ggaatctctgatgccacagctc and 5'-accttcaagggctgtcaaagatg (BAFF-R); 5'-agcatcctgagtaatgagtggcc and 5'-gagcttgttctcacagaagtatgc (TACI); 5'-aaaactttggcactggggcacttg and 5'-catctttaagcgattactcagggc (NF-IL6); 5'-agatgcagcagaagttggtggag and 5'-tagcttctctcgcagtttagtgg (NF-IL6β); 5'-atgggatctgcactgtaactgc and 5'-tcatagatggcgtctgataccacg (NF-κB1); 5'-cctgactttgagggactgtatcca and 5'-gcagcatttagcagcaaggtcttc (NF-κB2). Primer sets for BAFF and GAPDH were designed as described previously [6]. The expression level of each gene underwent dual normalization against GAPDH expression and expression of the same gene in unstimulated normal monocytes.

FACS analysis

FACS and data analyses were carried out on a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). FACS analysis of cells in whole blood was carried out according to methods recommended by the manufacturer of the antibodies (BD Biosciences/Pharmingen).

Statistical analysis

Differences between groups were examined for statistical significance by using the two-tailed Student's *t*-test for single comparisons. Two-way analysis of variance (ANOVA) was also employed when appropriate. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Aberrant production of sBAFF by pSS monocytes

Peripheral monocytes were prepared from primary pSS patients and normal individuals. The clinical characteristics of the pSS patients involved in this study are listed in Table 1. The cells were cultured for 96 hours in the absence or presence of IFN-γ (200 ng/ml), which is known to activate monocytes [34] and upregulate the expression of BAFF [2]. Stimulation of the cells was confirmed by the induction of interferon-gamma inducible protein 10 (data not shown). pSS monocytes released a significantly higher amount of sBAFF (5.4 ± 0.8 ng/ml) into the culture media than normal monocytes did (1.6 ± 0.3 ng/ml), even in the absence of stimulation, suggesting dysregulated production of sBAFF in pSS monocytes (Figure 1A, "Normal -" and "pSS -"). IFN-γ stimulation (Figure 1A, "Normal +" and "pSS +") resulted in an increase in sBAFF in both normal (6.6 ± 1.6 ng/ml) and pSS monocytes (21.1 ± 2.1 ng/ml).

RT-PCR analysis indicated that the expression of the *BAFF* gene in pSS monocytes was distinctly elevated upon stimulation with IFN-γ (Figure 1B). Quantitative RT-PCR analysis indicated that the relative expression

Table 1 Clinical characteristics of primary Sjögren's syndrome patients involved in this study

Patient characteristics	Clinical data
Female (%)	100
Mean age ± SD (years)	50.5 ± 10.2
Subjective ocular dryness (%)	100
Subjective oral dryness (%)	100
Presence of anti-SSA/Ro (%)	61.5
Presence of anti-SSB/La (%)	23.1
Presence of rheumatoid factor (%)	53.8
Mean serum IgG ± SD (μg/ml)	1,979.6 ± 870.5
Steroid medication (%)	15.4

IgG = immunoglobulin G.

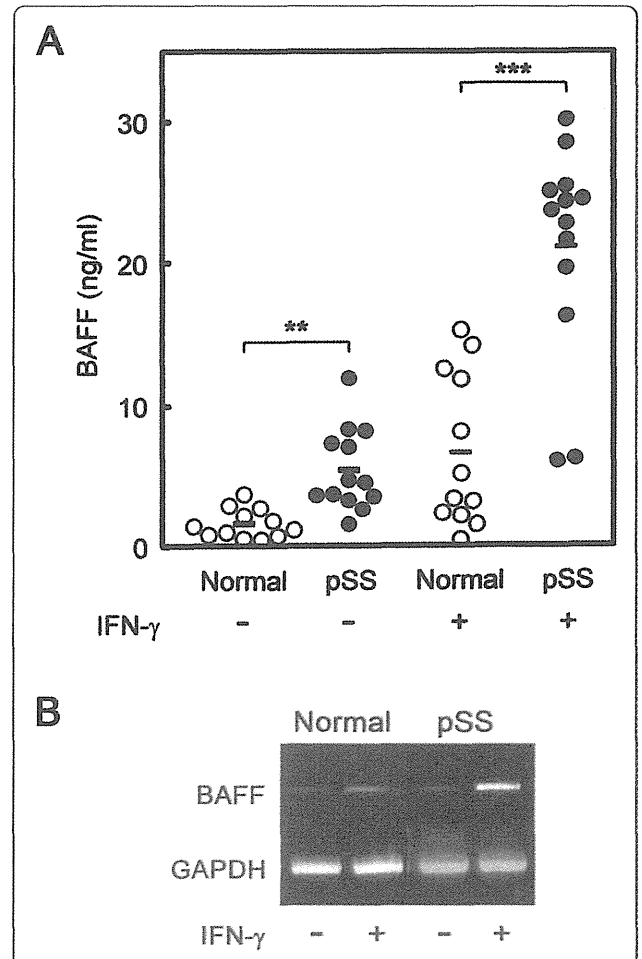

Figure 1 Production of sBAFF by peripheral monocytes. Monocytes (2.5 × 10^5/ml) were cultured for 96 hours in the absence (-) or presence (+) of recombinant human IFN-γ (200 ng/ml). **(A)** The amounts of soluble B-cell-activating factor of the TNF family (sBAFF) in the culture supernatants of normal individuals (open circles) and primary Sjögren's syndrome (pSS) patients (closed circles) were quantitated in triplicate measurements by sandwich ELISA. Bars indicate means. $^{**}P < 0.01$ and $^{***}P < 0.001$. **(B)** Total RNAs were prepared from normal and pSS monocytes and subjected to RT-PCR for BAFF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Representative data are shown.

level of the *BAFF* gene was about three times higher in pSS monocytes than in normal monocytes under unstimulated conditions. The expression levels increased about sixfold in both normal and pSS monocytes upon stimulation with IFN-γ. These data are basically consistent with the results derived by ELISA. Therefore, we postulated that the elevated production of sBAFF was the consequence of the enhanced expression of the *BAFF* gene.

Aberrant production of IL-6 by pSS monocytes

We also investigated whether the production of IL-6 by pSS monocytes was abnormal. As indicated in Figure 2, pSS monocytes produced significantly higher amounts of IL-6 than normal monocytes without stimulation (Figure 2, open column vs checkerboard column; $P < 0.01$). Stimulation of pSS monocytes with 200 ng/ml IFN-γ induced a striking increase (8.6-fold; $P < 0.001$) in IL-6 production (Figure 2, checkerboard column vs closed column). Since IFN-γ induced the expression of BAFF (Figure 1) and BAFF is able to activate monocytes [35,36], these results suggest that BAFF produced by monocytes may act in an autocrine fashion to augment the expression of IL-6. To test this hypothesis, we stimulated pSS monocytes with IFN-γ in the presence of an anti-BAFF mAb [6]. Interestingly, the mAb suppressed IL-6 production in part, but significantly so ($P < 0.05$) (Figure 2, closed column vs hatched column, whereas a control antibody had no effect (Figure 2, closed column vs gray column). These results suggest that the signal transduction pathway mediated by BAFF is implicated in the regulation of IL-6 production by IFN-γ-primed monocytes.

If this is really the case, then exogenously supplemented sBAFF should affect the production of IL-6 by monocytes. As expected, recombinant human sBAFF induced the production of IL-6 by both normal (Figure 3, closed circles) and pSS (Figure 3, open circles) monocytes in a dose-dependent manner. pSS monocytes produced approximately six times more abundant IL-6 than normal monocytes in the presence of 2 μg/ml sBAFF (Figure 3). It should be noted that two-way ANOVA revealed that disease status (normal or pSS) had significantly stronger effects than stimulation with sBAFF ($P <$

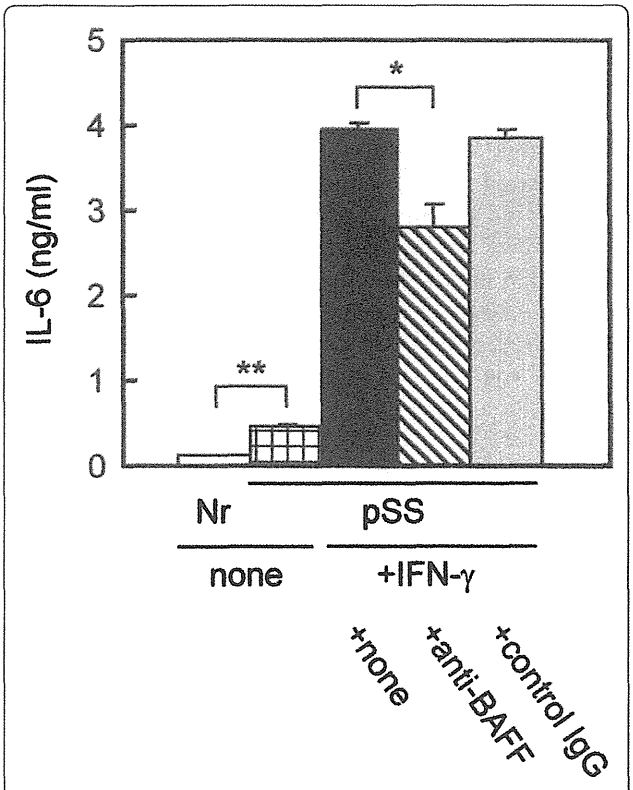

Figure 2 Production of IL-6 by peripheral monocytes stimulated with IFN-γ. Monocytes (2.5 × 10^5/ml) prepared from normal individuals (Nr) (open column) and primary Sjögren's syndrome (pSS) patients (checkerboard column) were cultured for 96 hours without stimulation. pSS monocytes were similarly cultured in the presence of 200 ng/ml of recombinant human IFN-γ (closed, hatched and gray columns), and simultaneously exposed to none (closed column), an anti-BAFF antibody (10 μg/ml; hatched column) or a control IgG (10 μg/ml; gray column). The amounts of IL-6 in the culture supernatants were measured by sandwich ELISA. BAFF = B-cell-activating factor of the TNF family; IgG = immunoglobulin G. Data represent means ± SEM. *$P < 0.05$ and **$P < 0.01$.

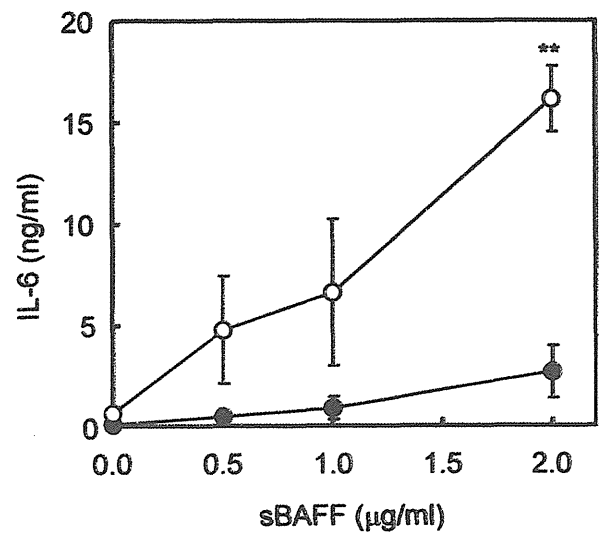

Figure 3 Production of IL-6 by peripheral monocytes stimulated with sBAFF. Monocytes (2.5 × 10^5/ml) prepared from normal individuals (closed circles) and primary Sjögren's syndrome (pSS) patients (open circles) were cultured for 96 hours in the presence of 0, 0.5, 1.0 and 2.0 μg/ml of recombinant human soluble B-cell-activating factor of the TNF family (sBAFF). The amounts of IL-6 in the culture supernatants were measured by sandwich ELISA. Data represent means ± SEM. **$P < 0.01$.

0.001 for cell type × stimulation interaction) on IL-6 production by monocytes.

A 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assay in a separate experiment indicated that 2 μg/ml sBAFF supported the survival of both normal and pSS monocytes during the culture period. However, there was no significant difference in survival rates between normal and pSS monocytes (data not shown), suggesting that higher production of IL-6 was not simply a consequence of enhanced survival of monocytes. These data suggest that the regulatory mechanism for IL-6 production is aberrant in pSS monocytes.

Aberrant expression of BAFF receptors in pSS monocytes

Although it has been reported that BAFF receptors are mainly expressed in lymphocytes [37], our results suggest that BAFF receptors are also expressed in monocytes. RT-PCR detected mRNA for BAFF-R in monocytes. Notably, the expression level of BAFF-R was significantly elevated in pSS monocytes (2.1-fold; $P < 0.001$) compared to normal monocytes (Table 2). In accordance with these data, FACS analysis indicated that approximately 60% of pSS monocytes were BAFF-R-positive (mean fluorescence intensity (MFI) = 50), whereas only about 25% of normal monocytes were positive to the same antibody (MFI = 20) (Figure 4A). The proportion of TACI-positive monocytes was relatively low compared to BAFF-R-positive cells (Figure 4B). Although the population of TACI-positive monocytes seemed to increase slightly in pSS compared to control monocytes (Figure 4B), the expression level of the *TACI* gene was not significantly increased (Table 2).

FACS analysis of lymphocytes in whole blood indicated that there were no significant differences between pSS patients and normal individuals in the population of BAFF-R-positive B and T cells (Figures 4C and 4D). All these data suggest that the expression of BAFF-R is dysregulated in pSS monocytes and that this dysregulation seems to be specific to monocytes among the cells examined thus far.

Table 2 Expression of BAFF receptors in peripheral monocytes

Receptor	Normal	pSS
TACI	100.0 ± 17.6	121.7 ± 20.4
BAFF-R	100.0 ± 5.9	213.8 ± 14.9***

BAFF = B-cell-activating factor of the TNF family; pSS = primary Sjögren's syndrome; TACI = transmembrane activator and calcium-modulator and cyclophilin ligand interactor. Monocytes (2.5×10^5/ml) prepared from normal individuals ("Normal") and pSS patients ("pSS") were cultured for 96 hours without stimulation. Total RNAs were extracted from the cells, and the expression levels of TACI and BAFF-R were quantitated. The relative expression levels of the genes are indicated. Data represent means ± SEM. Asterisk denotes statistically significant difference between "Normal" and "pSS." ***$P < 0.001$.

Aberrant expression of transcription factors in pSS monocytes

In an attempt to elucidate a possible mechanism of sBAFF-mediated overproduction of IL-6 by pSS monocytes, we investigated the expression levels of transcription factors involved in the expression of the IL-6 gene (that is, NF-IL6 (CCAAT/enhancer-binding protein β), NF-IL6β (CCAAT/enhancer-binding protein δ), NF-κB1 and NF-κB2). The relative expression levels of all the transcription factors were significantly elevated in pSS monocytes compared with the control (Table 3). Remarkably, the relative expression level of NF-IL6 was more than six times higher in pSS monocytes than in normal monocytes. These data indicate that the expression of IL-6-regulating transcription factors was abnormally upregulated in pSS monocytes.

Discussion

Several lines of circumstantial evidence have suggested that BAFF and IL-6 are implicated in the development of primary pSS [19-23,27,28,38]. In addition, these cytokines are produced by monocytes [2,4,39,40]. These findings prompted us to investigate the possibility of aberrations in the monocytes of pSS patients. We hypothesized that the production of these cytokines is dysregulated in pSS monocytes. To address this issue, we examined the production of these cytokines by peripheral pSS monocytes *in vitro* in response to IFN-γ, a cytokine known to upregulate BAFF expression [2,41]. As expected, pSS monocytes produced a higher amount of sBAFF than normal monocytes, even in the absence of stimulation (Figure 1A).

IFN-γ also induced the production of IL-6 by pSS monocytes. Interestingly, the induction was suppressed in part, but significantly, by an anti-BAFF antibody (Figure 2). In addition, exogenously supplemented sBAFF induced a striking increase in the production of IL-6 by pSS monocytes (Figure 3), whereas exogenously supplemented IL-6 had no effects on the production of sBAFF by the cells (data not shown). These data, together with the results shown in Figure 1A, collectively imply that BAFF produced by monocytes act in an autocrine fashion and that signal transduction pathways mediated by BAFF are likely involved in the regulation of IL-6 production by monocytes. Notably, two-way ANOVA indicated that pSS monocytes were more susceptible than normal monocytes to stimulation by sBAFF. This increased susceptibility may be due to an exaggeration of signals in pSS monocytes triggered by sBAFF.

BAFF is known to bind to several receptors, such as TACI, BAFF-R and BCMA [8,10,11,13]. BAFF binds TACI [42] and BAFF-R [43,44] with high affinity, whereas the binding affinity of BAFF to BCMA is very low [44,45]. We found that a relatively small population

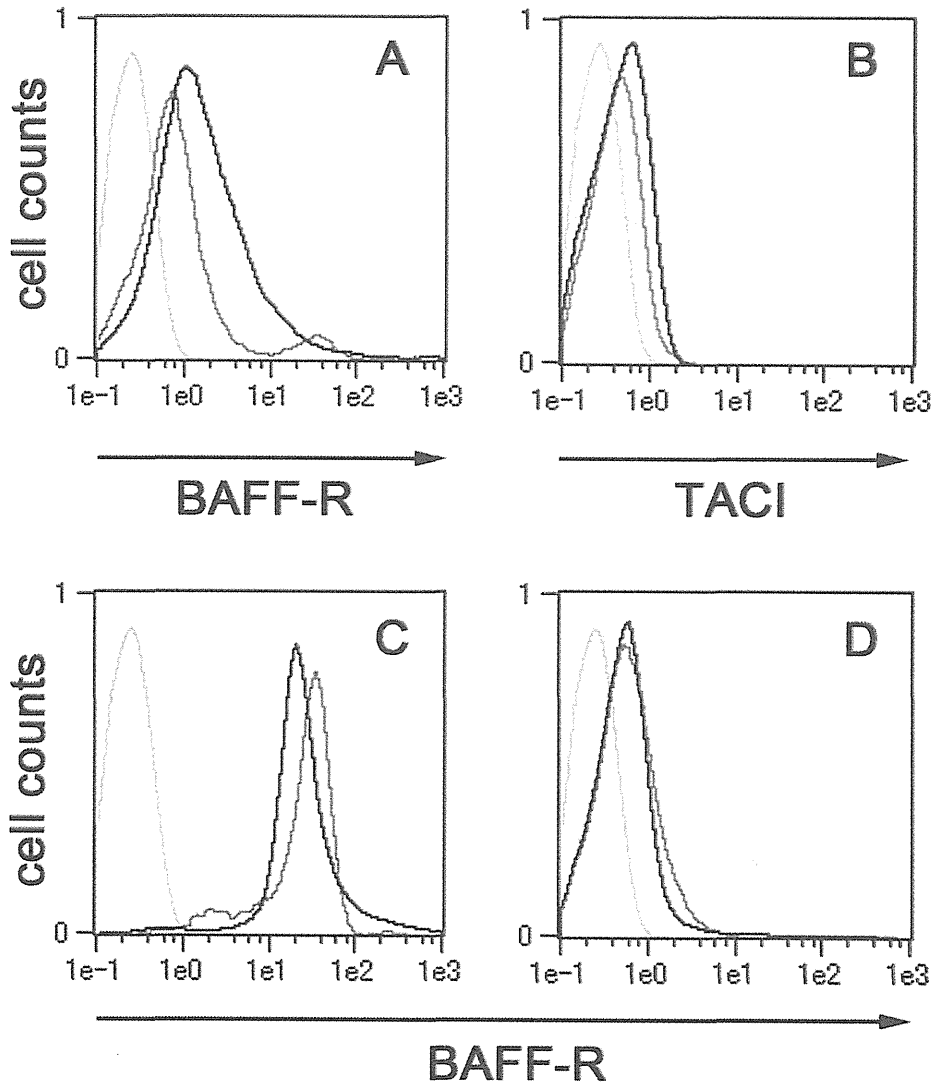

Figure 4 Fluorescence-activated cell sorting analysis of monocytes and lymphocytes. (A) and **(B)** Normal (gray line) and primary Sjögren's syndrome (pSS) (black line) monocytes were cultured for 96 hours without stimulation, and $CD14^+$/BAFF-R^+ cells **(A)** and $CD14^+$/$TACI^+$ cells **(B)** were examined by fluorescence-activated cell sorting (FACS) analysis. **(C)** and **(D)** Lymphocytes in whole blood samples of a normal individual (gray line) and a pSS patient (black line) were examined by FACS analysis for $CD20^+$/BAFF-R^+ cells (B cells in part **(C)**) and $CD4^+$/BAFF-R^+ cells (T cells in part **(D)**). Light gray lines represent isotype controls. Representative data derived by FACS analysis are shown. BAFF-R = B cell activating factor receptor; TACI = transmembrane activator and calcium modulator and cyclophilin ligand interactor.

of normal monocytes was TACI-positive (Figure 4B) and that the expression level of TACI did not increase in pSS patients (Table 2). Interestingly, expression of BAFF-R, a BAFF-specific receptor, was significantly elevated in pSS monocytes compared to the control (Table 2). FACS analysis suggested that this elevation may be the consequence of an increase not only in the population of BAFF-R-positive cells but also in the expression of the *BAFF-R* gene in individual pSS monocytes (Figure 4A). Considering all of this information together, we believe that abnormally overexpressed BAFF-R may have contributed to the enhanced production of IL-6 by pSS monocytes upon stimulation with sBAFF (Figure 3). The increase in the population of BAFF-R-positive cells was specific to pSS monocytes among the cells examined thus far, and no significant differences were observed in the population of BAFF-R-positive lymphocytes between pSS and the normal control (Figures 4C and 4D).

To shed light on the aberrant production of IL-6 by pSS monocytes, we examined the expression levels of several transcription factors involved in the expression of IL-6. Interestingly, the expression levels of all the transcription factors examined in the present study were significantly elevated compared to normal monocytes (Table 3). The

expression of these transcription factors was generally constitutive and insensitive to stimulation, in particular with regard to sBAFF (data not shown). The expression level of NF-IL6 was especially high among the transcription factors examined. The higher expression of these factors may have amplified a signal triggered by sBAFF which resulted in overproduction of IL-6 by pSS monocytes. On the basis of the results shown in Figure 2 andTable 3 we suppose that IFN-γ induces the production of IL-6 in pSS monocytes through at least two distinct pathways: one is direct activation of the IL-6 gene and the other is indirect activation of the gene mediated by sBAFF.

The relationship between the aberration of pSS monocytes and the clinical manifestations of the disease remains unclear. There was no significant correlation between the presence of rheumatoid factor, anti-SSA/Ro or anti-SSB/La in pSS patients and the amounts of IL-6 and sBAFF produced by pSS monocytes. However, dendritic cells have been observed in the salivary glands of pSS patients [46-48], and peripheral monocytes can migrate to the salivary glands and develop into dendritic cells [49-51]. In addition, the local concentration of IFN-γ in the salivary glands of pSS patients seems to be increased because of T cells' infiltrating the tissue [51,52]. Therefore, we hypothesize that monocyte-derived dendritic cells infiltrating the salivary glands of pSS patients are stimulated by IFN-γ to produce excessive amounts of BAFF and IL-6.

Conclusions

Although the number of the patients involved in the current study was small, the data strongly suggest that monocytes of pSS patients are abnormally activated. We hypothesize that stimulation of pSS monocytes by IFN-γ is partly mediated by BAFF as a result of the abnormal overexpression of BAFF-R and that the signals are amplified by abnormal overexpression of transcription factors that regulate IL-6 production. We speculate that these aberrations may underlie the pathogenesis of pSS.

Table 3 Expression of transcription factors in peripheral monocytes

Transcription factor	Normal	pSS
NF-IL6	100.0 ± 16.0	623.6 ± 85.8***
NF-IL6β	100.0 ± 18.5	252.8 ± 51.5*
NF-κB1	100.0 ± 11.4	167.5 ± 23.4*
NF-κB2	100.0 ± 14.9	342.6 ± 45.4***

pSS = primary Sjögren's syndrome. Total RNAs were extracted from monocytes prepared as described in the Table 2 footnote, and the expression levels of NF-IL6, NF-IL6β, NF-κB1 and NF-κB2 were quantitated. The relative expression levels of the genes are indicated. Data represent means ± SEM. Asterisk denotes statistically significant difference between "Normal" and "pSS." *$P < 0.05$ and ***$P < 0.001$.

Abbreviations

ANOVA: analysis of variance; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; IFN: interferon; IL: interleukin; mAb: monoclonal antibody; MFI: mean fluorescence intensity; PCR: polymerase chain reaction; RT: reverse transcriptase; pSS: primary Sjögren's syndrome; sBAFF: soluble BAFF; TNF: tumor necrosis factor.

Acknowledgements

We thank Dr Makoto Yoshimoto for his help in the preparation of this manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20591193).

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Authors' contributions

KY and TT were responsible for the study design; the acquisition, analysis and interpretation of data; and manuscript preparation. MT, YS and MK contributed to the acquisition, analysis and interpretation of data. HK, KS, KeT, YO and KaT participated in the enrollment of patients into the study and assisted in the acquisition and interpretation of data. TA was involved in data interpretation and manuscript preparation. All authors read and approved the final manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

Received: 11 March 2011 Revised: 15 April 2011
Accepted: 21 October 2011 Published: 21 October 2011

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doi:10.1186/ar3493
Cite this article as: Yoshimoto *et al.*: **Regulatory mechanisms for the production of BAFF and IL-6 are impaired in monocytes of patients of primary Sjögren's syndrome.** *Arthritis Research & Therapy* 2011 **13**:R170.

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Skewed Production of IL-6 and TGFβ by Cultured Salivary Gland Epithelial Cells from Patients with Sjögren's Syndrome

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Abstract

Objective: To determine the cytokine production profile of cultured salivary gland epithelial (SGE) cells obtained from patients with Sjögren's syndrome (SS).

Methods: SGE cells obtained from 9 SS patients and 6 normal controls were cultured in the presence of exogenous IFNγ. Cell proliferation and apoptosis in response to IFNγ were determined by WST1 assay and by FACS analysis. The concentrations of IL-6 and TGFβ secreted into culture supernatants were analyzed by ELISA.

Results: IFNγ did not significantly affect the proliferation or apoptosis of SGE cells. However, IL-6 concentrations were higher, and TGFβ concentrations were lower, in culture supernatants of SGE cells from SS patients than from normal controls.

Conclusion: Cytokine production by SGE cells from SS patients showed a skewed balance compared with normal controls, with increased IL-6 and decreased TGFβ secretion. This imbalance may be critical in the regulation of Treg/Th17 cells and may foster a pathogenic milieu that may be causative and predictive in SS.

Citation: Kawanami T, Sawaki T, Sakai T, Miki M, Iwao H, et al. (2012) Skewed Production of IL-6 and TGFβ by Cultured Salivary Gland Epithelial Cells from Patients with Sjögren's Syndrome. PLoS ONE 7(10): e45689. doi:10.1371/journal.pone.0045689

Editor: Silke Appel, University of Bergen, Norway

Received January 11, 2012; **Accepted** August 23, 2012; **Published** October 30, 2012

Funding: This work was supported by grants 15024236, 15390313 and 22249041 from The Japanese Ministry of Education and Science and Culture, Uehara Memorial Foundation, The Vehicle Racing Commemorative Foundation, and Kanazawa Medical University Research Foundation to T.K. (S-2010-4) and H.U. (C2009-4, C2010-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands [1,2]. This chronic inflammation leads to destruction of the salivary glands and may ultimately result in salivary hypofunction. Although the mechanisms underlying this salivary gland destruction are not clearly understood, a better understanding of the precise molecular mechanisms may lead to the development of specific therapies for SS, similar to cytokine-targeted therapies in patients with rheumatoid arthritis (RA) [3,4].

Cytokines are key molecules that mediate chronic autoimmune inflammatory reactions in the salivary glands of SS patients [5]. Proinflammatory cytokines, such as interferon (IFN) γ, interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF) α, are produced by infiltrating lymphocytes and are involved in the maintenance of chronic inflammation [6–9]. In SS patients, these cytokines can induce the expression of HLA-DR, BAFF, costimulatory molecules such as CD80 and CD86, and/or chemokines in salivary gland epithelial (SGE) cells [10,11]. In addition, we have reported that the production of IFNs can further perpetuate the homing and activation of lymphocytes and the apoptosis of glandular cells [12,13]. In contrast, the absence of transforming growth factor (TGF) β has been reported to lead to systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and SS in TGFβknockout mice [14,15]. TGFβ promotes the differentiation of regulatory-T cells (Treg) [16] and, together with IL-6, plays a crucial role in the induction of Th17 cells [17,18]. Taken together, these findings suggest that cytokine balance plays an important role in chronic inflammation of the salivary glands in SS patients [5]. Moreover, long-term exposure to pro-inflammatory cytokines such as IFNγ and TNFα can result in salivary epithelium dysfunction, leading to hyposalivation. We therefore evaluated cytokine expression profiles in salivary gland epithelial (SGE) cells from SS patients stimulated with IFNγ.

Materials and Methods

Patients and controls

We evaluated 15 patients at Kanazawa Medical University Hospital (Ishikawa, Japan) who were enrolled in the Sjögren's International Collaborative Clinical Alliance (SICCA) Registry; the complete details of this registry have been described [19]. In brief, SICCA is an ongoing longitudinal multisite observational study of a large and growing cohort of uniformly evaluated individuals from ethnically diverse populations, designed to develop standardized classification/diagnostic criteria for SS [20,21]. Each participant in the SICCA cohort is assessed, systemically and extensively, for symptoms and signs related to SS. Of the 15 patients, nine (all women; mean age, 48±14 years) met both the 2002 American-European consensus group (AECG) and the SICCA criteria for SS [21,22], whereas six (all women; mean age, 57±8 years) did not meet either set of criteria and had no objective findings indicative of SS (Table 1). All experimental protocols were approved by the independent ethics committee of Kanazawa Medical University, and all participants provided written informed consent.

Labial minor salivary gland (MSG) biopsies were taken from each patient for diagnostic evaluation of SS, with SG tissue samples processed for further culture of primary epithelial cells. None of these participants had taken any immune suppressants or steroids.

Cell lines and primary cultures of SGE cells from MSGs

Human airway epithelial cells (HBTEC) and human umbilical vein endothelial cells (HUVEC) were obtained from Kurabo Co. Ltd., Osaka Japan. Epithelial cells obtained from the MSGs were cultured immediately after biopsy, as described [13]. In brief, each tissue sample was rinsed with cold sterile phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin and minced into small pieces of approximately 1–2 mm^3. One tissue sample from each subject was placed in a well of a collagen type 1-coated 12-well plate (Iwaki, Tokyo, Japan) and cultured in keratinocyte serum-free medium (SFM; Invitrogen Corp., Carlsbad, CA) containing 0.4 μg/ml hydrocortisone and 25 μg/ml bovine pituitary extract (Sigma Chemical Co., St. Louis, MO). The epithelial cell outgrowth of each explant was assessed after 1–2 weeks. Upon attaining confluence, the monolayer cells were subcultured. Cells were rinsed twice with PBS and detached from the substrate by incubation with 0.05% trypsin (Invitrogen) for no longer than 10 min, with cell detachment monitored by light microscopy. Trypsin was inactivated by adding an equal volume of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS). The detached cells were centrifuged at 1500 rpm for 5 min, washed once with PBS, resuspended in culture medium, and reseeded, at a concentration of 8×10^4 cells per well, in a fresh collagen type I-coated 6-well

Table 1. Profile of patients included in the study.

	Sex	Age	Diagnosis	Focus Score (/4 mm^2)	ANA*	Anti SS-A	Anti SS-B
SS.1	F	64	SS	2.3	40	+	−
SS.2	F	67	SS	3.9	1280	−	−
SS.3	F	56	SS	1.8	160	+	+
SS.4	F	44	SS	2.8	320	+	+
SS.5	F	28	SS	3.2	160	+	−
SS.6	F	32	SS	2.4	80	+	+
SS.7	F	58	SS	2.7	160	+	−
SS.8	F	36	SS	2.9	160	+	+
SS.9	F	52	SS	1.2	−	+	−
No.1	F	67	non-SS	0	320	−	−
No.2	F	67	non-SS	0	−	−	−
No.3	F	51	non-SS	0	−	−	−
No.4	F	57	non-SS	0.33	−	−	−
No.5	F	46	non-SS	0	−	−	−
No.6	F	58	non-SS	0	640	−	−

Nine patients (all women; mean age, 48±14 years) met both the 2002 American-European consensus group (AECG) criteria and the SICCA criteria for Sjögren's syndrome (SS), whereas the other six (all women; mean age, 57±8 years) did not (No).
*Titers of anti-nuclear antibody (ANA).
doi:10.1371/journal.pone.0045689.t001

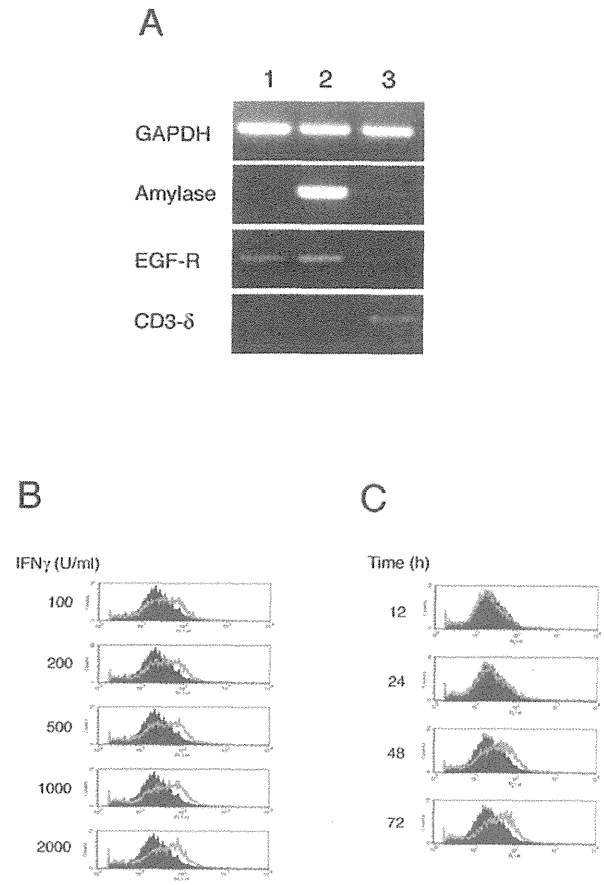

Figure 1. (A) Expression of mRNA in salivary gland epithelial cells. Salivary gland epithelial cells were isolated from SS patients and cultured. Total RNA was isolated from these cells, and EGF-R, α amylase-1, and CD3δ mRNAs were assayed by RT-PCR, as described in the *Materials and Methods section*. Lane 1: salivary gland epithelial cells, Lane 2: labial salivary gland of the same patient, Lane 3: normal lymph node as a control for CD3δ. **(B and C) Effects of IFNγ on human SGE cells.** SGE cells were incubated with various concentration of IFNγ for 48 hours (B) or with 1000 U/ml of IFNγ for the indicated times (C), and the surface expression of CD40 was examined by FACS analysis.
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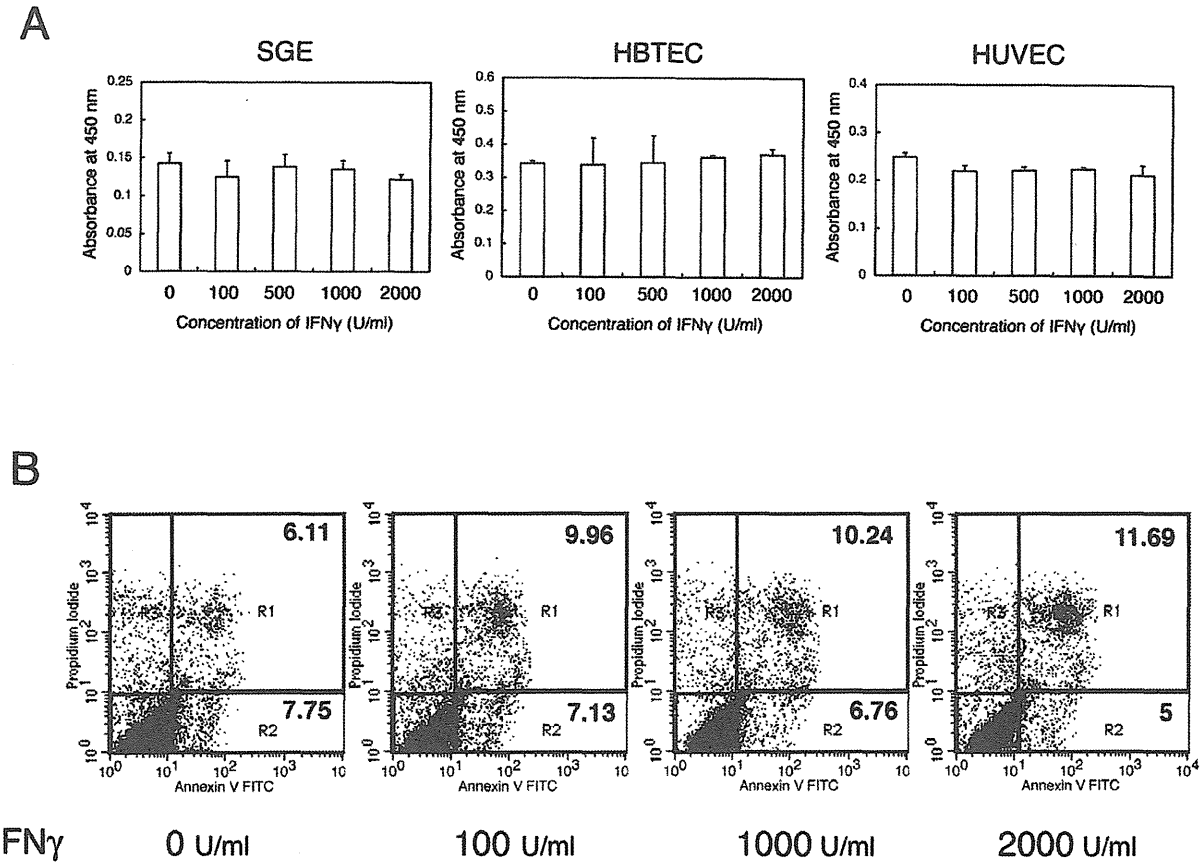

Figure 2. Effects of IFNγ on the proliferation and apoptosis of SGE cells. (A) SGE cells, human airway epithelial cells (HBTEC) and human umbilical vein endothelial cells (HUVEC)were incubated with the indicated concentration of IFNγ, and proliferative responses were assessed at 48 h. Each bar shows mean + SD. IFNγ did not significantly affect the proliferation of any of these cells. The results shown are representative of three independent experiments. (B) SGE cells were incubated with the indicated concentration of IFNγ, and apoptosis was determined at 12 h by flow cytometry. Numbers in R1 and R2 indicate early and late apoptosis, respectively.
doi:10.1371/journal.pone.0045689.g002

plate. Fibroblasts were routinely removed from the cultures by treating the cells with 0.02% ethylenediaminetetraacetic acid (EDTA; Invitrogen).

Immunocytochemistry

The SGE cells were harvested, washed once with PBS, and allowed to adhere to a glass slide in a monolayer. The cells were air dried for 30 min, fixed in 4% paraformaldehyde (PFA) acetone at 4°C for 30 sec, washed 3 times with purified water, incubated in Tris-buffered saline (TBS) for 5 min, and blocked with bovine serum albumin at room temperature for 5 min. After washing, the cells were incubated overnight at 4°C in a moist chamber with primary antibodies against epithelial membrane antigen (EMA, clone E29), cytokeratin 8 (35βH11), and cytokeratin 18 (DC10; Dako, Kyoto, Japan), each at a concentration of 1 μg/ml. Antigen-antibody complexes were detected using a labeled polymer conjugated with alkaline phosphatase (Envision/AP kit, Dako), according to the manufacturer's instructions, visualized by treatment for 10 min with the New Fuchsin chromogen-substrate solution and counterstained with Mayer's hematoxylin. Control slides were incubated with isotype-matched antibodies in TBS in place of the primary antibody; these invariably yielded negative results (data not shown).

Proliferation assay and detection of cell apoptosis

The proliferative responses of SGE cells to IFNγ (R&D Systems) were determined using a WST-1 cell counting kit, and cell apoptosis was determined by flow cytometry as described [23]. Briefly, SGE cells were cultured in collagen type I-coated plates in the presence of IFNγ at 37°C for the indicated times. Ethanol-fixed cell suspensions were centrifuged, followed by the addition of 50 μl RNase solution and 450 μl propidium iodide (final concentration 50 mg/ml). The cells were washed twice and subsequently analyzed by flow cytometry (BD Biosciences, Palo Alto, CA, USA).

Quantification of cytokines in the culture supernatants by ELISA

The concentrations of IL-6 and TGFβ1 secreted into culture supernatants were quantified using ELISA test kits for IL-6 (Immunotech, Marseille, France) and TGFβ1 (R&D Systems, Minneapolis, MN), according to the manufacturers' instructions.

Statistical analysis

Median cytokine concentrations, calculated to minimize the effects of extreme outliers, were compared using non-parametric

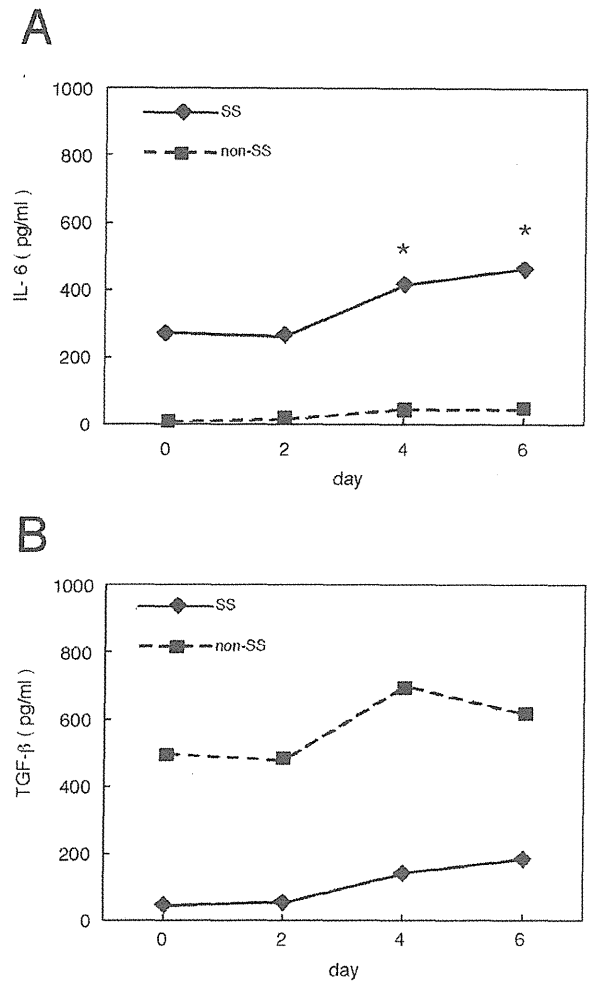

Figure 3. Quantification of cytokines secreted into the culture supernatants of SGE cells. Confluent SGE cells obtained from 9 SS patients and 6 normal controls (non-SS) were incubated in the presence of IFNγ (1000 U/ml), and the culture supernatants were collected on days 0, 2, 4, and 6. Median concentrations (pg/ml) of IL-6 (A) and TGFβ (B) in the supernatants were determined by ELISA and compared by non-parametric Mann-Whitney tests (*, $p<0.05$).
doi:10.1371/journal.pone.0045689.g003

Mann-Whitney tests. A p value<0.05 was considered statistically significant.

Results

Primary cultures of human SGE cells from MSGs

To establish primary cultures of human SGE cells, MSG biopsy samples were washed with PBS, cut into pieces of approximately 1–2 mm^3, and explanted onto collagen type 1-coated plastic plates. Within a few weeks, the epithelial cells gradually grew out from the explants. Most of these cells were cuboidal, round, or spindle shaped. RT-PCR showed that these cells expressed EGF-R, but not α-amylase 1 or CD3δ, mRNA (Figure 1A). Immunohistochemical analysis showed that these cells were positive for EMA and cytokeratins-8 and -18 (data not shown), indicating that these cells were primarily ductal epithelial cells, with small amounts of acinar or lymphocytic components. These SGE cells could be maintained in culture medium for at least a few months.

Effects of IFNγ on activation of SGE cells

Previous studies have shown that the concentrations of cytokines such as IFNγ, IL-1β, IL-6, IL-10 and TNFα are increased in SS patients, and that long-term exposure to pro-inflammatory cytokines such as IFNγ and TNFα can lead to dysfunction of the salivary epithelium. To assess the effects of IFNγ on SGE cell activation, SGE cells were incubated with various concentration of IFNγ for 48 hours (Figure 1B) or with 1000 U/ml of IFNγ for the indicated times (Figure 1C), and the expression of CD40 was examined by FACS analysis. We found that CD40 expression on SGE cells was increased in a dose- and time-dependent manner (Figure 1B and C).

Effects of IFNγ on the proliferation and apoptosis of SGE cells

To test the effects of IFNγ on the proliferation of SGE cells, these cells, as well as human airway epithelial cells (HBTEC) and human umbilical vein endothelial cells (HUVEC), were incubated in the presence of the indicated concentrations of IFNγ for 48 h. We found that IFNγ had no effects on the proliferation these three cell types (Figure 2A). To assess the effects of IFNγ on SGE cell apoptosis, these cells were incubated with the indicated concentrations of IFNγ for 12 h. We observed that IFNγ did not significantly affect the early (R1) or late (R2) apoptosis of SGE cells (Figure 2B).

Production of IL-6 and TGFβ by IFNγ stimulated SGE cells

TGFβ induces Foxp3 in naïve T cells, resulting in their differentiation to regulatory T cells (Treg). In contrast, IL-6 switches T cell differentiation from a Treg to a Th17 pathway. We therefore incubated confluent SGE cells with IFNγ (1000 U/ml), and assayed the concentrations of IL-6 and TGFβ secreted into culture supernatants on days 0, 2, 4 and 6. We found that IL-6 concentrations on days 4 and 6 were significantly higher (Figure 3A), and TGFβ concentrations on days 2 and 4 were significantly lower (Figure 3B), in the supernatants of cells from SS patients than from controls.

Discussion

Although the production of cytokines in the salivary glands of SS patients has been evaluated [6–9,12,13], the most important cytokines involved in the pathogenesis of SS have not yet been identified. Since it is difficult to distinguish between cause and effect relationships of cytokine production, due to their complex interactions and the presence of various cells in tissues, we attempted to minimize this problem by using cultured SGE cells. Although previous studies have been hampered by the lack of a suitable in vitro culture system for SGE cells, our culture system, using a non-serum-containing medium, enabled us to examine cellular functions including cytokine production and to maintain human SGE cells as ductal epithelial cells for at least a few months.

Because the salivary glands of SS patients contain T cells that express IFNγ and Stat1 mRNA [24], with these T cells being predominantly Th1 cells [25], the proinflammatory cytokine IFNγ has been considered a principle mediator of inflammation in SS patients, similar to TNFα in patients with RA [3]. Local IFNγ production in the salivary glands may perpetuate inflammation by inducing SGE cells to express HLA-DR, co-stimulatory molecules, cytokines and chemokines, leading to secretory gland dysfunction [2]. We therefore comprehensively examined the effects of IFNγ

on the functions of SGE cells obtained from SS patients. We found that IFNγ activated SGE cells, leading to the increased expression of CD40, but did not significantly affect the proliferation or apoptosis of SGE cells.

Next we examined cytokine production by IFNγ-stimulated SGE cells obtained from SS patients. Although the differences were not significant, IFNγ induced a skewed expression of mRNAs encoding several cytokines, including IL-6, TNFα, TGFα and TGFβ, in SGE cells from both SS patients and normal controls (data not shown). We hypothesized that TGFβand IL-6 may play key roles in the pathogenesis of SS by affecting the balance between Treg and Th17 cells. TGFβ can have pro- or anti-inflammatory effects, depending on the context; i.e., TGFβ promotes the differentiation of naive T cells to Treg cells in the presence of IL-2, while inducing Th17 cells in the presence of IL-6 [16,26,27].

IL-6 is another pleiotropic cytokine that regulates immune responses, hematopoiesis and bone metabolism [28]. IL-6 overproduction has been found to be involved in the pathogenesis of several human autoimmune diseases, including RA and Castleman's disease [29]. Recently, IL-6 was shown to play an important role in T helper differentiation [26]. Stimulation of cultured CD4 T cells with IL-6 and TGFβ potently induced Th17 differentiation. IL-17 has been reported to be involved in the chronic inflammatory processes that occur in many autoimmune diseases, including SS [27,30]. Whereas TGFβ was found to induce naive CD4 T cells to differentiate into Foxp3+ Treg cells, this Treg induction was potently inhibited by IL-6. Rather, IL-6 was found to promote their differentiation into inflammatory Th17 cells, providing further evidence that IL-6 is critical in the regulation of the Treg/Th17 balance [26].

To identify the cytokines that play major roles in the development of salivary gland lesions in SS, we assayed the concentrations of IL-6 and TGFβ secreted into the culture supernatants of IFNγ-stimulated SGE cells. We found that IL-6 concentrations were higher in the supernatants of cells from SS patients than from normal controls, suggesting that IL-6 may be important in maintaining chronic inflammation in SS. These findings are consistent with results showing that IL-6 is highly expressed in SGE, with high focal scores [31]. Moreover, increased IL-6 production may contribute to the presence of abundant IL-17-bearing cells in the salivary glands of SS patients [30].

In contrast, we found that the secretion of TGFβ was lower in supernatants of SGE cells from SS patients than from controls. Since TGFβ has been linked to the generation of Treg cells, it is likely that Th1 cell differentiation and IFNγ production are not suppressed in the salivary glands of SS patients during the initial stages of inflammation.

Our findings suggested that the skewed production of IL-6 and TGFβ in the salivary glands of SS patients may shift the milieu to one more favorable for the propagation of Th17 cells, with correspondingly fewer Foxp3+ Treg cells, and may foster a pathogenic milieu that may be causative and predictive of infiltrative injury in SS patients.

Although the mechanism underlying this cytokine imbalance in SGE cells of SS patients, including reduced TGFβ and increased IL-6 production is currently unknown, modulation of this imbalance may help control the chronic inflammation of the salivary glands occurring in SS patients.

Acknowledgments

We thank Dr. N. Ogawa for providing critical feedback and Mr. K. Yoshida for providing assistance with immunohistochemistry.

Author Contributions

Conceived and designed the experiments: TK YH TO HU. Performed the experiments: TK T. Sawaki T. Sakai T. Sato MM HI AN. Analyzed the data: TK TN T. Sawaki T. Sakai T. Sato. Contributed reagents/materials/analysis tools: TK YF M. Tanaka M. Taniguchi YM TF NS. Wrote the paper: TK HU.

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