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Role of Sphingosine 1-Phosphate in the Pathogenesis of Sjögren’s Syndrome

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Primary Sjögren’s syndrome (SS) is an autoimmune disease characterized by inflammatory mononuclear cell infiltration and destruction of epithelial cells of lacrimal and salivary glands. Sphingosine 1-phosphate (SIP) and signaling through its receptor SIP1, have been implicated in many critical cellular events including inflammation, cancer, and angiogenesis. This study was undertaken to examine the role of SIP1, signaling in the pathogenesis of primary SS. SIP1 and sphingosine kinase 1, which converts sphingosine to SIP, were detected in the cytoplasm of inflammatory mononuclear cells, vascular endothelial cells, and epithelial cells in all labial salivary glands by immunohistochemistry. The expression of SIP1 in inflammatory mononuclear cells was enhanced in advanced stages of primary SS. SIP enhanced proliferation and IFN-γ production by CD4+ T cells. The enhancing effect of SIP on IFN-γ production by CD4+ T cells was stronger in patients with primary SS than in healthy controls. SIP also enhanced Fas expression and Fas-mediated caspase-3 induction in salivary gland epithelial cells. IL-6 expression was detected in the cytoplasm of inflammatory mononuclear cells and ductal epithelial cells and was enhanced in advanced stages of primary SS. Furthermore, both IFN-γ and SIP augmented IL-6 secretion by salivary gland epithelial cells. These effects of SIP were inhibited by pretreatment of pertussis toxin. Our data reveal that SIP1, signaling may modulate the autoimmune phenotype of primary SS by the action of immune as well as epithelial cells. The Journal of Immunology, 2008, 180: 1921–1928.

Materials and Methods

Patients and samples

Peripheral bloods for CD4+ T cell isolations were obtained from six healthy volunteers (four women and two men) with a mean age of 39.5 years (range 31–55 years) and five patients with primary SS (four women and one man) with a mean age of 53.5 years (range 23–79 years). The healthy controls were subjected who had experienced subjective symptoms of oral dryness, but met none of the objective criteria for a diagnosis of primary SS. Informed consent was obtained from all subjects before CD4+...
T cells sampling and LSG biopsy were performed, and the institutional medical ethics committee approved the study protocol. All patients fulfilled the American-European Consensus Group criteria for a diagnosis of primary SS (16). Grading of LSG biopsies was performed based on size and degree of lymphoid organization of the infiltrates (17). Briefly, lymphocytes and plasma cells per 4 mm² were counted. Cellular aggregate with 50 or more lymphocytes, histiocytes, and plasma cells were defined as focus. Grade 1 is characterized by slight infiltration. Grade 2 displayed moderate infiltrate or less than one focus per 4 mm². Grade 4 displayed more than one focus per 4 mm².

**Immunohistochemistry for S1P, SK1, CD4, IFN-γ, and IL-6 expression in LSG**

LSG tissue specimens were preserved in 10% formalin, embedded in paraffin, and serially sectioned onto microscope slides at a thickness of 4 μm. Immuno-reactive protein was detected using an indirect immunoperoxidase method. All procedures were performed at room temperature. Tissue sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 5 min. S1P staining was performed as follows. The sections were preincubated with 1% FBS in PBS for 60 min followed by incubation overnight with the primary antibody against human S1P1 (puriﬁed IgG from a rabbit immunized with human S1P1), or preimmune chicken serum (1/200 dilution in PBSS) (19). Sections were then washed in PBS and incubated with HRP-conjugated anti-chicken IgG (1/200 dilution in PBS) for 60 min. The sections were further washed with PBS, and the color was developed by immersing the sections in a solution of 0.05% (w/v) 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.01% hydrogen peroxide in 0.05 M Tris (pH 7.4) for 3 min.

The sections were counterstained with Mayer’s hematoxylin solution (Wako Pure Chemicals). SK1, CD4, IFN-γ, or IL-6 staining was performed with the Vectastain ABC kit (Vector Laboratories), according to the manufacturer’s suggested protocol using the primary Ab against human SK1 (1/250 dilution in PBS) (puriﬁed polyclonal Ab from a rabbit immunized with human SK1) (20), the primary Ab against human CD4 (1/20 dilution in PBS) (NovoCasta Laboratories) (21), the primary Ab against human IFN-γ (1/40 dilution in PBS) (R&D Systems) (22), or the primary Ab against human IL-6 (1/100 dilution in PBS) (Santa Cruz Biotechnology), respectively (23). Positive staining was indicated by a brownish deposit, and background staining was purple.

**Cell line**

NS-SV-DC cell line (24) is immortalized normal salivary gland ductal cells established by transfection with SV40, cultured in serum-free keratinocyte medium (Invitrogen Life Technologies). HUVeCs were purchased from Cambrex Bio Science and were cultured in EGM Bulletkit (Takara Shuzo).

**DNA preparation and analysis of S1P, Fas mRNA**

Preparations of total RNA from CD4⁺ T cells and NS-SV-DC cells were performed using Isogen (Nippon Gene) according to the manufacturer’s protocol. Reverse transcription and cDNA ampliﬁcation techniques were performed using Isogen (Nippon Gene) according to the manufacturer’s protocol. RNA sample (1 μg) was converted to cDNA using M-MLV reverse transcriptase (Promega) in the presence of oligo-dT(18) primer. The reverse-transcribed products were stored at -20°C. Q-PCR ampliﬁcation was performed with the TaKaRa RNA PCR kit (Takara) (25). The following primers were used for the RT-PCR analysis: human S1P1 (429 bp product), sense 5’-CTACTGCCCGGACACGGAAGAACG-3’ and antisense 5’-ATAG GGACGGCCACCCAGGTGAG-3’; human Fas (500 bp product), sense 5’-TTCCAGGAGGTGCTCAACA-3’ and antisense 5’-GGTTAGGTGTG CTATCCTTGC-3’; and GAPDH (246 bp product), sense 5’-GATGACATACTGAGCTGCTT-3’ and antisense 5’-GTCTTACTCGCGCCATG-3’. Ampliﬁcation was performed at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min in a DNA thermal cycler (PerkinElmer Cetus Instruments) for 26, 29, and 32 cycles to ensure linearity. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The S1P1 and Fas PCR products were normalized in relation to the GAPDH internal control.

**CD4⁺ T cell isolation**

CD4⁺ T cells were isolated from peripheral blood from healthy volunteers and from patients with primary SS. Briefly, mononuclear cells were isolated from peripheral blood by Ficoll-Paque density gradient centrifugation. Mononuclear cells (1 × 10⁷) were labeled with biotin-conjugated anti-CD4 mAb and magnetic anti-biotin microbeads according to the manufacturer’s protocol (CD4⁺ T cell isolation kit II; Milteny Biotec) and CD4⁺ T cells were separated using miniMACS separator (Milteny Biotec). Purity of the CD4⁺ T cell population was 95%.

**Cell proliferation studies**

The 96-well plates were coated overnight at 4°C with 0.5 μg/ml anti-CD3 mAb and washed three times with PBS. CD4⁺ T cells (2 × 10⁵/100 μl well) and 30 Gy-irradiated autologous PBMC (1 × 10⁷/100 μl/well) were cultured in serum-free medium (AIM-V, Invitrogen Life Technologies) without or with S1P (0.01–0.5 μM) in anti-CD3 mAb-coated 96-well plates. After 3 days of culture, 1 μl/well [H]thymidine (Amersham Biosciences) was added to each well, and 16 h later incorporation was assessed.

**Measurement of IFN-γ secretion**

The 24-well plates were coated overnight at 4°C with 0.5 μg/ml anti-CD3 mAb and washed three times with PBS. CD4⁺ T cells (2 × 10⁵/ml well) and 30 Gy-irradiated autologous PBMC (1 × 10⁷/ml/well) were cultured in serum-free medium (AIM-V, Invitrogen Life Technologies) without or with S1P (0.01–0.5 μM) in anti-CD3 mAb-coated 24-well plates. After 48 h of culture, the supernatants were collected, and IFN-γ concentrations in the supernatants were measured by ELISA.

**Measurement of IL-6 secretion**

The 96-well plates were coated overnight at 4°C with 0.5 μg/ml anti-CD3 mAb and washed three times with PBS. CD4⁺ T cells (2 × 10⁵/ml well) and 30 Gy-irradiated autologous PBMC (1 × 10⁷/ml/well) were cultured in serum-free medium (AIM-V, Invitrogen Life Technologies) without or with S1P (0.01–0.5 μM) in anti-CD3 mAb-coated 24-well plates. After 48 h of culture, the supernatants were collected, and IL-6 concentrations in the supernatants were measured by ELISA.

**IFN-γ and IL-6 ELISA**

The concentrations of IFN-γ and IL-6 in the culture supernatants were measured by ELISA using Quantikine ELISA kit according to the manufacturer’s protocol (R&D Systems).

**Measurement of caspase-3 activity**

Caspase-3 activity was measured using Caspase-3 Colorimetric Assay (R&D Systems) according to manufacturer’s protocol. NS-SV-DC cells (1 × 10⁶) were cultured in serum-free keratinocyte medium in the absence or presence of S1P (0.01–0.5 μM) or IFN-γ (0.2 μg/ml) in 6-well plates. After 72 h of culture, anti-Fas mAb (100 ng/ml, Medical Biological Laboratories) was added and cultured an additional 6 h, and the cells were collected by centrifugation. The cell lysates were transferred into 96-well plate and mixed with buffer and caspase-3 colorimetric substrate (DEVD-pNA). After 2 h of incubation at 37°C, caspase-3 activity was determined by a microplate spectrophotometer (SpectraMax, Molecular Devices).

**Detection of apoptosis**

DNA morphology was assessed using HÖECHST staining. NS-SV-DC cells treated with S1P were labeled with 8 mg/ml HÖECHST 33342 (Sigma-Aldrich) for 10 min and the cells were examined by fluorescence microscopy.

**Measurement of the effect of pertussis toxin (PTX) on S1P-enhanced IFN-γ production by CD4⁺ T cells and Fas mRNA expression by NS-SV-DC cells**

CD4⁺ T cells were preincubated for 24 h in the presence of 100 ng/ml PTX (Sigma-Aldrich). After rigorous washing, cells were stimulated with anti-CD3 mAb (0.5 μg/ml) in the presence of S1P. After 48 h of culture, IFN-γ concentrations in the culture supernatants were measured. PTX-pretreated NS-SV-DC cells were treated with S1P or IFN-γ (0.2 μg/ml) for 6 h, and Fas mRNA expression in NS-SV-DC cells was analyzed by RT-PCR.

**Statistical analysis**

Results are expressed as mean ± SD. Student’s t test was used to compare individual treatments with their respective control values. A value for p < 0.05 was considered statistically significant.

**Results**

**Tissue distribution of SK1 and S1P expression in salivary glands from patients with primary SS**

SIP promotes cell proliferation and survival, whereas sphingosine and ceramide inhibit cell proliferation and stimulate apoptosis. The balance of these three important lipid-signaling molecules is critically regulated by SK, which converts sphingosine to SIP by

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approach to various stages of sialoadenitis and examined the extent of clear cells in LSG biopsy specimens (Fig. 2). We next extended this analysis by representative SK1 and S1P1 immunohistochemistry in LSG biopsies from patients with primary SS. Immunohistochemistry for SK1 and S1P1, which were expressed within cytoplasm of inflammatory mononuclear cells, vascular endothelial cells, and salivary gland epithelial cells in LSG biopsy specimens (Fig. 2). We next extended this approach to various stages of sialoadenitis and examined the extent and intensity of both SK1 and S1P1 immunostaining. Fig. 3 shows representative SK1 and S1P1 immunohistochemistry in LSG biopsy specimens. Although SK1 staining intensity was not different between grade 1 and grade 4 LSG biopsy specimens, S1P1 staining intensity was more extensive in the grade 4 LSG biopsy specimens than in the grade 1 LSG biopsy specimens (Fig. 3, E and G). These results indicate that S1P-S1P1 interactions occur in salivary glands from patients with primary SS.

**FIGURE 1.** Pathways of S1P metabolism. Graphic representation of S1P metabolism. Graph shows S1P is generated by metabolism of sphingomyelin with S1P levels being tightly regulated by a variety of enzymes including SK and S1P phosphatase. S1P acts as an extracellular mediator by binding to GPCRs and promotes cell proliferation and differentiation, whereas sphingosine and ceramide inhibit cell proliferation and stimulate apoptosis. The balance of these three important lipid-signaling molecules is critically regulated by SK1, the agonist-inducible isoform that can be activated by a variety of growth factors, cytokines and mitogens. S1P1 is one of the GPCRs of S1P, which promotes angiogenesis and the recruitment of lymphocytes.

**FIGURE 2.** Patterns of S1P1 and SK1 expression in grade 4 LSG biopsy specimens from patients with primary SS. Immunohistochemistry for SK1 (B and E) and for S1P1 (C and F) was performed on 4-μm thick sections of grade 4 LSG biopsy specimens from patients with primary SS. Control staining with pre-immune chicken serum (A and D) was also performed. The same biopsy sections were used to analyze both SK1 and S1P1, which were expressed within cytoplasm of inflammatory mononuclear cells, vascular endothelial cells, and salivary gland epithelial cells in LSG biopsy specimens. Original magnification, ×200, with insets, ×1000 (B and C). Grade 4 displayed more than one focus per 4 mm². Cellular aggregate with 50 or more lymphocytes, histiocytes, and plasma cells was defined as one focus. BV, Blood vessel; EP, epithelial cell; MN, mononuclear cell.

**FIGURE 3.** Characterization of SK1 and S1P1 expression and histologic grading in LSG biopsy specimens from patients with primary SS. Immunohistochemistry for SK1 (B, D, and F) and for S1P1 (C, E, and G) was performed on 4-μm thick sections of control (B and C), grade 1 (D and E) and grade 4 (F and G) LSG biopsy specimens. Control staining with pre-immune chicken serum (A) was also performed. The same biopsy sections were used to analyze both SK1 and S1P1. Original magnification, ×400, with insets, ×1000. A trend toward an increase of S1P1 expression in inflammatory mononuclear cells with increasing histologic grading (E and G) is shown. Grade 1 is characterized by slight infiltration and grade 4 displayed more than one focus per 4 mm². Cellular aggregate with 50 or more lymphocytes, histiocytes, and plasma cells was defined as one focus. EP, Epithelial cell; MN, mononuclear cell.

**FIGURE 4.** Effect of S1P on CD4+ T cell functions. Inflammatory mononuclear cells in primary SS are mainly represented by CD4+ T cells (1). Because S1P1, which was significantly over-expressed in infiltrating inflammatory mononuclear cells in the salivary glands from advanced stages of primary SS (Fig. 3, E and G), we investigated the effect of S1P on CD4+ T cell functions. First, we examined whether CD4+ T cells from peripheral blood expressed S1P1, using RT-PCR analysis. As shown in Fig. 4A, magnetically purified CD4+ T cells from both healthy volunteers and patients with primary SS expressed S1P1 (Fig. 4A). Next, we examined the effect of S1P on CD4+ T cell proliferation. The proliferation of CD4+ T cells was not affected by S1P alone (data not shown). However, when CD4+ T cells were stimulated with plate-bound anti-CD3 mAb, addition of S1P (0.01–0.5 μM) enhanced the proliferation of CD4+ T cells (Fig. 4B). We also examined whether S1P had a role in the secretion of IFN-γ by CD4+ T cells.
Interestingly, S1P, at the same concentrations used in the proliferation assay (0.01–0.5 μM), enhanced IFN-γ secretion by CD4⁺ T cells (Fig. 4C). We compared enhancing effects of S1P on proliferation and IFN-γ secretion by CD4⁺ T cells from healthy controls and from patients with primary SS. The effect of S1P to enhance IFN-γ secretion by CD4⁺ T cells was stronger in patients with primary SS than in healthy controls, although the effect of S1P to enhance proliferation by CD4⁺ T cells was not different between these two groups (Fig. 5). To characterize the IFN-γ-secreting CD4⁺ T cells present in LSG tissue, serial sectioned LSG biopsy samples from patients with primary SS were labeled with anti-CD4 or anti-IFN-γ Abs. Fig. 6 shows a representative section from LSG tissue. The majority of CD4⁺ T cells accumulated at LSG stained positive for IFN-γ (Fig. 6). These results indicate that S1P enhances TCR-stimulated CD4⁺ T cell proliferation and IFN-γ secretion and that the effect of S1P to enhance IFN-γ secretion by CD4⁺ T cells is augmented in patients with primary SS.

**Effect of S1P on Fas and caspase-3 expression in salivary gland epithelial cells**

Because S1P₁ was strongly expressed in epithelial cells in the salivary glands of patients with primary SS (Figs. 2 and 3), we next examined the effect of S1P on a salivary gland ductal epithelial cell line, the effect of S1P on Fas and caspase-3 expression in these cells was examined (Fig. 7). The majority of these cells had a strong positive Fas expression, and S1P had no effect on Fas expression. Caspase-3 expression was not altered by S1P treatment. These results suggest that S1P may play a role in the regulation of T cell function in the salivary glands of patients with primary SS.
line (NS-SV-DC). Apoptosis of the ductal epithelial cells of salivary and lacrimal glands has been proposed as a possible mechanism responsible for primary SS. Apoptotic cell death may be induced by interaction between Fas on epithelial cells and Fas ligand expressed by T cells. There are in vitro findings that an unstimulated human salivary gland cell line constitutively expressed low levels of Fas, and IFN-γ secreted by lymphocytes may up-regulate Fas expression on epithelial cells, thus increasing their sensitivity to apoptotic death signals (29). We observed that IFN-γ significantly increased Fas mRNA expression in NS-SV-DC. Fas mRNA expression was also significantly increased by S1P (0.1–0.5 μM). Furthermore, S1P enhanced IFN-γ-induced Fas mRNA expression in NS-SV-DC cells (Fig. 7A). These results indicate that both S1P and IFN-γ secreted by infiltrating CD4+ T cells increase Fas expression on salivary gland epithelial cells. The activation of caspase-3 appears to be indispensable for the apoptotic process (30). Therefore, we examined caspase-3 expression in NS-SV-DC cultured with IFN-γ or S1P. As shown in Fig. 7B, both IFN-γ and S1P (0.1–0.5 μM) significantly increased caspase-3 expression in NS-SV-DC in the presence of anti-Fas mAb (Fig. 7B). We also confirmed apoptosis of NS-SV-DC cells treated with anti-Fas mAb and S1P by HOECHST staining (data not shown). These results indicate that both IFN-γ and S1P induce apoptosis via Fas signaling by salivary gland epithelial cells.

**IL-6 expression in salivary glands from patients with primary SS**

IL-6 is known to be a B cell growth and differentiation factor and is generally found to be highly expressed in many autoimmune diseases (31). Although serum levels of IL-6 have generated conflicting results, analysis of tear and salivary IL-6 levels in patients with primary SS are elevated when compared with those in healthy controls (32–34). We examined IL-6 expression in the salivary glands from patients with primary SS by immunohistochemistry. IL-6 was detected in the cytoplasm of most inflammatory mononuclear cells and ductal epithelial cells. Furthermore, the extent and intensity of IL-6 expression in ductal epithelial cells correlated with mononuclear cell infiltration grade (Fig. 8A). These results indicate that IL-6 expression in ductal epithelial cells is increased in more advanced stage of primary SS.

**Effect of S1P on IL-6 secretion by epithelial cells**

Because SK1, S1P1, and IL-6 are expressed in ductal epithelial cells from patients with primary SS (Figs. 2, 3, 8A), we examined the role of S1P1 signaling for the secretion of IL-6 by ductal epithelial cells. IFN-γ significantly augmented IL-6 secretion by NS-SV-DC. Although low levels of S1P (0.01–0.1 μM) did not affect IL-6 secretion, higher levels of S1P (0.5 μM) significantly augmented IL-6 secretion by NS-SV-DC (Fig. 8B). These results indicate that IFN-γ and high concentrations of S1P induce IL-6 secretion by ductal epithelial cells.

**Mediation of S1P-enhanced IFN-γ production by CD4+ T cells and Fas mRNA expression by NS-SV-DC cells via PTX-sensitive pathway**

It has been reported that S1P1 couples to PTX-sensitive G proteins of G12/G13 family (35–37). Because we demonstrated that both...
CD4⁺ T cells and salivary gland epithelial cells expressed S1P₁ (Figs. 2, 3, 4A), we investigated the role of PTX-sensitive G proteins in S1P enhancement of IFN-γ production by CD4⁺ T cells and Fas mRNA expression by NS-SV-DC cells. Peripheral blood CD4⁺ T cells were preincubated with 100 ng/ml PTX for 24 h. After rigorous washing, the cells were stimulated with plate-bound anti-CD3 mAb in the presence of S1P (0.01–0.5 μM). After 72 h, IFN-γ concentrations in culture supernatants were measured. Pretreatment with PTX inhibited S1P-enhanced IFN-γ secretion by CD4⁺ T cells (Fig. 9A). We next examined the effect of PTX-sensitive G proteins on S1P-enhanced Fas mRNA expression by NS-SV-DC cells. NS-SV-DC cells were preincubated with 100 ng/ml PTX for 24 h before S1P or IFN-γ stimulation. Pretreatment with PTX inhibited S1P enhancement of Fas mRNA expression by NS-SV-DC cells. However, preincubation with PTX did not affect the enhancing effect of IFN-γ on Fas mRNA expression by NS-SV-DC cells (Fig. 9B). These results indicate that S1P enhancements of IFN-γ production by CD4⁺ T cells and Fas mRNA expression by salivary gland epithelial cells depend on G₁/G₀-dependent pathway.

Discussion

The sphingolipid metabolites ceramide, sphingosine, and S1P have recently emerged as a new class of lipid messengers that regulate cell proliferation, differentiation, and survival in opposite directions (35–37). The balance of these three lipid-signaling molecules is critically regulated by SK, which converts sphingosine to S1P by phosphorylating sphingosine. Recent studies have demonstrated that the agonist-inducible SK, SK1, is up-regulated in azoxymethane-induced colon cancer cells and in B cells resistant to Fas-mediated apoptosis from patients with rheumatoid arthritis (38, 39). The mechanisms by which SK1 promotes carcinogenesis and resistance to Fas-mediated apoptosis probably depend on its ability to phosphorylate sphingosine to produce S1P. In the present study we demonstrated that both SK1 and S1P₁ were expressed within cytoplasm of inflammatory mononuclear cells, vascular endothelial cells, and salivary gland epithelial cells in LSG biopsy specimens. Furthermore, S1P₁ staining in inflammatory mononuclear cells was significantly more extensive and intense in more advanced stage of primary SS, suggesting that the increased S1P₁ signaling in inflammatory mononuclear cells may contribute the inflammation of salivary glands from patients with primary SS.

S1P concentrations in the lymph node and tissues (0.005–0.02 μM) are lower than those in lymph (0.03–0.3 μM) and in blood (0.1–1 μM). S1P concentrations in the lymph node and tissues are

**FIGURE 8.** IL-6 expression and secretion by salivary gland epithelial cells. A, Characterization of IL-6 expression and histologic grading in LSG biopsy specimens from patients with primary SS. Immunohistochemistry for IL-6 was performed on 4-μm thick sections of grade 1 (left) and grade 4 (right) LSG biopsy specimens from patients with primary SS. Original magnification, ×100. Inset magnification is ×1000. A trend toward an increase of IL-6 expression in ductal epithelial cells (EP) with increasing histologic grading is shown. Grade 1 is characterized by slight infiltration and grade 4 displayed more than one focus per 4 mm². Cellular aggregates with 50 or more lymphocytes, histiocytes, and plasma cells were defined as one focus. MN, mononuclear cells. B, Effect of S1P on IL-6 secretion from salivary gland epithelial cells. NS-SV-DC cells were treated with 0.01–0.5 μM S1P or with (+) IFN-γ (0.2 μg/ml) or left untreated (−). After 48 h of culture, culture supernatants were collected and IL-6 levels were measured by ELISA. Data represent mean ± SD from three independent experiments. *, p < 0.05.

**FIGURE 9.** Effect of PTX on S1P-enhanced IFN-γ production by CD4⁺ T cells and Fas mRNA expression by salivary gland epithelial cells. A, IFN-γ production by CD4⁺ T cells with (■) or without (□) PTX pretreatment was measured using cells incubated with plate-bound anti-CD3 mAb (0.5 μg/ml) in the absence (−) or presence (+) of 0.01–0.5 μM S1P as described in Fig. 4C. After 48 h culture, culture supernatants were collected, and IFN-γ levels were measured by ELISA. Data represent mean ± SD from three independent experiments. *, p < 0.05. NS, Not significant. B, Fas mRNA expression by NS-SV-DC cells with (■) or without (□) PTX pretreatment was determined by treatment with S1P or IFN-γ for 6 h or left untreated as described in Fig. 7. Semiquantitative RT-PCR for the expression of Fas mRNA in NS-SV-DC cells was measured and were determined by normalizing expression with respect to GAPDH mRNA expression levels. Data represent mean ± SD from three independent experiments. *, p < 0.05. NS, Not significant.
thought to enhance, whereas those in the blood suppress migration of T cells in murine model (14, 15). We observed that S1P (0.01–0.1 μM) enhanced proliferation and IFN-γ secretion by anti-CD3 mAb-stimulated CD4⁺ T cells, suggesting that concentrations of S1P in the tissues enhance IFN-γ production by TCR-stimulated CD4⁺ T cells. In contrast to our findings, Dorsam et al. (40) reported that proliferation and IFN-γ secretion by mouse CD4⁺ T cells stimulated with anti-CD3 mAb plus anti-CD28 mAb was inhibited by 0.001–1 μM S1P. This discrepancy may be explained by the different sensitivity of CD4⁺ T cell responses to S1P between mice and humans. Furthermore, we used a suboptimal dose of anti-CD3 mAb (0.5 μg/ml), whereas they used a much higher concentration of anti-CD3 mAb (2 μg/ml) for CD4⁺ T cell activation. Therefore, the enhancing effect of S1P on proliferation and IFN-γ secretion by CD4⁺ T cells may not be observed because of maximal CD4⁺ T cell activation by anti-CD3 mAb. Supporting our findings, Jin et al. (41) reported that S1P enhanced IL-2 and IFN-γ production by human peripheral blood T cells stimulated with anti-CD3 and anti-CD28 mAbs. We also observed that the enhancing effect of S1P on IFN-γ secretion by CD4⁺ T cells was stronger in patients with primary SS than in healthy controls. Taken together, increased S1P₁ signaling in infiltrating CD4⁺ T cells may enhance IFN-γ secretion by CD4⁺ T cells in the salivary glands of patients with primary SS.

Apoptosis of the acinar and ductal epithelial cells of the salivary and lacrimal glands has been proposed as a possible mechanism of primary SS. Apoptotic cell death may be induced by either CTLs through the release of proteases, such as perforin and granzyme B, or the interaction of Fas ligand expressed by T cells (42). Matsumura et al. (29) showed that IFN-γ and TNF-α up-regulated Fas expression on human salivary gland epithelial cells. We also observed that IFN-γ up-regulated Fas mRNA expression in salivary gland epithelial cell line (NS-SV-DC). Interestingly, S1P (0.1–0.5 μM) up-regulated Fas mRNA expression in NS-SV-DC. Furthermore, S1P augmented IFN-γ-induced Fas mRNA expression in NS-SV-DC. Caspase-3 appears to be indispensable for the apoptotic process (30). Both IFN-γ and S1P induced caspase-3 expression by NS-SV-DC in the presence of anti-Fas mAb. These results indicate that both IFN-γ and S1P augment Fas expression on salivary gland epithelial cells, thereby inducing apoptosis of the cells via Fas signaling.

The presence of various autoantibodies such as rheumatoid factor and anti-SSA/SSB Abs, as well as hyperγmagnoglobulinemia, is considered to reflect B cell hyperactivity in primary SS. IL-6 is known to be a B cell growth and differentiation factor and it is considered to reflect B cell hyperactivity in primary SS. IL-6 is generally found to be highly expressed in many autoimmune diseases (31). IL-6 mRNA is highly expressed in salivary gland of patients with primary SS (8). We observed IL-6 expression in ductal epithelial cells and inflammatory mononuclear cells. IL-6 expression in ductal epithelial cells was augmented in more advanced stages of primary SS. Furthermore, both IFN-γ and S1P augmented IL-6 secretion from a salivary gland epithelial cell line. These results suggest that S1P signaling augments IL-6 secretion from ductal epithelial cells in the salivary glands in patients with primary SS.

We investigated whether S1P-enhanced IFN-γ production by CD4⁺ T cells and Fas mRNA expression by salivary gland epithelial cells used S1P₁ signaling pathways. Both of these actions of S1P were inhibited by PTX. Because S1P₁ couples only to G₁α, most of its effects are PTX sensitive, these results indicate that S1P₁ signaling pathways play an important role in modulating T cell as well as epithelial cell functions.

In conclusion, we have demonstrated increased S1P₁ expression in inflammatory mononuclear cells in the salivary glands of patients with primary SS. S1P₁ signaling enhanced proliferation and IFN-γ production by CD4⁺ T cells, Fas expression and Fas-mediated apoptosis by salivary gland epithelial cells, and IL-6 secretion by ductal epithelial cells. Therefore, S1P₁ signaling may play an important role in the pathogenesis of primary SS by potentiating IFN-γ secretion by CD4⁺ T cells, ductal epithelial cell apoptosis, and IL-6 secretion by ductal epithelial cells. Regulation of S1P₁ signaling may be a novel therapeutic strategy in primary SS.

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Disclosures
The authors have no financial conflict of interest.

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