IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Achaete-Scute Homologue 2—Regulated Follicular Helper T Cells Promote Autoimmunity in a Murine Model for Sjögren Syndrome

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Follicular helper T (Tfh) cells contribute to various immune responses as well as to the pathogenesis of several immune diseases. However, the precise mechanism underlying the onset or development of autoimmunity via Tfh cells remains unclear. Herein, the detailed relationship between autoimmune disease and Tfh cells was analyzed using a murine model for Sjögren syndrome (SS) wherein the mice underwent neonatal thymectomy. Germinal center (GC) development was promoted in this SS model along with an increase of Tfh cells and GC B cells. The severity of the autoimmune lesions was correlated with the number of Tfh cells detected in the spleen of the SS model mice. In addition, treatment with an anti-CD20 monoclonal antibody effectively suppressed the autoimmune lesions with a reduction of Tfh cells and GC B cells. Comprehensive gene analysis revealed that several genes associated with Tfh cell differentiation, including achaete-scute homologue 2 (*Ascl2*), were up-regulated in peripheral CD25⁺ CD4⁺ T cells in SS model mice compared with those in control mice. Moreover, an experiment using *CD4^{Cre} Bcl6^{fl/fl}* mice that received neonatal thymectomy treatment demonstrated that *Ascl2* contributes to the Tfh cell differentiation associated with autoimmunity during the early stages, independent of *Bcl6*. In conclusion, our results indicate that abnormal Tfh cell differentiation via *Ascl2* regulation might contribute to the pathogenesis of autoimmunity. (*Am J Pathol* 2019, 189: 2414–2427; <https://doi.org/10.1016/j.ajpath.2019.08.008>)

Follicular helper T (Tfh) cells are essential for germinal center (GC) formation, affinity maturation, and high-affinity antibody production.^{1–3} Several studies have demonstrated that Tfh cells as well as other helper T-cell subsets influence various immune responses, including autoimmunity and infection.^{4,5} However, the precise mechanism underlying the onset or development of autoimmunity related to Tfh cells in the peripheral immune system remains unclear.

Sjögren syndrome (SS) is an autoimmune disease, which is generally considered as T cell and B cell mediated, affecting the lacrimal and salivary exocrine glands. The primary clinical symptoms consist of sicca syndrome, including dry eyes and mouth.^{6,7} In addition, SS often accompanies other systemic autoimmune diseases, such as pulmonary lesions, rheumatoid arthritis, and lupus.^{8,9} Several autoantigens have

been reported in human and murine SS models,^{10–13} and the relationship between autoreactive T cells and autoantigens within SS has previously been described.^{10–13} Moreover, anti-Ro/SSA and anti-La/SSB autoantibodies are widely known to be clinically useful in SS diagnosis.¹⁴ The complicated mechanisms underlying SS further complicate the radical therapy, on the basis of the etiology for patients with SS.

Tfh cells are increased in the peripheral blood and target organs of patients with SS, along with enhanced memory B cells and GC B cells in the target tissues, but not in the

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peripheral blood.^{15–18} Therefore, Tfh cells have been emphasized on for understanding the pathogenesis of SS. On histologic evaluation, ectopic GC formation is observed in the salivary gland tissues of patients with SS by histologic analysis¹⁹; this formation is associated with the development and outcome of B-cell lymphoma.^{20–22} However, a recent report demonstrates that the presence of GC formation in minor salivary glands of patients with SS is not correlated to the development of non-Hodgkin lymphoma.²³ In addition, a previous study has demonstrated that a large number of Tfh cells were detected in the peripheral blood of patients with SS at the time of disease onset, with aberrations of serum anti-Ro/SSA and anti-La/SSB levels.¹⁸ Moreover, C-X-C chemokine receptor type 5 (CXCR5)⁺ CD4⁺ Tfh cells are significantly elevated in the salivary gland tissues and peripheral blood of patients with SS, along with aberrant B cells and plasma cells, suggesting that CXCR5⁺ CD4⁺ Tfh cells contribute to the pathogenesis of SS by promoting B-cell maturation.²⁴ In addition, programmed cell death-1 (PD-1)⁺ inducible costimulatory–positive Tfh cells in peripheral blood are associated with disease activity of primary SS.²⁵ Recent studies have described *Bcl6* mRNA expression levels as being significantly higher in the ectopic GC of the salivary gland tissues from patients with SS.²⁶ In addition to CXCR5, CD84 and PD-1 expression were also detected on infiltrating lymphocytes in the salivary gland tissues of these patients.²⁶ However, the precise cellular or molecular mechanism underlying the pathogenesis of SS via Tfh cells has not yet been clarified.

In the present study, we investigated the mechanism via which Tfh cells contribute to the breakdown of peripheral immune tolerance and the formation of autoimmune lesions in an SS murine model. Our results indicate an autoreactive response mechanism of Tfh cells in an SS murine model, which has not yet been reported, to the best of our knowledge; these results would be supportive for understanding the pathogenesis of autoimmunity.

Materials and Methods

Mice

NFS/*sld* mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). C57BL/6 mice were obtained from the Japan SLC Laboratory (Shizuoka, Japan). *CD4^{Cre}* mice were obtained from Taconic (Germantown, NY), and *Bcl6^{fl/fl}* mice²⁷ were obtained from RIKEN BioResource Center (Tsukuba, Japan). *CD4^{Cre}* and *Bcl6^{fl/fl}* mice were mated to generate *CD4^{Cre} Bcl6^{fl/fl}* mice. Thymectomy (Tx) surgery was performed on female NFS/*sld*, C57BL/6, *CD4^{Cre} Bcl6^{+/+}*, and *CD4^{Cre} Bcl6^{fl/fl}* mice at day 3 after birth via a suction technique employing an aspirator. At 4 weeks after the Tx surgery, peripheral blood mononuclear cells from the tail vein were analyzed using flow cytometry. The completeness of Tx surgery was confirmed by evaluating the proportion of CD90.2⁺ T cells (<7%). NFS/*sld* mice that

underwent Tx surgery are defined as SS model mice in the present study. Sham operation (control) in this research is thoracotomy without excision of thymus tissue. The present study was conducted following the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The protocol was approved by the Committee on Animal Experiments of Tokushima University and the Biological Safety Research Center (permit number T29-115). Experiments were performed under general anesthesia, and all efforts were made to minimize suffering. Mice ages 4 to 30 weeks (NFS/*sld*) and 20 to 24 weeks (*C57BL/6*, *CD4^{Cre} Bcl6^{+/+}*, and *CD4^{Cre} Bcl6^{fl/fl}*) were used in the present study.

Histologic Evaluations

After the mice were sacrificed, all the organs were removed and fixed using 10% phosphate-buffered formalin (pH 7.2). Formalin-fixed tissue sections of salivary glands, cervical lymph nodes, and spleen were stained using hematoxylin and eosin. The number of lymphocytes that infiltrated into submandibular gland tissues per mm² was determined using hematoxylin and eosin-stained sections.

Immunohistochemistry

Deparaffinized sections from spleen were used. Heat-induced antigen retrieval was performed in ImmunoActive antigen retrieval solution (pH 6.0) (Matsunami Glass, Osaka, Japan) with microwave thrice for 5 minutes. Biotinylated peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) was applied to the sections; the sections were then incubated overnight at 4°C. After washing with phosphate-buffered saline, the sections were incubated with horseradish peroxidase–conjugated streptavidin solution (Dako, Carpinteria, CA). Horseradish peroxidase reacted with the 3,3'-diaminobenzidine substrate using the Histofine 3,3'-diaminobenzidine substrate kit (Nichirei Biosciences, Tokyo, Japan). The sections were counterstained with hematoxylin.

Confocal Analysis

Frozen spleen tissue sections were fixed using 4% paraformaldehyde in phosphate-buffered saline and blocked using Blocking One Histo (Nacalai Tesque, Kyoto, Japan). For GC staining, sections were stained using fluorescein-labeled PNA (Vector Laboratories) and biotinylated antibody against IgD (BioLegend, San Diego, CA), followed by Alexa Fluor 568–conjugated streptavidin (Invitrogen, Carlsbad, CA). After washing thrice with phosphate-buffered saline, nuclear DNA was stained using DAPI (Invitrogen). Sections were observed with a PASCAL confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) at ×400 magnification.

The laser-scanning microscope image browser version 3.5 (Carl Zeiss) was used for image acquisition.

Enzyme-Linked Immunosorbent Assay

The autoantibody titers in mice sera were measured using enzyme-linked immunosorbent assays, as previously described.²⁶ In brief, the Ro/SS-A (ImmunoVision, Springdale, AR), La/SS-B (ImmunoVision), or α -fodrin (JS-1)²⁸ antigens were added to microtiter wells at a concentration of 0.5, 1, or 10 μ g/mL, respectively, in carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO). Non-specific sites were absorbed using Blocking One (Nacalai Tesque). Plates were washed, and diluted sera were added. Horseradish peroxidase-conjugated anti-mouse IgG (H + L) (Vector Laboratories) was used as the secondary antibody, and phosphate-citrate buffer containing o-phenylenediamine dihydrochloride tablets (Sigma-Aldrich) and hydrogen peroxide (Wako Pure Chemical Industries, Osaka, Japan) was added. The OD at 490 nm for each well was measured using a microplate reader (Bio-Rad Laboratories, Richmond, CA).

Flow Cytometry Analysis

Lymphocytes from the spleen were stained with fluorescein-labeled PNA (Vector Laboratories) and antibodies against fluorescein isothiocyanate-conjugated anti-mouse CD62L (eBioscience, San Diego, CA) and CD21 (BD Biosciences, San Jose, CA); phycoerythrin-conjugated anti-mouse CD23 (TONBO Biosciences, San Diego, CA); peridinin chlorophyll protein-cyanine 5.5 (Cy5.5)-conjugated anti-mouse CD44 (TONBO Biosciences), CD3 (TONBO Biosciences), and CD45.2 (TONBO Biosciences); phycoerythrin-Cy7-conjugated anti-mouse CD45.2 (TONBO Biosciences); allophycocyanin-conjugated anti-mouse CD4 (TONBO Biosciences), GL-7 (BioLegend), and CD11c (BioLegend); allophycocyanin-Cy7-conjugated CD19 (BioLegend) and CD62L (BioLegend); and Alexa Fluor 780-conjugated anti-mouse PD-1 (eBioscience; using Armenian hamster IgG as an isotype control; eBioscience). For CXCR5 staining, biotinylated anti-mouse CXCR5 (BD Biosciences; using rat IgG2b as an isotype control; BioLegend) and peridinin chlorophyll protein-Cy5.5-conjugated streptavidin (eBioscience) were used. Intracellular forkhead box protein P3 (Foxp3) and achaete-scute homologue 2 (*Ascl2*) expression was analyzed using an intracellular Foxp3 detection kit (eBioscience), according to the manufacturer's instructions. For Foxp3 intracellular staining, fluorescein isothiocyanate- or phycoerythrin-conjugated anti-mouse/human Foxp3 (eBioscience) was used, whereas for *Ascl2* intracellular staining, rabbit anti-mouse/human *Ascl2* (Biorbyt, Cambridge, UK) was used as primary antibody (with rabbit IgG as an isotype control; MBL, Nagoya, Japan) and phycoerythrin-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) was used as

secondary antibody. A FACSCanto flow cytometer (BD Biosciences) was used for identifying the cell populations on the basis of the expression profiles. Data were analyzed using FlowJo FACS Analysis software version 10.5.3 (R&D Systems, Minneapolis, MN). Cell numbers of each subset were calculated by cell counter and flow cytometer and showed within total CD45.2⁺ cells in a spleen tissue.

Real-Time Quantitative RT-PCR

The splenic CD4⁺ T cells were purified using EasySep mouse CD4⁺ T-cell purification kits (Stemcell Technologies, Vancouver, BC, Canada), and splenic CD25⁻ CD4⁺ T cells were isolated using purified rat anti-mouse CD25 (eBioscience) and Dynabeads sheep anti-rat IgG (Invitrogen). Total RNA was extracted from purified CD25⁻ CD4⁺ T cells using RNeasy Plus Micro kits (Qiagen, Hilden, Germany) and was reverse transcribed. The transcriptions of target genes and β -actin were generated with a Light Cycler 96 System (Roche, Basel, Switzerland) using TB Green Premix Ex Taq II (Takara Bio, Shiga, Japan) and the following primers: *Ascl2*, 5'-CGCTGCCCAGACTCATGCCC-3' (forward) and 5'-GCTTTACGCGTTGCGCTCG-3' (reverse); and β -actin, 5'-GTGGGCCGCTCTAGGCACCA-3' (forward) and 5'-CGGTTGGCCTTAGGGTTCAGGGGG-3' (reverse). Relative *Ascl2* mRNA expression was normalized against β -actin mRNA.

Stimulation of CD25⁻ CD4⁺ T Cells with Anti-CD3/CD28 Antibody

Purified CD25⁻ CD4⁺ T cells (1×10^5 cells/well) were cultured with or without Dynabeads mouse T-activator CD3/CD28 (Thermo Fisher Scientific, Rockford, IL) at a CD25⁻ CD4⁺ T cell per bead ratio of 1:0, 1:0.5, or 1:1 for 3 days.

Administration of Anti-CD20 Monoclonal Antibody

Ultra-LEAF purified anti-mouse CD20 antibody (100 μ g/dose; BioLegend) was administered weekly via i.v. injection to female SS model mice, aged 7 to 10 weeks. As a control, LEAF purified rat IgG2b, κ isotype control antibody (100 μ g/dose; BioLegend), was administered to SS model mice following the same regimen.

Microarray Analysis

Total RNA from splenic CD25⁻ CD4⁺ T cells of control and SS model mice was isolated using an RNeasy Mini Kit (Qiagen). Samples were pooled from four mice for each treatment. The RNA quality was checked using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Cy3-labeled cRNA was prepared from 150 ng RNA using the One-Color Low Input Quick Amp Labeling kit (Agilent Technologies), according to the manufacturer's instructions. Cy3-labeled cRNA was hybridized to Agilent Whole Mouse Genome Microarrays 4 $\times$ 44K version 2 (Agilent

Technologies). Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (Agilent Technologies). The scanned images were analyzed with Feature Extraction software version 10.7.1.1 (Agilent Technologies). All analyses were supported by the Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences

(Tokushima, Japan). The data are available online (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE123332).

Statistical Analysis

Group means were compared using an unpaired *t*-test, and $P < 0.05$ was considered significant. The correlations

Figure 1 Germinal center (GC) reaction in SS model mice. **A:** Histology of autoimmune lesions of submandibular glands in SS model mice at 8 weeks of age. Representative images with hematoxylin and eosin staining of control and SS model mice are shown. **B:** Ectopic lymphoid structure in autoimmune lesions of parotid glands from SS model mice is observed at 18 weeks of age. **White dotted line** shows GC area. **C:** GCs of spleen in control and SS model mice at 10 weeks of age. **Top panels:** Hematoxylin and eosin staining. **Bottom panels:** Peanut agglutinin (PNA)⁺ GCs. Images are representative of each group. **D:** PNA⁺ GC area is presented. **E:** GCs of cervical lymph nodes from control and SS model mice at 10 weeks of age. Images are representative of each group. **White dotted lines** show GC area. **F:** GC reaction was evaluated by confocal microscopic analysis of PNA and IgD expression in the spleen from control and SS model mice at 10 weeks of age. Data are expressed as means $\pm$ SD (**D**). $n = 4$ mice in each group (**D**); $n = 5$ mice in each group (**F**). * $P < 0.05$. Scale bar = 100 μ m (**A–C**, **E**, and **F**).

between two variables were evaluated with Spearman's rank correlation coefficient.

Results

GC Reaction in SS Model Mice

We have previously established an SS murine model using *NFS/sld* mice that received neonatal Tx.^{10,29} Autoimmune lesions, including lymphocyte accumulation surrounding the extended duct along with the acinar cell destruction, were observed in the submandibular glands, resembling those in patients with SS (Figure 1A). Among the aged SS model mice, inflammatory lesions with GCs were occasionally observed in the parotid glands (Figure 1B), resembling the inflammatory lesions with GC formation observed within the minor salivary glands in patients with SS.

GC formation is detectable in lymphoid tissues, such as the spleen and lymph nodes. The PNA⁺ GCs in the spleen of SS model mice were observed to be expanded when compared with those of control mice (Figure 1C). The areas of these expanded GCs were significantly increased compared with those of control mice (Figure 1D). In addition, the GCs within the cervical lymph nodes of SS model mice were expanded compared with those of control mice (Figure 1E). Accumulation of PNA⁺ IgD⁺ GC B cells and PNA⁺ IgD⁺ B cells within the spleen in the SS model mice was considerably increased compared with that of control mice (Figure 1F). Furthermore, the autoantibody levels of anti-Ro/SSA, anti-La/SSB, and anti- α -fodrin antibodies in the sera of SS model mice were significantly elevated compared with those from control mice (Supplemental Figure S1). These results suggest that the enhanced GC reaction observed in this SS murine model contributes to the

Figure 2 Germinal center (GC) reaction—associated subsets in SS model mice. **A:** Programmed cell death-1 (PD-1)⁺ C-X-C chemokine receptor type 5 (CXCR5)⁺ CD62L⁻ CD4⁺ follicular helper T (Tfh) cells from the spleen of control and SS model mice were analyzed using flow cytometry. Results are representative of mice at 8 weeks of age. **B:** The proportion and number of Tfh cells are presented. **C:** The number of forkhead box protein P3 (Foxp3)⁺ PD-1⁺ CXCR5⁺ CD62L⁻ CD4⁺ follicular regulatory T (Tfr) cells is presented. **D:** Peanut agglutinin (PNA)⁺ GL-7⁺ CD19⁺ GC B cells from the spleen of control and SS model mice were analyzed using flow cytometry. Results are representative of mice at 8 weeks of age. **E:** The proportion and number of GC B cells are presented. **F:** CD21⁺ CD23⁺ CD11c⁺ follicular dendritic cells (FDCs) from the spleen of control and SS model mice were analyzed using flow cytometry. Results are representative of mice at 8 weeks of age. **G:** The proportion and number of FDCs are presented. Data are expressed as means $\pm$ SD (**B**, **E**, and **G**) or averages $\pm$ SD (**C**). $n = 5$ mice in each group (**A–G**). * $P < 0.05$, *** $P < 0.0005$.

autoimmunity, including inflammatory lesions, in the target organ as well as to autoantibody production.

GC Reaction and Autoimmunity

Immune cell populations associated with GC reactions, including Tfh cells, GC B cells, follicular regulatory T cells, and follicular dendritic cells, were isolated from the spleen of control and SS model mice and analyzed using flow cytometry, according to the gating strategy (Supplemental Figure S2). The proportion and cell number of PD-1⁺ CXCR5⁺ Foxp3[−] CD62L[−] CD4⁺ Tfh cells were significantly higher in SS model mice than in control mice (Figure 2, A and B). No change was observed in the number of PD-1⁺ CXCR5⁺ Foxp3⁺ CD62L[−] CD4⁺ follicular regulatory T cells between the two groups (Figure 2C). In addition, the proportion and cell number of PNA⁺ GL-7⁺ CD19⁺ GC B cells were significantly enhanced in SS model mice compared with control mice (Figure 2, D and E). Moreover, a significant increase in the number of CD23⁺ CD21⁺ CD3[−] CD19[−] CD11c⁺ follicular dendritic cells of SS model mice was observed compared with that of control mice, but there was no difference in the proportion (Figure 2, F and G). Therefore, the expansion of Tfh cells

and GC B cells enhances the GC reaction in the SS model mice. On the other hand, cell numbers of type 1, 2, and 17 helper T cells were not changed between control and SS model mice (Supplemental Figure S3). Otherwise, the cell number of T-regulatory cells in SS model mice was significantly decreased compared with that in control mice (Supplemental Figure S3). Thus, Tfh cells may play a key role in the pathogenesis of autoimmunity in this SS model.

Correlation of Autoimmunity with Tfh Cells

To elucidate the role of Tfh cells in the severity of autoimmune lesions, correlation between the number of Tfh cells in the spleen and the number of lymphocytes that infiltrated into submandibular glands per mm² was analyzed using SS model mice from 8 to 30 weeks of age. The number of Tfh cells in the spleen was positively correlated with the severity of autoimmune lesions ($r = 0.53$, $P < 0.05$) (Figure 3A). Conversely, no significant correlation was observed between the number of GC B cells in the spleen and the number of lymphocytes that infiltrated into the submandibular glands per mm² in this model ($P > 0.05$) (Figure 3B). Furthermore, no correlation was observed between the number of lymphocytes that infiltrated into the submandibular glands per

Figure 3 Relationship between the severity of inflammation and follicular helper T (Tfh) cells in SS model mice. Using cells harvested from SS model mice at 8 to 30 weeks of age, statistical analyses were performed concerning the relationships between numbers of lymphocytes that infiltrated into the submandibular gland tissues per mm² and the number of Tfh cells in the spleen (A), the number of germinal center (GC) B cells in the spleen (B), and serum autoantibody titers against anti-Ro/SSA, anti-La/SSB, and anti-α-fodrin (C). R^2 is the coefficient of determination. Black line shows the approximate curve.

Figure 4 Effect of anti-CD20 monoclonal antibody (mAb) administration on autoimmune lesions and follicular helper T (Tfh) cells in SS model mice. **A:** Anti-CD20 mAb or isotype control mAb (100 µg/dose) was intravenously injected into SS model mice once a week from 7 to 9 weeks of age, with analysis at 10 weeks of age. **B:** The proportion of CD19⁺ B cells within CD45.2⁺ cells was analyzed using flow cytometry. **C:** Histopathologic analysis was performed using the submandibular gland tissues from mice that received isotype control mAb or anti-CD20 mAb-administered mice. **D:** The number of lymphocytes that infiltrated into the submandibular gland tissues per mm² was determined from the hematoxylin and eosin-stained sections. **E:** Germinal centers (GCs) of the spleen in isotype control mAb- or anti-CD20 mAb-administered mice. **F:** GC area is presented. **G:** The numbers of Tfh cells, GC B cells, follicular regulatory T (Tfr) cells, and follicular dendritic cells (FDCs) in the spleen of isotype control mAb- or anti-CD20 mAb-administered mice were analyzed using flow cytometry. Data are expressed as means ± SD (**B**, **D**, and **F**) or average ± SD (**G**). *n* = 4 mice in each group (**B** and **D–G**); *n* = 5 mice in each group (**C**). **P* < 0.05, ***P* < 0.005, and ****P* < 0.0005. Scale bar = 100 µm (**C** and **E**). PNA, peanut agglutinin.

mm² and the titer level of autoantibodies, such as anti-Ro/SSA, anti-La/SSB, and anti- α -fodrin autoantibodies ($P > 0.05$) (Figure 3C). These results suggest that Tfh cells within GCs play a key role in the formation of autoimmune lesions within the SS model mice.

Effect of B-Cell Depletion on Tfh Cells in SS Model Mice

A previous report has demonstrated that therapy using anti-CD20 monoclonal antibody (mAb) for B-cell depletion was effective for patients with SS and resulted in a reduced number of Tfh cells.³⁰ Therefore, we hypothesized that B-cell depletion may suppress a GC reaction in the SS model mice. Anti-CD20 mAb or rat IgG2b mAb (ie, isotype control) was intravenously administered thrice into SS model mice from 7 to 9 weeks of age, and the mice were analyzed at 10 weeks of age (Figure 4A). The proportion of CD19⁺ B cells within CD45.2⁺ cells from anti-CD20 mAb-treated mice was significantly reduced compared with that from isotype control mAb-treated mice (Figure 4B). Interestingly, autoimmune lesions within the salivary glands of SS model mice were suppressed by anti-CD20 mAb administration (Figure 4C). The number of lymphocytes that infiltrated into the submandibular glands per mm² of anti-CD20 mAb-treated mice was significantly reduced compared with that of isotype control mAb-treated mice (Figure 4D). In addition, PNA⁺ GCs in the spleen of anti-CD20 mAb-treated mice were considerably diminished compared with those of isotype control mAb-treated mice (Figure 4E). The PNA⁺ GC areas of anti-CD20 mAb-treated mice were significantly decreased compared with those of isotype control mAb-treated mice (Figure 4F). Furthermore, the numbers of both Tfh cells and GC B cells in the spleen of anti-CD20 mAb-treated mice were significantly reduced compared with those of isotype control mAb-treated mice (Figure 4G). In contrast, no differences were observed in number of follicular regulatory T cells and follicular dendritic cells between anti-CD20 mAb-treated mice and isotype control mAb-treated mice (Figure 4G). Furthermore, no changes were noted in the autoantibody levels of anti-Ro/SSA, anti-La/SSB, and anti- α -fodrin within the sera between anti-CD20 mAb-treated and isotype control mAb-treated mice (Supplemental Figure S4). These results suggest that anti-CD20 mAb treatment is effective on autoimmune lesions in SS model mice and that B-cell depletion suppresses the GC reaction with a reduction of Tfh cells in these mice.

Tfh Cell–Related Gene Signature in SS Model Mice

Tfh cell–related gene expression of splenic CD25⁺ CD4⁺ T cells in SS model mice was comprehensively analyzed by comparison with that in control mice using DNA microarray analysis. Regarding genes that are associated with Tfh cell differentiation, positive regulator genes, such as *Ascl2*, *Il21* isoform 2 precursor, *Cxcr5*, and *Il6* isoform 1 precursor, were

greater than threefold up-regulated in SS model mice compared with those in control mice (Figure 5A). In contrast, negative regulator genes, such as inhibitor of DNA binding-3 (*Id3*), PR domain-containing protein 1 (*Prdm1*)/B lymphocyte-induced maturation protein 1 (*Blimp1*), and *STAT5A* isoform 1, remained unchanged (Figure 5A). A significantly up-regulated level of *Ascl2* mRNA in CD25⁺ CD4⁺ T cells of SS model mice was confirmed in comparison with that in control mice from 4 to 20 weeks of age by real-time quantitative RT-PCR (Figure 5B). A significantly increased *Ascl2* mRNA expression of CD4⁺ T cells was observed in SS model mice compared with control mice at 4 weeks of age when the autoimmune lesions are not observed yet in the target organ (Figure 5B). *Ascl2* is known as one of the key transcription factors involved in the early stage of Tfh cell differentiation for controlling *Cxcr5* expression.³¹ The proportion of *Ascl2*⁺ CD4⁺ T cells in the spleen of the SS model mice was significantly higher in SS model mice than in control mice (Figure 5, C and D). In addition, CXCR5 expression on *Ascl2*⁺ CD4⁺ cells in the spleen of the SS model mice was also significantly increased compared with that of control mice (Figure 5, E and F). *Ascl2* expression is controlled by AKT and Wnt signaling under the CD3/CD28 pathway.³¹ On stimulating CD25⁺ CD4⁺ T cells from the spleen of control and SS model mice using anti-CD3/CD28 mAb-coated beads for 3 days, the intracellular *Ascl2* expression of CD4⁺ T cells was significantly enhanced within SS model mice compared with that within controls (Figure 5, G and H). In our experimental system, the suitable condition for *Ascl2* expression was cell/bead ratio of 1:0.5. Perhaps a strong signaling (1:1) of CD3/CD28 may reach to the peak of *Ascl2* expression of T cells in both control and SS model mice. These results suggest that the *Ascl2* up-regulation enhances Tfh cell differentiation to influence an autoimmune reaction in this SS model.

Ascl2⁺ CD4⁺ Cells in Thymectomized *CD4*^{Cre} *Bcl6*^{fl/fl} Mice

Although *Bcl6* is recognized as a master regulatory molecule for Tfh cell differentiation,³² the relationship between *Bcl6* and *Ascl2* within the autoimmune response associated with Tfh cells remains unclear. Therefore, *Ascl2* expression of CD4⁺ T cells was analyzed in C57BL/6-background wild-type (WT) and *CD4*^{Cre} *Bcl6*^{fl/fl} mice treated with or without neonatal Tx, which resemble the SS model mice in the present study. Sham-WT or Tx-WT and *CD4*^{Cre} *Bcl6*^{fl/fl} mice, aged 20 to 24 weeks, were used to analyze *Ascl2* expression in the spleen cells. In WT mice, *Ascl2* expression of splenic CD4⁺ T cells in Tx mice was significantly higher than that of sham mice (Figure 6A); this finding is consistent with that in SS model mice. In *CD4*^{Cre} *Bcl6*^{fl/fl} mice, *Ascl2* expression of splenic CD4⁺ T cells in Tx mice was also significantly higher than that of sham mice (Figure 6, A and B). Moreover, the proportion and number of Tfh cells in the spleen of Tx-WT mice were significantly

higher than those in sham-WT mice (Figure 6, C and D); these values from both sham-*CD4^{Cre} Bcl6^{fl/fl}* and Tx-*CD4^{Cre} Bcl6^{fl/fl}* mice were considerably lower (Figure 6, C and D). Furthermore, the proportion and number of GC B cells in the spleen were significantly higher within Tx-WT than within sham-WT mice (Figure 6, E and F). In contrast, the proportion and number of the splenic GC B cells obtained from both sham-*CD4^{Cre} Bcl6^{fl/fl}* and Tx-*CD4^{Cre} Bcl6^{fl/fl}* mice remained decreased (Figure 6, E and F). Expression of *Ascl2* was increased in PD-1⁺ CXCR5⁺ Foxp3⁺ CD4⁺ pre-Tfh cells, and the expression was not changed in Tfh cells (Figure 6, G and H). Therefore, *Ascl2* plays a key role in the early stage of Tfh differentiation. In addition, CXCR5 expression of *Ascl2*⁺ CD4⁺ T cells was not changed between WT and *CD4^{Cre}-Bcl6^{fl/fl}* mice (Supplemental Figure S5). This result suggests that CXCR5 expression is controlled by *Ascl2*, independent of *Bcl-6*. Thus, these results suggest that *Ascl2* plays a key role in the early stage of Tfh cell differentiation, whereas *Bcl6* is a potent factor during the terminal stage of Tfh cell differentiation.

Discussion

In the present study, the relationship between autoimmunity and the GC reaction was investigated using SS model mice. The GC reaction was considerably enhanced along with the increase in the severity of autoimmune lesions in SS model mice. In particular, Tfh cells play a key role in the pathogenesis within these model mice. Positive regulators for Tfh cell differentiation, including *Ascl2*, of peripheral CD4⁺ T cells were up-regulated in SS model mice. The control of the peripheral Tfh cell differentiation may contribute to the onset and development of autoimmunity.

Tfh cells, one of the CD4⁺ T-cell subsets capable of activating B cells within the lymphoid organs, play a key role in the formation and maintenance of GCs in secondary lymphoid organs as well as in the regulation of memory B and plasma cell differentiation.^{1,2} Several studies have demonstrated that Tfh cells contribute to a variety of immune responses, including autoimmunity and infection.⁴ Increased numbers of Tfh cells have been reported in the peripheral blood and target organs of patients with SS.^{33,34} CXCR5 is highly expressed on Tfh cells and is a critical chemokine receptor for CXCL13 within homing and signaling pathways.³⁵ *Cxcr5* expression in Tfh cells is

directly controlled by *Ascl2*, which is one of the important transcription factors during the early stage of Tfh cell differentiation.³¹ Herein, both *Ascl2* and CXCR5 protein and gene expression of CD25⁺ CD4⁺ T cells were up-regulated in the SS model mice. In addition, gene expression of the other positive regulators of Tfh cell differentiation, such as *Il21* and *Il6*, was enhanced in SS model mice compared with that in control mice. The gene expression of various factors in peripheral T cells may be impaired in these neonatally thymectomized models. The expansion of T-regulatory cells is impaired via the down-regulation of transforming growth factor- β receptor genes in SS model mice.³⁶ These findings suggest that the thymic function during the neonatal stage of typical T-cell differentiation contributes to the normal peripheral T-cell differentiation.

Ascl2 is a basic helix-loop-helix transcription factor essential for various cell development processes, such as the trophoblast proliferation maintenance during placental development, nervous system development, somatic stem cell population maintenance, sensory organ development, and Tfh cell differentiation.^{31,37} *Ascl2* directly initiates Tfh cell development by controlling CXCR5 expression. Although *Ascl2* may play a key role in the pathogenesis of autoimmunity, no clear evidence concerning the association of *Ascl2*-regulated Tfh cells with the onset or development of autoimmune disease is available. A recent report has demonstrated that *Ascl2* overexpression accelerated SS lesion development in nonobese diabetic/ShiLtJ model mice and that the *Ascl2* level was higher in the peripheral blood mononuclear cells obtained from patients with SS compared with those from healthy controls.³⁸ Herein, both *Ascl2* gene and protein levels within the peripheral CD4⁺ T cells from SS model mice were higher than those of control mice.

Ectopic lymphoid structures are often observed within the inflammatory lesions in the target organs of various autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and SS.³⁹ Ectopic GCs develop in these chronic inflammatory lesions, thereby controlling antibody diversification, isotype class switching, B-cell differentiation, and oligoclonal expansion, wherein the Tfh cells, GC B cells, follicular dendritic cells, and follicular regulatory T cells form a network to contribute to an autoimmune response in the target tissues.³⁹ These ectopic lymphoid structures are often observed in the salivary gland tissues obtained from patients with SS^{19,26} and elicit the selection and differentiation of autoreactive B cells

Figure 5 Achaete-scute homologue 2 (*Ascl2*) expression of CD4⁺ T cells in SS model mice. **A:** The mRNA expression of follicular helper T cell–related genes was analyzed with DNA microarray using CD25⁺ CD4⁺ splenic T cells in control and SS model mice. Data are presented as pooled samples. **B:** *Ascl2* mRNA expression of CD25⁺ CD4⁺ T cells in control and SS model mice was analyzed using real-time quantitative RT-PCR. **C:** Intracellular *Ascl2* expression of CD4⁺ T cells from the spleen was analyzed using flow cytometry. **D:** *Ascl2*⁺ CD4⁺ T cells from the spleen are presented. **E:** C-X-C chemokine receptor type 5 (CXCR5)⁺ cells in *Ascl2*⁺ CD4⁺ T cells were analyzed using flow cytometry. Results are representative of five mice in each group. **F:** CXCR5⁺ cells in *Ascl2*⁺ CD4⁺ T cells are presented. **G:** *Ascl2* expression of CD4⁺ T cells stimulated with anti-CD3/CD28 monoclonal antibody–coated beads (cell/bead ratio = 1:0, 1:0.5, or 1:1) was analyzed using flow cytometry. **H:** *Ascl2* expression is presented as mean fluorescein intensity (MFI) in CD4⁺ T cells from triplicate samples. Data are expressed as means $\pm$ SD (**A**, **B**, **D**, **F**, and **H**). $n = 4$ mice in each group (**A**); $n = 5$ mice in each group (**B–D** and **F**); $n = 3$ independent experiments (**G**, using 3 mice in each group, and **H**). * $P < 0.05$, ** $P < 0.005$.

Figure 6 Change of achaete-scute homologue 2 (Ascl2) expression of CD4⁺ T cells in wild-type (WT) and *CD4^{Cre} Bcl6^{fl/fl}* mice treated with neonatal thymectomy (Tx). **A**: Ascl2 expression of CD4⁺ T cells in the spleen from WT and *CD4^{Cre} Bcl6^{fl/fl}* mice treated with sham or Tx. **B**: Ascl2 expression is shown as mean fluorescence intensity (MFI) in CD4⁺ T cells. **C**: The proportion of follicular helper T (Tfh) cells in the spleen from WT and *CD4^{Cre} Bcl6^{fl/fl}* mice treated with sham or Tx. **D**: The number of Tfh cells in the spleen from WT and *CD4^{Cre} Bcl6^{fl/fl}* mice treated with sham or Tx. **E**: The proportion of germinal center (GC) B cells in the spleen from WT and *CD4^{Cre} Bcl6^{fl/fl}* mice treated with sham or Tx. **F**: The number of GC B cells in spleen from WT and *CD4^{Cre} Bcl6^{fl/fl}* mice treated with sham or Tx. **G** and **H**: Ascl2 expressions of non-Tfh [programmed cell death-1 (PD-1)⁻ C-X-C chemokine receptor type 5 (CXCR5)⁻ forkhead box protein 3 (Foxp3)⁻ CD4⁺], pre-Tfh (PD-1⁻ CXCR5⁺ Foxp3⁻ CD4⁺), and Tfh cells of spleen in Tx C57BL/6 mice were analyzed and were expressed as MFI. Data are expressed as means $\pm$ SD (**B–F** and **H**). $n = 3$ to 5 mice in each group (**A–F**); $n = 3$ mice (**G** and **H**). * $P < 0.05$, ** $P < 0.005$, sham versus Tx; †† $P < 0.005$, non-Tfh cell versus pre-Tfh or Tfh cell. PNA, peanut agglutinin.

into high-affinity plasma cells reacting against the ribonucleoproteins anti-Ro/SSA and anti-La/SSB in SS.²⁴ In addition, B-cell lymphoma (ie, mucosa-associated lymphoid tissue lymphoma) occurs at a significantly higher rate in patients with SS.⁴⁰ Moreover, it has been reported that IL-6 and inducible costimulatory ligand expression by salivary gland epithelial cells contributes to naïve CD4⁺ T-cell differentiation into Tfh cells.³⁴ In our model, the ectopic GCs were observed in the salivary glands of aged mice, in which severe inflammatory lesions were observed. The proportion of Tfh cells in the spleen of the SS model mice was closely related to the disease severity of autoimmune lesions, suggesting that Tfh cells play a key role in the formation or acceleration of these autoimmune lesions, including the ectopic lymphoid structures within interactions with the salivary gland epithelial cells in the target organ. However, it is difficult to explain the detailed relationship between the increased number of Tfh cells in the periphery and the autoimmune lesions of SS model mice. Flow cytometric analysis showed that Tfh cells were hardly detected in the salivary gland tissues of the SS model mice (K.O., unpublished data), suggesting that Tfh cells play an indirect role in the formation of autoimmune lesions of the target organ. Increased Tfh cells in the periphery may promote autoimmune response in the lymphoid organs, but not the target organ.

In this model, inflammatory lesions are found in the salivary and lacrimal glands from 6 weeks of age. In addition, pulmonary lesions, including lymphocyte infiltration in the interstitial regions around bronchi and blood vessel, are observed from 8 weeks of age, in addition to the inflammatory lesions in lacrimal and salivary glands (A.U., unpublished data). Moreover, rheumatoid arthritis-like lesions are also observed in this aged model of >12 months of age.⁴¹ Autoimmune lesions are well known to be overlapped with multiple organs, such as lung, kidney, and joint. Thus, the inflammatory lesions in this model closely resemble those in patients with SS, and this mouse model has been used for analyzing the pathogenesis and therapeutic studies of SS.^{36,42,43} Moreover, a significant decrease in tear and saliva secretion of this model is observed compared with that in control mice.^{28,44} In addition, although the cell number of Tfh cells in SS model mice was significantly increased compared with that in control mice, cell numbers of type 1, 2, and 17 helper T cells were not changed between control and SS model mice. Otherwise, cell number of T-regulatory cells in SS model mice was significantly decreased compared with that in control mice described in a previous report.³⁶ However, it has been still unclear why cell number of Tfh cells relates to the severity of autoimmune lesions of salivary glands in this model.

As the pathogenesis of SS is extremely complex, fundamental treatments for this syndrome have not yet been established.^{6,7,45} Treatment with anti-CD20 mAb (eg, rituximab) exhibits effectiveness for improving the lacrimal gland function but not the salivary flow. In addition, no

differences were observed at the 24-week evaluation regarding fatigue reduction (30% visual analog scales), serious adverse event occurrence, quality of life improvement, or disease activity by treatment of anti-CD20 mAb for patients with primary SS.⁴⁶ Moreover, a report has demonstrated that the number of peripheral Tfh cells in patients with SS was significantly reduced by rituximab treatment.³⁰ In the present study, the treatment with anti-CD20 mAb in SS model mice resulted in autoimmune lesion suppression as well as Tfh and GC B-cell reduction. However, autoantibody levels in the sera of SS model mice remained unchanged by anti-CD20 mAb treatment. Furthermore, no significant correlation was observed between the number of GC cells in the spleen and the disease severity of autoimmune lesions in the SS model mice. These findings suggest that Tfh cells closely contribute to the formation of the inflammatory lesions in SS target organs. Moreover, neonatal Tx diminished T-cell maintenance in the periphery and resulted in *Ascl2* up-regulation of CD4⁺ T cells after an increase in Tfh cells within both SS model mice and C57BL/6-WT mice. The study using *CD4^{Cre} Bcl6^{fl/fl}* mice demonstrated that the terminal Tfh cell differentiation is determined by *Bcl6*, as described previously.³¹ *Ascl2* is a potent factor for controlling Tfh cell polarization from CD4⁺ T cells at the early stages during the T-cell activation process. In SS model mice, an autoimmune response may be enhanced by a promoted GC reaction contributed to by an increase of *Ascl2*-controlled Tfh cells.

In conclusion, the GC reaction was enhanced in SS model mice. The formation of autoimmune lesions was closely associated with the number of Tfh cells in the present model. In addition, Tfh cell-related genes, such as *Ascl2*, were observed to be up-regulated in SS model mice. *Ascl2* is a key molecule for the pathogenesis of SS, and targeting Tfh cells could be a useful therapeutic strategy for the treatment of autoimmune diseases.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.08.008>.

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