces increased susceptibility to AIG in mice

Finding increased elevated histopathology of AIG, including CD4⁺ T cell infiltration in the gastric mucosa in 12-wk-old NTx-TSLPR^{-/-} mice (Figs. 3, 4), we next examined whether TSLPR deficiency induced earlier onset and/or increased incidence of AIG. The gastric mucosa in mice at the ages of 6, 9, and 12 wk were evaluated histologically. We determined the incidence of AIG as chronic gastric inflammation greater than grade 2, characterized by moderately dense infiltration of mononuclear cells. These infiltrates were further confirmed to be CD4⁺ cells by immunohistological staining using FITC-conjugated anti-CD4. We found that the incidence of AIG in TSLPR^{-/-} NTx mice was significantly higher than that of NTx-TSLPR^{+/+} mice at the age of 6 wk (Fig. 5). These data suggested that TSLPR deficiency induces earlier onset of AIG.

FIGURE 3. TSLPR deficiency exacerbates histopathology in the mouse model of AIG. **A**, Histology of the gastric mucosa in 12-wk-old TSLPR^{+/+} and TSLPR^{-/-} mice, with or without neonatal thymectomy (H&E). Non-NTx TSLPR^{+/+} and TSLPR^{-/-} mice did not exhibit gastritis. Scale bars, 100 μ m. **B**, Degree of gastritis in NTx-TSLPR^{+/+} ($n = 9$) and NTx-TSLPR^{-/-} mice ($n = 7$) was determined by a semiquantitative scoring system, as described in *Materials and Methods* (upper panels and lower left panel). Data are presented as mean and SD. Lower left panel, Local pH in the corpus area of the stomach. Each symbol represents an individual mouse. * $p < 0.05$, Student t test for unpaired data.

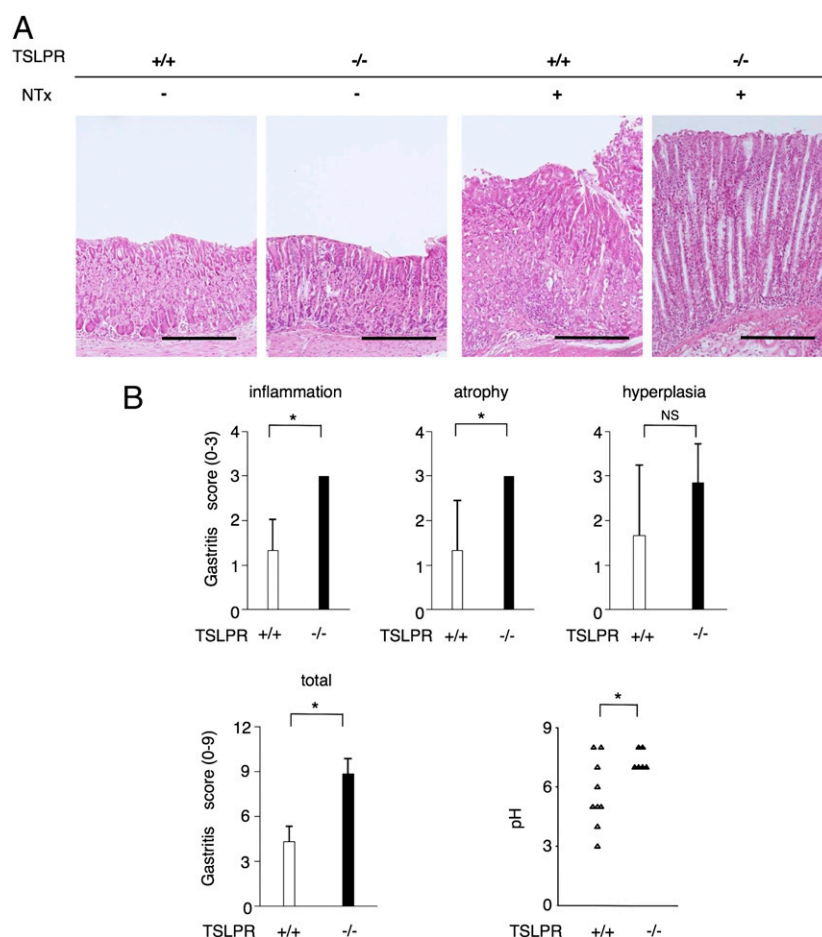
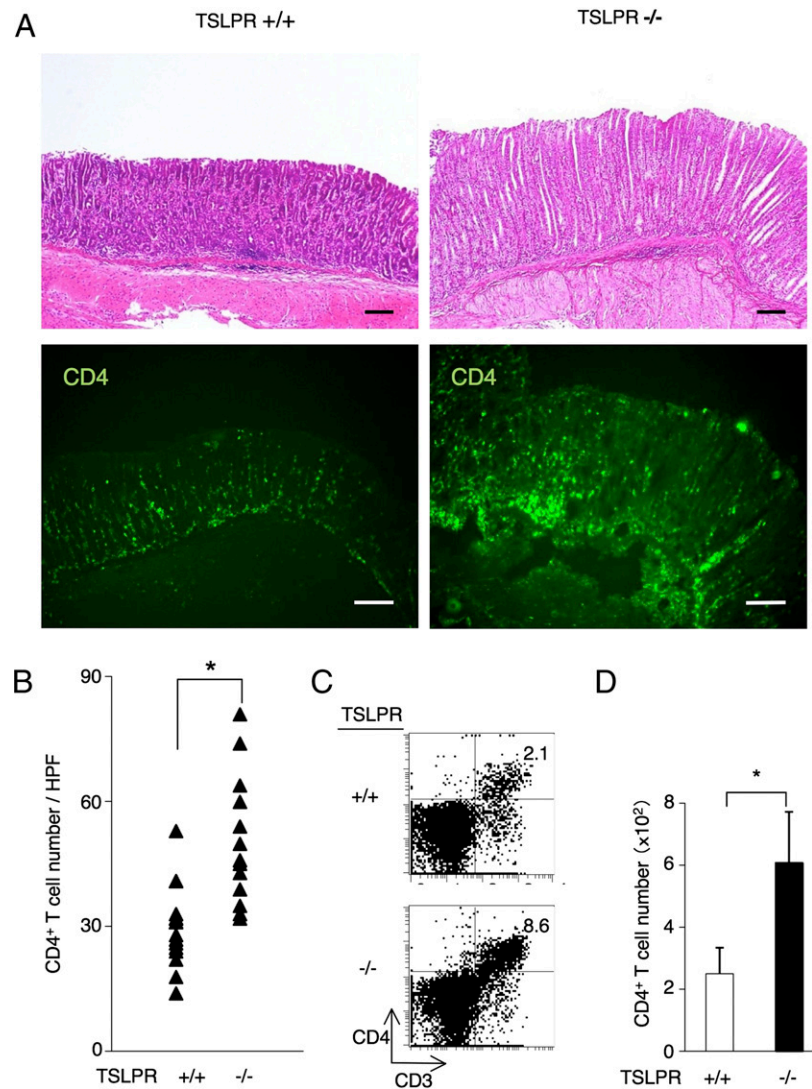


FIGURE 4. TSLPR deficiency exaggerates CD4⁺ T cell infiltration in the gastric mucosa in the mouse model of AIG. *A*, Fluorescence immunohistology of the stomach, using FITC-anti-CD4, in 12 wk-old NTx-TSLPR^{+/+} and NTx-TSLPR^{-/-} mice (*lower panels*). Histologic findings using H&E in the same mice are shown in the *upper panels*. Scale bars, 100 μ m. *B*, Numbers of CD4⁺ T cells in the gastric mucosa. Each symbol represents an individual mouse. **p* < 0.05, Student *t* test for unpaired data. *C* and *D*, Isolated mononuclear cells from the stomach of 12-wk-old NTx-TSLPR^{+/+} and NTx-TSLPR^{-/-} mice were analyzed by flow cytometry. Cells were stained with allophycocyanin-Cy7-conjugated anti-CD4 and PE-conjugated anti-CD3. *C*, Numbers in quadrants indicate percentage of cells in that gate. Data represent one of five separate experiments. *D*, Numbers of CD4⁺ T cells in the gastric mucosa were calculated using the equation (percentage of CD3⁺CD4⁺ cells in viable cells) $\times$ (number of viable cells). Data are the mean and SD of five separate experiments. **p* < 0.05, Student *t* test for paired comparisons.



TSLPR deficiency exhibits exaggerated Th1 responses

Because inflammatory Th1 responses are critical for the development of AIG, and TSLP regulates Th1-type inflammation in

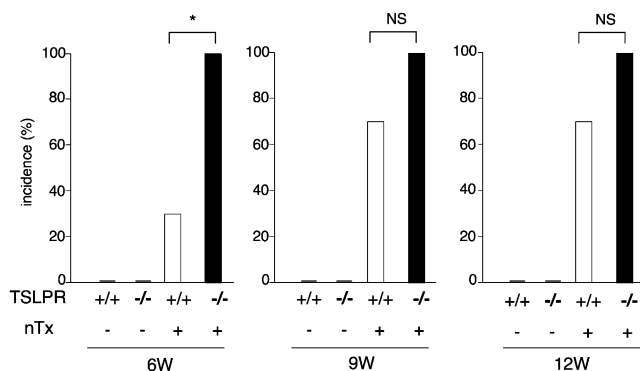
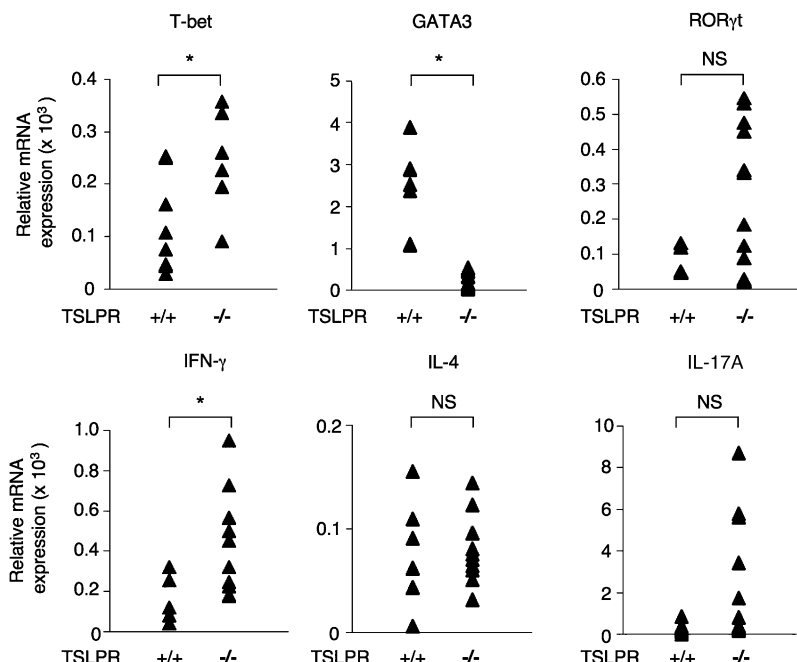


FIGURE 5. TSLPR deficiency induces increased susceptibility to AIG in mice. The gastric mucosa in mice at 6, 9, and 12 wk of age (*n* = 10 in each group) were evaluated histologically. The incidence of AIG was determined by chronic gastric inflammation greater than grade 2, characterized by moderately dense infiltration of mononuclear cells. These infiltrates were confirmed to be CD4⁺ cells by immunohistological staining using FITC-conjugated anti-CD4. **p* < 0.05, Fisher exact test.

mouse models of colitis (11–14, 21), we examined whether TSLPR deficiency enhanced Th1 responses in the inflamed stomach in AIG. We performed real-time quantitative RT-PCR analysis to measure the expression levels of mRNA encoding T cell lineage-specific transcription factors, such as T-bet, GATA3, and ROR γ t, and cytokines, such as IFN- γ , IL-4, and IL-17A. Inflamed gastric tissues of TSLPR^{-/-} NTx mice at 12 wk of age expressed a significantly increased level of mRNA expression of Th1 lineage-specific transcription factor T-bet, together with IFN- γ , in comparison with those in NTx-TSLPR^{+/+} mice (Fig. 6). In contrast, inflamed gastric tissues of NTx-TSLPR^{-/-} mice expressed reduced levels of mRNA expression of Th2 lineage-specific transcription factor GATA3. Levels of mRNA expression of IL-4, as well as Th17-related ROR γ t and IL-17A, were not significantly affected by TSLPR deficiency (Fig. 6). To confirm enhanced expression of IFN- γ by infiltrated CD4⁺ T cells, we performed flow cytometry using intracellular cytokine staining of CD4⁺ T cell infiltrates restimulated with PMA plus ionomycin. We observed significantly increased numbers of CD4⁺ T cells in the stomach of NTx-TSLPR^{-/-} mice (Fig. 4). In addition, we found significantly increased percentages of IFN- γ -expressing cells in gastric CD4⁺ T cells of NTx-TSLPR^{-/-} mice (Fig. 7A). Taken together, these data suggested that TSLPR deficiency enhances Th1 responses in the inflamed gastric tissues in AIG.

FIGURE 6. TSLPR deficiency exhibits exaggerated Th1 responses. Real-time quantitative RT-PCR analysis was used to measure the expression levels of mRNA encoding T cell lineage-specific transcription factors, such as T-bet, GATA3, or ROR γ t, and cytokines, such as IFN- γ , IL-4, and IL-17A, in the stomach of 12-wk-old NTx-TSLPR $^{+/+}$ or NTx-TSLPR $^{-/-}$ mice. Each symbol represents an individual mouse. * $p < 0.05$, Student t test for unpaired data.



TSLPR deficiency induces enhanced IL-12/23p40 production by DCs in GLN

In mice, TSLP was reported to negatively regulate production of IL-12/23p40 by DCs in vivo and in vitro and to suppress Th1 responses (21). In AIG-bearing mice, the draining lymph node of the stomach GLN is the induction site for activation of autoreactive CD4 $^{+}$ T cells by DCs capturing gastric tissue-specific self-Ag (29). Next, we examined whether TSLP deficiency may enhance DC activation to produce IL-12/23p40 in GLNs. Anti-mouse IL-12/23p40 Abs (clones C15.6 and C17.8) can react with both free and complexed heterodimer p70 forms of the p40 subunit of mouse IL-12, and they have been used to enumerate IL-12-producing cells (21, 30, 31). We isolated cells from the GLN, MLN, and spleen and enumerated IL-12-producing cells using anti-mouse IL-12/23p40 by flow cytometry. In contrast to NTx-TSLPR $^{+/+}$ mice, NTx-TSLPR $^{-/-}$ mice showed significantly increased percentages of IL-12/23p40 $^{+}$ cells in CD11c $^{+}$ CD11b $^{+/-}$ DCs isolated from the GLN, MLN, and spleen.

In addition, these increased percentages were observed in CD11c $^{+}$ CD11b $^{+/-}$ DCs but not in CD11c $^{-}$ CD11b $^{+}$ macrophages (Fig. 7B). These data suggested that TSLPR deficiency induced enhanced IL-12/23p40 production by DCs in the GLN, the induction sites for activation of autoreactive CD4 $^{+}$ T cells in AIG-bearing mice.

Discussion

In the current study, we demonstrated that TSLP was expressed in the inflamed stomach of AIG-bearing mice and that TSLPR deficiency enhanced production of anti-parietal cell Abs. Importantly, NTx-TSLPR $^{-/-}$ mice exhibited early onset of AIG and exaggerated inflammation of gastric mucosa, with enhanced Th1 responses. These data suggested that TSLPR-mediated signaling negatively regulates development of Th1-dependent autoimmunity in the gastric mucosa.

We showed in this study that inflamed gastric tissues in AIG-bearing mice exhibited increased levels of expression of TSLP.

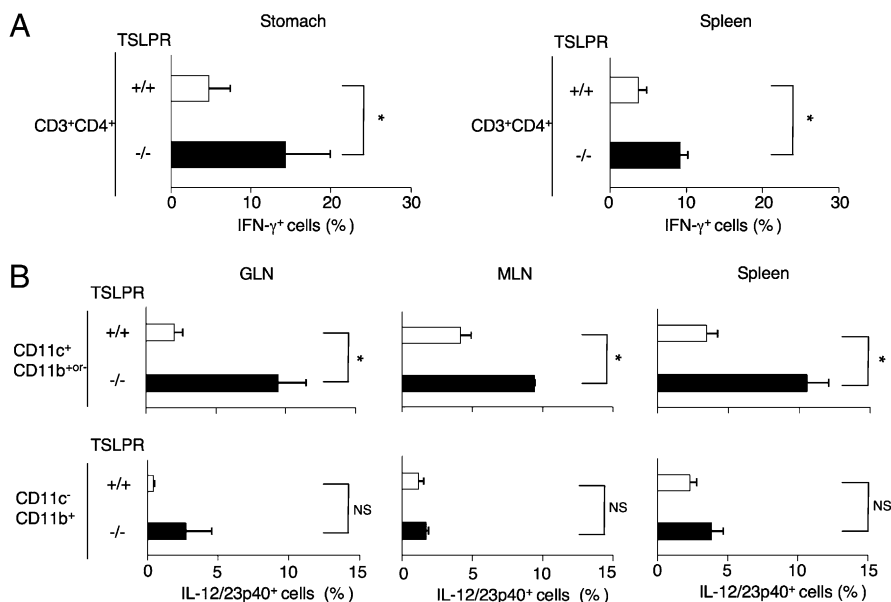


FIGURE 7. TSLPR deficiency enhances production of IFN- γ and IL-12/23p40. A, Intracellular cytokine staining of CD3 $^{+}$ CD4 $^{+}$ T cells in the stomach and spleen of 12-wk-old NTx-TSLPR $^{+/+}$ and NTx-TSLPR $^{-/-}$ mice. The cells were isolated from stomachs and spleens and then stimulated with PMA plus ionomycin. Percentages of IFN- γ $^{+}$ cells in CD3 $^{+}$ CD4 $^{+}$ T cells are shown. B, Intracellular cytokine staining of CD11c $^{+}$ CD11b $^{+/-}$ cells and CD11c $^{-}$ CD11b $^{+}$ cells in the GLN, MLN, and spleen of 12-wk-old NTx-TSLPR $^{+/+}$ and NTx-TSLPR $^{-/-}$ mice. Percentages of IL-12/23p40 $^{+}$ cells in indicated cells are shown. Data are the mean and SD of five separate experiments. * $p < 0.05$, Student t test for paired comparisons.

In a previous study in mice, mRNA expression of TSLP could be detected throughout the small and large intestines (20, 21). In patients with eosinophilic esophagitis, mRNA expression of TSLP in the inflamed esophageal tissues was significantly overexpressed (18, 19). TSLP expression was also found in gastric mucosal lesions from patients with *Helicobacter pylori*-induced chronic gastritis and *H. pylori* colonization-induced TSLP production in gastric epithelial cells (27). In the intestine, mRNA expression of TSLP was overexpressed in patients with ulcerative colitis, and proinflammatory cytokines induced overexpression of TSLP in colonic epithelial cells (32, 33). Therefore, TSLP can be expressed in the intestinal tract throughout the esophagus, stomach, and small and large intestines, both in humans and mice, suggesting that TSLP is involved in various immune responses in the gastrointestinal tract.

We also demonstrated in this study that TSLPR deficiency induced early onset of AIG and that the increased susceptibility was associated with exacerbated histopathology accompanied by enhanced Th1 responses. In addition, in AIG-bearing mice, TSLP negatively regulated production of IL-12/23p40 by DCs, as described (21). Therefore, these data suggested that TSLP expression in inflamed mucosa negatively modulates DC activation to promote Th1-type autoimmunity in the stomach. In mice, TSLP is involved in allergic diarrhea and Th2-type intestinal immunity against helminth infection, and it regulates Th1-type inflammation in colitis induced by dextran sodium sulfate (17, 21). In humans, TSLP is a candidate gene critically involved in susceptibility to eosinophilic esophagitis. TSLP overexpression is thought to be involved in *H. pylori*-induced chronic gastritis with formation of B cell follicles (18, 27). In addition, mRNA expression of TSLP was significantly reduced in patients with Crohn's disease, whereas TSLP was overexpressed in patients with ulcerative colitis (32, 33). Taking these results together, although TSLP is deeply involved in the pathophysiology of some allergic or Th2-dependent inflammatory conditions, its primary role in physiological conditions of the gastrointestinal tract may be to maintain intestinal immune homeostasis, including regulation of autoimmunity.

Although inflammatory Th1 responses induced by autoreactive CD4⁺ T cells have the potential to develop AIG in any mice, various regulatory mechanisms in normal mice suppress the development of AIG. NTx-BALB/c mice possess disease-relevant Tregs, but these cannot prevent AIG development (7–9), suggesting that Tregs are critically involved in negatively regulating the development of AIG. In addition, programmed cell death 1 (PD-1) provides negative costimulation to lymphocytes. PD-1-deficient BALB/c mice spontaneously develop AIG (25, 34), suggesting that PD-1-mediated signaling is critical for the negative regulation of AIG development. These regulatory mechanisms are primarily involved in suppressing the development of AIG, whereas although TSLP may be secondarily induced after triggering inflammation of AIG, induced TSLP did not regress the inflammation of AIG. However, it is not clear whether TSLP might have a regulatory function if there were more of it. Further studies are required to ascertain whether TSLP might be used as a therapeutic target for treating patients with AIG.

A previous report showed that adding recombinant TSLP enhanced the proliferation capacity of TCR-stimulated CD4⁺ T cells in vitro and that CD4⁺ T cells from TSLPR-deficient mice expanded less efficiently than did CD4⁺ T cells from wild-type mice in irradiated γ c/Rag2-deficient hosts (35). However, we found that infiltrating mononuclear cells in the gastric mucosa of NTx-TSLPR^{-/-} mice were also mainly CD4⁺ T cells and that TSLPR deficiency increased the cell numbers of CD4⁺ T cells in the

gastric mucosa (Fig. 5). These data suggested that the enhanced proliferative capacity of CD4⁺ T cells in a Th1-dominant autoimmune setting may overcome a deficiency of direct action of TSLP on CD4⁺ T cells.

In adult NTx mice, CD4⁺Foxp3⁺ Tregs were observed in the periphery, as described (7–9). We also found that 12-wk-old NTx-TSLPR^{-/-} mice had a number of CD4⁺Foxp3⁺ Tregs in the periphery, comparable to those found in NTx-TSLPR^{+/+} mice (data not shown). These findings suggested that it is not likely that impaired generation and expansion of induced Tregs enhance CD4⁺ T cell infiltration in the gastric mucosa of NTx-TSLPR^{-/-} mice.

In conclusion, we demonstrated that TSLP was expressed in the inflamed stomach of AIG-bearing mice and that TSLPR deficiency both increased susceptibility to AIG and exacerbated its severity. These data suggested that TSLPR-mediated signaling negatively regulates organ-specific Th1-dependent autoimmunity in the gastric mucosa.

Acknowledgments

We thank Dr. Dovie Wylie for assistance with preparation of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

- Strickland, R. G., and I. R. Mackay. 1973. A reappraisal of the nature and significance of chronic atrophic gastritis. *Am. J. Dig. Dis.* 18: 426–440.
- Stockbrügger, R. W., G. G. Menon, J. O. Beilby, R. R. Mason, and P. B. Cotton. 1983. Gastroscopic screening in 80 patients with pernicious anaemia. *Gut* 24: 1141–1147.
- Mosbech, J., and A. Videbaek. 1950. Mortality from and risk of gastric carcinoma among patients with pernicious anaemia. *BMJ* 2: 390–394.
- Mori, Y., K. Fukuma, Y. Adachi, K. Shigeta, R. Kannagi, H. Tanaka, M. Sakai, K. Kuribayashi, H. Uchino, and T. Masuda. 1989. Parietal cell autoantigens involved in neonatal thymectomy-induced murine autoimmune gastritis. Studies using monoclonal autoantibodies. *Gastroenterology* 97: 364–375.
- Jones, C. M., J. M. Callaghan, P. A. Gleeson, Y. Mori, T. Masuda, and B. H. Toh. 1991. The parietal cell autoantigens recognized in neonatal thymectomy-induced murine gastritis are the alpha and beta subunits of the gastric proton pump [corrected]. [Published erratum appears in 1992 *Gastroenterology* 102: 1829.] *Gastroenterology* 101: 287–294.
- Alderuccio, F., J. W. SENTRY, A. C. Marshall, M. Biondo, and B. H. Toh. 2002. Animal models of human disease: experimental autoimmune gastritis—a model for autoimmune gastritis and pernicious anemia. *Clin. Immunol.* 102: 48–58.
- Dujardin, H. C., O. Buren-Defranoux, L. Boucontet, P. Vieira, A. Cumano, and A. Bandeira. 2004. Regulatory potential and control of Foxp3 expression in newborn CD4⁺ T cells. *Proc. Natl. Acad. Sci. USA* 101: 14473–14478.
- Ang, D. K., T. C. Brodnicki, M. A. Jordan, W. E. Wilson, P. Silveira, B. L. Gliddon, A. G. Baxter, and I. R. van Driel. 2007. Two genetic loci independently confer susceptibility to autoimmune gastritis. *Int. Immunol.* 19: 1135–1144.
- Samy, E. T., K. M. Wheeler, R. J. Roper, C. Teuscher, and K. S. Tung. 2008. Cutting edge: Autoimmune disease in day 3 thymectomized mice is actively controlled by endogenous disease-specific regulatory T cells. *J. Immunol.* 180: 4366–4370.
- Fukuma, K., S. Sakaguchi, K. Kuribayashi, W. L. Chen, R. Morishita, K. Sekita, H. Uchino, and T. Masuda. 1988. Immunologic and clinical studies on murine experimental autoimmune gastritis induced by neonatal thymectomy. *Gastroenterology* 94: 274–283.
- Sakaguchi, S., K. Fukuma, K. Kuribayashi, and T. Masuda. 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J. Exp. Med.* 161: 72–87.
- De Silva, H. D., I. R. Van Driel, N. La Gruta, B. H. Toh, and P. A. Gleeson. 1998. CD4⁺ T cells, but not CD8⁺ T cells, are required for the development of experimental autoimmune gastritis. *Immunology* 93: 405–408.
- Barrett, S. P., P. A. Gleeson, H. de Silva, B. H. Toh, and I. R. van Driel. 1996. Interferon-gamma is required during the initiation of an organ-specific autoimmune disease. *Eur. J. Immunol.* 26: 1652–1655.
- Suri-Payer, E., and H. Cantor. 2001. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. *J. Autoimmun.* 16: 115–123.
- Liu, Y. J., V. Soumelis, N. Watanabe, T. Ito, Y. H. Wang, R. W. Malefyt, M. Omori, B. Zhou, and S. F. Ziegler. 2007. TSLP: an epithelial cell cytokine

- that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu. Rev. Immunol.* 25: 193–219.
16. Ziegler, S. F., and D. Artis. 2010. Sensing the outside world: TSLP regulates barrier immunity. *Nat. Immunol.* 11: 289–293.
 17. Blázquez, A. B., L. Mayer, and M. C. Berin. 2010. Thymic stromal lymphopoietin is required for gastrointestinal allergy but not oral tolerance. *Gastroenterology* 139: 1301–1309.
 18. Rothenberg, M. E., J. M. Spergel, J. D. Sherrill, K. Annaiah, L. J. Martin, A. Cianferoni, L. Gobar, C. Kim, J. Glessner, E. Frackelton, et al. 2010. Common variants at 5q22 associate with pediatric eosinophilic esophagitis. *Nat. Genet.* 42: 289–291.
 19. Sherrill, J. D., P. S. Gao, E. M. Stucke, C. Blanchard, M. H. Collins, P. E. Putnam, J. P. Franciosi, J. P. Kushner, J. P. Abonia, A. H. Assa'ad, et al. 2010. Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis. *J. Allergy Clin. Immunol.* 126: 160–165.e3.
 20. Zaph, C., A. E. Troy, B. C. Taylor, L. D. Berman-Booty, K. J. Guild, Y. Du, E. A. Yost, A. D. Gruber, M. J. May, F. R. Greten, et al. 2007. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* 446: 552–556.
 21. Taylor, B. C., C. Zaph, A. E. Troy, Y. Du, K. J. Guild, M. R. Comeau, and D. Artis. 2009. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J. Exp. Med.* 206: 655–667.
 22. Carpino, N., W. E. Thierfelder, M. S. Chang, C. Saris, S. J. Turner, S. F. Ziegler, and J. N. Ihle. 2004. Absence of an essential role for thymic stromal lymphopoietin receptor in murine B-cell development. *Mol. Cell. Biol.* 24: 2584–2592.
 23. Zhou, B., M. R. Comeau, T. De Smedt, H. D. Liggitt, M. E. Dahl, D. B. Lewis, D. Gyarmati, T. Aye, D. J. Campbell, and S. F. Ziegler. 2005. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat. Immunol.* 6: 1047–1053.
 24. Uchida, K., K. Okazaki, A. Debrecceni, T. Nishi, H. Iwano, M. Inai, S. Uose, H. Nakase, M. Ohana, C. Oshima, et al. 2001. Analysis of cytokines in the early development of gastric secondary lymphoid follicles in *Helicobacter pylori*-infected BALB/c mice with neonatal thymectomy. *Infect. Immun.* 69: 6749–6754.
 25. Kido, M., N. Watanabe, T. Okazaki, T. Akamatsu, J. Tanaka, K. Saga, A. Nishio, T. Honjo, and T. Chiba. 2008. Fatal autoimmune hepatitis induced by concurrent loss of naturally arising regulatory T cells and PD-1-mediated signaling. *Gastroenterology* 135: 1333–1343.
 26. Ohana, M., K. Okazaki, C. Oshima, K. Kawasaki, T. Fukui, H. Tamaki, M. Matsuura, M. Asada, T. Nishi, K. Uchida, et al. 2003. Inhibitory effects of *Helicobacter pylori* infection on murine autoimmune gastritis. *Gut* 52: 1102–1110.
 27. Kido, M., J. Tanaka, N. Aoki, S. Iwamoto, H. Nishiura, T. Chiba, and N. Watanabe. 2010. *Helicobacter pylori* promotes the production of thymic stromal lymphopoietin by gastric epithelial cells and induces dendritic cell-mediated inflammatory Th2 responses. *Infect. Immun.* 78: 108–114.
 28. Alderuccio, F., B. H. Toh, P. A. Gleeson, and I. R. Van Driel. 1995. A novel method for isolating mononuclear cells from the stomachs of mice with experimental autoimmune gastritis. *Autoimmunity* 21: 215–221.
 29. Scheinecker, C., R. McHugh, E. M. Shevach, and R. N. Germain. 2002. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J. Exp. Med.* 196: 1079–1090.
 30. Bliss, S. K., B. A. Butcher, and E. Y. Denkers. 2000. Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. *J. Immunol.* 165: 4515–4521.
 31. Mason, N., J. Aliberti, J. C. Caamano, H. C. Liou, and C. A. Hunter. 2002. Cutting edge: identification of c-Rel-dependent and -independent pathways of IL-12 production during infectious and inflammatory stimuli. *J. Immunol.* 168: 2590–2594.
 32. Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G. M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat. Immunol.* 6: 507–514.
 33. Tanaka, J., K. Saga, M. Kido, H. Nishiura, T. Akamatsu, T. Chiba, and N. Watanabe. 2010. Proinflammatory Th2 cytokines induce production of thymic stromal lymphopoietin in human colonic epithelial cells. *Dig. Dis. Sci.* 55: 1896–1904.
 34. Okazaki, T., Y. Otaka, J. Wang, H. Hiai, T. Takai, J. V. Ravetch, and T. Honjo. 2005. Hydronephrosis associated with antiurothelial and antinuclear autoantibodies in BALB/c-*Fcgr2b*^{-/-}-*Pdcd1*^{-/-} mice. *J. Exp. Med.* 202: 1643–1648.
 35. Al-Shami, A., R. Spolski, J. Kelly, T. Fry, P. L. Schwartzberg, A. Pandey, C. L. Mackall, and W. J. Leonard. 2004. A role for thymic stromal lymphopoietin in CD4(+) T cell development. *J. Exp. Med.* 200: 159–168.