with 0.3 nM ET-1. As shown in Fig. 2, 10 μM EIPA and 30 μM KB-R7943 partly inhibited the ET-1-induced sustained increases in $[Ca^{2+}]_i$ to 52.0 ± 1.9% and 37.3 ± 1.7%, respectively. EIPA had no further inhibitory effect on the $[Ca^{2+}]_i$ level after the inhibitory effect of KB-R7943 reached the maximum. These findings indicate that NHE functionally couples to NCX, which operates in the reverse mode to induce Ca^{2+} influx in exchange for Na^+ efflux, leading to an increase in $[Ca^{2+}]_i$. Furthermore, molecular machinery other than the NHE/NCX coupling may be involved in NCX-mediated Ca^{2+} influx, since the NCX inhibitor showed further suppression of the EIPA-resistant Ca^{2+} response to ET-1 (Fig. 2). Although such machinery remains to be determined, there are two possibilities, which will be described below.

One possibility is that an increase in $[Na^+]_i$ by activation of NHE following stimulation of ET_AR with ET-1 facilitates the Na^+,K^+-ATPase (Na^+/K^+ pump) that maintains the concentration gradients of Na^+ and K^+ ions across the surface membrane of cells by exporting 3 Na^+ and importing 2 K^+ (15). The operation of Na^+,K^+-ATPase is known to result in a change in membrane potential that modulates Ca^{2+} handling via certain Ca^{2+} channels and NCX (15). However, the possible involvement of a Na^+/K^+ pump affecting membrane potential in ET-1-induced Ca^{2+} influx could be ruled out since the Ca^{2+} response to ET-1 was unaffected by treatment with 30 μM ouabain, an inhibitor of Na^+,K^+-ATPase (data not shown).

The second possibility is a non-selective cation channel pathway. Our recent studies using whole-cell patch clamp and $[Ca^{2+}]_i$ measurement have shown that stimulation of ET_AR expressed in CHO cells activates two types of Ca^{2+}-permeable nonselective cation channels such as ROCs (designated as nonselective cation channel type 1 and type 2) and one type of SOCs (6). Although the precise molecular entities of SOCs and ROCs have been unknown, several studies with molecular and pharmacological techniques have indicated that transient receptor potential canonical channels, which function as voltage-independent, nonselective cation channels, are potential candidates for SOCs and ROCs (13). Unlike voltage-gated Ca^{2+} channels, these nonselective cation channels allow the entry of Na^+ in addition to Ca^{2+} with varying degrees of Ca^{2+}/Na^+ permeability. Therefore, the functional coupling of Na^+ entry via nonselective cation channels such as SOCs and ROCs with Ca^{2+} influx via NCX may be involved in the ET-1-induced Ca^{2+} entry, although this possibility must be further elucidated in the future.

As described above, NHE transports extracellular Na^+ for intracellular H^+, while NCX exchanges intracellular

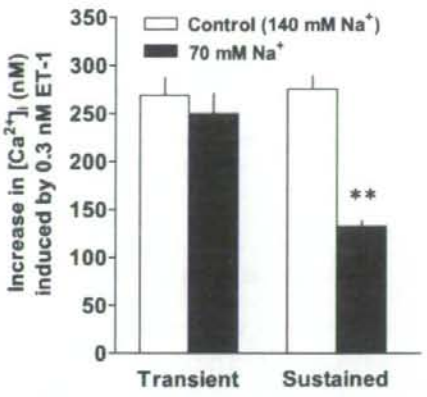

Fig. 3. Effects of reducing extracellular concentrations of Na^+ on the transient and sustained increases in $[Ca^{2+}]_i$ induced by 0.3 nM ET-1. The extracellular concentration of Na^+ was reduced by replacing Na^+ with Li^+. Data are presented as means ± S.E.M of the results obtained from 4 separate experiments. $^{**}P<0.01$.

Na^+ in exchange for extracellular Ca^{2+}. This implies that Na^+ transport via either NHE or NCX plays an important role in Ca^{2+} influx. However, the functional significance of Na^+ transport in the ET-1-induced increases in $[Ca^{2+}]_i$ has been unknown. To assess the role of Na^+ transport in the Ca^{2+} response to ET-1, the effects of reduction of extracellular Na^+ concentrations ($[Na^+]_e$) were examined. As shown in Fig. 3, reduction of $[Na^+]_e$ to 70 mM by replacing NaCl with equimolar concentration of LiCl did not affect the transient increase but significantly inhibited the sustained increase. This result suggests that Na^+ transport is involved in the ET-1-induced sustained increases in $[Ca^{2+}]_i$.

In summary, the present study provides evidence that ET-1 facilitates NHE activity, resulting in the sustained Ca^{2+} influx via the reverse mode of NCX, in CHO cells expressing human ET_AR. Furthermore, it is also suggested that Na^+ transport via both NHE and NCX plays a significant role in the regulation of $[Ca^{2+}]_i$.

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Biotin deficiency up-regulates TNF-α production in murine macrophages

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Abstract: Biotin, a water-soluble vitamin of the B complex, functions as a cofactor of carboxylases that catalyze an indispensable cellular metabolism. Although significant decreases in serum biotin levels have been reported in patients with chronic inflammatory diseases, the biological roles of biotin in inflammatory responses are unclear. In this study, we investigated the effects of biotin deficiency on TNF-α production. Mice were fed a basal diet or a biotin-deficient diet for 8 weeks. Serum biotin levels were significantly lower in biotin-deficient mice than biotin-sufficient mice. After i.v. administration of LPS, serum TNF-α levels were significantly higher in biotin-deficient mice than biotin-sufficient mice. A murine macrophage-like cell line, J774.1, was cultured in a biotin-sufficient or -deficient medium for 4 weeks. Cell proliferation and biotinylation of intracellular proteins were decreased significantly in biotin-deficient cells compared with biotin-sufficient cells. Significantly higher production and mRNA expression of TNF-α were detected in biotin-deficient J774.1 cells than biotin-sufficient cells in response to LPS and even without LPS stimulation. Intracellular TNF-α expression was inhibited by actinomycin D, indicating that biotin deficiency up-regulates TNF-α production at the transcriptional level. However, the expression levels of TNF receptors, CD14, and TLR4/myeloid differentiation protein 2 complex were similar between biotin-sufficient and -deficient cells. No differences were detected in the activities of the NF-κB family or AP-1. The TNF-α induction by biotin deficiency was down-regulated by biotin supplementation in vitro and in vivo. These results indicate that biotin deficiency may up-regulate TNF-α production or that biotin excess down-regulates TNF-α production, suggesting that biotin status may influence inflammatory diseases. *J. Leukoc. Biol.* 83: 912–920; 2008.

Key Words: *cytokine · inflammation · nutrition*

INTRODUCTION

Biotin is a water-soluble vitamin of the B complex found in all organisms [1]. Biotin functions as a cofactor of five carboxylases—pyruvate carboxylase (EC 6.4.1.1), two forms of acetyl-CoA carboxylase (ACC1 and ACC2; EC 6.4.1.2), propionyl-CoA carboxylase (PCC; EC 6.4.1.3), and methylcrotonoyl-CoA carboxylase (EC 6.4.1.4)—enzymes that catalyze the metabolism of glucose, amino acids, and fatty acids. As these biotin-dependent enzymes are indispensable for cellular metabolism, biotin starvation or deficiency is potentially lethal [2]. Biotin is covalently attached to specific lysine residues of carboxylases by the enzyme holocarboxylase synthetase (HCS; EC 6.3.4.10) [2]. During the turnover of carboxylases, biotin is released from biotinylated peptides by biotinidase (EC 3.5.1.12) and recycled in the biotinylation of new carboxylases [2]. The defect of this biotin cycle causes a neonatal form of life-threatening ketoacidosis and organic acidemia, known as multiple carboxylase deficiency [2].

In addition to this classical function as a cofactor of carboxylases, biotin is involved in various cellular events. It was reported that biotin regulates the mRNA expression of HCS and biotin-dependent carboxylases via a cGMP-dependent pathway [3]. Biotin regulates transcription factors, such as NF-κB, specificity protein 1 (Sp1), and Sp3, in human T cell line Jurkat cells [4, 5]. Moreover, biotinylation of histones in human cells was also reported [6]. In a human hepatoblastoma cell line, biotin regulates the expressions of asialoglycoprotein receptor and insulin receptor at the post-transcriptional level [7]. These reports clearly indicated that biotin regulates the various cellular events at transcriptional and post-transcriptional levels.

Immunological effects of biotin have also been studied. In vitro biotin supplementation induces IL-2Rγ expression and decreases the net secretion of IL-2 from Jurkat cells [8, 9]. In vivo supplementation of a pharmacologic dose of biotin decreases the proliferation rate of PBMC and the release of IL-1β and IL-2 [10]. Moreover, biotin deficiency changes the number and subpopulations of spleen lymphocytes and blocks thymocyte maturation in mice [11, 12]. On the other hand, contradictory results have been reported, namely that lymphocyte subpopulations, mitogen-induced cytokine production, IgG responses, and NK cell activity do not differ significantly be-

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tween mild to moderately biotin-deficient and biotin-sufficient rats [13]. Therefore, the immunological effects of biotin have not been clarified.

Moderately severe biotin deficiency causes alopecia and scaly erythematous dermatitis [11, 14, 15]. In addition to applications as a dietary supplement, biotin is prescribed for chronic dermatitis. It was reported that biotin has a therapeutic effect on pustulosis palmaris et plantaris, a type of chronic dermatitis that is restricted to the palms and soles and is related to metal allergy [16, 17]. Moreover, Makino et al. [18] reported that serum biotin levels are significantly lower in atopic dermatitis patients than in healthy subjects. These reports suggest that biotin deficiency is involved in inflammatory diseases. However, few reports are available about the biological roles of biotin in inflammatory responses.

In this study, we investigate the in vivo effects of biotin deficiency using a mouse model of LPS-induced TNF-α production. We also investigated the in vitro effects of biotin deficiency on the production of TNF-α by the murine macrophage cell line J774.1. We showed that biotin status affects the production of TNF-α, and biotin-supplementation down-regulated it in vivo and in vitro.

MATERIALS AND METHODS

Reagents

LPS from *Escherichia coli* O55:B5, prepared by Westphal's method, was purchased from Difco Laboratories (Detroit, MI, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Mice

Female BALB/c mice (4 weeks old), obtained from the Institute for Experimental Animals of the Tohoku University Graduate School of Medicine (Sendai, Japan), were used for the experiments. Mice were divided into two groups: biotin-sufficient and -deficient groups, which received a basal diet (AIN-76, containing 0.8 mg *d*-biotin per kg) or a biotin-deficient (biotin-free) AIN-76 diet (Nosan Corp., Yokohama, Japan), respectively. Preliminary experiments showed that 8-week feeding led to a significant decrease in the serum biotin level in mice. For in vivo biotin supplementation, biotin-deficient mice were provided with biotin-supplemented drinking water (15 μM) for 2 weeks. The dose of biotin was based on the dose of human biotin therapy (10–40 mg/day) and drinking volume of mice (5 ml/day) [19, 20]. The Ethical Board for Nonhuman Species of the Tohoku University Graduate School of Medicine approved the experimental procedure followed in this study.

Measurement of biotin in serum

Biotin in serum was purified by HPLC and measured by ELISA, as originally described by Mock [21]. Briefly, serum (1 ml; the serum was pooled from five mice) was ultrafiltrated using a centrifugal ultrafiltration unit (a MW cutoff of 30 kDa) at 1500 *g* for 90 min at 4°C. The filtrates were adjusted to pH 2.5 by 6 M HCl and then separated by reversed-phase HPLC on a Capcelpak AG120A C18 (5 μm, 4.6×250 mm, Shiseido, Tokyo, Japan) column at a flow rate of 1.0 ml/min at 40°C. Elution was carried out with solution A [0.1% trifluoroacetic acid (TFA)] and solution B (80% acetonitrile in 0.8% TFA). The linear gradient was started at solution A 100% and solution B 0% and was reached at solution A 81% and solution B 19% at 30 min and then solution A 0% and solution B 100% at 60 min. The column eluent was collected every minute in test tubes and dried by a centrifugal concentrator. After drying, each fraction was dissolved in 500 μl H_2O. From preliminary experiments with commercial biotin standard, we confirmed that biotin elutes at 32 min. For ELISA, 96-well, flat-bottomed plates (Nunc-Immuno modules, MaxiSorp, Nalge-Nunc International, Rochester, NY, USA) were coated with biotinyl-BSA, synthesized with BSA and biotin *N*-hydroxysuccinimide ester (Pierce Biotechnology, Inc., Rockford, IL, USA), and then blocked with 0.01% (wt/vol) BSA. Samples (100 μl) and 50 μl HRP-conjugated avidin (Calbiochem, Darmstadt, Germany) were mixed and incubated for 2 h at room temperature. Then, 100 μl of the mixture was transferred to a well of a biotinyl-BSA-coated plate and incubated for 4 h at room temperature. The ELISA was developed with 3,3',5,5' tetramethylbenzidine (substrate reagent set, BD Biosciences, San Diego, CA, USA) as a substrate. The detection range of biotin was 3–10,000 nM.

Measurement of PCC activity

PCC activity was measured as originally described by Zempleni et al. [22]. Briefly, the liver was homogenized on ice in buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM 2-ME, and 0.25 M sucrose and centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was used as a crude enzyme fraction. For PCC assay, a 5-μl tenfold-diluted crude enzyme fraction was incubated with a 95-μl reaction mixture for 5 min at room temperature. The reaction mixture consisted of 100 mM Tris-HCl (pH 8.0), 0.75 mM DTT, 6 mM $MgCl_2$, 3.14 mM Na_2-ATP, 100 mM KCl, 1% Triton X-100, 1 mM propionyl-CoA, and 3.5 mM $NaH[^{14}C]O_3$ (2.11 kBq/nmol). The reaction was stopped by the addition of 30 μl 1 M $HClO_4$. After centrifugation at 1000 *g* for 15 min, 100 μl aliquot was transferred into a vial and dried. Finally, the sample was resolved in a scintillation cocktail, and the bound [^{14}C]bicarbonate was quantified by a liquid scintillation counter. PCC activity was expressed in units: 1 unit = 1 nmol HCO_3^- fixed/min/mg protein.

Measurement of TNF-α in serum

LPS (1 μg/kg) was injected i.v. into mice. After 90 min, blood was collected and coagulated on ice for 1 h, and sera were recovered by centrifugation. The amounts of TNF-α in sera were measured with an OptEIA mouse TNF-α (Mono/Mono) set (BD Biosciences).

Biotin depletion from FCS

Biotin in FCS was depleted with immobilized avidin-agarose (Pierce Biotechnology, Inc.). Briefly, FCS (Tissue Culture Biologicals, Tulare, CA, USA) was mixed with avidin-agarose and gently stirred overnight at 4°C. After centrifugation, FCS was sterilized by filtration (0.22 μm pore size) and stored at −30°C until use. Biotin depletion was confirmed by ELISA as described above. The biotin concentration in FCS was 8.6 ± 0.25 nM, and that in biotin-deficient FCS was not detectable (less than 3 nM).

Cells and cell culture

J774.1, a murine macrophage-like cell line, was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. J774.1 cells were grown in biotin-sufficient and -deficient medium. The biotin-sufficient medium was RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) containing *d*-biotin (0.2 μg/ml) supplemented with 10% FCS. The biotin-deficient medium was biotin-free RPMI 1640 supplemented with 10% biotin-deficient FCS.

Western blotting

J774.1 cells were lysed in SDS-PAGE sample buffer. Cell lysates were separated by SDS-PAGE under reducing conditions. After SDS-PAGE, gel proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blot was blocked for 1 h with 3% (wt/vol) skim milk and 0.05% Tween 20 in PBS and incubated with HRP-conjugated avidin (Calbiochem). After washing, the blot was analyzed with SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Inc.) and a Chemi Imager (Alpha Innotech Corp., San Leadro, CA, USA).

[^{3}H] TdR incorporation assay

J774.1 cells were suspended in RPMI 1640 with 10% FCS and seeded in a 96-well, flat-bottomed plate (Nalge-Nunc International) at the indicated cell

numbers. The cells were incubated at 37°C for 3 h, and each well was pulsed with 1 kBq [^{3}H] TdR (MP Biomedicals, Irvine, CA, USA) for 2 h. After the pulse, 100 μl 3% Triton X-100 was added to each well, and the cells were harvested onto a glass fiber filter. The counts of [^{3}H] TdR per minute (cpm) were measured with a liquid scintillation counter (Packard Instrument Company, Meriden, CT, USA).

Measurement of TNF-α in culture supernatants of J774.1 cells

J774.1 cells were suspended in RPMI 1640 with 10% FCS and seeded in a 96-well flat-bottomed plate at 2×10^5 cells/200 μl/well. The cells were stimulated with LPS for 24 h at 37°C. The amount of TNF-α in the culture supernatant was measured with an OptEIA mouse TNF-α (Mono/Mono) set (BD Biosciences).

Flow cytometry

For TNF-α staining, J774.1 cells were seeded in a six-well, flat-bottomed plate (Nalge-Nunc International) at 1×10^6 cells/2 ml/well with RPMI 1640 with 10% FCS. The cells were cultured for 6 h at 37°C in the presence or absence of 1 μg/ml actinomycin D (Act-D). Then, the cells were stained with FITC-conjugated anti-mouse TNF mAb (clone MP6-XT22, BD Biosciences) for cell surface cytokine staining. For intracellular cytokine staining, the cells were incubated with a protein transport inhibitor (GolgiPlug, BD Biosciences). After fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences), the cells were stained with FITC-conjugated anti-mouse TNF mAb. For cell surface receptor staining, J774.1 cells were stained with PE-conjugated anti-mouse CD120a (TNFR type I) mAb (clone 55R-286, BioLegend, San Diego, CA, USA), PE-conjugated anti-mouse CD120b (TNFR type II) mAb (clone TR75-89, AbD Serotec, Oxford, UK), PE-conjugated anti-mouse CD14 mAb (clone rmC5-3, BD Biosciences), or FITC-conjugated anti-TLR4/myeloid differentiation protein 2 (MD2) complex mAb (clone MTS510, Stressgen Bioreagents, Ann Arbor, MI, USA). Expression of each molecule was measured using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Measurement of lactate dehydrogenase (LDH) activity

For the quantification of plasma membrane damage, LDH activity in the culture supernatants of J774.1 cells was measured with a cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

Cells were lysed in 1 ml Isogen (Nippon Gene, Toyama, Japan), and total RNA was extracted as described in the instruction manual. Total RNA was dissolved in 30 μl diethyl pyrocarbonate-treated water (Nippon Gene) and incubated at 65°C for 10 min. cDNA synthesis was carried out with a first-strand cDNA synthesis kit (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA). Real-time PCR was performed with a LightCycler FastStart DNA Master SYBR Green I and a LightCycler 1.5 system (Roche Diagnostics). The primers used for PCR were as follows: TNF-α, forward 5′-AGCCTCTTCTCATTCCTGC-3′ and reverse 5′-GGAGGCCATTTGGGAACT-3′; β-actin, forward 5′-CGTTGACATCCGTAAAGACCTC-3′ and reverse 5′-AGCCACCGATCCACACAGA-3′ (Nihon Gene Research Labs Inc., Sendai, Japan). The PCR conditions were 35 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The product sizes for TNF-α and β-actin were 106 bp and 173 bp, respectively. The mRNA expression levels were expressed as relative units after normalization by the β-actin level. The specificity of the PCR was confirmed by the molecular weight of the products and melting curve analysis for each data point. PCR products were electrophoresed using 3% agarose (Nusieve 3:1 agarose, BMA, Rockland, ME, USA). After staining with ethidium bromide, amplified DNA bands were analyzed with a Chemi Imager.

Measurement of NF-κB and AP-1 activity

Activities of NF-κB family and AP-1 in nuclear extracts were measured with a NF-κB assay kit specific for the p65, p50, and RelB, p52 subunits, and an AP-1 assay kit specific for the phospho c-Jun, according to the manufacturer's instructions (Active Motif, Carlsbad, CA), respectively. The nuclear extractions were performed with a nuclear extract kit (Active Motif), according to the manufacturer's instructions. Protein concentrations of each fraction were determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc.). Each activity was expressed as absorbance at 450 nm ($A_{450\ nm}$) per 100 μg protein.

Data analysis

All of the experiments in this study were performed at least three times to confirm the reproducibility of the results. The data shown are representative results. Experimental values are given as the mean ± SD of triplicate assays. Statistical analysis was performed with the unpaired *t*-test or one-way ANOVA using Dunnett's method, and $P < 0.05$ was considered significant.

RESULTS

Augmentation of LPS-induced serum TNF-α levels in biotin-deficient mice

As it is well known that LPS induces the elevation of the serum concentration of TNF-α in mice, we first examined the in vivo effects of biotin deficiency on LPS-induced TNF-α production. Mice were fed biotin-sufficient or -deficient diets. After 8 weeks of feeding, the serum concentrations of biotin were significantly ($P<0.001$) lower in biotin-deficient mice than biotin-sufficient mice (**Fig. 1A**). However, PCC activities in the liver were comparable between biotin-sufficient and -deficient mice (sufficiency: 2.99±0.49 nmol HCO_3^- fixed/min/mg protein; deficiency: 2.80±0.34 nmol HCO_3^- fixed/min/mg pro-

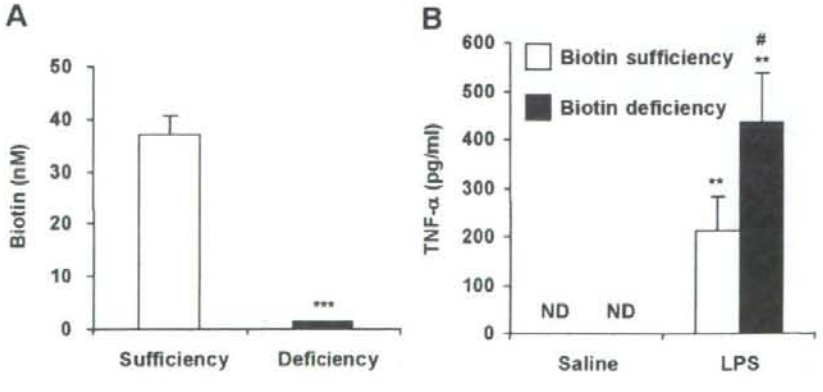

Fig. 1. Serum levels of biotin and TNF-α in biotin-sufficient and -deficient mice. Female BALB/c mice (4 weeks old) were fed a biotin-sufficient or -deficient diet for 8 weeks. (A) Concentrations of biotin in sera were measured by ELISA after prior separation by HPLC. The results were expressed as mean ± SD of triplicate assay. ***, $P < 0.001$, compared with biotin sufficiency. (B) The mice were challenged i.v. with LPS (1 μg/kg) or saline alone, and blood was taken at 90 min after injection. Concentrations of TNF-α in sera were measured by ELISA. The results were expressed as mean ± SD for four mice. ND, Not detected; **, $P < 0.01$, compared with saline; #, $P < 0.05$, compared with biotin sufficiency.

tein; $n=5$; $P=0.517$). No clinical symptoms were detected in the biotin-deficient mice, and no significant differences of body weights were detected between biotin-sufficient and -deficient mice (data not shown). A significant ($P<0.01$) increase of the serum TNF-α level was induced 90 min after i.v. injection of LPS (1 μg/kg) in both groups (Fig. 1B). In biotin-deficient mice, the concentration of TNF-α was significantly ($P<0.05$) higher than that in biotin-sufficient mice. These results indicated that biotin deficiency augments TNF-α production in vivo.

In vitro effects of biotin deficiency on murine macrophages

Macrophages are the major cells that produce TNF-α in response to LPS. Therefore, we next examined the in vitro effects of biotin deficiency using J774.1 cells. In biotin-sufficient J774.1 cells, 130- and 80-kDa polypeptides were detected by Western blotting with HRP-conjugated avidin. On the other hand, these polypeptides were not detected after 2 weeks cultivation in biotin-deficient medium (**Fig. 2A**). On the basis of molecular weight, it is likely that the 130-kDa polypeptide is pyruvate carboxylase, and the 80-kDa polypeptide is propionyl-CoA carboxylase and/or methylcrotonyl-CoA carboxylase. Moreover, [^{3}H] TdR incorporation was significantly ($P<0.01$) lower in biotin-deficient cells than biotin-sufficient cells (Fig. 2B). These results clearly indicated that biotinylation of cellular proteins and cell proliferation was reduced by biotin deficiency.

Augmentation of LPS-induced TNF-α production in biotin-deficient J774.1 cells

Next, we analyzed the production of TNF-α from biotin-sufficient and -deficient J774.1 cells. As shown in **Figure 3A**, both types of cells produced TNF-α in response to LPS in a dose-dependent manner. The concentration of TNF-α in the culture supernatant of biotin-deficient cells was significantly ($P<0.01$) higher than that of biotin-sufficient cells, even without LPS stimulation. A similar pattern of TNF-α production was observed when the type of medium was replaced with the opposite type during LPS stimulation for 24 h, which excluded the possibility of contamination with stimulatory or inhibitory factors in either type of medium. LDH activity was not detected in the culture supernatant of biotin-deficient cells (data not shown), indicating that the augmentation of TNF-α production in biotin-deficient cells was not a result of plasma membrane damage. The levels of TNF-α mRNA were significantly ($P<0.01$) elevated in biotin-deficient cells compared with those in biotin-sufficient cells with or without LPS stimulation (Fig. 3B). Moreover, flow cytometric analysis revealed that the expression levels of cell surface and intracellular TNF-α were also higher in biotin-deficient cells than in biotin-sufficient cells (Fig. 3, C and D).

We also analyzed the effects of Act-D, an inhibitor of RNA polymerase II. In the presence of Act-D, intracellular TNF-α was decreased slightly in biotin-sufficient cells (**Fig. 4A**). On the other hand, it was markedly decreased in biotin-deficient cells (Fig. 4B) and in LPS-stimulated, biotin-sufficient cells (Fig. 4C). These results clearly indicated that biotin deficiency induces the augmentation of TNF-α production in vitro at the transcriptional level.

Cell surface expression of TNFRs

It was reported that biotin supplementation induces IL-2Rγ expression and decreases the net secretion of IL-2 by endocytosis of the cytokine [9]. Therefore, we measured cell surface expressions of TNFR types I and II on biotin-sufficient and -deficient J774.1 cells using a flow cytometer. As shown in **Figure 5, A** and **B**, the expression levels of TNF-R types I and II were similar between biotin-sufficient and -deficient cells. Mean fluorescence intensities of TNFR type I in biotin-sufficient and -deficient cells were 33.3 and 30.6, respectively; those of TNFR type II in biotin-sufficient and -deficient cells were 19.4 and 17.7, respectively. These results indicated that the augmentation of TNF-α production in biotin-deficient cells was not a result of down-regulation of the cell surface expression of TNFR and endocytosis of TNF-α.

Cell surface expression of CD14 and TLR4

To examine the possibility that biotin deficiency up-regulates LPS responsiveness through augmentation of LPS receptors, we measured the cell surface expression of CD14 and the TLR4/MD2 complex on biotin-sufficient and -deficient J774.1 cells.

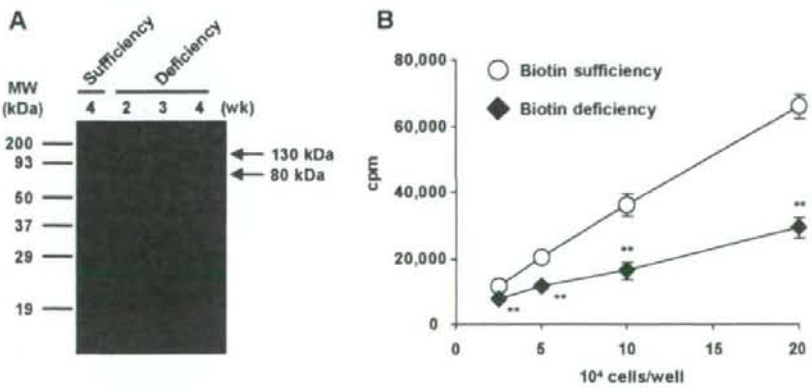

Fig. 2. In vitro effects of biotin deficiency on J774.1 cells, which were (A) cultured with biotin-sufficient or -deficient medium for the time indicated. Cells were lysed in SDS-PAGE sample buffer. Cell lysate (1×10^4 cells each) was subjected to Western blotting with HRP-conjugated avidin. (B) J774.1 cells were cultured with biotin-sufficient or -deficient medium for 4 weeks. Biotin-sufficient and -deficient cells in the medium with and without biotin, respectively, were seeded in 96-well flat-bottomed plates and incubated at 37°C for 3 h. Cells were pulsed with 1 kBq/well [^{3}H] TdR for 2 h. After the pulse, 100 μl 3% Triton X-100 was added to each well, and cells were harvested onto a glass fiber filter. **, $P < 0.01$, compared with biotin sufficiency.

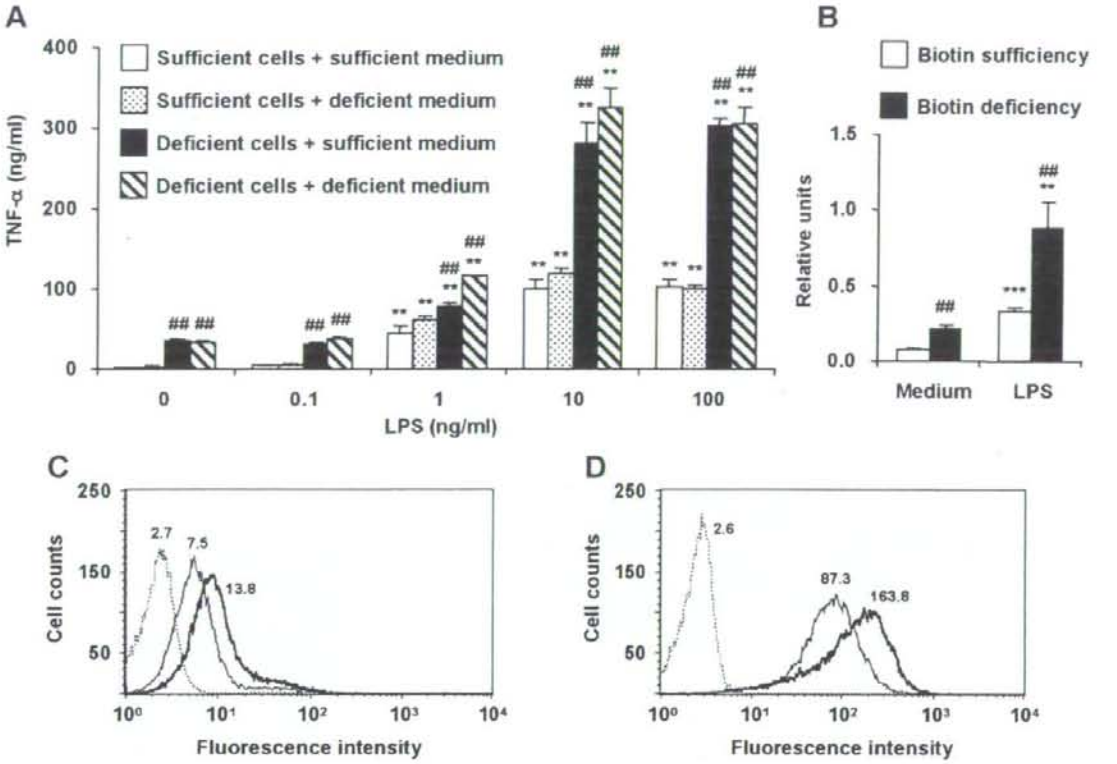

Fig. 3. TNF-α production of biotin-sufficient and -deficient J774.1 cells, which were cultured with biotin-sufficient or -deficient medium for 4 weeks. (A) Cells were seeded in 96-well flat-bottomed plates at 2×10^5 cells/200 μl/well with biotin-sufficient and -deficient medium and then stimulated with LPS at 37°C for 24 h. Concentrations of TNF-α in culture supernatants were measured by ELISA. **, $P < 0.01$, compared with medium alone (0 ng/ml LPS); ##, $P < 0.01$, compared with biotin sufficiency. (B) Biotin-sufficient and -deficient cells in medium with and without biotin, respectively, were seeded in 24-well flat-bottomed plates at 5×10^5 cells/500 μl/well and then stimulated with LPS (10 ng/ml) at 37°C for 4 h. TNF-α mRNA expression levels were determined by qRT-PCR. The results were expressed as relative units after normalization by the β-actin level. **, $P < 0.01$; ***, $P < 0.001$, compared with medium alone; ##, $P < 0.01$, compared with biotin sufficiency. (C and D) Cell surface (C) and intracellular TNF-α (D) of biotin-sufficient and -deficient cells were analyzed by flow cytometry. Dotted line, Unstained J774.1 cells (control); thin line, biotin sufficiency; bold line, biotin deficiency. Numbers in histograms indicate mean fluorescence intensity.

No differences were detected in the expression of these LPS receptors between biotin-sufficient and -deficient cells. Mean fluorescence intensities of CD14 in biotin-sufficient and -deficient cells were 9.0 and 9.2, respectively (Fig. 5C). Those of the TLR4/MD2 complex in biotin-sufficient and -deficient cells were 2.5 and 2.2, respectively (Fig. 5D). These results indicated that up-regulation of TNF-α production in biotin-deficient cells was not a result of augmentation of LPS receptors.

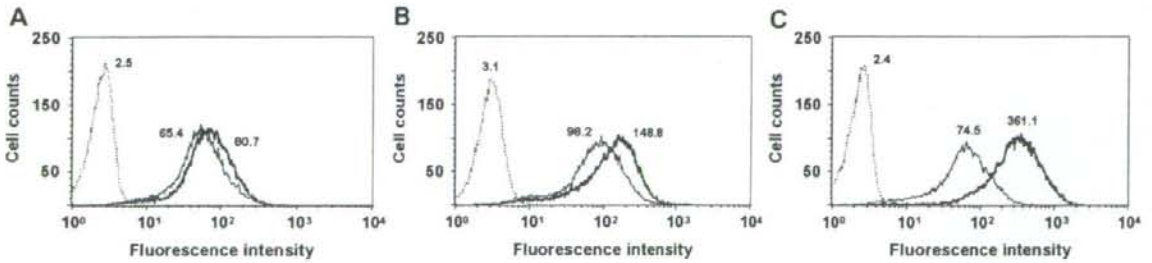

Fig. 4. Effects of Act-D on intracellular TNF-α levels of biotin-sufficient and -deficient J774.1 cells, which were cultured with biotin-sufficient or -deficient medium for 4 weeks. Biotin-sufficient and -deficient cells in the medium with and without biotin, respectively, were seeded in six-well flat-bottomed plates at 1×10^6 cells/2 ml/well with (thin line) or without (bold line) Act-D (1 μg/ml) at 37°C for 6 h. Intracellular TNF-α was analyzed by flow cytometry. The dotted line shows unstained J774.1 cells (control). (A) Biotin sufficiency without LPS; (B) biotin deficiency without LPS; (C) biotin sufficiency with LPS (10 ng/ml). Numbers in histograms indicate mean fluorescence intensity.

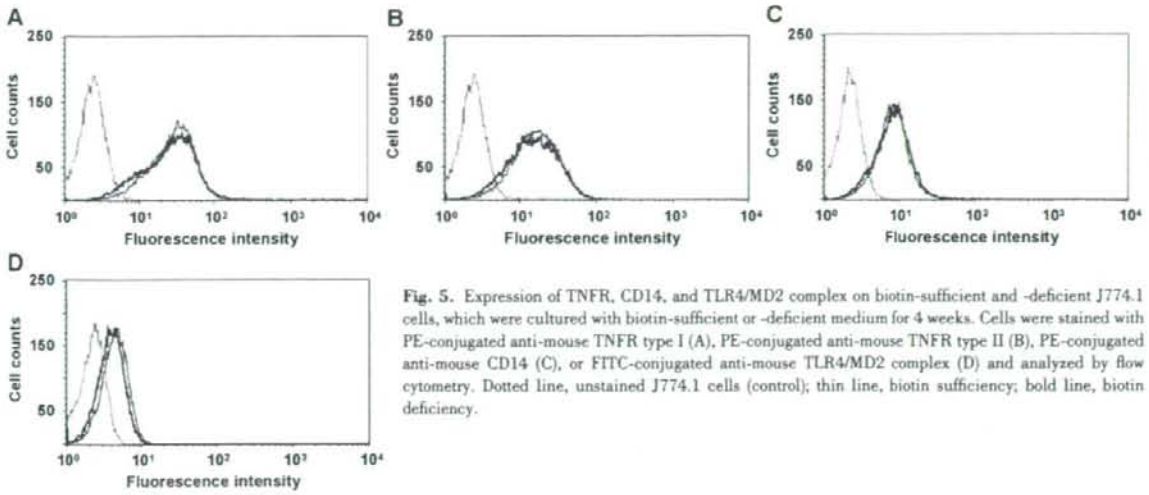

Fig. 5. Expression of TNFR, CD14, and TLR4/MD2 complex on biotin-sufficient and -deficient J774.1 cells, which were cultured with biotin-sufficient or -deficient medium for 4 weeks. Cells were stained with PE-conjugated anti-mouse TNFR type I (A), PE-conjugated anti-mouse TNFR type II (B), PE-conjugated anti-mouse CD14 (C), or FITC-conjugated anti-mouse TLR4/MD2 complex (D) and analyzed by flow cytometry. Dotted line, unstained J774.1 cells (control); thin line, biotin sufficiency; bold line, biotin deficiency.

Activities of transcriptional factors in biotin-sufficient and -deficient J774.1 cells

We also analyzed the activities of two major transcriptional factors, which are responsible for TNF-α mRNA transcription—the NF-κB family (p65, p50, RelB, and p52) and AP-1 (phospho c-Jun)—in nuclear fractions of biotin-sufficient and -deficient J774.1 cells. The activities of NF-κB p65, p50, and AP-1 were increased significantly with LPS stimulation. However, no significant differences were detected in NF-κB family and AP-1 activities between biotin-sufficient and -deficient cells (**Fig. 6**), indicating that other factors are involved in the augmentation of TNF-α mRNA transcription in biotin-deficient cells.

Regulation of TNF-α levels by biotin supplementation in biotin-deficient J774.1 cells and biotin-deficient mice

To further confirm the effects of biotin deficiency, J774.1 cells were cultured with biotin-deficient medium for 4 weeks and then further incubated with biotin-sufficient medium for 2 weeks (biotin-supplement cells). [^{3}H] TdR incorporation in biotin-supplemented cells was still lower than that in biotin-

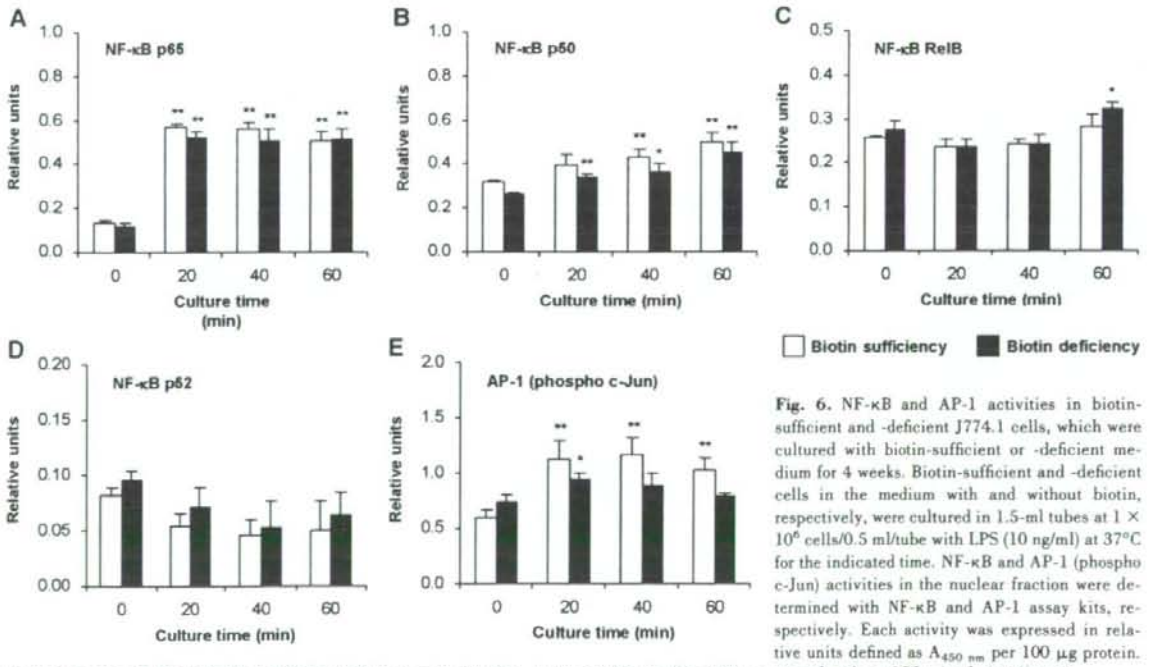

Fig. 6. NF-κB and AP-1 activities in biotin-sufficient and -deficient J774.1 cells, which were cultured with biotin-sufficient or -deficient medium for 4 weeks. Biotin-sufficient and -deficient cells in the medium with and without biotin, respectively, were cultured in 1.5-ml tubes at 1×10^6 cells/0.5 ml/tube with LPS (10 ng/ml) at 37°C for the indicated time. NF-κB and AP-1 (phospho c-Jun) activities in the nuclear fraction were determined with NF-κB and AP-1 assay kits, respectively. Each activity was expressed in relative units defined as $A_{450\ nm}$ per 100 μg protein. (A) NF-κB p65; (B) NF-κB p50; (C) RelB; (D) NF-κB p52; (E) AP-1. *, $P < 0.05$; **, $P < 0.01$, compared with no LPS stimulation (0 min).

sufficient cells but was significantly ($P<0.01$) higher than that in biotin-deficient cells (**Fig. 7A**). The concentrations of TNF-α in the culture supernatants of biotin-supplemented cells with and without LPS stimulation were significantly ($P<0.01$) reduced to near the levels in the supernatants of biotin-sufficient cells (Fig. 7B). The TNF-α production of biotin-deficient cells was significantly ($P<0.01$) decreased, even in the presence of 50 μM biotin during LPS stimulation (Fig. 7C). When biotin-deficient mice were provided with biotin-supplemented drinking water, the LPS-induced serum TNF-α levels of the mice were significantly ($P<0.05$) decreased (Fig. 7D). These results indicated that biotin supplementation restored the TNF-α production to the basal level in vitro and in vivo.

DISCUSSION

In this study, we clearly demonstrated that TNF-α production is up-regulated under biotin-deficient conditions and that biotin supplementation down-regulates the TNF-α production to the basal level in vivo and in vitro. These results suggest that biotin contributes to the regulation of inflammatory responses.

As biotin is produced by intestinal bacteria, many studies analyzed biotin-deficient animals that were fed a biotin-deficient diet containing egg-white protein, which contains avidin, a glycoprotein that forms a noncovalent and nonabsorbed complex with biotin [11–13, 23]. It was reported that biotin-deficient mice fed egg white for more than 7 weeks showed body weight loss and clinical symptoms, such as alopecia and

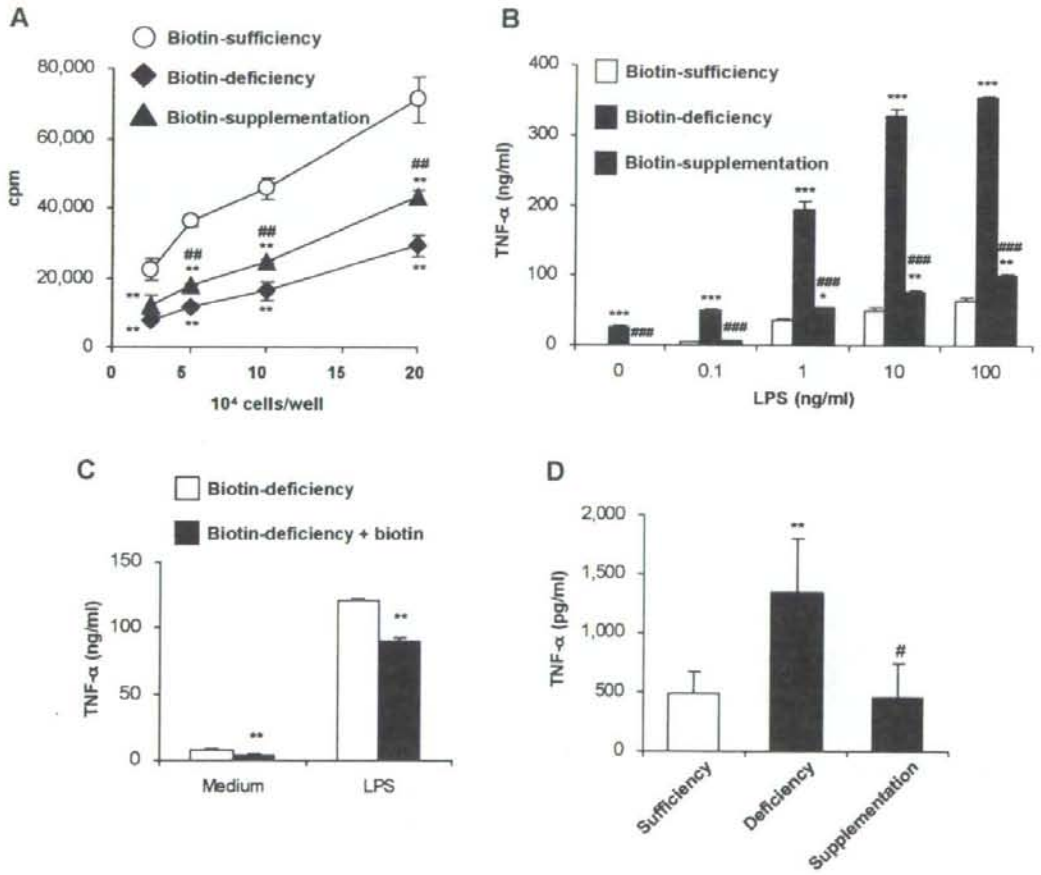

Fig. 7. Effects of biotin supplementation in vitro and in vivo. (A) J774.1 cells were cultured with biotin-deficient medium for 4 weeks and then incubated further in medium without biotin (biotin deficiency) or with biotin (biotin supplementation) for 2 weeks. J774.1 cells were also cultured in biotin-sufficient medium for 6 weeks (biotin sufficiency). [^{3}H] TdR incorporation is shown in Figure 2B. **, $P < 0.01$, compared with biotin sufficiency; ##, $P < 0.01$, compared with biotin deficiency. (B) Cells (2×10^5 cells/200 μl/well) in A were stimulated with LPS at 37°C for 24 h. Concentrations of TNF-α in culture supernatants were measured by ELISA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with biotin sufficiency; ###, $P < 0.001$, compared with biotin deficiency. (C) Cells (2×10^5 cells/200 μl/well) in A were stimulated with 10 ng/ml LPS in the presence of 50 μM biotin at 37°C for 24 h. Concentrations of TNF-α in culture supernatants were measured by ELISA. **, $P < 0.01$, compared with biotin deficiency (without biotin). (D) Mice were fed a biotin-deficient diet for 10 weeks (deficiency). Biotin-supplemented drinking water (15 μM) was administered to the mice for the last 2 weeks (biotin supplementation). The mice were then challenged i.v. with LPS (1 μg/kg) or saline alone, and blood was collected at 90 min after injection. Concentrations of TNF-α in sera were measured by ELISA. The results were expressed as mean ± SD for four mice. **, $P < 0.01$, compared with biotin sufficiency; #, $P < 0.05$, compared with biotin deficiency.

squatting posture [11]. Biotin-deficient rats fed egg white for 40 days showed partial alopecia and neurologic signs, such as kangaroo gait and irritability [13]. Moreover, severe biotin deficiency causes alopecia and scaly erythematous dermatitis in humans [11, 14, 15]. In this study, biotin-deficient mice were fed a biotin-deficient AIN-76 basal diet for 8 weeks, and the biotin concentrations in sera from biotin-deficient mice were significantly ($P<0.001$) lower than those from biotin-sufficient mice (Fig. 1A). However, PCC activities in the liver were comparable in biotin-sufficient and -deficient mice, and no body weight loss or clinical symptoms were detected. Therefore, we considered that our method causes mild biotin deficiency in mice.

Biotin-deficient J774.1 cells exhibited lower proliferation than biotin-sufficient cells (Fig. 2B). It was reported that intracellular uptake of biotin increases with cell proliferation [24]. As mentioned in Introduction, biotin-dependent carboxylases are essential for cellular metabolism, indicating that biotin deficiency causes inactivation of biotin-dependent carboxylases followed by the down-regulation of cellular metabolism and proliferation.

TNF-α production was augmented in biotin-deficient mice (Fig. 1B) and cells (Fig. 3). Biotin-deficient J774.1 cells produced a significantly higher amount of TNF-α, even without LPS stimulation. Moreover, the cell surface expression levels of the CD14 and TLR4/MD2 complex were not significantly different between biotin-sufficient and -deficient cells (Fig. 5, C and D). These results exclude the possibility that the augmentation of TNF-α production was mediated by up-regulation of LPS receptors. The TNF-α mRNA expression was significantly higher in biotin-deficient cells than -sufficient cells, and intracellular TNF-α was decreased markedly in the presence of Act-D (Fig. 4). It was reported that biotin supplementation up-regulates the expression level of the IL-2R and decreases the net production of IL-2 by endocytosis of cytokines [9]. However, no differences were detected in the expression levels of TNFR types I and II between biotin-sufficient and -deficient cells (Fig. 5, A and B). Furthermore, no differences were observed in the TNF-α-converting enzyme activities or mRNA levels of the tissue inhibitor of metalloproteinase-3, an inhibitor of the TNF-α-converting enzyme, between biotin-sufficient and -deficient cells (data not shown). The expression of intracellular and cell surface TNF-α was higher in biotin-deficient than -sufficient cells (Fig. 3, C and D). These results indicate that the augmentation of TNF-α production was regulated at the transcriptional level.

On the other hand, the biotin content in the AIN-76 diet (0.8 mg/kg) is much higher than the metabolic requirement of biotin. It was reported that the adequate intake of biotin for adult humans (~60 kg body weight) is 45 μg/day (0.75 μg/kg/day, Ministry of Health, Labor and Welfare, Japan). Based on the biotin content (0.8 mg/kg) and the total intake of diet (~5 g/day), biotin-sufficient mice (~25 g body weight) received 4 μg biotin per day (160 μg/kg/day). Moreover, the concentration of biotin in RPMI-1640 medium (0.2 μg/ml, ~800 nM) is much higher than biotin levels in normal mouse (40 nM) and human sera (0.2 nM) [11, 25]. Therefore, it is possible that the excess of biotin is involved in the down-regulation of TNF-α production.

No differences were detected in the activities of two major transcriptional factors involved in regulating TNF-α expression, namely NF-κB family members and AP-1 (Fig. 6). This result conflicts with the finding reported by Rodriguez-Melendez et al. [5] that the nuclear translocation and transcriptional activities of NF-κB were increased by biotin deficiency in Jurkat cells. The discrepancy may have been caused by the differences of cell line (murine macrophage cell line J774.1 and human T cell line Jurkat) or assay conditions. J774.1 cells were stimulated with LPS up to 60 min. On the other hand, Jurkat cells were stimulated with PMA and phytohemagglutinin for 3 h. Further studies are needed to clarify this point. It was reported that lysine residues in histones are modified by biotinylation, and the biotinylation of histones was enriched in transcriptionally inactive chromatin [6, 26, 27]. It is well known that some histone modifications, such as acetylation and methylation, correlate with transcriptional activation [28]. These observations suggest that the mechanism by which biotin deficiency contributes to up-regulation of TNF-α in J774.1 cells might be through biotin deficiency, producing reduced biotinylation of critical histones, leading to increased gene transcription.

Because of the uncertain immunological and pharmacological mechanisms, few studies have been reported about biotin treatment of inflammatory diseases [16]. On the other hand, it was reported that 10 nM biotin inhibited IL-2 production by Jurkat cells [8, 9]. Moreover, Zempleni et al. [10] reported that in vivo supplementation of biotin in healthy human subjects (3.1 μmol/day) inhibited IL-1β and IL-2 production of PBMCs. In this study, we demonstrated clearly that biotin supplementation down-regulated the augmented TNF-α production induced by biotin deficiency in vivo and in vitro (Fig. 7). Therefore, we speculate that a pharmacological dose of biotin might have some potentially therapeutic effects on inflammatory diseases, but this remains to be tested empirically.

TNF-α production of biotin-deficient J774.1 cells was drastically decreased by biotin supplementation in experiments in which biotin-deficient cells were cultured with biotin-sufficient medium (containing ~0.5 μM biotin) for 2 weeks (Fig. 7B). On the other hand, TNF-α production was similar in biotin-deficient cells cultured with biotin-sufficient and -deficient medium (Fig. 3A). A high dose of biotin (50 μM) slightly but significantly decreased TNF-α production by biotin-deficient cells (Fig. 7C). These results indicate that long-term supplementation of biotin is more effective for the inhibition of TNF-α up-regulation than stimulation with a high dose of biotin. Therefore, we considered that TNF-α up-regulation in biotin-deficient cells is regulated via various metabolic pathways that are affected by biotin rather than via the direct effects of biotin about TNF-α production.

It is well known that biotin deficiency causes cutaneous abnormalities [11, 14, 15]. In addition, it was reported that the biotin concentration in serum correlates with inflammatory diseases [11, 14, 15, 18]. Although several studies reported the contribution of abnormalities in lipid metabolism to cutaneous abnormalities (reviewed in ref. [14]), the pathological mechanisms of disease conditions caused by biotin deficiency remain to be clarified. In this study, we clearly demonstrated that biotin regulates TNF-α production in vivo and in vitro. TNF-α

plays important roles in the pathogenesis of inflammatory diseases, which have been reported to be correlated with biotin [29–31]. Therefore, biotin status may be involved in the pathological mechanisms of dermatitis and other inflammatory diseases. Our results should encourage further investigations on biotin treatment for various inflammatory diseases.

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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[1]

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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)[3] 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 an 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 linage 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 naive 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, Sjögren's syndrome; proIL-18, 24-kDa precursor form of IL-18; EAE, experimental autoimmune encephalomyelitis; GVHD, graft-versus-host disease.

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 focus histopathologically in their labial salivary gland biopsy and were positive for autoantibodies against SS-A or SS-B, or at 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 mouse and dry eye without focus histopathologically in their labial salivary gland biopsy results. 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 labial salivary gland biopsy specimen and had been receiving immunosuppressive drugs.

Immunohistochemistry

Labial salivary glands tissues were obtained with informed consent from healthy volunteers ($n = 3$), patients with primary SS ($n = 10$), patients with sicca syndrome ($n = 10$), and patients with chronic GVHD ($n = 10$). Tissues were fixed in periodate-lysine-4% paraformaldehyde for 4 h at 4°C. After washing in PBS containing sucrose, fixed tissues were embedded in OCT compound (Sakura) and immediately frozen. Six-micrometer frozen tissue sections were incubated with anti-human IL-18 mAb 25-2G (mouse IgG1; Medical & Biological Laboratories), rabbit anti-human IL-17 polyclonal Ab (Santa Cruz Biotechnology), goat anti-human IL-17 polyclonal Ab, anti-human IFN-γ mAb (mouse IgG2a; R&D Systems), anti-human CD4 mAb SK3 (mouse IgG1), or anti-human CD8 mAb SK1 (mouse IgG1; BD Biosciences) overnight at 4°C. Subsequently, sections were treated with secondary Ab such as goat anti-mouse EnVision+ system/HRP kit, goat anti-rabbit EnVision+ system/HRP kit (DAKOCytomation), or rabbit anti-goat simple stain MAX PO (Nichirei) for 1 h at room temperature. The chromogen used was 3',3-diaminobenzidine tetrahydrochloride (DAKOCytomation). The sections were counterstained with hematoxylin. As negative controls, mouse IgG1, mouse IgG2a, normal rabbit serum (DAKOCytomation), and normal goat serum (Zymed Laboratories) were used.

Double immunofluorescence staining was performed, as follows. Tissues were embedded in OCT compound (Sakura) and immediately frozen in acetone dry ice at −70°C. Six-micrometer frozen tissue sections were fixed in acetone for 10 min and dried in air 15 min. The sections were incubated with anti-human IL-18 mAb 25-2G (mouse IgG1) and anti-human CD68 mAb PG-M1 (mouse IgG3; DAKOCytomation) overnight at 4°C. After three washes with PBS, sections were incubated with Alexa Fluor 488 goat anti-mouse IgG1 and Alexa Fluor 546 goat anti-mouse IgG3 (Molecular Probes). After drying, the specimens were mounted.

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 before 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 microscope using the application Leica Qfluoro 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 (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 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 AZA3 (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 (GE Healthcare). PCR mixtures contained 0.25 μl of cDNA mixture, 2.5 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'-GAAGATTCAAATTGCATCTTAT-3' (7); caspase-1 (forward) 5'-ATCCGTTCCATGGGTGAAGGTACA-3' and (reverse) 5'-CAAATGCCTCCAGCTCTGTAATCA-3' (24); and β-actin (forward) 5'-GTGGGGCGCCCCAGGCACCCA-3' and (reverse) 5'-CTCCTTAATGTCACGCACGATTTC-3' (25). The PCR conditions were as follows: IL-18, 40 cycles at 94°C for 30 s, 52°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).

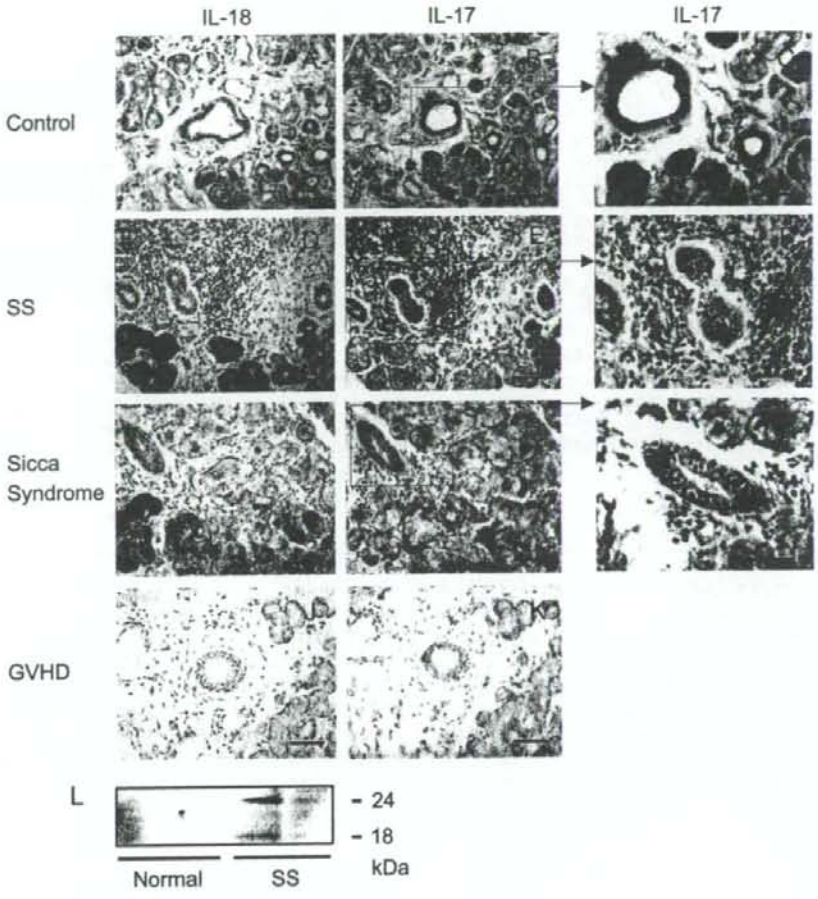

FIGURE 1. Detection of IL-18 and IL-17 in the salivary glands of SS patients. *A–K*, Sequential cryosections of labial salivary glands of control subjects (*A–C*), patients with SS (*D–F*), patients with sicca syndrome (*G–I*), and patients with chronic GVHD (*J* and *K*) were stained with anti-IL-18 mAb 25-2G (*A*, *D*, *G*, and *J*) or rabbit anti-human IL-17 polyclonal Ab (*B*, *C*, *E*, *F*, *H*, *I*, and *K*) (brown). The sections were counterstained with hematoxylin (blue). The same results were obtained with goat anti-human IL-17 polyclonal Ab. High magnification of areas (square) in *B*, *E*, and *H* was shown as *C*, *F*, and *I*, respectively. Scale bar represents 100 μm (*A*, *B*, *D*, *E*, *G*, *H*, *J*, and *K*) and 50 μm (*C*, *F*, and *I*). *L*, Lysates of the labial salivary glands of control subjects ($n = 2$) and patients with SS ($n = 2$) were subjected to Western blotting with anti-IL-18 mAb 25-2G.

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% CO_2 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-18Rα mAb H44 (mouse IgG2b), anti-IL-18Rβ 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

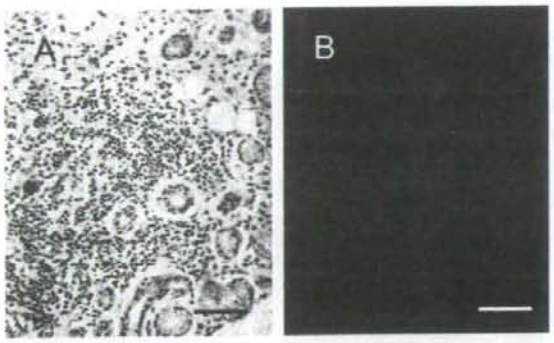

FIGURE 2. Detection of IL-18-expressed $CD68^+$ macrophages in the salivary glands of SS patients. Cryosections of labial salivary glands of SS patients were stained with anti-CD68 mAb PG-M1 (brown) (*A*) or double stained with anti-CD68 mAb PG-M1 (red) and anti-IL-18 mAb 25-2G (green) (*B*). Scale bar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

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.

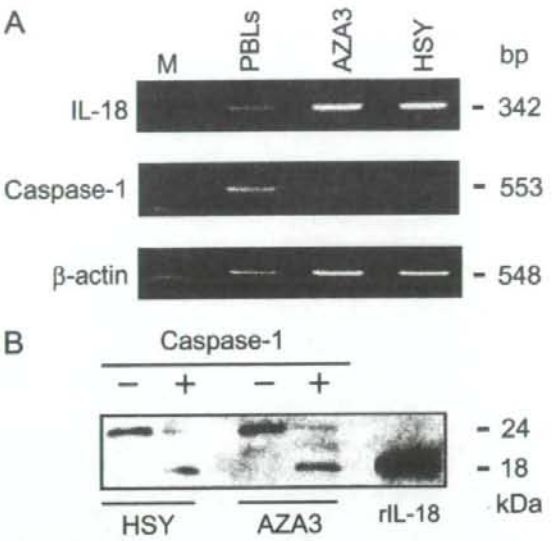

FIGURE 4. Expression of IL-18 in human salivary gland cells in culture. *A*, Total RNA was extracted from human salivary gland AZA3 and HSY cells. Human PBLs were used as a positive control. The m.w. marker (M) is also shown. cDNA was prepared and analyzed for the expression of IL-18, caspase-1, and 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.

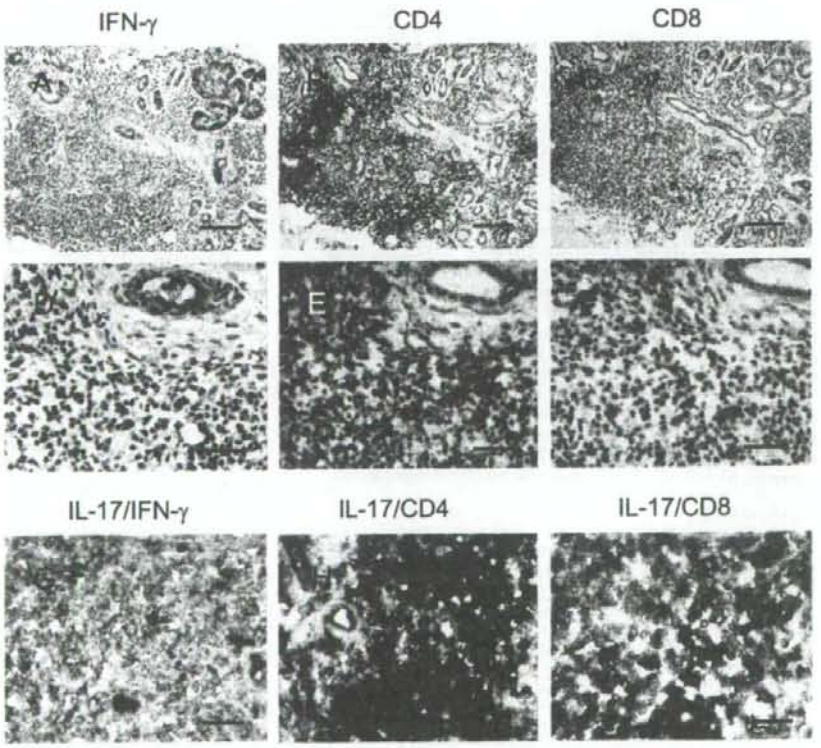

FIGURE 3. Detection of IL-17 in infiltrating $CD4^+$ and $CD8^+$ T cells in the salivary glands of SS patients. Sequential cryosections of labial salivary glands of SS patients were stained with anti-IFN-γ (*A* and *D*), anti-CD4 mAb SK3 (*B* and *E*), and anti-CD8 mAb SK1 (*C* and *F*) (brown). The 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*).

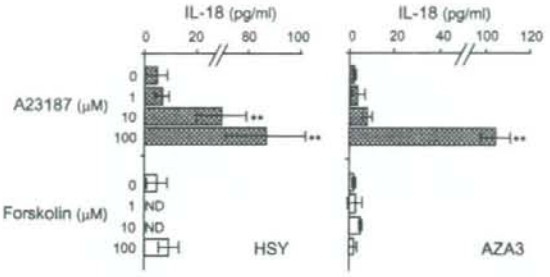

FIGURE 5. Induction of IL-18 secretion from salivary gland cells by activation of second messengers. HSY and AZA3 cells were incubated with or without the indicated concentrations of A23187 or forskolin for 3 h. Supernatants were then collected, and the concentrations of IL-18 in the supernatants were determined using ELISA. Results are expressed as mean ± SD for triplicate cultures. **, $p < 0.01$ compared with the unstimulated control. ND, Not detected.

1*K*). These results indicated that expression of IL-18 and IL-17 in the salivary glands is correlated with SS, but not sicca syndrome or chronic GVHD.

As immunohistochemistry could not distinguish between 24-kDa proIL-18 and 18-kDa mature IL-18, we next examined which type of IL-18 is expressed in the salivary glands of SS patients by Western blot analysis. The results showed that both proIL-18 and mature IL-18 were detected in the salivary glands of SS patients (Fig. 1*L*). Samples from healthy subjects were used as a negative control. These results indicate that the mature IL-18 as well as proIL-18 is expressed in the salivary gland of SS patients and is involved in pathogenesis of SS.

Detection of IL-17 in infiltrating CD4$^+$ and CD8$^+$ T cells in the salivary glands of SS patients

The majority of the infiltrating cells in the salivary glands of SS patients were CD4$^+$ T cells (Fig. 3, *B* and *E*), and CD8$^+$ T cells were also infiltrated to a lesser extent (Fig. 3, *C* and *F*), which is consistent with a previous report (2). Therefore, we next examined which type of infiltrating T cells expressed IL-17 in the salivary glands of SS patients. The results showed that the predominant expression of IL-17 was found in infiltrating CD4$^+$ T cells (Fig. 3*H*), whereas a small number of infiltrating CD8$^+$ T cells expressed IL-17 (Fig. 3*I*). IFN-γ-expressing cells were detected in the salivary glands of SS patients, but the number of IFN-γ$^+$ cells was smaller than that of CD4$^+$ T cells (Fig. 3, *A* and *D*). Furthermore, IFN-γ$^+$ cells did not coexpress IL-17 (Fig. 3*G*). These results indicate that the majority of infiltrating T cells in the salivary glands of SS patients are Th17 cells, and that a small number of IL-17-expressing CD8$^+$ T cells are also infiltrated.

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. 4*A*). 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. 4*B*). Incubation of the proIL-18 in cell lysates of HSY and AZA 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 Ca^{2+} 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 Ca^{2+} by A23817 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

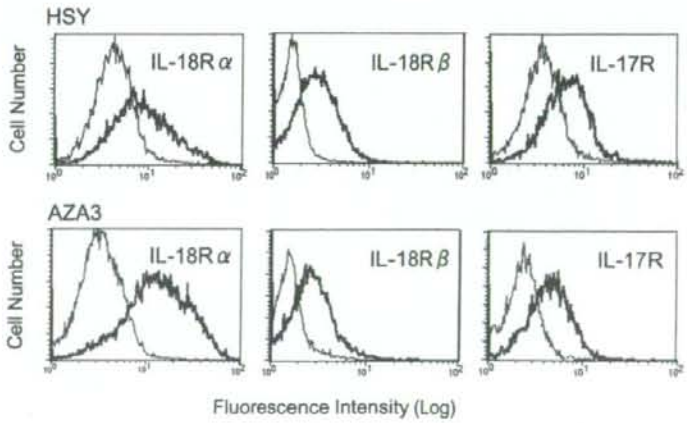

FIGURE 6. Expression of IL-18R and IL-17R on salivary gland cells. HSY and AZA3 cells were stained with anti-IL-18Rα mAb H44, anti-IL-18Rβ mAb 132029, or anti-IL-17R mAb 133617 (thick line histogram) or with control Ab (solid lines) and were analyzed by flow cytometry. The results presented are representative of three different experiments with similar results.

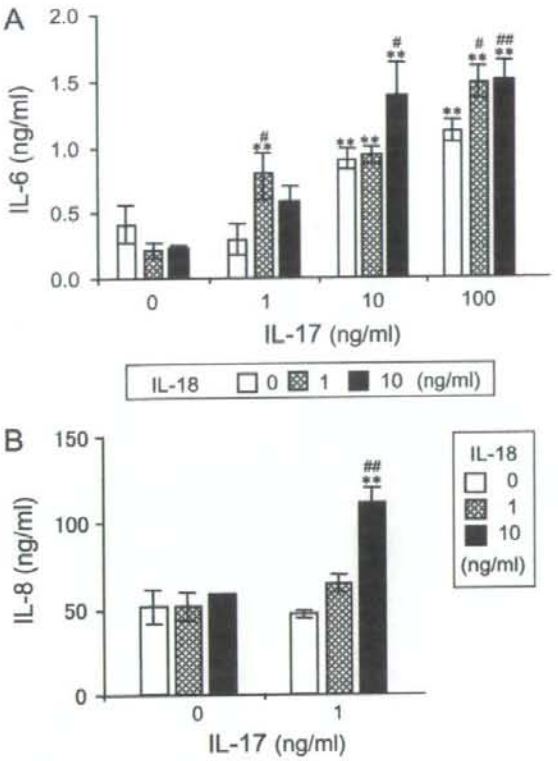

FIGURE 7. Cooperation between IL-17 and IL-18 for the secretion of IL-6 and IL-8 by salivary gland cells. HSY cells were stimulated with the indicated concentrations of IL-18 and IL-17 for 24 h. After the incubation, the supernatants were collected, and the concentrations of IL-6 (*A*) and IL-8 (*B*) were determined by ELISA. Results are expressed as mean ± SD for triplicate cultures. **, $p < 0.01$ compared with 0 ng/ml IL-17. #, $p < 0.05$, and ##, $p < 0.01$ compared with 0 ng/ml IL-18.

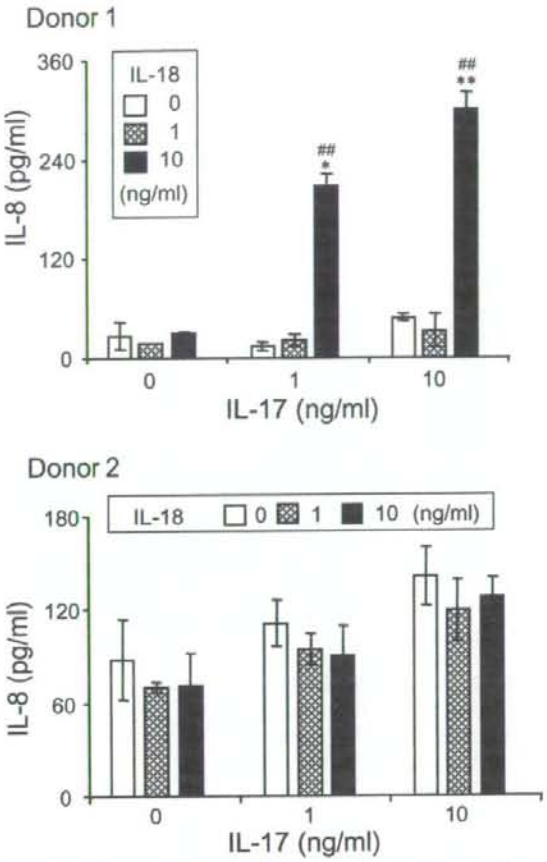

FIGURE 8. Effect of IL-17 and IL-18 on secretion of IL-8 by salivary gland epithelial cells in primary culture. Primary cultures of salivary gland epithelial cells were prepared from the labial minor salivary glands from healthy subjects. The cells were collected and stimulated with the indicated concentrations of IL-18 and IL-17 for 24 h. After the incubation, the supernatants were collected, and the concentration of IL-8 was determined by ELISA. The results are expressed as mean ± SD for triplicate cultures. *, $p < 0.05$, and **, $p < 0.01$ compared with 0 ng/ml IL-17. ##, $p < 0.01$ compared with 0 ng/ml IL-18.

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. 7*A*). 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. 7*B*). However, in the presence of 10 ng/ml IL-18, IL-17 at 1 ng/ml markedly augmented the production of IL-8. Higher concentrations of IL-17 induced IL-8 production, but IL-18 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 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). 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. 1*L*). 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 epithelial 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). Therefore, 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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Disclosures

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