

## Th2 and Regulatory Immune Reactions Contribute to IgG4 Production and the Initiation of Mikulicz Disease

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**Objective.** Mikulicz disease has been considered to be a subtype of Sjögren's syndrome (SS). However, recent studies have suggested that Mikulicz disease is an IgG4-related disease and is distinguishable from SS. In addition, it has been reported that both interleukin-4 (IL-4) and IL-10 induce IgG4 production and inhibit IgE. This study was undertaken to examine the expression of these cytokines in patients with Mikulicz disease and patients with SS.

**Methods.** Labial salivary gland (LSG) sections from 15 patients with Mikulicz disease and 18 patients with SS were examined for subsets of the infiltrating lymphocytes, expression patterns of messenger RNA (mRNA) for cytokines/chemokines, and relationships between the IgG4:IgG ratio and the expression of mRNA for IL-4 or IL-10.

**Results.** Immunohistochemical analysis showed lymphocyte infiltration of various subsets in the LSGs of SS patients, and the selective infiltration of IgG4-positive plasma cells and Treg cells in the LSGs of Mikulicz disease patients. The levels of mRNA for both Th1 and Th2 cytokines and chemokines in LSGs from

patients with SS were significantly higher than in controls, while the expression of both Th2 and Treg cells was significantly higher in the patients with Mikulicz disease than in controls. Furthermore, the expression of IL-4 or IL-10 in the LSGs was correlated with the IgG4:IgG ratio.

**Conclusion.** These results suggest that the pathogenesis of Mikulicz disease is different from that of SS. Mikulicz disease is a unique inflammatory disorder characterized by Th2 and regulatory immune reactions that might play key roles in IgG4 production.

Mikulicz disease has been considered to be a subtype of Sjögren's syndrome (SS), based on the histopathologic similarities between the two diseases (1). However, Mikulicz disease shows several differences in comparison with typical SS. In Mikulicz disease, enlargement of the lacrimal and salivary glands is persistent, salivary secretion is either normal or moderately dysfunctional, patients have a good response to corticosteroid treatment, and hypergammaglobulinemia and low frequencies of anti-SSA and anti-SSB antibodies are found on serologic analyses. Since Yamamoto et al (1–3) reported that serum IgG4 levels are elevated and IgG4-positive plasma cells infiltrate into the gland tissue in Mikulicz disease, these symptoms have also been recognized in autoimmune pancreatitis (4), primary sclerosing cholangitis (5), tubulointerstitial nephritis (6), interstitial pneumonia (7), Ridel's thyroiditis (8), and Küttner's tumor (9). These diseases are now called "IgG4-related diseases" (2,10). IgG4 is a Th2-dependent Ig and has low affinity for target antigen. Interleukin-4 (IL-4) directs naive human B cells to switch to IgG4 and IgE production (11).

CD4+ T helper cells including at least 5 subsets have been identified. Th0, Th1, Th2, Th17, and Treg cells are generally considered to maintain the balance

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and homeostasis of the immune system and possibly to induce various diseases by their impaired regulation. The difference in the functions of Th1 and Th2 cells has been characterized by the patterns of cytokines secreted by these Th cells. Th1 cells induced by IL-12 are mainly responsible for cell-mediated immunity, while Th2 cells induced by IL-4 are responsible for humoral immunity. These Th subsets are then mutually controlled by the cytokine that each produces. Several studies have indicated that many autoimmune diseases or allergic diseases are caused by the collapse of the Th1/Th2 balance. Th0 cells are produced by both Th1 and Th2 cytokines and are considered to be precursors of Th1 and Th2 cells. Treg cells are essential for the maintenance of immunologic self-tolerance and immune homeostasis. Recently, a subset of IL-17-producing T cells (Th17 cells) distinct from Th1 and Th2 cells was described and was shown to play a crucial role in the induction of autoimmunity and allergic inflammation (12). Furthermore, it has been demonstrated that chemokines are intimately involved in the Th1/Th2 balance and immune responses in various diseases, such as rheumatoid arthritis (13), systemic lupus erythematosus (13,14), SS (13,15), systemic sclerosis (13,16), idiopathic inflammatory myopathy (13), and atopic dermatitis (17).

The relationship of Th1/Th2 imbalance to the pathogenesis of SS has been investigated widely, and a polarized Th1 balance has been associated with the immunopathology of the disease (18–20). Numerous interferon- $\gamma$  (IFN $\gamma$ )-positive CD4+ T cells are detected in the salivary glands of SS patients, and an intracellular cytokine assay demonstrated subsequent promotion of Th1 cells in SS (21). We have previously shown that SS was initiated and/or maintained by Th1 cytokines and subsequently progressed in association with Th2 cytokines (22). Ogawa et al (15) reported that Th1 chemokines, such as IFN $\gamma$ -inducible 10-kd protein (IP-10) and monokine induced by IFN $\gamma$ , are involved in the accumulation of T cell infiltrates in the salivary glands of patients with SS. These findings suggest that Th1 cells play a central role in the pathogenesis of SS.

In contrast, patients with Mikulicz disease frequently have a history of bronchial asthma and allergic rhinitis and show severe eosinophilia and elevated serum IgE levels. We previously reported that peripheral CD4+ T cells from patients with Mikulicz disease revealed deviation of the Th1/Th2 balance to Th2 and elevated the expression of Th2-type cytokines (23,24). Moreover, recent studies have indicated that peripheral blood CD4+ T cells in patients with IgG4-related lacrimal gland enlargement showed a Th2 bias and elevated

serum IgE levels (24). Therefore, it is suggested that Mikulicz disease has a Th2-predominant phenotype. The findings of a previous study showing that autoimmune pancreatocholangitis, which is an IgG4-related disease, could also be characterized by the overproduction of Th2 and regulatory cytokines (25) deserve our attention.

To date, pathogenetic differences between immune responses in SS and Mikulicz disease are not well understood. In this study, we identified the expression patterns of cytokines, chemokines, and chemokine receptors in the salivary glands of these diseases to clarify the involvement of characteristic immune responses in the development of Mikulicz disease.

## PATIENTS AND METHODS

**Patients.** Fifteen patients with Mikulicz disease (12 women and 3 men with a mean  $\pm$  SD age of  $56.3 \pm 13.0$  years) and 18 patients with SS (16 women and 2 men with a mean  $\pm$  SD age of  $54.6 \pm 12.8$  years) who were referred to the Department of Oral and Maxillofacial Surgery at Kyushu University Hospital were included in the study. Mikulicz disease was diagnosed according to the following criteria (3): persistent symmetrical swelling (lasting longer than 3 months) of  $>2$  lacrimal and major salivary glands, elevated serum levels of IgG4 ( $>135$  mg/dl), and infiltration of IgG4-positive plasma cells in the tissue (ratio of IgG4-positive cells:IgG-positive cells  $>40\%$ ) on immunostaining. SS was diagnosed according to the criteria of both the Research Committee on SS of the Ministry of Health and Welfare of the Japanese Government (1999) (26) and the American-European Consensus Group criteria for SS (27).

All patients exhibited objective evidence of salivary gland involvement based on the presence of subjective xerostomia and a decreased salivary flow rate, abnormal findings on parotid sialography, and focal lymphocytic infiltrates in the labial salivary glands (LSGs) and submandibular glands. All patients with SS had primary SS with strong lymphocytic infiltration in the LSGs, had no other autoimmune diseases, and had never been treated with corticosteroids or any other immunosuppressants. LSG biopsies were performed as described by Greenspan et al (28). As controls, LSGs biopsy specimens were obtained from 18 patients with mucocoeles who had no clinical or laboratory evidence of systemic autoimmune disease. These control LSGs were all histologically normal. Written informed consent was obtained from all patients and healthy controls.

**Histologic analysis of LSGs.** Formalin-fixed and paraffin-embedded sections (4  $\mu$ m) of LSG specimens were prepared and stained with hematoxylin and eosin for conventional histologic examinations. The degree of lymphocytic infiltration in the specimens was judged by focus scoring (28,29). One standardized score is the number of focal inflammatory cell aggregates containing 50 or more mononuclear cells in each 4-mm<sup>2</sup> area of salivary gland tissue (30). All of the patients with Mikulicz disease and patients with SS

in this study had strong lymphocytic infiltration (focus scores of 10–12).

**Immunohistochemical analysis of LSGs.** For the immunohistochemical analysis of lymphocyte subsets, 4- $\mu$ m formalin-fixed and paraffin-embedded sections were prepared and stained by a conventional avidin–biotin complex technique as previously described (31). The mouse monoclonal antibodies used to analyze lymphocyte subsets were anti-CD4 (clone B12; MBL), anti-CD20 (clones L26 and M0755; Dako), and anti-FoxP3 (clone mAbcam 22510; Abcam). The mouse monoclonal antibody and rabbit polyclonal antibody used to analyze IgG4-positive and IgG-positive plasma cells were anti-IgG (A0423; Dako) and anti-IgG4 (The Binding Site). HDP-1 (antidinitrophenyl [anti-DNP] IgG1) was used as a control mouse monoclonal antibody. The polyclonal antibodies used to analyze the cytokines were anti-IL-4 (clone ab9622), anti-IL-10 (clone ab34843), anti-IFN $\gamma$  (clone ab9657) (all from Abcam), and anti-IL-17 (clone sc-7927; Santa Cruz Biotechnology). SS1 (anti-sheep erythrocyte IgG2a), NS8.1 (anti-sheep erythrocyte IgG2b), and NS4.1 (anti-sheep erythrocyte IgM), were used as control rabbit polyclonal antibodies. The mouse monoclonal antibodies used to analyze the chemokines and chemokine receptors were anti-IP-10 (clone ab73837; Abcam), anti-CXCR3 (clone ab64714; Abcam), anti-thymus and activation-regulated chemokine (anti-TARC) (54015; R&D Systems), anti-macrophage-derived chemokine (anti-MDC) (57203; R&D Systems), and anti-CCR4 (MAB1567; R&D Systems). HDP-1 (anti-DNP IgG1) was used as a control mouse monoclonal antibody. The sections were sequentially incubated with primary antibodies, biotinylated anti-mouse IgG secondary antibodies (Vector Laboratories), avidin–biotin–horseradish peroxidase complex (Vector Laboratories), and 3,3'-diaminobenzidine (Vector Laboratories). Mayer's hematoxylin was used for counterstaining. Photomicrographs were obtained using a light microscope equipped with a digital camera (CoolSNAP; Photometrics). Stained IgG4-positive cells and IgG-positive cells were counted in 1-mm<sup>2</sup> sections from 5 different areas, and the ratio of IgG4-positive cells to IgG-positive cells was calculated.

**RNA extraction and complementary DNA (cDNA) synthesis.** Total RNA was prepared from the LSG specimens by the acid guanidinium–phenol–chloroform method as previously described (32–34). Three micrograms of the total RNA preparation was then used for the synthesis of cDNA. Briefly, RNA was incubated for 1 hour at 42°C with 20 units of RNasin ribonuclease inhibitor (Promega), 0.5  $\mu$ g of oligo(dT)<sub>12–18</sub> (Pharmacia), 0.5 mM of each dNTP (Pharmacia), 10 mM of dithiothreitol, and 100 units of RNase H reverse transcriptase (Life Technologies).

**Quantitative estimation of messenger RNA (mRNA) by real-time polymerase chain reaction (PCR).** Quantitative cDNA amplification was performed according to the recommendations of the manufacturer and as previously described (32–34). The cDNAs for the cytokines, chemokines, and chemokine receptors were analyzed by real-time PCR using LightCycler FastStart DNA Master SYBR Green 1 (Roche Diagnostics) in a LightCycler real-time PCR instrument (version 3.5; Roche Diagnostics). The cytokines, chemokines, and chemokine receptors examined were IL-2, IFN $\gamma$ , IL-12, IP-10, CXCR3, IL-4, IL-5, TARC, MDC, CCR4, IL-10, transforming

growth factor  $\beta$  (TGF $\beta$ ), FoxP3, IL-17, and IL-6. The markers of lymphocytes examined were IgG and IgG4.

The primer sequences used were as follows: for  $\beta$ -actin (260 bp), forward 5'-GCAAAGACCTG-TACGCCAAC-3', reverse 5'-CTAGAAGCATTTTGGCGTGGA-3'; for CD3 $\delta$  (184 bp), forward 5'-GATGTCATTGCCACTCTGC-3', reverse 5'-ACTTGTTCCGAGCCAGTT-3'; for IL-2 (416 bp), forward 5'-ACTCACCAGGATGCTCACAT-3', reverse 5'-AGGTAATCCATCTG-TTCAGA-3'; for IFN $\gamma$  (355 bp), forward 5'-AGTTATATCTTGGCTTTTCA-3', reverse 5'-ACCGAATAATTAGTCAGCTT-3'; for IL-4 (203 bp), forward 5'-CTGCCTCCAAGAACAACACT-3', reverse 5'-CACAGGACAGGAATTCAAGC-3'; for IL-5 (104 bp), forward 5'-ATGAGGATGCTTCTGCATTTG-3', reverse 5'-TCAACTTTCTATTATCCACTCG-3'; for IL-6 (115 bp),

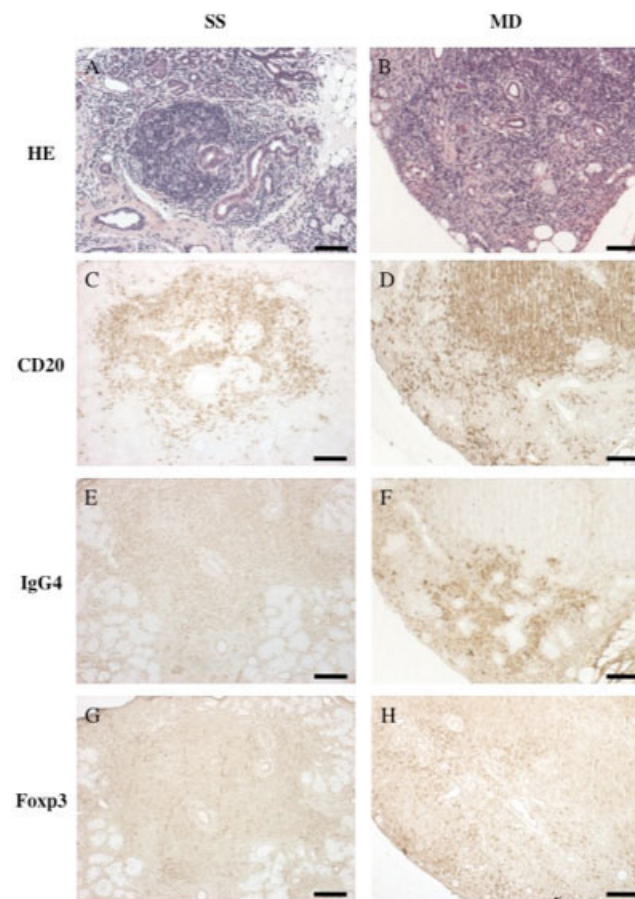


**Figure 1.** Selective infiltration of IgG4-positive plasma cells and FoxP3-positive Treg cells in the labial salivary glands (LSGs) of patients with Mikulicz disease (MD). Sections from the LSGs of a representative patient with Sjögren's syndrome (SS) and a representative patient with Mikulicz disease were immunostained with hematoxylin and eosin (H&E) (A and B) and anti-CD20 (C and D), anti-IgG4 (E and F), and anti-FoxP3 (G and H) monoclonal antibodies. Counterstaining with Mayer's hematoxylin was subsequently performed. Bars = 100  $\mu$ m; original magnification  $\times$  100.

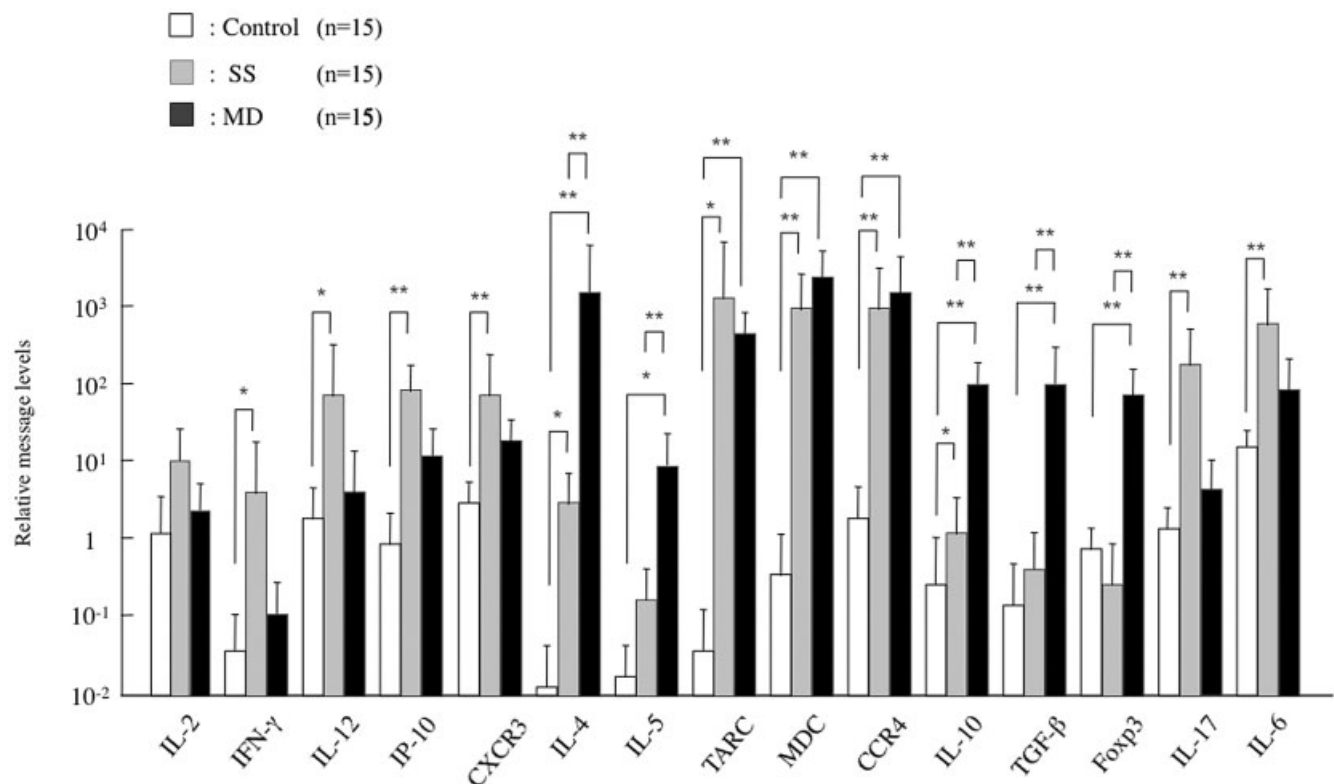


**Figure 2.** Expression patterns of mRNA for cytokines, chemokines, and chemokine receptors in LSGs from controls, patients with SS, and patients with Mikulicz disease. Levels of interferon- $\gamma$  (IFN $\gamma$ ), interleukin-2 (IL-2), IL-12, IFN $\gamma$ -inducible 10-kd protein (IP-10), and CXCR3 (Th1 type); IL-4, IL-5, thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), and CCR4 (Th2 type); IL-10, transforming growth factor  $\beta$  (TGF $\beta$ ), and FoxP3 (Treg cell type); and IL-6 and IL-17 (Th17 type) were quantitatively estimated as described in Patients and Methods. Levels of mRNA in LSGs from SS patients and patients with Mikulicz disease were compared with those in control LSGs. Bars show the mean  $\pm$  SD. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$  by Mann-Whitney U test. See Figure 1 for other definitions.

forward 5'-GGCACTGGCAGAAAACAA-3', reverse 5'-CTCCAAAAGACCAGTGATGA-3'; for IL-10 (351 bp), forward 5'-ATGCCCCAAGCTGAGAACCAA-3', reverse 5'-TCTCAAGGGGCTGGGTGAGCTA-3'; for IL-12 (187 bp), forward 5'-CCTGACCCACCCAAGAAGTT-3', reverse 5'-GTGGCTGAGGTCTGTGTCGT-3'; for IL-17 (186 bp), forward 5'-GCAGGAATCACAATCCAC-3', reverse 5'-TCTCTCAGGGTCCTCATTGC-3'; for FoxP3 (207 bp), forward 5'-CCCCTTGCCCCACTTACA-3', reverse 5'-GCCACGTTGATCCCAGGT-3'; for TGF $\beta$  (142 bp), forward 5'-GCCCCCTACATTTGGAGCCTG-3', reverse 5'-TTGCGGCCACGATAGTACAC-3'; for IgG (129 bp), forward 5'-CAAGTGCAAGGTCTCCAACA-3', reverse 5'-TGTTCTTGGTCAGCTCATC-3'; for IgG4 (132 bp), forward 5'-ACTCTACTCCCTCAGCAGCG-3', reverse 5'-GGGGGACCATATTTGGAC-3'; for IP-10 (288 bp), forward 5'-CCTTAAACAGAGGGGAGC-3', reverse 5'-AGCAGGTGAGAACATCCAC-3'; for CXCR3 (184 bp), forward 5'-CTGGTGGTGCTGGTGGACAT-3', reverse 5'-AGAGCAGCATCCACATCCG-3'; for MDC (253 bp), forward 5'-CGCGTGGTGAAACACTTCTA-3', reverse 5'-GAATGCAGAGAGTTGGCACA-3'; for TARC (140 bp), forward 5'-TAGAAAGCTGAAGACGTGGT-3', reverse 5'-

GGCTTTGCAGGTATTTAACT-3'; for CCR4 (214 bp), forward 5'-GTGCTCTGCCAATACTGTGG-3', reverse 5'-CTTCCTCCTGACACTGGCTC-3'; and for CD3 $\alpha$  (184 bp), forward 5'-GATGTCATTGCCACTCTGC-3', reverse 5'-ACTTGTTCCGAGCCAGTT-3'.

In order to provide a meaningful comparison between different individuals or samples, we calculated the relative amounts of the PCR products of these molecules to the amounts of the PCR products of CD3 $\delta$  (for the standardization of T cell mRNA) or the PCR products of  $\beta$ -actin (for the standardization of total cellular mRNA) in each sample, as previously described (22,23,35,36). The CD3 $\delta$  PCR product levels were used for T cell-specific molecules, such as IL-2, IL-5, IL-12, and IFN $\gamma$ , while the  $\beta$ -actin PCR product levels were used for T cell-nonspecific molecules, such as IL-4, IL-6, IL-10, IL-17, and chemokines, which were produced by a variety of cell types.

**Statistical analysis.** The statistical significance of the differences between the groups was determined by the Mann-Whitney U test and Spearman's rank correlation. All statistical analyses in this study were performed using JMP software, version 8 (SAS Institute).  $P$  values less than 0.05 were considered significant.

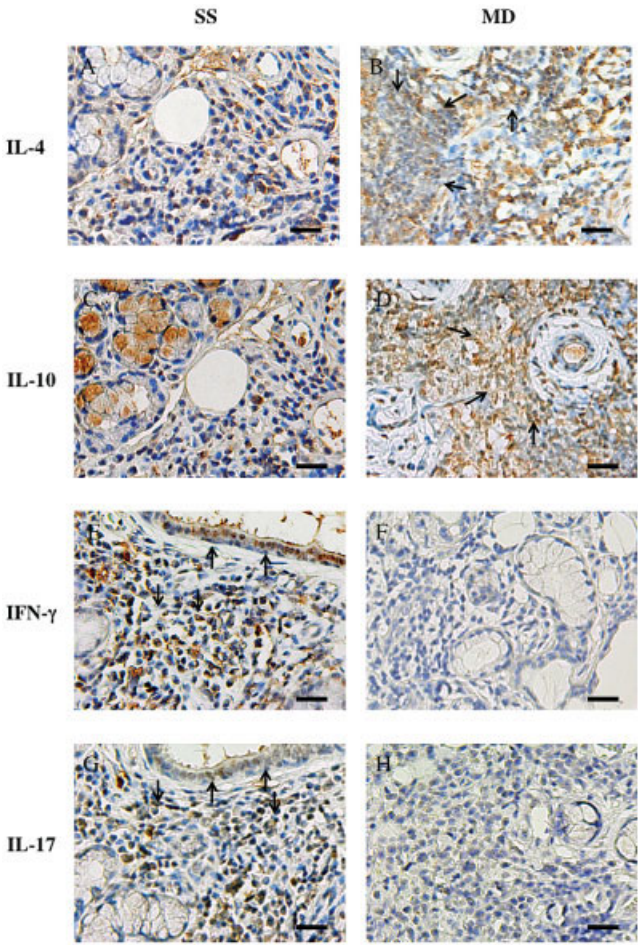


**Figure 3.** Expression of interleukin-10 (IL-10), IL-4, interferon- $\gamma$  (IFN $\gamma$ ), and IL-17 in LSG specimens from patients with SS and patients with Mikulicz disease. IL-10 and IL-4 were prominently expressed around the germinal centers in LSG specimens from patients with Mikulicz disease. Sections from LSGs of a representative patient with SS and a representative patient with Mikulicz disease were immunostained with anti-IL-10 (A and B), anti-IL-4 (C and D), anti-IFN $\gamma$  (E and F), and anti-IL-17 (G and H) polyclonal antibodies. Counterstaining with Mayer's hematoxylin was subsequently performed. **Arrows** indicate key features of infiltrating cells. Bars = 50 mm; original magnification  $\times$  400. See Figure 1 for other definitions.

**RESULTS**

**Results of histologic analysis of lymphocyte subsets in the LSGs.** Representative sections showing histologic findings and lymphocyte subsets in LSG specimens from patients with Mikulicz disease and patients with SS are shown in Figure 1. Specimens from patients with SS showed lymphocytic infiltration of various subsets with atrophy or severe destruction of the acini, while

specimens from patients with Mikulicz disease showed selective infiltration of IgG4-positive plasma cells and FoxP3-positive Treg cells around the acinar and ductal cells with a lot of lymphoid follicles and mild destruction of the acini.

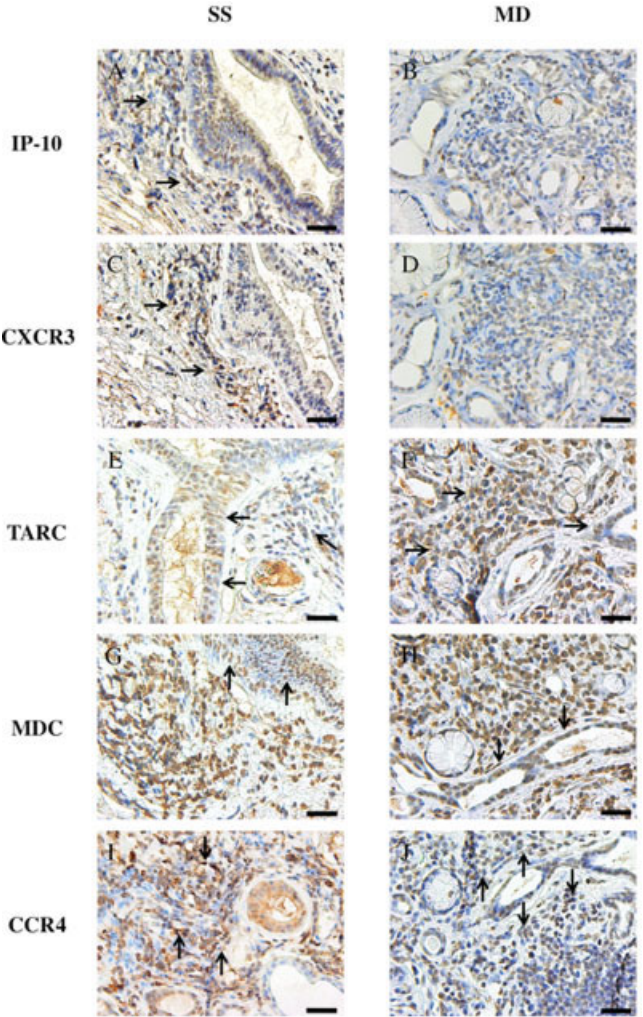


**Figure 4.** Expression of interferon- $\gamma$ -inducible 10-kd protein (IP-10), CXCR3, thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), and CCR4 in LSG specimens from patients with SS and patients with Mikulicz disease. TARC and MDC were strongly expressed around the germinal centers in LSG specimens from patients with Mikulicz disease. Sections from LSGs of a representative patient with SS and a representative patient with Mikulicz disease were immunostained with anti-IP-10 (A and B), anti-CXCR3 (C and D), anti-TARC (E and F), anti-MDC (G and H), and anti-CCR4 (I and J) monoclonal antibodies. Counterstaining with Mayer's hematoxylin was subsequently performed. **Arrows** indicate key features of infiltrating cells. Bars = 50 mm; original magnification  $\times$  400. See Figure 1 for other definitions.

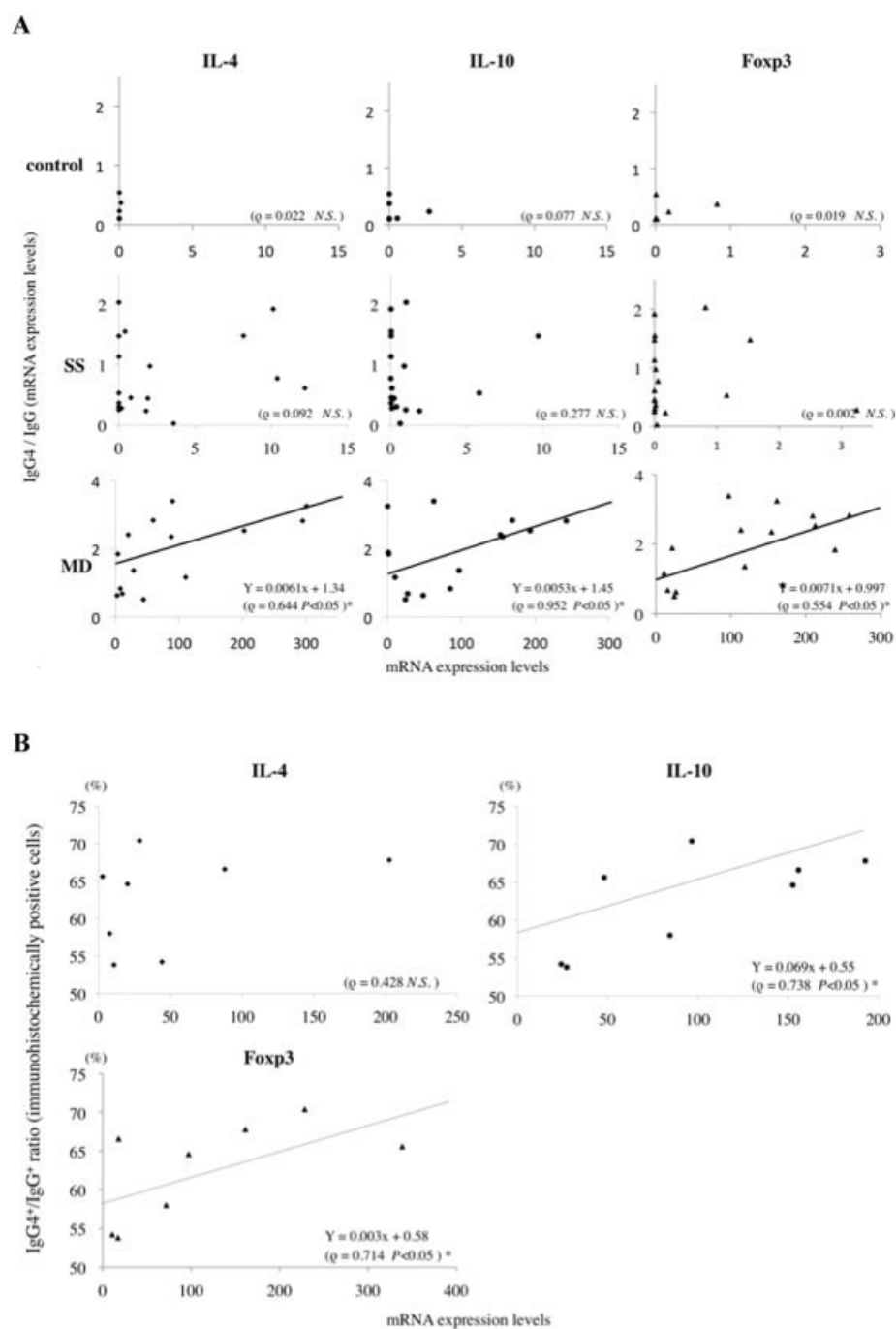


**Figure 5.** Correlation of the production of IgG4 with the production of interleukin-4 (IL-4), IL-10, and FoxP3 in LSGs from patients with Mikulicz disease. **A**, Correlations between the ratio of IgG4 mRNA to IgG mRNA and the expression of mRNA for IL-4, IL-10, and FoxP3 in LSGs from patients with SS, patients with Mikulicz disease, and healthy controls. Real-time polymerase chain reaction products for IL-4, IL-10, FoxP3, IgG, and IgG4 were quantitatively estimated as described in Patients and Methods. The ratio was calculated as IgG4-positive cells/IgG-positive cells  $\times$  100. The counts were obtained in 1-mm<sup>2</sup> sections from 5 different areas. **B**, Correlations between the frequencies of IgG4-positive cells and the expression of mRNA for IL-4, IL-10, and FoxP3 in LSGs from patients with Mikulicz disease. \* =  $P < 0.05$  by Spearman's rank correlation. NS = not significant (see Figure 1 for other definitions).

**Expression of mRNA for cytokines, chemokines, and chemokine receptors in the LSGs.** In order to compare the expression of mRNA for cytokines, chemokines, and chemokine receptors in LSGs from patients with Mikulicz disease and LSGs from patients with SS, the relative expression compared to CD3 $\delta$  was estimated and compared for cytokines and chemokine receptors primarily expressed by T cells, and the relative expression compared to  $\beta$ -actin was estimated for cytokines and chemokines produced by a variety of cell types.

The expression of mRNA for IFN $\gamma$ , IL-12, IP-10, CXCR3, IL-4, TARC, MDC, CCR4, IL-10, IL-17, and IL-6 in LSGs from SS patients were higher than those in control LSGs (Figure 2). The expression of mRNA for IL-4, IL-5, TARC, MDC, CCR4, IL-10, TGF $\beta$ , and FoxP3 in LSGs from patients with Mikulicz disease were higher than those in control LSGs (Figure 2). In addition, the levels of expression of mRNA for IL-4, IL-5, IL-10, TGF $\beta$ , and FoxP3 in LSGs from patients with Mikulicz disease were higher than in LSGs from patients with SS (Figure 2).

**Protein levels of cytokines, chemokines, and chemokine receptors in the LSGs.** The specimens were immunohistochemically examined to evaluate the distributions of these proteins in LSGs from patients with SS and patients with Mikulicz disease. The Th1-type cytokine IFN $\gamma$  and Th17-type cytokine IL-17 were strongly expressed and detected in and around the ductal epithelial cells in LSGs from SS patients only (Figures 3E and G). Although IL-10 and IL-4 were detected in LSGs from both patients with Mikulicz disease and patients with SS, they were prominently expressed around germinal centers in LSGs from Mikulicz disease patients but not in LSGs from SS patients (Figures 3B and D). In LSGs from patients with SS, IP-10 and CXCR3 were detected in a higher number of infiltrating lymphocytes than in LSGs from patients with Mikulicz disease (Figures 4A and C). In LSGs from patients with Mikulicz disease, TARC and MDC were strongly expressed, especially around germinal centers (Figures 4F and H). In LSGs from both patients with SS and patients with Mikulicz disease, CCR4 was detected in high numbers of infiltrating lymphocytes (Figures 4I and J).

**Relationship between IgG4 production and cytokine expression in the LSGs.** The relationships between IgG4 production and the expression of mRNA for IL-4, IL-10, and FoxP3 in the LSGs were examined. These molecules were all positively correlated with the ratio of IgG4 mRNA to IgG mRNA in LSGs from patients with Mikulicz disease, but no relationships were confirmed in those from SS patients (Figure 5A). Furthermore, IL-10

mRNA and FoxP3 mRNA in LSGs from patients with Mikulicz disease were correlated with the ratio of IgG4 to IgG in immunohistochemically positive cells (Figure 5B).

## DISCUSSION

Mikulicz disease presents with bilateral and persistent swelling of the lacrimal and salivary glands, and it has been considered to be part of primary SS or a subtype of primary SS since the findings by Morgan and Castleman were published in 1953 (37). However, Yamamoto et al (38,39) reported differences in the clinical and histopathologic findings between Mikulicz disease and SS. Serologically, Mikulicz disease patients show hypergammaglobulinemia, hypocomplementemia, and high levels of serum IgG4, but are negative for anti-SSA and anti-SSB antibodies. Immunohistologic analysis of samples from patients with Mikulicz disease revealed the selective infiltration of IgG4-positive plasma cells, which was not observed near acinar and ductal cells. In contrast, similar specimens from SS patients showed no IgG4-positive plasma cells (38,39). In this study, samples from patients with Mikulicz disease showed selective infiltration of IgG4-positive plasma cells and FoxP3-positive cells around acinar and ductal cells with mild destruction of the acini, while samples from patients with SS showed no infiltration of IgG4-positive plasma cells and FoxP3-positive cells, and had atrophy or severe destruction of acini (Figure 1).

In order to examine the differences in infiltrating lymphocytes between LSGs from patients with SS and LSGs from patients with Mikulicz disease, we analyzed the levels of cytokines, chemokines, and chemokine receptors. The levels of Th1-, Th2-, and Th17-type molecules in LSGs from SS patients were significantly higher than those in LSGs from controls. The levels of Th2 and Treg-type molecules in LSGs from patients with Mikulicz disease were significantly higher than those in LSGs from controls. Furthermore, immunohistochemical staining indicated that IFN $\gamma$  and IL-17 were strongly detected in and around ductal epithelial cells in LSGs from SS patients only, while IL-4 and IL-10 were detected in LSGs from both patients with SS and patients with Mikulicz disease. In particular, these cytokines were prominently expressed around germinal centers in specimens from patients with Mikulicz disease but not in specimens from patients with SS.

It is generally accepted that CD4 $^{+}$  Th cells play a crucial role in the pathogenesis of SS. Several studies of autoimmune diseases have demonstrated pathoge-

netic roles for Th1 cells and the possible protective role for Th2 cells (40,41). Our previous studies of SS suggested that the mutual stimulation of Th1 cells and their target organs via the production of various cytokines plays a key role in the induction and maintenance of SS and results in the eventual destruction of the target organ (22,42,43). Subsequently, additional Th2 cells then stimulate B cells to differentiate, proliferate, and produce immunoglobulins and, thus, play a role in the lymphoaggressiveness of SS. Regarding the possible roles of Th2 cells in the induction of B cell abnormalities, these cells might have an important association with the progression of SS. In contrast, Zen et al (25) reported that autoimmune pancreatocholangitis, an IgG4-related disease, is characterized by immune reactions that are predominantly mediated by Th2 cells and Treg cells.

The results of the present study concerning the levels of cytokines, chemokines, and chemokine receptors in the LSGs are consistent with the model of SS and Mikulicz disease as distinct diseases. Immunohistochemical staining indicated that MDC and TARC were detectable in and around the ductal epithelial cells and germinal centers, while CCR4 was expressed on the infiltrating lymphocytes in the LSGs in both SS patients and patients with Mikulicz disease. The interactions of CCR4 with MDC and TARC are suggested to play a critical role in the accumulation of Th2 cells and, consequently, the progression of SS and Mikulicz disease. TARC and MDC are natural ligands for CCR4 on Th2 cells (44,45). In contrast, IP-10 was detected in and around the ductal epithelial cells, while CXCR3 was expressed on the infiltrating lymphocytes in the LSGs in SS patients only. IP-10 is a natural ligand for CXCR3 on Th1 cells (15).

It is well known that allergic immune responses are the development of allergen-specific Th2-type cytokines IL-4 and IL-13, which are responsible for IgG4 and IgE induced by B cells (46). In our previous studies, we demonstrated that Th2 immune reactions contributed to Mikulicz disease and IgG4-related tubulointerstitial nephritis (23,24,35). The expression profile of cytokines demonstrated in this study suggested that Mikulicz disease was characterized by an intense expression of Th2 and regulatory cytokines (Figure 2). In addition, recent studies have shown that class switching of IgG4 is caused by costimulation with IL-4 and IL-10, and that IL-10 decreased IL-4-induced IgE switching but elevated IL-4-induced IgG4 production (47).

Treg cells exert their effects through the modulation of both T and B cell responses, and two subsets of

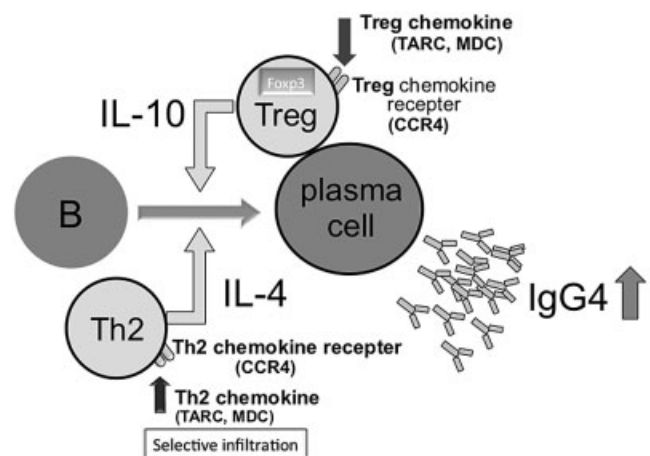


**Figure 6.** Schematic model of the mechanisms underlying IgG4 production. TARC = thymus and activation-regulated chemokine; MDC = macrophage-derived chemokine; IL-10 = interleukin-10.

Treg cells, CD4+CD25+FoxP3+ Treg cells (48) and IL-10-producing Tr1 cells (49), are crucial in regulating effector T cell function. CD4+CD25+FoxP3+ Treg cells are known to affect the pathogenesis of cases of autoimmune hepatitis and primary biliary cirrhosis (50). Miyoshi et al (51) showed a positive correlation between the number of mature Treg cells (CD4+CD25<sup>high</sup> Treg cells) and IgG4. These results indicated that increased numbers of CD4+CD25<sup>high</sup> Treg cells may influence IgG4 production in autoimmune pancreatocholangitis, whereas decreased numbers of naive Treg cells (CD4+CD25+CD45RA+) may be involved in the pathogenesis of the disease (51). Therefore, we examined the relationships between IgG4 and IL-4, IL-10, and FoxP3.

We found that IL-4, IL-10, and FoxP3 were positively correlated with the ratio of IgG4 mRNA to IgG mRNA in samples from patients with Mikulicz disease analyzed by real-time PCR and comparison with the IgG4 to IgG ratio of immunohistochemically positive cells. In particular, IL-10 and FoxP3 levels were strongly correlated with IgG4 production. These results suggested that Th2 and Treg cells might be involved in the pathogenesis of Mikulicz disease. The findings of the present study provided additional support for the model of Mikulicz disease as distinct from SS (Figure 6). However, accumulation of case reports and further examinations are required to elucidate the pathogenesis of the disease.

In this study, we clarified the pathogenesis of Mikulicz disease and found that it is a unique IgG4-related disease, characterized by Th2 and regulatory

immune reactions, which apparently differs from SS. A more thorough understanding of the complex mechanisms of the disease might lead to pharmacologic strategies to interrupt the interactions between chemokines and chemokine receptors or to disrupt the cytokine network as a further means of inhibiting the initiation and/or progression of Mikulicz disease.

### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nakamura had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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