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Identification of IL-18 and Th17 Cells in Salivary Glands of Patients with Sjögren’s Syndrome, and Amplification of IL-17-Mediated Secretion of Inflammatory Cytokines from Salivary Gland Cells by IL-18

Azusa Sakai,*† Yumiko Sugawara,† Toshinobu Kuroishi,* Takashi Sasano,† and Shunji Sugawara‡*

IL-18 is a proinflammatory cytokine and plays an important pathogenic role in inflammatory and autoimmune disorders. IL-17 is also a proinflammatory cytokine and IL-17-secreting Th17 cells are involved in autoimmunity. However, the pathological roles of IL-18 and Th17 cells in Sjögren’s syndrome (SS) remain to be elucidated. This study showed that the expression of IL-18 was detected in acinar cells, intraducts, and CD68+ macrophages in salivary glands of SS patients, but not in those of healthy subjects or patients with chronic graft-vs-host disease, by immunohistochemistry, and immunoblot analysis revealed that 24-kDa precursor form of IL-18 (proIL-18) and 18-kDa mature IL-18 were detected in SS salivary glands. The majority of the infiltrating cells in the salivary glands of SS patients were CD4+ T cells, and CD8+ T cells were infiltrated to a lesser extent. The predominant expression of IL-17 was found in infiltrating CD4+ T cells, whereas a small number of infiltrating CD8+ T cells expressed IL-17. Human salivary gland HSY and acinar AZA3 cells constitutively expressed proIL-18 and caspase-1, and a calcium ionophore A23187 induced the secretion of IL-18 from the cells. HSY and AZA3 cells expressed IL-18R and IL-17R on the cell surface, and IL-18 amplified the secretion of IL-6 and IL-8 that were induced by low amounts of IL-17. Primary salivary gland cells from normal subjects partially confirmed these findings. These results suggest that IL-18 and Th17 cells detected in the salivary glands in SS patients are associated with the pathogenesis of SS in the salivary glands. *The Journal of Immunology, 2008, 181: 2898–2906.

Sjögren’s syndrome (SS) is a chronic autoimmune disease of the exocrine glands with infiltration of lymphocytes, and with a female predominance (1). Dryness of the mouth and eyes results from destruction of the salivary and lachrymal glands. The exocrinopathy can be encountered alone (primary SS) or in the presence of other autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, or progressive systemic sclerosis (secondary SS). Histologically, SS is characterized by extensive lymphocytic infiltration of the salivary and lachrymal glands (1), and the majority of infiltrating cells in the salivary glands of SS patients are T cells, predominantly CD4+ T cells but also CD8+ T cells (2). However, the pathological role of T cells in SS remains to be elucidated.

IL-18 is a multifunctional regulator of innate and acquired immune responses through its activation of Th1 and Th2 responses (3–6). IL-18 is also suggested to be a potent proinflammatory cytokine that regulates autoimmune and inflammatory diseases (4–6). IL-18 is produced intracellularly as an inactive 24-kDa precursor form (proIL-18) and secreted as a 18-kDa active mature form after cleavage by caspase-1 (4–6). Recent studies have identified IL-18 not only in activated macrophages, including dendritic cells and Kupffer cells, but also in nonimmune cells, such as keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells of various organs and tissues, microglial cells, and synovial fibroblasts (4–7). Increased levels of IL-18 have been reported in the sera from patients with a wide variety of diseases, including autoimmune and inflammatory disorders, allergy, allograft rejection, and infectious diseases (4–6), and the elevated serum IL-18 levels are considered to be a parameter for the disease severity and a diagnostic marker. We have shown recently that the induction of serum IL-18 is independent of phagocytic macrophages in a murine model (8). These observations imply that IL-18 plays pathophysiological roles and acts as a component of systemic immune regulation. Recent studies revealed that a proinflammatory cytokine, IL-17 (also known as IL-17A), is involved in several inflammatory and autoimmune diseases (9–11) and that IL-17-producing CD4+ T cells comprise a distinct lineage of proinflammatory Th cells, termed Th17 cells, that contribute critically to autoimmune diseases (12, 13). TGF-β induces the differentiation of Th17 cells and regulatory T cells, a subset of immunosuppressive T cells, from naïve T cells (14, 15), and IL-6 and IL-2 act as switch factors for

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3 Abbreviations used in this paper: SS, Sjo ¨gren’s syndrome; proIL-18, 24-kDa precur- sor form of IL-18; EAE, experimental autoimmune encephalomyelitis; GVHD, graft-versus-host disease.

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the development of Th17 cells and regulatory T cells, respectively (16, 17). Most studies on Th17 cells are performed using murine models, such as experimental autoimmune encephalomyelitis (EAE), and the involvement of Th17 cells in human autoimmune diseases is still unclear.

There are no reports regarding infiltration of Th17 cells in the salivary glands of SS patients to date. Expression of IL-18 was detected in SS salivary glands in correlation with increases in serum IL-18 levels (18, 19), but the pathological role of IL-18 expressed in the salivary glands of SS patients is still unclear. We hypothesized that the infiltrating T cells in the salivary glands of SS patients express IL-17 and that there are relations between IL-17 and IL-18 in the pathogenesis of SS. To address these issues, this study examined the expression of IL-18 and IL-17 in the salivary glands of SS patients. The role of IL-17 and IL-18 in the induction of proinflammatory cytokines, IL-6 and IL-8, was also examined using human salivary gland cells in culture.

Materials and Methods

Reagents

Human rIL-18 and rIL-17 were obtained from Medical & Biological Laboratories and Acris Abs, respectively. A23187 was obtained from Calbiochem. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Patients and healthy controls

Ten patients with primary SS, 10 patients with sicca syndrome, and 10 patients with chronic graft-versus-host disease (GVHD) were included in this study. Three healthy volunteers were included as controls. SS patients were diagnosed at Tohoku University Hospital (Sendai, Japan) based on the Japanese Ministry of Health criteria for the classification of SS (20). All patients with SS had histopathologically in their salivary gland biopsy specimens, a score of greater than or equal to 2 for autonomic loss and SS-A or SS-B, more than stage I by sialography of the parotid glands. None of the patients had any other associated autoimmune disease, evidence of lymphoma, HIV or hepatitis C virus infection at the moment of the study. Patients with sicca syndrome had dry mouth and dry eye without focus histopathologically in their salivary gland biopsy specimens. Patients with chronic GVHD who received allogeneic hematopoietic stem cell transplantation more than 60 days before had lichenoid lesions in their oral mucosa and lymphocytic infiltration in their salivary gland biopsy specimens and had been receiving immunosuppressive drugs.

Immunohistochemistry

Labial salivary gland tissues were homogenized in a buffer (200 µl) containing 1% SDS under reducing conditions, according to the method of Lavender et al. (21). Proteins were transferred to a polyvinylidene difluoride membrane by a semidry transblot system (Bio-Rad). The blot was blocked for 90 min with 1.5% horse serum and 0.05% Tween 20 in PBS and incubated with anti-IL-18 mAb Ab2 25-2G at 1 µg/ml in 0.05% Tween 20 in PBS overnight at 4°C. The blot was washed five times with 0.05% Tween 20 in PBS and then reacted with VECTASTAIN Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. After being washed, IL-18 was visualized with SuperSignal West Femto maximum sensitivity substrate (Pierce) in a Chemi Imager (Alpha Innotech). The relative molecular mass of the proteins was estimated by comparison with the position of protein standards (Bio-Rad).

Cells and cell culture

A human parotid gland cell line HSY (22) and human salivary acinar cell line A2A3 (23) were prepared by M. Sato (Tokushima University, Tokushima, Japan) as previously described (22, 23), and grown in DMEM (Nissui Pharmaceutical) with 10% FCS (Tissue Culture Biological). Human PBLs were isolated from heparinized peripheral blood of healthy adult donors by Lympholyte H (Cedarlane Laboratories) gradient centrifugation at 800 × g for 20 min at room temperature (7). The isolated PBLs were washed three times with PBS.

RT-PCR

Cells were lysed in 1 ml of ISOGEN (Nippon Gene), and total RNA was extracted as described in the instruction manual. Total RNA was dissolved in 30 µl of diethyl pyrocarbonate-treated water (Nippon Gene) and incubated at 65°C for 10 min. cDNA synthesis was conducted with a first-strand cDNA synthesis kit (AMV Reverse Transcriptase) (GE Healthcare). PCR reactions contained 5.0 µl of 10X PCR buffer (Applied Biosystems), 200 µM dNTP (Applied Biosystems), 25 pmol each of primer, and 0.625 U of AmpliTaq DNA polymerase (Applied Biosystems) in a total volume of 25 µl. Amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems). The primers used for PCR were as follows: IL-18 (forward) 5'-GCTTGAATCTAAATTATCAGTC-3' and (reverse) 5'-GAAGAAATTCTTAAATTTGATC-3'; IL-17 (forward) 5'-TCTCATGGGTAAGGTACA-3' and (reverse) 5'-CCTTCTTAATCTGACAGTTGACC-3'; and caspase-1, 32 cycles at 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s for amplifying a 342-bp product; caspase-1, 35 cycles at 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s for amplifying a 553-bp product; and β-actin, 32 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for amplifying a 548-bp product. PCR products were electrophoresed using 3% agarose gels (Nusieve 3:1 agarose; BMA).

Double enzyme-linked immunohistochemistry was performed as follows. Firstly, rabbit anti-human IL-17 polyclonal Ab (Santa Cruz Biotechnology) was applied overnight with the goat anti-rabbit EnVision+ system/HRP. The chromogen used was 3-amino-9-ethylcarbazole (DAKOCytomation). Then, anti-IFN-γ mAb, anti-CD4 mAb SK3, or anti-CD8 mAb SK1 was applied overnight, followed by application of goat anti-mouse EnVision System-Alkaline Phosphatase (DAKOCytomation). The chromogen in this second system was Vector blue, alkaline phosphatase substrate kit (Vector Laboratories). Endogenous alkaline phosphatase activity was inhibited by the addition of levamisole (Vector Laboratories) to the buffer prior to the preparation of the working solution. After drying, the specimens were mounted. As negative controls, rabbit and mouse control Abs were used in each step of double immunohistochemistry. Samples were photographed with a Leica DC 200 cooled charge-coupled device camera mounted on a Leica DMR DMR microscope using the Q fluoro system (Leica Microsystems). The Ethical Review Board of Tohoku University Graduate School of Dentistry approved the experimental procedures.

Western blotting

Labial salivary glands tissues were homogenized in a buffer (200 µl) containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM PMSF, 10 µg/ml soybean trypsin inhibitor, and 1 mg/ml leupeptin. After 1 h at 4°C, the homogenates were centrifuged at 10,000 × g for 10 min at 4°C. The supernatants were collected and then mixed with Laemmli sample buffer (21). Cultured cells (10 6 cells) were lysed with a buffer (100 µl) containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM PMSF, and 10 µg/ml soybean trypsin inhibitor for 30 min at 4°C. After centrifugation, the supernatants were treated with or without 20 U/ml caspase-1 for 1 h at 37°C and then mixed with Laemmli sample buffer.

SDS-PAGE was performed in a 15% polyacrylamide slab gel containing 0.1% SDS under reducing conditions, according to the method of Laemmli (24); and 10% SDS-PAGE was performed from heparinized peripheral blood of healthy adult donors by Lympholyte H (Cedarlane Laboratories) gradient centrifugation at 800 × g for 20 min at room temperature (7). The isolated PBLs were washed three times with PBS.

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After staining with ethidium bromide, amplified DNA bands were analyzed with a Chemi Imager (Alpha Innotech).

**Measurement of cytokines**

Salivary gland cells (4 × 10^5 cells/400 µl) in DMEM with 10% FCS were seeded into the wells of 24-well plates (Falcon; BD Labware) and incubated overnight at 37°C in a 5% CO2 atmosphere. The cells were then washed with PBS and incubated with or without A23187 or forskolin in 400 µl of DMEM without FCS for 3 h. After the incubation, the supernatants were collected. The levels of IL-18 in the culture supernatants were determined using a human IL-18 ELISA kit (Medical & Biological Laboratories).

The cells were also incubated with or without IL-18 and IL-17 in 400 µl of DMEM with 10% FCS for 24 h. After the incubation, the supernatants were collected, and the levels of IL-6 and IL-8 in the supernatants were determined using human IL-6 and IL-8 OptEIA ELISA kits (BD Biosciences), respectively. Each sample was assayed in triplicate.

**Flow cytometry**

Flow cytometric analyses were performed using a FACSCalibur cytometer (BD Biosciences). Cells were stained with anti-IL-18 mAb 25-2G (mouse IgG2b), anti-IL-18R mAb 132029 (mouse IgG2b), or anti-IL-17R mAb 133617 (mouse IgG1; R&D Systems) at 4°C for 30 min, followed by staining with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 4°C for another 30 min.

**Cultures of salivary gland epithelial cells**

Primary cultures of salivary gland epithelial cells were prepared from the labial minor salivary glands with informed consent as previously described (7). The tissue specimens were cut into pieces and cultured in keratinocyte serum-free medium (Life Technologies) containing bovine pituitary extract (0.05%) and recombinant human epidermal growth factor (820 µM) supplemented with kanamycin (100 µg/ml) with a medium change every 3–5 days until confluent cell monolayers were formed.

**Statistical analysis**

Experimental values were expressed as mean ± SD, and the statistical significance of differences between two mean values was evaluated by one-way ANOVA using the Bonferroni or Dunnett methods, for which values of *p* < 0.05 were considered to be statistically significant.

**Results**

**Detection of IL-18 and IL-17 in the salivary glands of SS patients**

We first examined the expression of IL-18 and IL-17 in the salivary glands of normal subjects and patients with SS, sicca syndrome, and chronic GVHD by immunohistochemistry. Mononuclear cells were severely infiltrated in the salivary glands of SS patients, and infiltrating mononuclear cells was also found diffusely and moderately in the salivary glands of sicca syndrome and chronic GVHD patients but not in those of normal subjects (Fig. 1, A–K). Expression of IL-18 was detected in acinar cells and intraducts in the salivary glands of SS patients, whereas some of acinar cells expressed IL-18 in the salivary glands of sicca syndrome patients (Fig. 1, D and G). Expression of IL-18 was also detected...
in infiltrating CD68+ macrophages in the salivary glands of SS patients, although the number of CD68+ cells was small in the field (Fig. 2). IL-18 expression was not detected in the salivary glands of normal subjects or chronic GVHD patients (Fig. 1, A and J). Expression of IL-17 was detected slightly in ductal epithelial cells in salivary glands from normal subjects as well as sicca syndrome patients (Fig. 1, B, C, H, and I), whereas the dominant expression of IL-17 was detected in the infiltrated cells, and ductal epithelial cells also expressed IL-17 in the salivary glands of SS patients (Fig. 1, E and F). No staining for IL-17 was found in acinar cells in the SS sample. In contrast, IL-17 expression was not detected in the salivary glands of chronic GVHD patients (Fig. 1).

**FIGURE 2.** Detection of IL-18-expressed CD68+ macrophages in the salivary glands of SS patients. Cryosections of labial salivary glands of SS patients were stained with anti-CD68 mAb PG-M1 (brown) (A) or double stained with anti-CD68 mAb PG-M1 (red) and anti-IL-18 mAb 25-2G (green) (B). Scale bar represents 100 μm (A) and 10 μm (B).

**FIGURE 3.** Detection of IL-17 in infiltrating CD4+ and CD8+ T cells in the salivary glands of SS patients. Sequential cryosections of labial salivary glands of SS patients were stained with anti-IFN-γ (A and D), anti-CD4 mAb SK3 (B and E), and anti-CD8 mAb SK1 (C and F) (brown). The sections were counterstained with hematoxylin in blue. The same sections were double stained with rabbit anti-human IL-17 polyclonal Ab (light brown) and with anti-IFN-γ (G), anti-CD4 mAb SK3 (H), and anti-CD8 mAb SK1 (I) (light blue). Dark blue indicates double positive cells. Scale bar represents 200 μm (A–C) and 50 μm (D–I).

**FIGURE 4.** Expression of IL-18 in human salivary gland cells in culture. A, Total RNA was extracted from human salivary gland AZA3 and HSY cells. Human PBLs were used as a positive control. The m.w. marker (M) is also shown. cDNA was prepared and analyzed for the expression of IL-18, caspase-1, and β-actin mRNA by RT-PCR. B, Cell lysates of HSY and AZA3 were either untreated or treated with caspase-1 (20 U/ml) for 1 h, and the treated samples were subjected to Western blotting with anti-IL-18 mAb 25-2G. IL-18 (10 ng) was loaded as a positive control.
were also infiltrated to a lesser extent (Fig. 3, C), cells expressed IL-17 (Fig. 3).

IFN-γ and IFN-β detected in the salivary glands of SS patients, but the number of MANUAL for table.

stimulated control. ND, Not detected.

FIGURE 6. Expression of IL-18R and IL-17R on the surface of salivary gland cells

To elucidate the biological functions of IL-18 and IL-17 in salivary gland cells, the expression of IL-18R and IL-17R on salivary gland cells was examined. Flow cytometric analyses showed that AZA3 and HSY cells express IL-18Rα, IL-18Rβ, and IL-17R on the cell surface (Fig. 6). Incubation of the cells with IL-18 or IL-17 did not alter the expression of IL-18R or IL-17R (data not shown). These results indicate that IL-18 secreted by salivary gland cells is able

Expression and secretion of IL-18 in salivary gland cells in culture

We then examined the expression and secretion of IL-18 using human salivary gland cells in culture. Salivary gland HSY and AZA3 cells constitutively expressed IL-18 and caspase-1 mRNA (Fig. 4A). Human PBLs were used as a positive control. Western blotting showed that the cells constitutively expressed proIL-18 but not mature IL-18 in the cells (Fig. 4B). Incubation of the proIL-18 in cell lysates of HSY and AZA3 cells with caspase-1 converted proIL-18 to 18-kDa mature IL-18, indicating that the IL-18 expressed in salivary gland cells was properly processed in the presence of caspase-1.

As the stimulation of mammalian cells by a wide variety of ligands induces second messengers, such as calcium mobilization or increase in cAMP, in the cells, we next examined the effect of Ca²⁺ ionophore A23187 and forskolin, which is an activator of adenylate cyclase, on IL-18 secretion from salivary gland cells. The results showed that elevation of intracellular Ca²⁺ by A23187 significantly induced IL-18 secretion in a dose-dependent manner in HSY and AZA3 cells, although the amount of IL-18 was low (Fig. 5). In contrast, forskolin did not induce the secretion of IL-18 from these cells. These results indicate that increases in intracellular cAMP do not contribute to the secretion of IL-18 from salivary gland cells, and that intracellular calcium mobilization induced by external stimuli induced the secretion of IL-18 from salivary gland cells in a microenvironment.

Expression of IL-18R and IL-17R on the surface of salivary gland cells

To elucidate the biological functions of IL-18 and IL-17 in salivary gland cells, the expression of IL-18R and IL-17R on salivary gland cells was examined. Flow cytometric analyses showed that AZA3 and HSY cells express IL-18Rα, IL-18Rβ, and IL-17R on the cell surface (Fig. 6). Incubation of the cells with IL-18 or IL-17 did not alter the expression of IL-18R or IL-17R (data not shown). These results indicate that IL-18 secreted by salivary gland cells is able
to bind to their own cells in an autocrine manner, and that IL-17 from T cells and IL-18 together may activate salivary gland cells.

Synergistic secretion of IL-6 and IL-8 in salivary gland cells by IL-17 with IL-18

Stimulation of HSY cells with IL-18 alone did not induce the secretion of IL-6 even at 100 ng/ml IL-18 (data not shown), whereas the secretion of IL-6 was induced by IL-17 alone at 10 and 100 ng/ml, but not at 1 ng/ml (Fig. 7A). However, in the presence of IL-18 at 1 and 10 ng/ml, IL-17 at 1 ng/ml induced the secretion of IL-6 from HSY cells, which was comparable to that by 10 ng/ml IL-17 alone. The IL-6 production induced by IL-17 at 10 and 100 ng/ml was augmented in the presence of IL-18. The basal level of IL-8 production was extremely high in HSY cells, and the production of IL-8 was not further augmented by IL-18 alone or by IL-17 at 1 ng/ml (Fig. 7B). However, in the presence of 10 ng/ml IL-8, IL-17 at 1 ng/ml markedly augmented the production of IL-8. Higher concentrations of IL-17 induced IL-8 production, but IL-17 did not further augment IL-8 production (data not shown). The same results were obtained with AZA3 cells. These results indicate that IL-18 amplifies the production of inflammatory cytokines induced by IL-17 in salivary gland cells.

To further confirm the results, we examined the effect of IL-17 and IL-18 on salivary gland cells in primary culture. As glandular cells from SS patients did not grow successfully in vitro, we studied glandular cells from normal subjects. Two representative results from four donors are shown in Fig. 8. The glandular cells from Donor 1 showed that IL-18 at 10 ng/ml amplified the production of IL-8 induced by IL-17, whereas the cells from Donor 2 did not show a synergy between IL-17 and IL-18. Production of IL-6 by IL-17 or IL-18 could not be detected in these primary glandular cells (data not shown). These findings suggest that susceptibility of glandular cells to IL-17 and IL-18 varied depending on donors or glandular cells in the salivary glands.

Discussion

Recent evidence indicates that Th17 cells rather than Th1 cells are the key effector Th cells in the induction and development of autoimmune disorders in murine models (12–17), and this study showed that Th17 cells are infiltrated in the salivary glands of SS patients, suggesting that Th17 cells are involved in a human autoimmune disease, SS.
SS is characterized by extensive lymphocytic infiltration of the salivary and lacrimal glands (1), and the majority of infiltrating cells in the salivary glands of SS patients are T cells, predominantly CD4⁺ T cells but also CD8⁺ T cells (2). Other infiltrating cells are B cells and monocytes. Salivary gland CD4⁺ T cells from SS patients express IL-2 and IFN-γ (26), and studies of salivary glands in SS patients also confirmed the expression of Th1-related cytokines (IL-2, IFN-γ, IL-12, and IL-18) by RT-PCR (27), indicating that SS is a Th1-related disease (1). In addition, salivary gland CD4⁺ T cells express IL-10 and salivary gland epithelial cells express IL-6 in SS patients (26), and other studies showed that salivary glands consistently express IL-10, IL-6, and TGF-β (27, 28). Consistent with this observation, overexpression of IL-10 in salivary glands using a human salivary amylase promoter in mice develop SS-like symptoms (29). IL-6 and TGF-β are necessary to induce the differentiation of Th17 cells (13–17), and our study has shown for the first time the infiltration of Th17 cells and IL-17-expressing CD8⁺ T cells in the salivary glands of SS patients (Fig. 3). It has been shown that IL-17 and IFN-γ or TNF-α cooperate for the secretion of IL-6 and GM-CSF by human rheumatoid synoviocytes in vitro (30). Recent studies revealed that both IFN-γ and IL-17 together synergize to trigger severe intestinal inflammation in murine inflammatory bowel disease models and suggest that both Th1 and Th17 cells may contribute to the pathogenesis (31, 32). This study confirmed the expression of IFN-γ in the salivary glands of SS patients and showed that the number of IFN-γ⁺ cells was smaller than that of IL-17⁺ cells and that IFN-γ⁺ cells did not coexpress IL-17 (Fig. 3). These observations suggest that both Th1 and Th17 cells together are involved in the pathogenesis of SS.

It is reported that expression of IL-18 was detected in SS salivary gland cells and infiltrating CD68⁺ macrophages in correlation with increases in serum IL-18 levels (18, 19), but expression of mature IL-18 in the salivary glands was unknown. Our study confirmed the previous observation (Figs. 1 and 2) and showed that the salivary glands of SS patients express mature IL-18 as well as proIL-18 (Fig. 1L). Salivary gland cells in culture constitutively expressed proIL-18 and caspase-1 (Fig. 4), and calcium mobilization in the cells induced the secretion of IL-18, although the amount of IL-18 was low (Fig. 5). These results suggest that external stimuli can induce the secretion of mature IL-18 from salivary gland cells of SS patients. IL-18 has been suggested to be a potent proinflammatory cytokine that regulates autoimmune and inflammatory diseases (3–6). IL-18 in concert with IL-12 induces production of IFN-γ and TNF-α from Th1 and NK cells with subsequent production of IL-1 (33). Inflammatory cytokines, such as IL-1α, IL-2, IL-6, TNF-α, and IFN-γ, were detected in salivary gland biopsy specimens at mRNA levels and parotid saliva at protein levels of SS patients (26). Thus, IL-18 expression in salivary gland cells may trigger a cytokine cascade in inducing IFN-γ, TNF-α, and IL-1. In addition to the proinflammatory properties, IL-18 acts as a chemoattractant of human CD4⁺ T cells (34). Furthermore, activation of APCs through IL-18Rα is required for the generation of pathogenic Th17 cells in the murine EAE model (35). Murine Th17 cells as well as Th1 cells express IL-18Rα (36) and IL-18 and IL-23 together promote IL-17 production from Th17 cells (36, 37). Therefore, it is also possible that IL-18 produced by salivary gland cells may contribute to the generation and activation of Th17 cells in SS patients.

Salivary gland cells express IL-18R and IL-17R on the cell surface (Fig. 6), and the expression of the receptors was not changed by stimulation with IL-18 or IL-17 (data not shown). Activation by IL-18 through IL-18R is mediated by MyD88 (38) and IL-1R-associated kinase 4 (39) adaptor molecules, which ultimately stimulate NF-κB (40). Activation by IL-17 through IL-17R leads to the activation of NF-κB and MAPKs in a TNFR-associated factor 6-dependent manner (41). IL-17 is produced by activated CD4⁺ and CD8⁺ memory T cells from human PBMCs in vitro (30, 42), and our study showed that the majority of the infiltrating CD4⁺ cells and a small number of the infiltrating CD8⁺ cells in the diseased lesion of autoimmune disease, SS, express IL-17 (Fig. 3). IL-17 does not seem to regulate T cell function but acts mostly on other cell types, such as fibroblasts, epithelial cells and endothelial cells. IL-17 treatment of these cells induces the expression of proinflammatory cytokines, such as IL-6 and IL-8, CSFs, chemokines, and matrix metalloproteinases (9–11). Overexpression of IL-17 has also been found to be associated with several inflammatory and autoimmune diseases in humans, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and asthma (9–11). Our study showed that IL-18 alone did not induce the secretion of IL-6 and IL-8 but augmented the IL-6- and IL-8-inducing activity of IL-17 when IL-17 was used at low amounts with minimal activity (Fig. 7) and that primary salivary gland cells from Donor 1 showed a synergy between IL-17 and IL-18 in induction of IL-8 (Fig. 8), possibly by amplification of MAPKs and NF-κB through IL-18R and IL-17R. However, IL-17 or IL-18 did not regulate the production of IL-6 and IL-18 in primary salivary gland cells from normal subjects (data not shown). These findings also suggest that susceptibility of glandular cells to IL-17 and IL-18 varied depending on donors or glandular cells in the salivary glands.

This study detected IL-17 in ductal epithelial cells from not only SS and sicca syndrome patients but also healthy subjects (Fig. 1). A recent report also showed IL-17 staining in ductal cells of the salivary glands from SS patients (43). High magnification showed that IL-17 was detected in cytoplasm, but not in the cell membrane, of ductal epithelial cells, which excluded the possibility that exogenous IL-17 binds to IL-17R on the cells and suggests that ductal epithelial cells produce IL-17 even under healthy conditions. It is suggested that IL-17 is important for host defense at the epithelial surface by inducing different classes of antimicrobial molecules (44). Therefore, it is conceivable that autocrine or paracrine activation of ductal epithelial cells by IL-17 is physiologically important for the protection of salivary glands against oral microbes.

IL-6 is a pleiotropic cytokine and plays a major role in the regulation of inflammation by eliciting proinflammatory effects (45). In addition, IL-6 inhibits the differentiation and function of regulatory T cells, and IL-6 with TGF-β promotes the development of Th17 cells (16, 17). Salivary gland epithelial cells of SS patients express IL-6 (26), and other studies showed that salivary glands consistently express IL-10, IL-6, and TGF-β (27, 28). IL-8 is a major chemokine that is responsible for the activation of neutrophils and the migration of neutrophils and T cells to the inflammatory sites (46). Thus, cooperation between IL-18 and IL-17 for the secretion of IL-6 and IL-8 by salivary epithelial cells may contribute to amplify the induction of Th17 cells as well as inflammation.

Chronic GVHD is a major cause of morbidity and mortality in long-term survivors of allogeneic stem cell transplantation, and clinical manifestations of chronic GVHD are similar to autoimmune diseases (47). The typical histological finding of oral chronic GVHD includes diffuse and periductal lymphocytic infiltration in labial salivary glands, similar to findings in SS (48). However, our study showed that IL-18 and IL-17 are expressed in the salivary glands of SS patients but not in chronic GVHD patients (Fig. 1).
These results suggest that IL-18 and IL-17 expressed in the salivary glands are associated with the pathogenesis of SS but not chronic GVHD, although the clinical features are similar between SS and chronic GVHD. As chronic GVHD patients examined in this study had been receiving immunosuppressive drugs, it is also possible that the immunosuppressive drugs inhibit Th17 infiltration and expression of IL-18 in the salivary glands. It is suggested that environmental factors, such as microbial infection with a genetic predisposition, are thought to trigger activation and alteration of glandular cells in SS (1), and it is still unclear what causes up-regulation of the expression of IL-18 and IL-17 in the salivary glands of SS patients. Further studies are needed to clarify this point.

In conclusion, our study showed that Th17 cells are infiltrated in the diseased region (the salivary glands) of a human autoimmune disease, SS, and suggests that Th17 cells, which are studied mostly in the murine EAE model, are involved in the pathogenesis of human autoimmune diseases. Our study also showed that IL-18 expressed in salivary gland cells achieves cis activation of the cells together with IL-17 for the secretion of inflammatory cytokines IL-6 and IL-8, and suggests that IL-18 and IL-17 expressed in the salivary glands are associated with the pathogenesis of SS in the microenvironment of the salivary glands. Our study also suggests that overexpression of IL-18 and IL-17 in the salivary glands is critically important therapeutic targets in SS.

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References


