several pathways [10,11,13,15,16]. It is noteworthy that transgenic mice that overexpress BAFF in lymphoid cells develop hyperplasia of mature B cells [8,17,18] or pSS-like pathology [19]. BAFF is also elevated in the serum of pSS patients [20,21] and strongly expressed in the lymphocytes infiltrating the salivary glands [22,23]. Moreover, elevated production of BAFF has been linked to the development of another autoimmune disease, systemic lupus erythematosus [24-26].

Notably, systemic and/or local concentrations of several other cytokines, such as IL-6, are also significantly elevated in pSS patients compared to normal individuals [27,28]. IL-6 promotes the differentiation of B cells [29], which play a pivotal role in the production of autoantibodies and hence in the development of pSS. Since monocytes produce both IL-6 [30] and BAFF [2,4,31], we hypothesized that the production of these cytokines is dysregulated in pSS monocytes. If that is the case, aberrations of pSS monocytes may be implicated in the abnormal production of autoreactive immunoglobulin G (IgG) by B cells, which is involved in the pathogenesis of autoimmune diseases such as pSS [32]. In the present study, we demonstrate that the regulatory mechanisms for the production of these cytokines are impaired in pSS monocytes.

Materials and methods

Patients and controls

Venous blood samples were collected from pSS patients (n = 13 females ages 32 to 64 years (average age = 50.5)) and normal individuals (n = 12 females ages 26 to 60 years (average age = 43.5)) after receiving their informed consent. Patients fulfilled the American-European Consensus Group criteria for pSS [33]. At the time of the collection of blood samples, two patients (15.4%) were receiving prednisolone at a daily dose < 5 mg. The remaining patients were free of medication. This study was approved by the ethics committees at Keio University School of Medicine and Saitama Medical University.

Stimulation of peripheral monocytes *in vitro*

Peripheral monocytes were isolated as follows: Whole blood was mixed with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, BC, Canada) and centrifuged over Ficoll-Hypaque (Beckman Coulter, Fullerton, CA, USA). A monocyte-enriched fraction was collected and cultured overnight in RPMI 1640 (American Tissue Culture Collection, Manassas, VA, USA) supplemented with 10% FCS in a humidified incubator (7% CO_2) at 37°C so that the expression of various stress-induced genes subsided. The cells were then washed once with the medium to remove debris. Fluorescence-activated cell sorting (FACS) analysis of the cells demonstrated that > 96% of the living cells were CD14-positive.

The monocytes were cultured in the absence or presence of various concentrations of IFN-γ or sBAFF, and the cumulative production of sBAFF and/or IL-6 was examined by ELISA. The production was dependent on the incubation period. The optimal incubation period was found to be 96 hours. The production of the cytokines increased almost in proportion to the concentration of stimuli up to 200 ng/ml IFN-γ or 2 μg/ml sBAFF.

Antibodies and recombinant proteins

An anti-BAFF mAb for ELISA was prepared in our laboratory [6]. A rabbit polyclonal anti-BAFF antibody and recombinant human sBAFF were purchased from Chemicon International (Temecula, CA, USA). Recombinant human IFN-γ, a control mouse IgG1, and mAbs for measurement of the amount of IL-6 by ELISA (MQ2-13A5 and MQ2-39C3 for capture and detection, respectively) and for FACS analysis (CD4-APC (RPA-T4) for T cells, CD14-PE-Cy7 (M5E2) for monocytes, CD20-APC-Cy7 (L27) for B cells and CD268-FITC (11C1) for BAFF-R) were purchased from BD Biosciences/Pharmingen (San Diego, CA, USA). An anti-TACI antibody for FACS analysis (CD267-PE (FAB1741P)) was purchased from R&D Systems (Minneapolis, MN, USA).

ELISA

Monocytes were cultured at 2.5 × 10^5/ml for 96 hours in a 24-well plate (2 ml/well) in the presence of stimuli (that is, recombinant human IFN-γ or recombinant human sBAFF). The amounts of sBAFF (in response to IFN-γ as a stimulus) and IL-6 (in response to IFN-γ or sBAFF as stimuli) in the culture supernatants were measured by sandwich ELISA according to previously described methods [6], except for the concentrations of capture and detection antibodies for IL-6, which were prepared at 0.5 μg/ml.

For quantitation of sBAFF, we used our own anti-BAFF mAb, which specifically detects sBAFF and does not react with a proliferation-inducing ligand (APRIL) [6]. In our hands, the sensitivity of our ELISA system was better than that of commercially available ELISA kits (R&D Systems) in the range of 0.4 to 100 ng/ml sBAFF (data not shown).

Quantitation of the gene expression levels

The expression levels of BAFF, BAFF-R, TACI, NF-IL6, NF-IL6β, NF-κB1, NF-κB2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantitated by using a method described previously [6]. The following oligonucleotides were used as primers for PCR: 5'-

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.

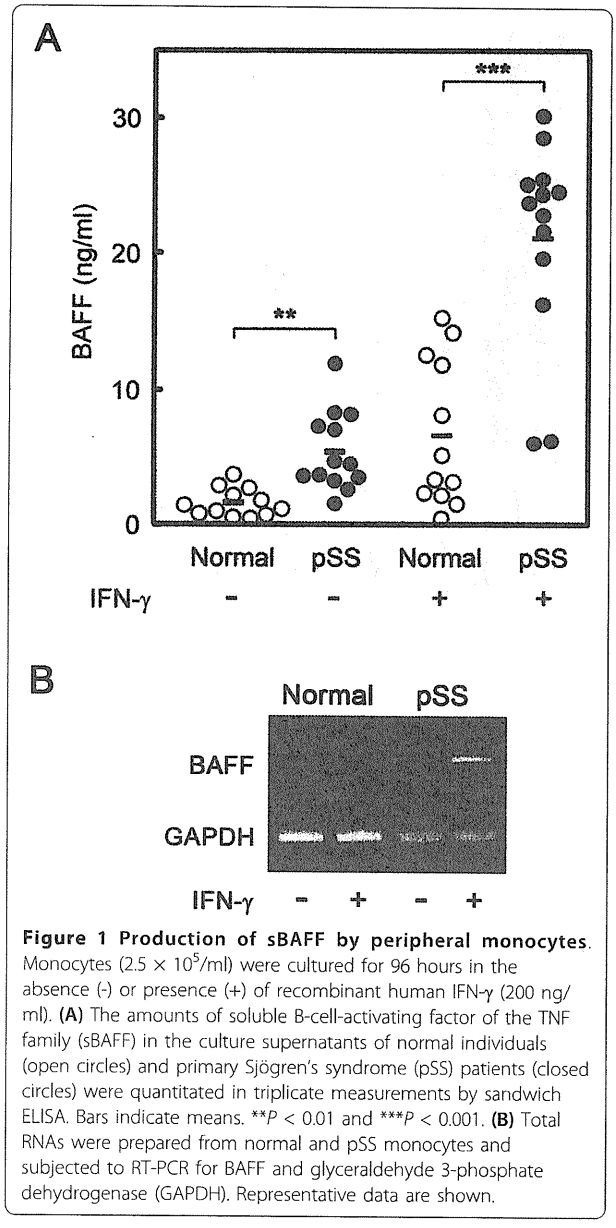

Figure 1 Production of sBAFF by peripheral monocytes. Monocytes (2.5×10^5/ml) were cultured for 96 hours in the absence (-) or presence (+) of recombinant human IFN-γ (200 ng/ml). **(A)** The amounts of soluble B-cell-activating factor of the TNF family (sBAFF) in the culture supernatants of normal individuals (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 ($P <$

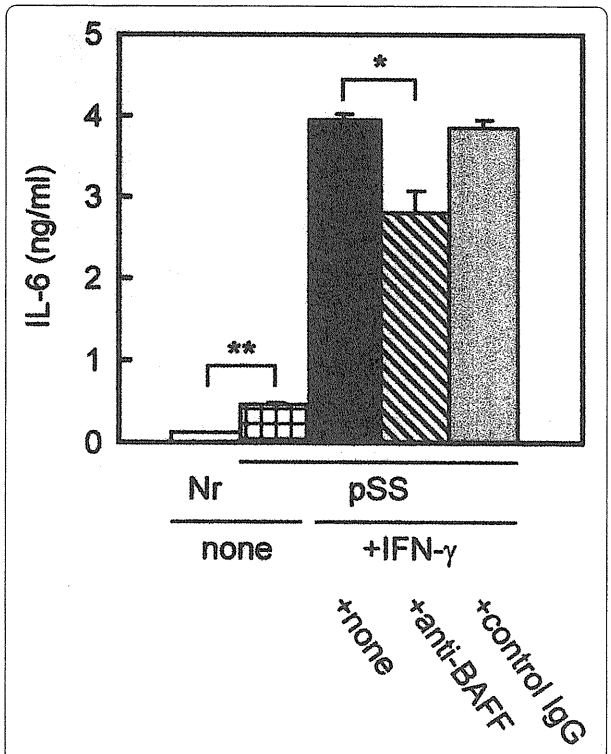

Figure 2 Production of IL-6 by peripheral monocytes stimulated with IFN-γ. Monocytes (2.5×10^5/ml) prepared from normal individuals (Nr) (open column) and primary Sjögren's syndrome (pSS) patients (checkerboard column) were cultured for 96 hours without stimulation. pSS monocytes were similarly cultured 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 **$P < 0.01$.

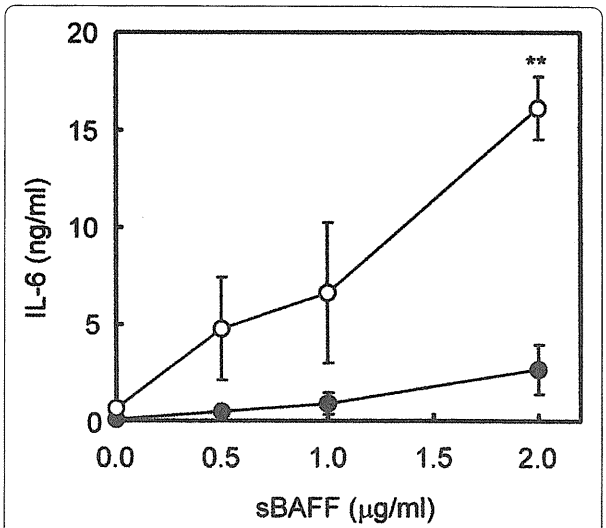

Figure 3 Production of IL-6 by peripheral monocytes stimulated with sBAFF. Monocytes (2.5×10^5/ml) prepared from normal individuals (closed circles) and primary Sjögren's syndrome (pSS) patients (open circles) were cultured for 96 hours in the presence of 0, 0.5, 1.0 and 2.0 μg/ml of recombinant human 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

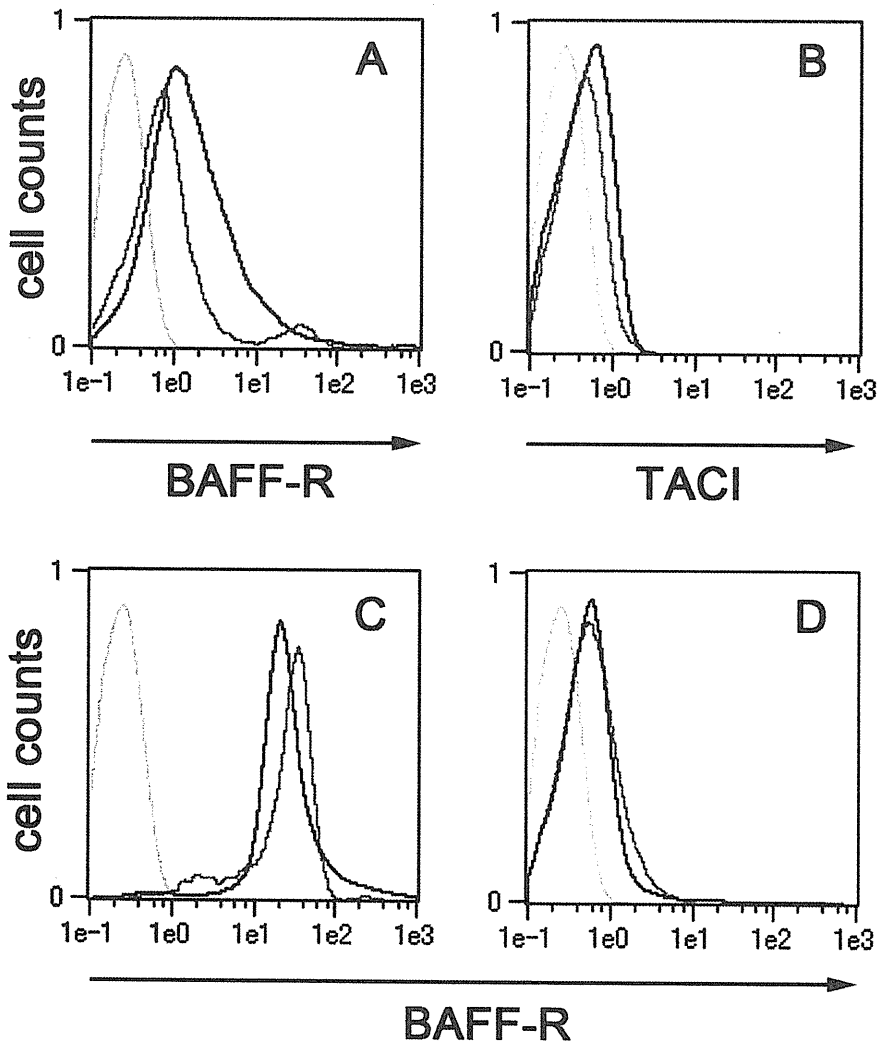

Figure 4 Fluorescence-activated cell sorting analysis of monocytes and lymphocytes. **(A)** and **(B)** Normal (gray line) and primary Sjögren's syndrome (pSS) (black line) monocytes were cultured for 96 hours without stimulation, and $CD14^+$/$BAFF\text{-}R^+$ cells **(A)** and $CD14^+$/$TACI^+$ cells **(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 $CD20^+$/$BAFF\text{-}R^+$ cells (B cells in part **(C)**) and $CD4^+$/$BAFF\text{-}R^+$ 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.

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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.

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DECREASED mRNA EXPRESSION OF TWO FOXP3 ISOFORMS IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS

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Both the number and functional capacity of T-regulatory (Treg) cells are known to be decreased in various autoimmune diseases. FOXP3, an essential transcription factor for Treg cells, has three isoforms in humans, wild, and exon 2- and exon 2-exon 7-lacking, although their role in autoimmunity is not clearly understood. Here, we investigated the messenger RNA (mRNA) expression of the major wild and exon-2 isoforms in peripheral mononuclear cells by quantitative PCR methods in 56 subjects, consisting of 23 rheumatoid arthritis (RA) and 25 systemic lupus erythematosus (SLE) patients, and 8 healthy controls (HCs). Although mRNA expression of the two isoforms did not directly correlate with clinical disease activity, relative expression of both was significantly lower in SLE and RA patients than in HCs. Furthermore, we found a significant statistical correlation between the two isoforms, suggesting that they are similarly regulated. Decreased expression of these isoforms in RA and SLE may reflect Treg cell abnormalities in these autoimmune diseases.

T-regulatory (Treg) cells were recently discovered as a distinct subset of helper T-cells that possess immunosuppressive activity. Although the mechanisms of these immunosuppressive effects are unknown, proposals include cell contact-dependent suppression, involvement of immunosuppressive cytokines such as IL-10 and TGF-beta, and functional modification of Treg by antigen-presenting cells (1). Treg cells also play an important role in the mechanisms of peripheral immune tolerance and the prevention of pathogenic immunity (2). While both the number and functional capacity of Treg cells are known to be decreased in various autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (3), their role and importance in the pathogenesis of RA and SLE have not been precisely determined.

In animal experiments, FOXP3, a transcription factor belonging to the fork head/winged-helix family, has been proved essential to the identification of Treg cells, as well as central to their regulatory function (1, 4). While only a single wild isoform occurs in mice, three FOXP3 isoforms are known in humans, wild, and exon-2 and exon 2-exon 7 lacking (5-6). Furthermore, FOXP3 has three discernible functional domains: a single C2H2 zinc-finger motif of unknown function, a leucine-zipper-like motif for dimerization, and a carboxy-terminal forkhead

Key words: T-regulatory cell, FOXP3, exon 2-lacking isoform, rheumatoid arthritis, systhemic lupus erythematosus

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domain for DNA binding. A repressor domain for transcriptional repression at the N-terminal which includes exon 2 has also been identified (7). While the functional role of the exon 2-lacking isoform is not fully defined, one proposal is that this isoform exerts transcriptional repressor activity towards the IL-2 promoter, and may have a distinct function from the wild isoform *in vivo* (8). In contrast, an alternative proposal suggests that the exon 2-lacking isoform works as a functional inhibitor of CD4-positive T cell activation (9). In any case, given the likely role for FOXP3 in human immune modulation, further investigation of the function and clinical significance of the exon 2-lacking isoform in autoimmune diseases is warranted.

Here, we analyzed the messenger RNA (mRNA) expression of both the wild and exon 2-lacking isoforms of FOXP3 in the peripheral blood of patients with RA and SLE to further elucidate the clinical significance of the two isoforms in these autoimmune diseases.

MATERIALS AND METHODS

Patients and controls

After informed consent was obtained, peripheral blood samples were taken from a convenience sample of 56 healthy volunteers and patients being seen at our division, namely 8 healthy controls (HCs), 23 patients with active RA according to the American Rheumatism Association's 1987 Revised Criteria for Disease Classification (10), and 25 patients with active SLE according to the American College of Rheumatology's 1997 Revised Criteria for the Classification of Systemic Lupus Erythematosus (11).

Peripheral blood mononuclear cells and mRNA isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (GE Healthcare, Bucks, UK) density-gradient centrifugation of heparinized venous blood. Messenger RNA was isolated from PBMCs with a Gene Elute Mammalian Total RNA Miniprep Kit (Sigma, MO, USA) immediately after separation from peripheral blood according to the manufacturer's instructions.

Quantitative PCR

The mRNA was converted to whole cDNA by reverse transcriptase. Using 5 μL of the whole cDNA derived from PBMCs as the template, the cDNA was quantified by detection of TaqMan probe fluorescence using the ABI Prism 7700 sequence detector system (Applied Biosystems, CA, USA). The relative quantification of the wild and exon-2 FOXP3 isoforms was determined by the comparative threshold cycle method using separate tubes according to the manufacturer's instructions (Applied Biosystems). To normalize gene expression, the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The schematic structure of FOXP3 in the human genome, including transcripts and the wild and exon-2 isoforms, is shown in Fig. 1. To discriminate the two isoforms, we designed an original series of primers and TaqMan probes specific to each using the Primer Express software (Applied Biosystems). Specificity of the oligo-nucleotide sequences was confirmed using The National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/), with the positions and directions shown in Fig. 1. The sequences of the primers and fluorescein phosphoramidite (FAM)-labeled probes were as follows: hFOXP3Ex2-3F, 5'-CTTGCCCCACTTACAGGCA-3'; hFOXP3Ex2-3R, 5'-GGGCATCCACCGTTGAGA-3'; hFOXP3Ex2-3PB, 5'-FAM-AGGACAGGCCACATT TCATGCACCAG-TAMRA-3'; hFOXP3Ex1-3F, 5'-TCCTCTTCTTCCTTGAACCCC-3'; hFOXP3Ex1-3R, 5'-GGGCGTGGGCATCCA-3'; and hFOXP3Ex1-3PB, 5'-FAM-CATCGCAGCTGCAGCTCTCAACG-TAMRA-3'. GAPDH, TaqMan control reagents, including JOE probe, and GAPDH forward and reverse primers (402869; primer and probe sequence information were not publicly available), were purchased from Applied Biosystems.

The TaqMan Universal Master Mix (2X) was used as the reaction solution with amplification conditions of 15 s at 95°C for denaturation, and 60 s at 60°C for annealing and primer extension (50 cycles). The nucleotide sizes of the generated PCR products were measured by electrophoresis using 3.0% agarose gels.

Collection of data for laboratory and clinical parameters

Laboratory data, including complete blood counts and leukocyte differentiation, were measured with a fully automated measurement analyzer used in routine clinical practice. Disease activity was evaluated at the point of blood sampling for RA by the EULAR Disease Activity Score 28 (DAS 28) (12) and C-reactive protein (CRP) formula (DAS28-3CRP), and for SLE by the SLE Disease Activity Index (SLEDAI) (13).

Statistical Analysis

For statistical analysis, we used the non-repeated ANOVA (nrANOVA) test for comparison of multiple groups and the Student-Newmann-Keulus (SNK) test for post-hoc tests. Statistical significance was set at $p < 0.05$.

RESULTS

Patients Demographics

Mean age for all subjects was 44.1 yr ± 15.8. Among groups, mean age was 38.4 yr ± 7.1 for HCs, 54.8 yr ± 13.9 for RA patients, and 36.1 yr ± 14.1 for SLE patients, with the age of RA patients significantly higher than that of the other two groups ($p=6.3 \times 10^{-6}$, two tail, nrANOVA). Male:female ratios were 11:45 for all subjects, and 2:5 for HCs, 5:18 for RA patients, and 4:21 for SLE patients.

The mean DAS28-3CRP score for RA patients was 4.94 ± 1.49, indicating moderate to high disease activity, while the mean SLEDAI for SLE patients was 9.60 ± 6.96, indicating high activity. Regarding prior treatment, 43% (10/23) of RA patients were newly diagnosed with no prior treatment. Of the remaining 13, 9 had received low-dose corticosteroids (CS) (mean dose 6.7 mg/day), 10 received methotrexates (mean dose 8.1 mg/week), and 5 received other DMARDs, with several patients receiving more than one medication. Among the SLE patients, 76% (19/25) were newly diagnosed with no prior treatment, and the remaining 24% (6/25) had received CS at a mean predonisone dose of 46.8 mg/day, including one patient treated with CS high-dose pulse therapy.

Measurement of mRNA expression of the two FOXP3 isoforms by quantitative PCR

We initially measured the gene expression of the two FOXP3 isoforms by conventional reverse transcription PCR using primer pairs targeting exons 1 and 3. Although we confirmed the presence of the two isoforms as reported elsewhere (9), quantification of expression was hampered by the low ratio of Treg to CD4-positive T cells. We therefore developed a new quantitative PCR protocol, as described in the Methods. The resultant quantitative PCR amplification curves were appropriate, and we confirmed the estimated sizes of the bands (71 and 66 base pairs for wild and exon 2-lacking isoforms, respectively) by electrophoresis (data not shown).

mRNA expression of the two FOXP3 isoforms in PBMCs from RA and SLE patients

The GAPDH-adjusted relative mRNA expressions of the two FOXP3 isoforms in PBMCs from RA, SLE, and HC subjects are shown in Figs. 2A (wild) and 2B (exon 2-lacking). In RA and SLE patients, the expression of both isoforms was significantly decreased compared to levels in HCs (wild isoform: $p=2.0 \times 10^{-5}$ (two tail, nrANOVA); post-hoc test, $p<0.01$ between HC and RA, and HC and SLE; and $p<0.05$ between RA and SLE (SNK test); exon 2-

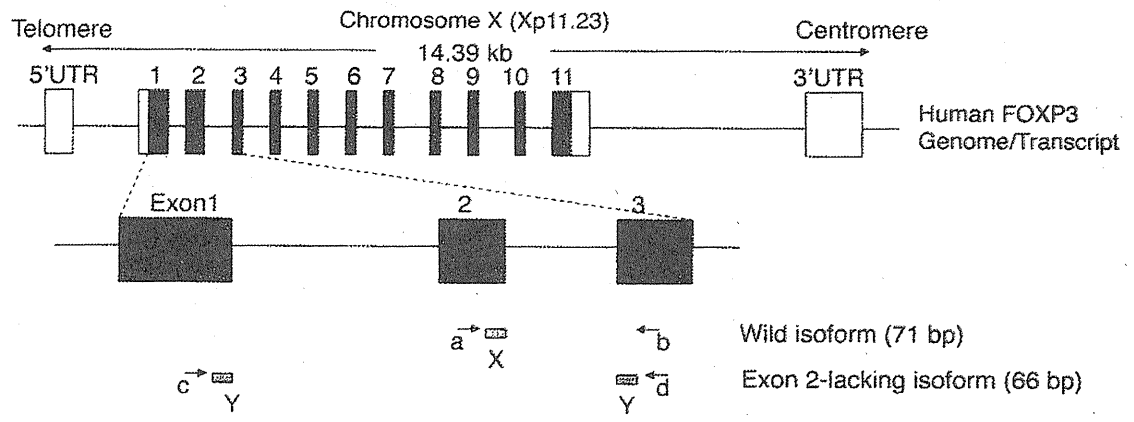

Fig. 1. *Schematic structure of FOXP3 in the human genome, its transcripts, and wild and exon 2-lacking isoforms. Transcripts were composed of 11 exons and are indicated as black square boxes, and introns as white square boxes. We created two sets of primers and probes for quantitative PCR to distinguish the two isoforms. The position and direction of the sequence-specific primers and probes are indicated as arrows (a-d) and grey square boxes (X and Y), respectively. By this protocol, length of PCR products was 71 bp (wild isoform) and 66 bp (exon 2-lacking isoform).*

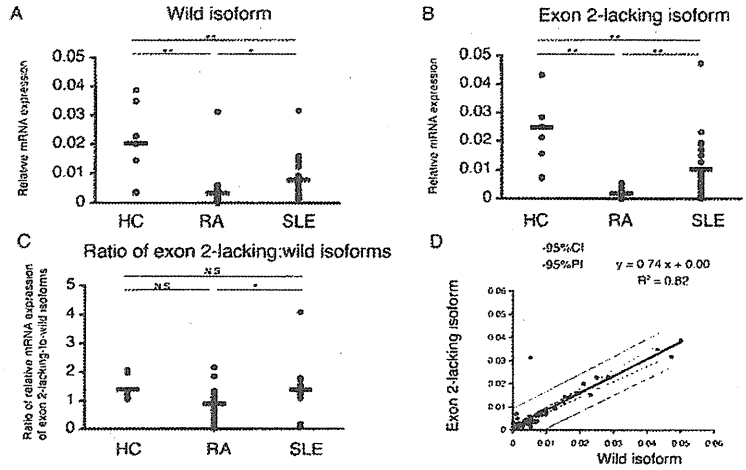

Fig. 2. *mRNA expression of FOXP3 wild and exon 2-lacking isoforms in PBMCs. Quantitative PCR analysis of GAPDH-adjusted relative mRNA expressions of two different isoforms, wild (A) and exon 2-lacking (B), in HC (n=8), RA (n=23), and SLE (n=25) patients. Statistic significances were calculated by non-repeated ANOVA (two tail) and SNK test. Ratio of GAPDH-adjusted relative mRNA expression of exon 2-lacking-to-wild isoforms is shown in C. Statistic significances were calculated by non-repeated ANOVA (two tail) and SNK test. Correlation between mRNA expression of FOXP3 wild and exon 2-lacking isoforms from the 56 cases is shown in D. Linear regression, 95% confidence interval (CI), and prediction interval (PI) are indicated as solid, dotted, and dashed lines, respectively. **: p<0.01, *: p<0.05.*

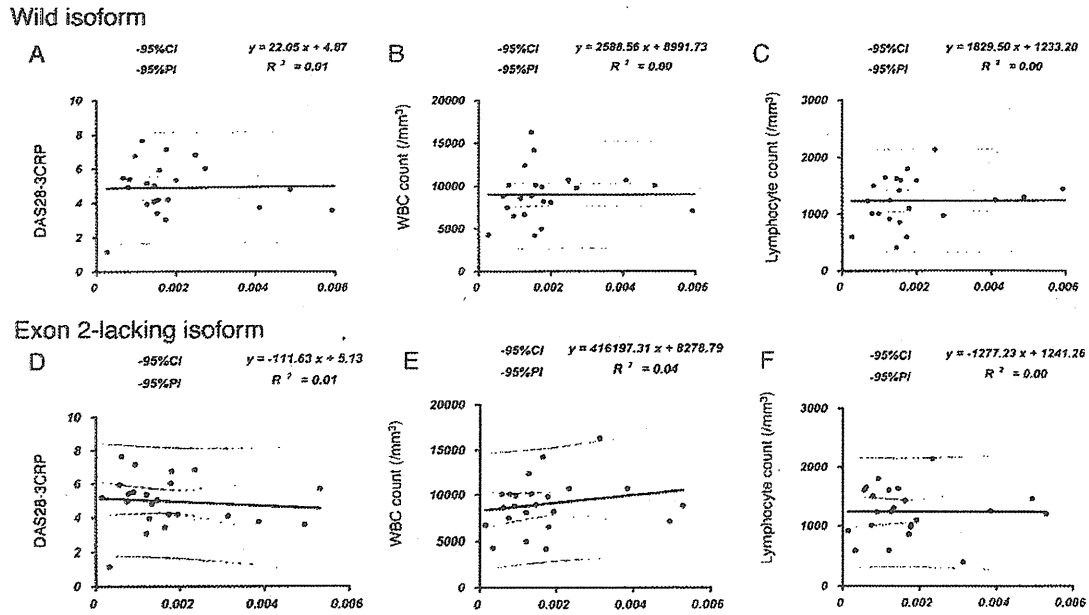

Fig. 3. *Relationship between FOXP3 mRNA expression in PBMCs and disease activity and laboratory data in RA patients. Correlations were calculated between GAPDH-adjusted FOXP3 mRNA expression in PBMCs and the clinical parameters DAS28-3CRP, peripheral white blood cell (WBC) count (/mm³), and peripheral lymphocyte counts (/mm³). A to C show results for the wild isoform, and D to F show results for the exon 2-lacking isoform. Statistical significances were calculated by correlation coefficient. Linear regression, 95% confidence interval (CI), and prediction interval (PI) are indicated as solid, dotted, and dashed lines, respectively.*

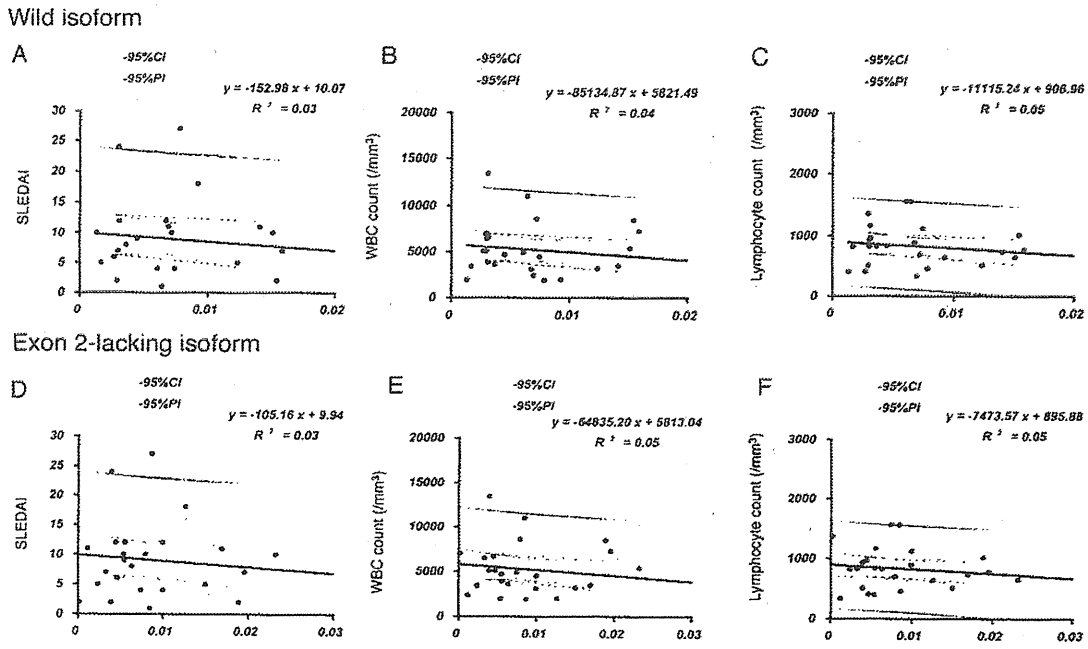

Fig. 4. *Relationship between FOXP3 mRNA expression in PBMCs and disease activity and laboratory data in SLE patients with SLE. Correlations were calculated between GAPDH-adjusted FOXP3 mRNA expression in PBMCs and the clinical parameters SLE Disease Activity Index (SLEDAI), peripheral white blood cell (WBC) count, (/mm³), and peripheral lymphocyte count (/mm³). A to C show results for the wild isoform, and D to F show results for the exon 2-lacking isoform. Statistical significances were calculated by correlation coefficient. Linear regression, 95% confidence interval (CI), and prediction interval (PI) are indicated as solid, dotted, and dashed lines, respectively.*

lacking isoform: $p=4.1x10^{-7}$ (two tail, nrANOVA); post-hoc test, $p<0.01$ for all three combinations (SNK test)). We next calculated the ratio of the relative gene expressions of the two isoforms. While the ratio of exon 2-lacking:wild isoforms in HCs and SLE patients was approximately 1:1, that in RA patients was slightly, but significantly, decreased ($p=0.025$ (two tail, nrANOVA), and post-hoc test $p<0.05$ between RA and SLE (SNK test)) (Fig. 2C), while both isoforms were significantly and positively correlated ($R^2=0.82$) in total patients (Fig. 2D).

Correlation of the two FOXP3 isoforms with laboratory and clinical parameters in RA and SLE patients

mRNA expressions of the two FOXP3 isoforms were not correlated with disease activity as determined by the DAS28-3CRP score for RA patients (Figs. 3A and D) and SLEDAI for SLE patients (Figs. 4A and D). Further, we also analyzed the relationship of their expression with peripheral leukocyte and lymphocyte counts, but identifiedno correlation for either isoform in either the RA (Figs. 3B, C, E, and F) or SLE patients (Figs. 4B, C, E, and F).

DISCUSSION

Here, we demonstrated by quantitative PCR methods that the expression of both wild and exon 2-lacking FOXP3 isoforms was markedly decreased in the peripheral blood of patients with RA and SLE when compared to HCs. Further, the mRNA expression of the exon 2-lacking isoform was positively correlated with that of the wild. This remarkable decrease in peripheral FOXP3 expression could not be simply explained by a decreased lymphocyte count, but likely rather indicated a quantitative decrease in FOXP3 expression in

individual lymphocytes. Our findings may therefore indicate that Treg cell-controlled peripheral tolerance in RA and SLE patients exhibits not only quantitative, but also qualitative abnormalities.

Previous reports on Treg cells in RA and SLE patients used CD4+ plus high CD25 expression as surface markers for the identification of Treg cells. However, this method does not completely exclude the possibility of contamination with activated T-cells, and findings using these markers to quantify peripheral Treg cells in patients with RA (14-15) and SLE (16-17) have been inconsistent. Fortunately, the recent discovery of FOXP3 has provided a more accurate marker of Treg cells that can be applied to their quantification, and it has been consequently demonstrated that Treg cells measured by FOXP3 are quantitatively decreased in PBMCs taken from patients with RA (18) and SLE (19-22). Here, given these findings indicating a decrease in FOXP3 expression in patients with RA and SLE, we focused on the role of the two major FOXP3 isoforms in these autoimmune diseases.

Regarding the technical aspects of Treg cell quantification, we found that the Treg cell population in CD4+T cells was too small (approximately 5%-10% in humans) for the accurate measurement of the FOXP3 isoforms by immunobloting. We therefore used a quantitative PCR method to discriminate both isoforms that proved highly sensitive, even when applied to blood samples of small volume.

Recently, the FOXP3 isoform lacking both exon 2 and exon7 was identified (5). The role of this isoform was subsequently analyzed using healthy human samples and an *in vitro* overexpression system (6), which revealed that the isoform is expressed at levels that are approximately 0.01 fold of those of the FoxP3 isoform lacking exon 2 in normal human PBMCs (6). Moreover, this isoform did not exhibit suppressive activity, unlike the wild and exon 2 lacking isoforms, and only displayed dominant negative effects in the overexpression experiments. After considering these findings, we speculated that the physiological role of this third isoform would be limited, although it might be potentially interesting to examine its role in autoimmune disorders in a future study.

Our results illustrating that mRNA levels of the wild and exon 2-lacking FOXP3 isoforms were decreased support recent reports which used flow cytometry to show decreased peripheral expression of FOXP3 (16-17, 20-22). Given that Treg cells may play a central role in RA and SLE, this notable decrease in FOXP3 expression may be expected to result in functional impairment. From our results, we speculate that the exon 2-lacking:wild isoform ratio is approximately one-to-one in HCs and SLE patients. Although the cause of the statistically significant, yet mildly decreased, ratio of exon 2-lacking:wild isoforms in the RA patients is unclear, one explanation may be that regulatory elements in the splicing process differ in individuals with RA and SLE.

We were interested in determining whether the two examined isoforms acted in an additive or competitive manner. While we found that expression levels of the two isoforms were decreased in the autoimmune disease patients, but almost expressed at equal ratios in all subjects, we detected no correlation with disease activity. Therefore, we could not determine which of the two hypotheses mentioned above is more likely *in vivo*. At a minimum, our findings may suggest that the roles of the two isoforms in RA and SLE do not significantly differ.

Quite recently, a study examining the expression of the full-length and two splice-variant isoforms of FOXP3 in CD4+ cells from RA patients found that full-length Foxp3 mRNA levels were elevated compared to healthy donors, no differences in the other two Foxp3 isoforms were detected. (23). Although the authors concluded that Tregs are increased in RA, they did not explain the discrepancy between this finding and the reduced FOXP3+Treg cells typically observed in RA patients.. In contrast, our present findings of reduced wild and exon 2-lacking FOXP3 expression in PBMCs are quite reasonable, and can explain the relationship with that of protein levels.

It has also been demonstrated that total FoxP3 mRNA is decreased by 0.7 fold (median) in the peripheral blood of patients with Juvenile Idiopathic Arthritis (JIA) compared to healthy controls ($p < 0.001$) (24). Compared with the levels in JIA patients, a larger decrease of total FOXP3 mRNA in RA and SLE patients may reflect the more significant role of FOXP3 regulation in these autoimmune diseases.

Comparative quantitative measurements of Foxp3 mRNA levels among various autoimmune diseases may aid in the understanding of different Treg-mediated pathophysiologies. With regard to the role of these isoforms in the overall clinical picture of RA and SLE, the lack of association in our study between the decreased mRNA expression of both isoforms and disease activity may indicate that FOXP3 mRNA levels reflect peripheral tolerance levels rather than disease activity, which is more likely multi-factorial.

Regarding immunological intervention, one possible approach to recovering impaired Treg cell function in RA and SLE is the peripheral up-regulation of FOXP3 mRNA expression. Possibilities include initial *ex vivo* Treg cell expansion, followed by mRNA delivery and pharmacological intervention. Although modulation of FOXP3 mRNA expression may be a reasonable approach to the reconstitution of peripheral tolerance, care in the selection of suitable sites and timing will be required.

In conclusion, we found that although wild and exon 2-lacking FOXP3 isoforms were markedly decreased in the peripheral blood of RA and SLE patients, and the mRNA expression levels of the exon 2-lacking isoform were positively correlated with those of the wild isoform, their expression was not correlated with clinical activity. Further investigation of the technical aspects of FOXP3 and Treg cell measurement, as well as the nature of FOXP3, including the role of induced Treg cells in autoimmune diseases, is warranted.

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Fcγ receptor IIIb polymorphism and use of glucocorticoids at baseline are associated with infusion reactions to infliximab in patients with rheumatoid arthritis

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ABSTRACT

Objective Infusion reaction is a major adverse event in patients with rheumatoid arthritis (RA) treated with infliximab. The possible factors including Fcγ receptor (FcγR) polymorphism associated with the development of infusion reactions in patients with RA receiving infliximab were prospectively examined.

Methods 96 patients with RA were enrolled and scheduled to receive infliximab at a dose of 3 mg/kg at weeks 0, 2 and 6 and every 8 weeks thereafter. Genetic polymorphisms for FcγR were examined in *FCGR3A* 176F/V and *FCGR3B* NA1/2 alleles by allele-specific PCR analysis.

Results An infusion reaction was observed in 17 patients (18%) during 52 weeks of treatment with infliximab. The *FCGR3B* NA1/NA1 genotype was found in 75% of the patients with infusion reactions and in only 37% of those without (p=0.01), whereas the *FCGR3A* 176F/V genotype was equally distributed in the patients with or without infusion reactions. Glucocorticoids were used in 53% of the patients who developed an infusion reaction and in 80% of those without an infusion reaction (p=0.02). A multivariable logistic regression model showed that the *FCGR3B* NA1/NA1 genotype and use of glucocorticoids at baseline could be used as independent predictive factors for infusion reactions (OR 6.1 (95% CI 1.9 to 24.3) and OR 0.26 (95% CI 0.08 to 0.84), respectively). The presence of anti-infliximab antibody during infliximab treatment was also associated with infusion reactions.

Conclusion *FCGR3B* NA1/NA1 genotype, use of glucocorticoids and the presence of anti-infliximab antibody accounted for nearly all patients with RA who developed infusion reactions.

INTRODUCTION

Biological agents targeting a specific molecule provide an effective means for therapeutic management of rheumatoid arthritis (RA) due to their specificity and powerful functional capabilities, which has resulted in a paradigm shift in the treatment strategy of this disease.[1–4] Despite their effectiveness, several adverse drug reactions associated with the use of biological agents have been identified, such as opportunistic infections and the development of hypersensitivity/infusion reactions. For example, approximately 10–23% of patients with RA experience infusion reactions (including fever, malaise, headaches, erythema and urticaria) to infliximab, a chimeric monoclonal IgG1 antibody against tumour necrosis factor α (TNFα), in combination with methotrexate (MTX).[5–9] Although much less frequent, severe infusion reactions such as anaphylactic/anaphylactoid symptoms are also observed in patients with RA.[8] Despite the fact that most of these reactions are only mild or moderate in severity, they may lead to discontinuation of treatment, which is of particular concern and highly relevant to daily clinical practice.[9] It is tempting to speculate that biological agents may induce these reactions in a portion of susceptible patients by causing hypersensitivity to the given biological agents,[10] in part through the immunogenicity of the agents or by direct effects on cellular functions through the Fc portion of the products.[11]

The Fc portion of IgG-based biological agents can bind to Fcγ receptors (FcγR) for clearing the agents and even affecting cellular functions including phagocytosis, antibody-dependent cellular cytotoxicity and neutrophil activation.[12] FcγR consist of three major families which are encoded by eight genes: *FCGR1A*, *FCGR1B*, *FCGR1C*, *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A* and *FCGR3B*. Functional allelic polymorphisms leading to distinct effector capabilities have been identified in the receptors FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb.[12]

Since the *FCGR2A* 131 H/R allele strongly influences the ability of FcγRIIa to bind human IgG2 but has only limited effects on IgG1 and IgG3 binding,[12] we focused on the *FCGR3A* 158F/V and *FCGR3B* NA1/NA2 alleles and prospectively examined the possible association of these *FCGR* alleles, development of antibody to infliximab and clinical parameters with development of infusion reactions to infliximab in patients with RA.

METHODS

Patients and treatment

Consecutive patients with RA who fulfilled the 1987 revised criteria of the American College of Rheumatology for the classification of RA[13] and satisfied the Japanese guidelines for the use of anti-TNF biological agents[14] were invited to participate in the study. Ninety-six patients showing incomplete response to MTX were enrolled after obtaining their written informed consent. The 2008 Declaration of Helsinki and the 2008 Ethical Guidelines for Clinical Research by the Japanese Ministry of Health, Labour and Welfare were

strictly followed in this study. The patients were scheduled to receive infliximab at a dose of 3 mg/kg at weeks 0, 2, 6 and subsequently every 8 weeks added to MTX at the Saitama Medical Center between September 2003 and March 2008. The management of infusion was performed using a previously reported protocol[15] approved by the University Institutional Review Board. We did not perform any premedication including histamine antagonists, paracetamol and additional glucocorticoids until the first infusion reaction developed in individual patients. Blood samples were taken for genotyping before initiation of infliximab, and anti-infliximab antibody (AIA) was measured at each infusion visit during the 52-week study.

Assessment of efficacy and safety

Patients were followed up longitudinally with examinations at baseline and at each regular infusion visit and emergency visit during the 52-week study. To monitor disease activity and disability, the 28-joint Disease Activity Score based on C reactive protein (DAS28-CRP) and serum levels of CRP, matrix metalloproteinase-3 (MMP-3) and Health Assessment Questionnaire-Disease Index (HAQ-DI) were determined. The attending physicians recorded any adverse drug reactions at baseline, at each regular infusion visit and emergency visit during the 52-week study period.

Measurement of AIA

AIA was measured using an ELISA kit (Immunodiagnostik, Bensheim, Germany). Briefly, serum samples were diluted with phosphate buffered saline (PBS) and added to 96-well plastic plates coated with the $F(ab)_2$ portion of infliximab to avoid interference with rheumatoid factor (RF). After incubating overnight at 4°C, the wells were washed with PBS and horseradish peroxidase-labelled infliximab was added. After incubation for 60 min, the plates were extensively washed with PBS, followed by addition of substrates into each well. The OD_{450}–OD_{620} was recorded using an ELISA reader.

Determination of FcγR polymorphisms

Heparinised venous blood was collected from patients and chromosomal DNA was isolated using phenol–chloroform extraction. Genetic polymorphisms for FcγR were examined in *FCGR3A* 176F/V and *FCGR3B* NA1/2 alleles by allele-specific PCR analysis, as previously described.[16 17] PCR products were separated on 3% agarose gels and visualised under ultraviolet light using a photoimager.

Statistical analysis

Baseline variables of patients with RA were analysed for association with development of infusion reactions using a χ^2 test for categorical variables and Student t test for continuous variables. Univariate logistic regression analysis was used to screen for potential predictor variables, and a stepwise selection process was used to generate a multivariate model for potential predictors of infusion reactions. All statistical analyses were performed on a Mac OS X platform (Sun Microsystems, Palo Alto, California, USA) using JMP Version 8.0.2.

RESULTS

Clinical characteristics of the patients with RA who developed infusion reactions

As shown in table 1, the development of infusion reactions, concomitant medications and the continuation of infliximab varied in individual patients. The observed infusion reactions were typically mild or moderate and included chills, fever, erythema and urticaria; however, one patient developed a severe anaphylactoid reaction and hypotension. Prior to the study, this patient had been enrolled in a clinical trial for infliximab, and the severe infusion reaction developed in this study after the second infusion, which represented a 3-year interval since he last received infliximab in the clinical trial. The most common manifestation in the patients was skin eruption (n=10), followed by fever (n=3), nausea/vomiting (n=3) and headache (n=2). Although these manifestations were not considered severe, nearly all of

Table 1 Demographic and clinical characteristics of patients with infusion reactions

No	Gender	Age (year)	Duration (month)	DAS28 at baseline	CRP (mg/dl)	RF (IU/ml)	ANA (×)	MMP-3 (ng/ml)	HAQ-DI	Clinical manifestation	Time of infusion reaction (weeks)	MTX (mg/week)	PRED (mg/day)	Adherence to infliximab
1	F	51	60	6.9	7.8	12	160	346	2.6	Chills, fever, dyspnoea	38	13	0	Discontinue
2	F	52	9	6.6	6.9	444	0	188	2.1	Hot flushes, headache, subfever	1	8	10	Continue
3	M	69	624	5.7	3.9	191	80	180	1.8	Anaphylactoid reaction, hypotension	2 (after 3-year interval)	6	5	Discontinue
4	F	40	50	4.8	2.9	864	80	200	1.3	Urticaria	14	12.5	0	Discontinue
5	F	50	97	3.5	0.3	23	80	121	0.3	Urticaria	30	10.5	5	Discontinue
6	F	68	48	4.5	3.2	42	80	95	0.13	Erythema	14	8	0	Discontinue
7	M	65	240	4.8	2.8	1080	320	85	2.6	Hot flushes, headache, nausea	14	10	3	Discontinue
8	F	52	255	5.7	3.3	845	160	191	1.3	Erythema	30	8	0	Discontinue
9	F	31	29	4.8	0.1	17	1280	98	0.5	Nausea	44	8	0	Discontinue
10	F	60	210	4.2	1.8	250	80	359	0.6	Urticaria	14	8	0	Discontinue
11	F	52	168	3.5	0.3	15	1280	121	0.3	Urticaria	2	6	0	Discontinue
12	F	57	360	6.6	6.4	98	1280	360	1.5	Erythema	14	6	4	Discontinue
13	F	33	21	6.6	5.2	122	80	612	1.1	Erythema	14	10	4	Continue
14	F	37	9	7.5	5.6	<5	0	280	1.9	Fever	14	6	10	Discontinue
15	F	37	60	4.3	1.4	446	80	ND	0.8	Erythema	30	8	0	Continue
16	F	63	4	7.4	11.0	1060	80	412	2.5	Nausea, vomiting	14	8	7.5	Discontinue
17	F	64	134	6.0	0.8	<5	1280	253	1.9	Erythema	22	6	0	Discontinue

ANA, antinuclear antibody; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score; HAQ-DI, Health Assessment Questionnaire-Disease Index; MMP, matrix metalloproteinase; MTX, methotrexate; ND, not done; PRED, prednisolone; RF, rheumatoid factor.

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the patients (15 of 17) discontinued infliximab due to the development of these infusion reactions.

Demographics and clinical characteristics of the patients

As shown in table 2, the mean age of the patients was 54 years and 83% were women. The mean disease duration was 8 years, RF positivity was 90%, the mean DAS28 score was 5.2 and the mean serum HAQ-DI level was 1.5, suggesting that the patients with RA enrolled in this study were established, active and disabled.

There were no significant differences in the demographics and clinical characteristics between patients who developed infusion reactions and those who did not, with the exception of the concomitant use of glucocorticoids. Glucocorticoids were used in 53% of patients with infusion reactions compared with 80% of patients without infusion reactions. In contrast, no significant differences in the demographics and clinical characteristics between patients who continued infliximab and those who discontinued its use were demonstrated in this study.

FcγR polymorphism

Allele distributions in the patients with RA for the *FCGR3A* 176F/V and *FCGR3B* NA1/NA2 polymorphisms are summarised in table 3. In this cohort of patients, the distribution of the *FCGR3A* high-affinity genotype V/V was 6% whereas the V/F and F/F genotypes were observed with a frequency of 48% and 46%, respectively. The occurrence of the V/V genotype was slightly lower than in the healthy Japanese population (8.6%).[18] The V/V genotype was enriched in patients with RA with infusion reactions (12%) over those without such reactions (5%), but the difference was not significant. The distributions of the genotypes for the *FCGR3A* 176F/V allele between patients with and without adherence to infliximab were comparable.

The high-affinity genotype of *FCGR3B* (NA1/NA1) was the most prevalent genotype in the patients with RA (44%), while the NA1/NA2 and NA2/NA2 genotypes were found in 33% and 23% of patients, respectively. The *FCGR3B* NA1/NA1 genotype was found in 70% of patients with infusion reactions but was present in only 37% of patients without infusion reactions, indicating that this genotype is associated with the development of infusion reactions. In contrast, the low-affinity genotypes NA1/NA2 and NA2/NA2 were only observed in 18% and 6%, respectively, of patients who developed infusion reactions, which is much lower than the 37% and 27%, respectively, of those without reactions. Analyses confirmed that the distribution of the *FCGR3B* genotypes between patients with and without infusion reactions was significantly different (p=0.01). On the other hand, such differences in distribution of the *FCGR3B* genotypes were not observed between those with and without adherence to infliximab.

The presence of AIA during each visit was found to be positive in 19% of the patients. The development of AIA was significantly higher in patients with infusion reactions than in those without (65% and 9%, respectively, p<0.001), whereas no difference was observed in patients with or without adherence to infliximab (15% and 22%, respectively, p=0.40).

Potential predictive variables for infusion reactions

The impact of the presence or absence of risk factors identified on the development of infusion reactions is summarised and compared in figure 1. In order to create a multivariate model of potential predictors of infusion reactions, we first screened a series of baseline variables which included age, gender, duration of RA, DAS score, levels of CRP, MMP-3, HAQ-DI, RF and antinuclear antibody, doses of MTX and prednisolone equivalent, glucocorticoid use and *FCGR3A* V/V+V/F and *FCGR3B* NA1/NA1 genotypes by univariate logistic regression. As shown in table 4, the *FCGR3B* NA1/NA1 genotype and use of glucocorticoids were significant predictive variables, consistent with the above analyses. Even by stepwise selection, these variables were identified as significant factors associated with infusion reactions (data not shown).

We next subjected these two variables to multivariate logistic regression analyses which allowed for differences in patients with or without infusion reactions to be adjusted. From these analyses, the *FCGR3B* NA1/NA1 genotype and use of glucocorticoids were finally identified as significant independent variables associated with the development of infusion reactions. The ORs of the *FCGR3B* NA1/NA1 genotype and the use of glucocorticoids were 6.1 (95% CI 1.9 to 24.3, p=0.002) and 0.26 (95% CI 0.08 to 0.84, p=0.025), respectively (table 5).

Finally, we examined the potential additive effect of the three identified factors on the development of infusion reactions. Patients with RA with only one factor had a rate of infusion

Table 2 Patient characteristics and infliximab-related outcome

		Infusion reaction during 52 weeks			Adherence to infliximab during 52 weeks		
Subject	Total (n=96)	Yes (n=17)	No (n=79)	p Value	Continued (n=46)	Discontinued (n=50)	p Value
Age (years)	54±13	52±12	55±13	0.82	56±12	53±14	0.16
Female (%)	83	88	81	0.55	89	78	0.14
Disease duration (years)	8±9	11±14	8±8	0.18	8±7	9±10	0.57
RF positivity (%)	90	88	90	0.84	91	88	0.60
RF (IU/ml)	213±321	324±394	189±300	0.10	190±319	145±324	0.79
ANA positivity (%)	32	41	30	0.39	30	34	0.71
ANA titre (×)	273±616	377±150	251±69	0.20	341±813	211±346	0.16
Stage I+II (%)	44	47	43	0.46	33	43	0.05
Class 1+2 (%)	65	82	62	0.18	65	64	0.53
DAS28	5.2±1.3	5.4±1.6	5.2±1.3	0.69	5.1±1.5	5.4±1.2	0.22
CRP (mg/dl)	4.0±3.3	4.1±3.0	3.9±3.4	0.87	3.5±3.4	4.3±3.2	0.26
MMP-3 (ng/ml)	293±287	146±38	301±309	0.36	340±374	255±189	0.17
HAQ-DI	1.5±0.8	1.4±0.9	1.5±0.7	0.76	1.5±0.8	1.5±0.7	0.96
MTX dose (mg/week)	8±3	8±2	8±3	0.35	8±4	9±3	0.95
Use of glucocorticoids (%)	75	53	80	0.02*	74	76	0.81
Glucocorticoid dose (mg/day)	4±3	3±4	5±3	0.92	5±4	4±3	0.19

ANA, antinuclear antibody; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score; HAQ-DI, Health Assessment Questionnaire-Disease Index; MMP, matrix metalloproteinase; MTX, methotrexate; RF, rheumatoid factor.

Table 3 Association of Fcγ receptor polymorphism and anti-infliximab antibody (AIA) with infliximab-related outcome

Subject	Total (n=96)	Infusion reaction during 52 weeks: Yes (n=17)	Infusion reaction during 52 weeks: No (n=79)	p Value	Adherence to infliximab during 52 weeks: Continued (n=46)	Adherence to infliximab during 52 weeks: Discontinued (n=50)	p Value
FCGR3A 176F/V							
F/F	44 (46)	7 (41)	37 (47)	0.58	22 (48)	22 (44)	0.53
F/V	46 (48)	8 (47)	38 (48)		20 (44)	26 (52)	
V/V	6 (6)	2 (12)	4 (5)		4 (9)	2 (4)	
FCGR3B NA1/NA2							
NA1/NA1	42 (44)	13 (77)	29 (37)	0.01*	16 (35)	26 (52)	0.23
NA1/NA2	32 (33)	3 (18)	29 (37)		18 (39)	14 (28)	
NA2/NA2	22 (23)	1 (6)	21 (27)		12 (26)	10 (20)	
AIA							
(+)	18 (19)	11 (65)	7 (9)	<0.01*	7 (15)	11 (22)	0.40
(−)	78 (81)	6 (35)	72 (91)		39 (85)	39 (78)	

Values are numbers (%) unless otherwise indicated.

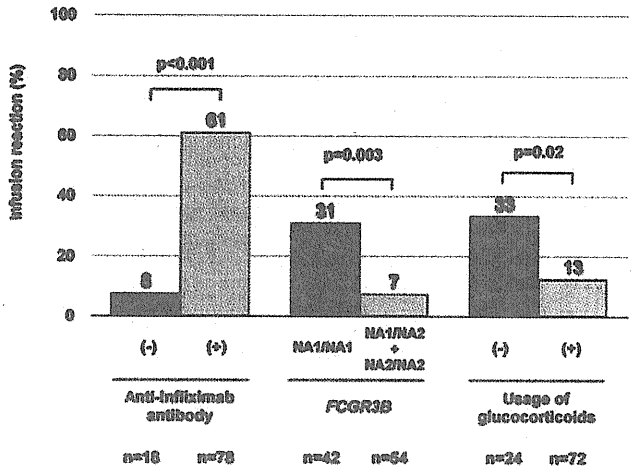

Figure 1 Percentage of patients with rheumatoid arthritis who developed an infusion reaction during the 52-week study in those with or without anti-infliximab antibody, those with or without *FCGR3B* NA1/NA1 genotype and those with or without concomitant use of glucocorticoids.

Table 4 Univariate logistic regression analysis for potential predictors of infusion reaction

Variables	Estimates	SE	OR	p Value
Age (years)	−0.02	0.02	0.98	0.37
Gender (female vs male)	0.24	0.40	1.62	0.55
Duration (years)	0.04	0.03	1.04	0.12
DAS28	0.08	0.21	1.08	0.69
CRP (mg/dl)	0.01	0.08	1.01	0.87
MMP-3 (per 10 ng/ml)	−0.01	0.01	0.99	0.55
HAQ-DI	−0.12	0.39	0.89	0.75
RF (per 10 IU/ml)	0.01	0.00	1.00	0.14
ANA (×)	0.00	0.00	1.00	0.46
MTX (mg/week)	0.03	0.09	1.03	0.77
MTX duration (months)	0.00	0.01	1.00	0.72
Use of glucocorticoids	−0.63	0.28	0.53	0.03*
Dose of prednisolone (mg/day)	−0.13	0.09	0.88	0.14
FCGR3A V/V+V/F vs F/F	0.11	0.27	1.12	0.67
FCGR3B NA1/1 vs NA1/2+2/2	0.86	0.31	2.37	<0.01*

ANA, antinuclear antibody; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score; HAQ-DI, Health Assessment Questionnaire-Disease Index; MMP, matrix metalloproteinase; MTX, methotrexate; RF, rheumatoid factor.

reactions of 11%, whereas those with two or three factors had rates of 53% and 100%, respectively (see figure in online supplement), demonstrating that these three factors additively account for the infusion reactions to infliximab observed in the 96 patients with RA examined in this study.

DISCUSSION

The results of this study show that the high-affinity *FCGR3B* NA1/NA1 genotype and the absence of glucocorticoid use at baseline are the main independent predictive factors for the development of infusion reactions to infliximab in Japanese patients with RA. This study is the first to demonstrate a role for the *FCGR3B* NA1/NA1 genotype in infusion reactions to infliximab.

Mild to moderate and severe infusion reactions were observed in 18% and 1%, respectively, of the Japanese patients with RA treated with infliximab during the 52-week course of this study. This result is consistent with previous studies showing that the incidence of infusion reactions to infliximab was approximately 10–23% of patients with RA per year.[5–7 8 15] The most frequent manifestations of the infusion reactions observed in this study were consistent with those reported for infusion reactions to infliximab in clinical practice settings in both the USA[5] and Japan.[8] A significant proportion of these patients subsequently discontinued infusion, implying that infusion reactions may account, as well as lack of efficacy, for the low retention rate of infliximab observed in the first year of treatment,[9 19–21] while others do not.[22 23] The inconsistency of the retention rate for infliximab among studies may result from the variable management strategies used to reduce infusion reactions, which include premedication,[5] gradual increases in infusion speed,[7] adjustment of infusion intervals[24] and dose increments,[25] among others.

It has been shown using radiolabelled infliximab in patients with RA that the formation of infliximab and anti-infliximab complexes were found in non-responders, one of which showed an anaphylactoid reaction, resulting in higher liver/spleen uptake and rapid clearance of infliximab.[11] It was recently observed that the IgE class of AIA is associated with the development of severe infusion reactions,[26] whereas true IgE-mediated hypersensitivity is not related to acute infusion reactions in most cases.[27] This possibility in one patient who developed an anaphylactoid reaction in this cohort remains to be addressed.