

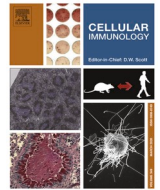
Type 1 helper T cells generate CXCL9/10-producing T-bet⁺ effector B cells potentially involved in the pathogenesis of rheumatoid arthritis

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Research paper

Type 1 helper T cells generate CXCL9/10-producing T-bet⁺ effector B cells potentially involved in the pathogenesis of rheumatoid arthritis

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ABSTRACT

Efficacy of B-cell depletion therapy highlights the antibody-independent effector functions of B cells in rheumatoid arthritis (RA). Given type 1 helper T (Th1) cells abundant in synovial fluid (SF) of RA, we have determined whether Th1 cells could generate novel effector B cells. Microarray and qPCR analysis identified CXCL9/10 transcripts as highly expressed genes upon BCR/CD40/IFN- γ stimulation. Activated Th1 cells promoted the generation of CXCL9/10-producing T-bet⁺ B cells. Expression of CXCL9/10 was most pronounced in CXCR3⁺ switched memory B cells. Compared with peripheral blood, SFRA enriched highly activated Th1 cells that coexisted with abundant CXCL9/10-producing T-bet⁺ B cells. Intriguingly, anti-IFN- γ antibody and JAK inhibitors significantly abrogated the generation of CXCL9/10-producing T-bet⁺ B cells. B cell derived CXCL9/10 significantly facilitated the migration of CD4⁺ T cells. These findings suggest that Th1 cells generate the novel CXCL9/10-producing T-bet⁺ effector B cells that could be an ideal pathogenic B cell target for RA therapy.

1. Introduction

Rheumatoid arthritis (RA) is among the most prevalent autoimmune disorders, characterized by joint inflammation and bone destruction. The emergence of autoantibodies such as anti-citrullinated protein antibodies (ACPA) and rheumatoid factors (RF) in the pre-clinical phase of RA indicates autoimmunity as an initial event in this disease [1,2]. In the clinical phase, the synovium of RA includes abundant immune and inflammatory cells such as dendritic cells, T cells, B cells, macrophages and neutrophils [3]. The crosstalk between these cells and non-immune cells ultimately forms the vicious circle leading to devastating bone destruction.

Genome-wide association studies (GWAS) on RA identify HLA class II as one of most susceptibility genes, suggesting a central role of CD4⁺ T cells in this disease [4]. Among helper T (Th) cell subsets, Th17 cells play an important role in murine arthritis model [5] however it remains somewhat elusive which T cell subset is pivotal in human RA. Notably, *STAT4*, a non-HLA gene associated with RA susceptibility, encodes the

signaling molecule via IL-12 receptor that is required for Th1 differentiation [6,7]. Indeed, we previously showed that interferon (IFN)- γ -producing Th1 cells dominate over Th17 cells in synovial fluids of RA (SFRA) [8]. In addition, a multi-ethnic meta-analysis identified a genetic variant in the *IFNGR2* region as a risk locus for RA [9] and gene expression of IFN- γ receptors (*IFNGR1* and *IFNGR2*) is associated with bone erosion in patients with RA [10]. These findings raise a possibility that Th1-derived IFN- γ plays a critical role in RA pathogenesis.

A classic tenet of B cell functions in autoimmune diseases is the generation of autoantibodies. However, B cell depleting agents, such as rituximab (RTX), significantly alleviate synovial inflammation and bone destruction without affecting the titers of autoantibodies, underscoring an antibody-independent effector function of B cells in RA [11–13]. Consistent with this idea, we previously identified RANKL⁺ effector B cells that directly promote osteoclastogenesis in patients with RA [14]. Intriguingly, RANKL⁺ B cells are significantly generated in vitro upon stimulation with BCR/CD40 and IFN- γ , a condition mimicking Th1 cell-B cell interaction in vivo.

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T-bet is the master transcription factor (TF) that dictates Th1 differentiation [15]. Recently, a novel B cell subset expressing T-bet was identified in infection, autoimmunity and aging [16]. Despite their heterogeneity to some extent, T-bet⁺ B cells are often defined as CD11c⁺CD21^{lo/-}CD19⁺ cells in humans [17]. This subset is proposed as a critical player in Th1 immunity and IFN- γ is an indispensable cytokine for its generation [18,19]. Indeed, T-bet⁺ B cells are found in peripheral blood (PB) and SF in patients with RA [17,20,21] however their role in RA pathogenesis remains to be fully elucidated.

CXCL9/MIG and CXCL10/IP10 belong to the CXC subfamily chemokine that plays a role in the recruitment and activation of CXCR3⁺Th1 cells in RA [22,23]. Besides, CXCR3⁺ memory B cells and CXCR3⁺ plasmablasts are accumulated in SFRA [14,24,25]. Notably, the levels of CXCL9 and CXCL10 are increased in PB and SF in patients with RA, and thus these chemokines are considered as biomarkers of disease activity of RA [26–28]. Along this line, CXCL9 and CXCL10 are both increased in amounts in untreated patients with early RA [26,29,30]. A phase II clinical trial of anti-CXCL10 mAb in patients with RA exerted a beneficial efficacy [31]. In early RA synovium, CXCL9 and CXCL10 are mainly produced by synovial fibroblasts, however it is also detected in lymphoid follicles or synovial macrophages [25]. This process is involved in the production of IFN- γ and tumor necrosis factor (TNF)- α by recruited Th1 cells [25]. Although B cells can produce CXCL10 upon CpG stimulation [32] it remains unclear whether they can do so in the context of RA pathogenesis.

In this study, we demonstrate that Th1 milieu promotes human B cells to express CXCL9/10 mRNAs and proteins. Notably, CXCL9/10-producing B cells expressed T-bet and were enriched in the fraction of CXCR3⁺ switched memory B cells. Besides, SFRA enriched highly activated Th1 cells that coexisted with abundant CXCL9/10-producing T-bet⁺ B cells that could in turn facilitate further recruitment of CXCR3⁺ cells into RA synovium. Anti-IFN- γ -antibody and JAK inhibitors significantly abrogated Th1-mediated generation of CXCL9/10-producing T-bet⁺ B cells. B cell derived CXCL9/10 significantly facilitated the migration of CD4⁺ T cells. These findings suggest that Th1 cells generate the novel CXCL9/10-producing T-bet⁺ effector B cells that could be an ideal pathogenic B cell target for RA therapy.

2. Methods

2.1. Patients and controls

Patients with RA met with the 1987 American College of Rheumatology classification criteria. PB was obtained from 30 patients with RA (PBRA: 4 males and 26 females, 33–88 years old, average age 64.0 years). PBRA was further divided into two groups, inactive RA (PBiRA: remission to low disease activity) and active RA (PBaRA: moderate to high disease activity). SF was obtained from 20 patients with RA (SFRA; 2 males and 18 females, 43–78 years old, average age 64.4 years). PB from healthy donors (HDs) matched to the RA patients' gender and age served as controls. Patient demographics are provided in [Supplementary Table 1](#). Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects (No. 29-544).

2.2. Reagents

An affiniPure F (ab') Fragment Goat Anti-Human IgA/IgG/IgM (H + L) (α BCR, 10 μ g/ml) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Anti-human CD40 monoclonal antibody (α CD40, 2 μ g/ml), recombinant human CD40L (100 ng/ml) and recombinant human IFN- γ (20 ng/ml) were from R&D Systems (Minneapolis, MN, USA). Neutralizing anti-IFN- γ antibody (100 ng/ml) and anti-CXCR3 antibody (100 ng/ml) was from Biolegend (San Diego, CA, USA). We used Dynabeads Human T-Activator CD3/CD28 from Thermo

Fisher Scientific (Waltham, MA, USA) for T cell activation with standard dose (25 μ l/1 \times 10⁶ cells). Tofacitinib and Baricitinib were from Cosmo Bio (Tokyo, Japan). Staphylococcal enterotoxin B from Staphylococcus aureus (SEB, 1 μ g/ml) and dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD28/49d antibody (1 μ g/ml) as a co-stimulator of SEB was from BD Biosciences (San Jose, CA, USA).

2.3. FACS analysis

Mononuclear cells (MNCs) were isolated from PB and SF using a density centrifugation with LSM (MP Biomedicals, LLC, Santa Ana, CA, USA). MNCs were stained with conjugated mouse monoclonal antibody (mAb), CD3, CD4, CD8, CD11a, CD11c, CD19, CD21, CD25, CD27, CD38, CD69, CD80, CD86, CD183 (CXCR3), CD196 (CCR6), CD307e (FCRL5), IgD, HLA-DR, T-bet, CXCL9, CXCL10 (all from BioLegend). In intracellular assays, we used Zombie NIR™ Fixable Viability Kit (Biolegend). For fixation, IC fixation buffer (eBioscience, San Diego, CA, USA) was used for CXCL9 and CXCL10, and Fixation/Permeabilization buffer (eBioscience) was used for T-bet.

2.4. Isolation and cell sorting

B cells were isolated by positive selection with Dynabeads M450 CD19 and DETACHa-BEAD CD19 (Invitrogen, Carlsbad, CA, USA). As we previously showed, only negligible levels of artificial activation of B cells occurred immediately after positive selection [33]. Isolated B cells exhibited greater than 99.5% viability and more than 95% purity, confirmed by flow cytometry. MNCs and isolated B cells were stained with mAb, and Th1 cells (CD3⁺CD4⁺CXCR3⁺CCR6⁻) and B cell subsets such as naïve (IgD⁺CD27⁻) and switched memory (IgD⁻CD27⁺) B cells were both purified using flow cytometry.

2.5. Cell culture

Cells were cultured in RPMI-1640 with 10% fetal bovine serum at 37 °C in a 5% CO₂ humidified atmosphere for the following conditions. In quantitative real-time polymerase chain reaction (qPCR) assays, whole B cells were cultured with or without stimulations for 24 h. In coculture assays, whole B cells or B cell subsets (1 \times 10⁵/well) were cocultured with Th1 (1 \times 10⁵/well) with or without T cell activator for 24 h in qPCR analysis and for 48 h in flow cytometry analysis, respectively. In flow cytometry analysis of CXCL9/10, PMA (Sigma), Ionomycin (Sigma) and brefeldin A (eBioscience) was added to the culture mediums for the last 4–5 h.

2.6. Microarray analysis

We extracted total RNA from cultured memory B cells derived from three healthy donors (HDs) using Isogen II reagent (Nippon Gene, Tokyo, Japan) and prepared amplified and biotinylated cRNA with the TotalPrep RNA Amplification Kit (Illumina, San Diego, CA, USA). After hybridization process of biotin-labeled cRNA, hybridized microarray chips (HumanHT-12 v4 Expression BeadChip) were stained with Cy3-conjugated streptavidin according to the manufacturer's instructions and scanned with a BeadArray Reader (Illumina). The obtained microarray data through BeadStudio software (Illumina) were analyzed with Gene Spring GX software (Agilent Technologies, Santa Clara, CA, USA). To identify differentially expressed genes between two experimental groups of memory B cells with or without IFN- γ stimulation ([Fig. 1A](#)), we performed a *t*-test and post-hoc test (Storey with bootstrapping correction). The microarray data were deposited in the Gene Expression Omnibus database (accession number GSE111475).

2.7. Quantitative real-time polymerase chain reaction (qPCR)

Total mRNA was extracted from primary B cells using Isogen II

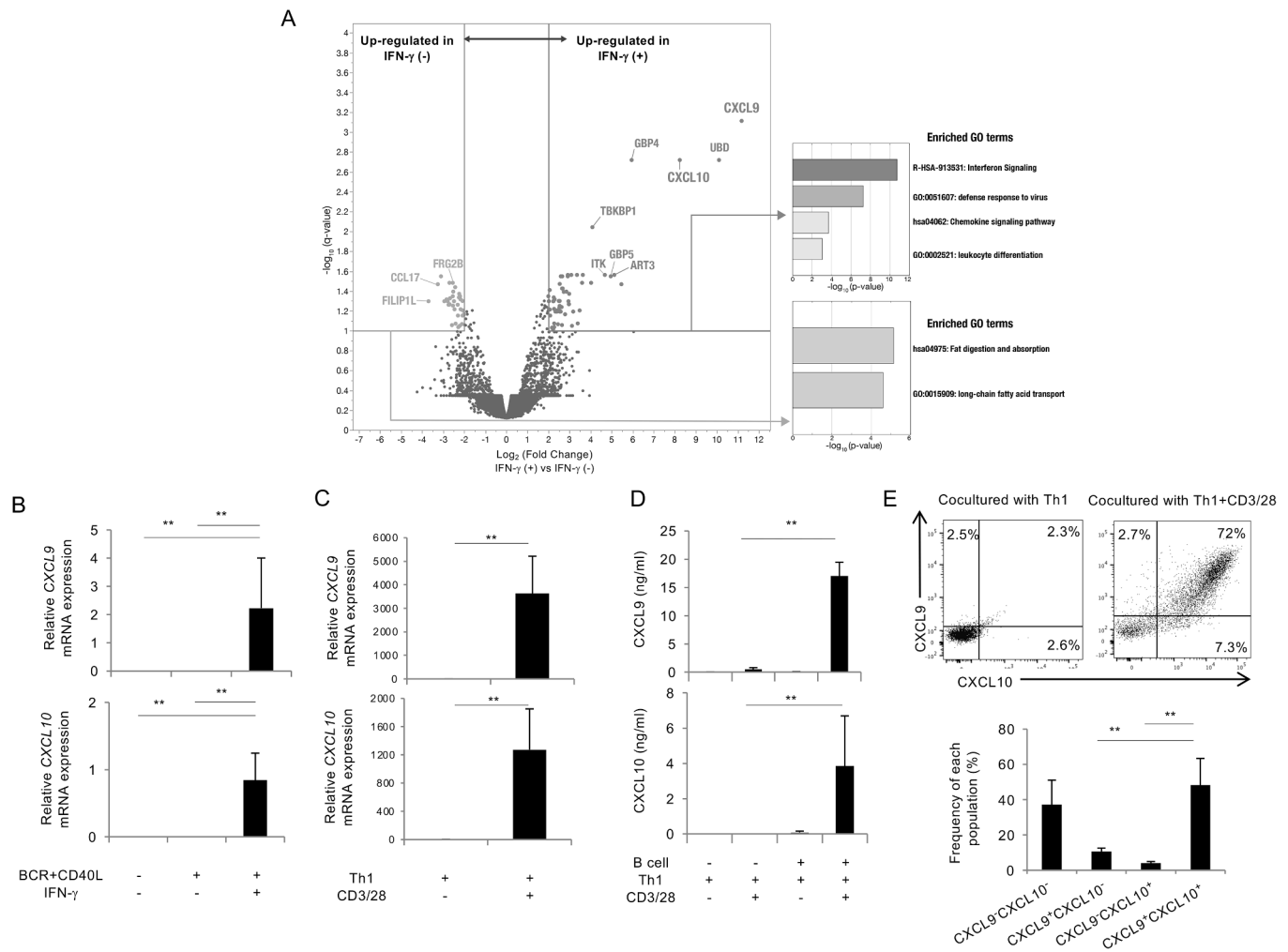


Fig. 1. B cells can produce CXCL9/10 in the presence of activated Th1 cells. (A) Microarray analysis. A volcano plot comparing gene expression in memory B cells from healthy donors (HDs) stimulated by BCR/CD40 and BCR/CD40/IFN- γ ($n = 3$). (B) Whole B cells from HDs were incubated with BCR/CD40L and IFN- γ for 24 h, and mRNA of CXCL9 and CXCL10 was analyzed ($n = 5$, $**P < 0.01$, with reference to IFN- γ stimulation). (C) Whole B cells from HDs were cocultured with CXCR3 $^{+}$ CCR6 $^{-}$ T cells (Th1) with or without T cell activator for 24 h, and mRNA of CXCL9 and CXCL10 in B cells was analyzed ($n = 5$, $**P < 0.01$). (D) Whole B cells from HDs were cocultured with Th1 in the presence or absence of T cell activator for 72 h. CXCL9 and CXCL10 in the supernatants were analyzed by ELISA ($n = 6$, $**P < 0.01$). (E) Whole B cells from HDs were cocultured with Th1 in the presence or absence of T cell activator for 48 h. Frequency of CXCL9 $^{+}$ and CXCL10 $^{+}$ B cells was determined by flow cytometry ($n = 5$, $**P < 0.01$).

reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using a SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA). qPCR was performed in the MX3000P Sequence Detector (Agilent technologies, Santa Clara, CA, USA). TaqMan target mixes for CXCL9 (Hs00171065_m1), CXCL10 (Hs01124251_g1), and TBX21 (Hs00203436_m1) were all purchased from Applied Biosystems. 18S ribosomal RNA was amplified as an internal control for variation in the amount of cDNA in PCR. The collected data were analyzed using Sequence Detector software (MX3000P). Data were expressed as the fold-change in gene expression relative to the expression in control cells.

2.8. ELISA

Concentrations of chemokines in the supernatant were measured with sandwich ELISA kits, human CXCL9/MIG Quantikine ELISA Kit and human CXCL10/IP-10 Quantikine ELISA Kit (R&D Systems), according to the manufacturer's instructions.

2.9. Chemotaxis assay

In vitro chemotaxis assays were performed using a 5 μm -pore CytoSelect 96-well assay plates (Cell Biolabs, San Diego, CA, USA). Briefly, 5×10^4 CD4 $^{+}$ T cells in 100 μl of chemotaxis medium (RPMI 1640, 10% BSA with penicillin and streptomycin) were added to the upper chamber. Conditioned medium as described were placed in the lower chamber. After 2 h incubation, cell numbers in the bottom chamber were determined by EnSpire Multimode Plate Reader (PerkinElmer, MA, USA).

2.10. Statistical analysis

Numerical data in in vitro experiments were presented as the mean of the different experiments and standard error of the mean (SEM). The significance of the differences was determined by Student's *t*-test for comparing differences between two groups, and one-way analysis of variance for comparing differences between multiple groups. Multiple comparisons were analyzed using Tukey-Kramer honestly significant difference test or Dunnett's test were applied. Numerical data in patient-sample analysis were analyzed using the non-parametric Wilcoxon

signed-rank test. For all tests, P values less than 0.05 were considered significant. All analyses were performed using JMP statistical software (SAS Institute, Cary, NC, USA).

3. Results

3.1. B cells can produce CXCL9/10 in the presence of activated Th1 cells

Since SFRA enriches IFN- γ -producing T-cells [8] it was of our interest to evaluate B cell functions in the presence of IFN- γ . To this end, we first determined gene expression in memory B cells from HCs with or without IFN- γ using cDNA microarray analysis. As shown in Fig. 1A, IFN- γ stimulation resulted in marked up-regulation of expression of chemokines CXCL9 and CXCL10 in memory B cells. To validate these results, we next determined expression levels of CXCL9 and CXCL10 mRNA by quantitative PCR analysis. Combination of IFN- γ with BCR/CD40 stimulation strongly induced CXCL9/10 mRNA in B cells (Fig. 1B). Since most of IFN- γ -producing T cells are CXCR3⁺ helper T (Th1) cells [34,35] we determined B cell expression of CXCL9/10 in coculture with activated Th1 cells. B cells significantly expressed CXCL9/10 at both mRNA and protein levels in coculture with activated Th1 (Fig. 1C and D). Under these conditions, a majority of B cells co-expressed CXCL9 and CXCL10 (Fig. 1E). The frequency of CXCL9⁺CXCL10⁺ B cells increased at the graded doses of T cell activator (Supplementary Fig. 1A). CXCL9⁺CXCL10⁺ B cells were also significantly generated in coculture with Th1 cells stimulated by SEB and anti-CD28/49d antibody (Supplementary Fig. 1B). These results suggest that B cells can produce CXCL9/10 in the presence of activated Th1 cells.

3.2. CXCL9/10-producing B cells express T-bet in the presence of activated Th1 cells

B cells can express TBX21 (T-bet), the master TF of Th1 cells, upon stimulation with IFN- γ in murine models [36]. We thus examined T-bet expression in human B cells in Th1 milieu. Activated Th1 remarkably

induced T-bet expression at both mRNA and protein levels in B cells (Fig. 2A). Next, we examined the relationship between CXCL9/10 and T-bet. B cells barely expressed T-bet as well as CXCL9/10 in coculture with unstimulated Th1 cells (Fig. 2B). On the other hand, almost all of CXCL9/10-producing B cells induced by activated Th1 cells expressed T-bet (Fig. 2B). These results suggest that CXCL9/10-producing B cells express T-bet in the presence of activated Th1 cells.

3.3. CXCR3⁺ switched memory B cells have high potential to give rise to T-bet⁺CXCL9/10-producing cells

To determine which B cell subsets have high potential to give rise to CXCL9/10-producing cells, we compared the expression of CXCL9/10 and T-bet between naïve and switched memory B cells in coculture with activated Th1 cells. The frequency of CXCL9/10-producing cells was higher in switched memory B cells than naïve B cells, while that of T-bet⁺ cells was comparable between these subsets (Fig. 3A). We next determined the frequency of CXCR3⁺ cells in B cell subsets because these cells potentially could localize in the vicinity of CXCR3-expressing Th1 cells at inflammatory sites. As shown in Fig. 3B, the frequency of CXCR3⁺ cells was significantly higher in switched memory B cells than naïve B cells. In addition, T-bet⁺ B cells expressed higher expression of CXCR3 (Fig. 3C). Furthermore, the frequency of CXCL9/10-producing and T-bet⁺ cells was higher in CXCR3⁺ than CXCR3⁻ switched memory B cells (Fig. 3D). These results suggest that CXCR3⁺ switched memory B cells have high potential to give rise to T-bet⁺CXCL9/10-producing cells.

3.4. B cells in SFRA exhibit spontaneous expression of CXCL9/10 and T-bet

Based on the above findings, we hypothesized that upon stimulation by activated T cells in vivo, B cells would exhibit spontaneous expression of CXCL9/10 and T-bet in patients with RA. We first compared mRNA expression of CXCL9/10 and TBX21 in B cells from PB in HCs (PBHC),

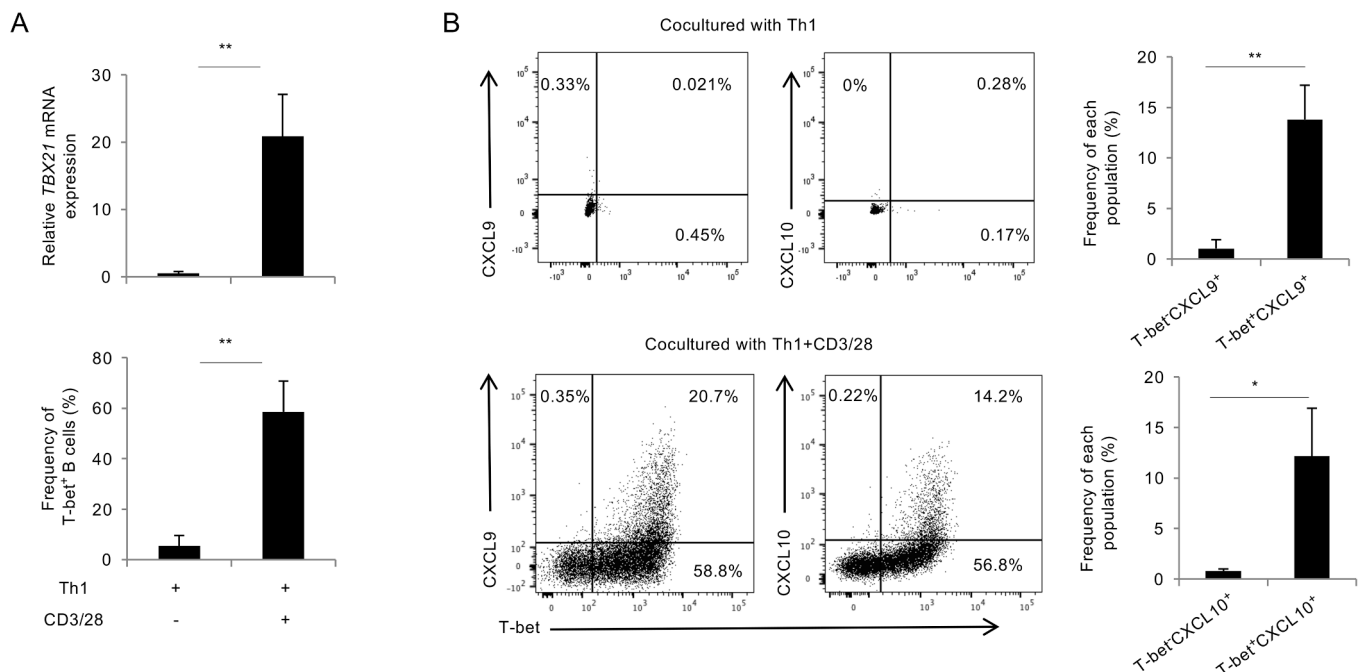


Fig. 2. CXCL9/10-producing B cells express T-bet in the presence of activated Th1 cells. (A) Whole B cells from HDs were cocultured with Th1 in the presence or absence of T cell activator. *TBX21* mRNA expression and intracellular staining of T-bet in B cells were analyzed for 24 h and 48 h, respectively ($n = 5$, $**P < 0.01$). (B) Whole B cells from HDs were cocultured with Th1 in the presence or absence of T cell activator for 48 h. Frequency of CXCL9, CXCL10 and T-bet in B cells was determined by flow cytometry. Right panels show the frequency of T-bet⁺CXCL9⁺ and T-bet⁺CXCL10⁺ B cells in the presence of T cell activator ($n = 4$, $*P < 0.05$, $**P < 0.01$).

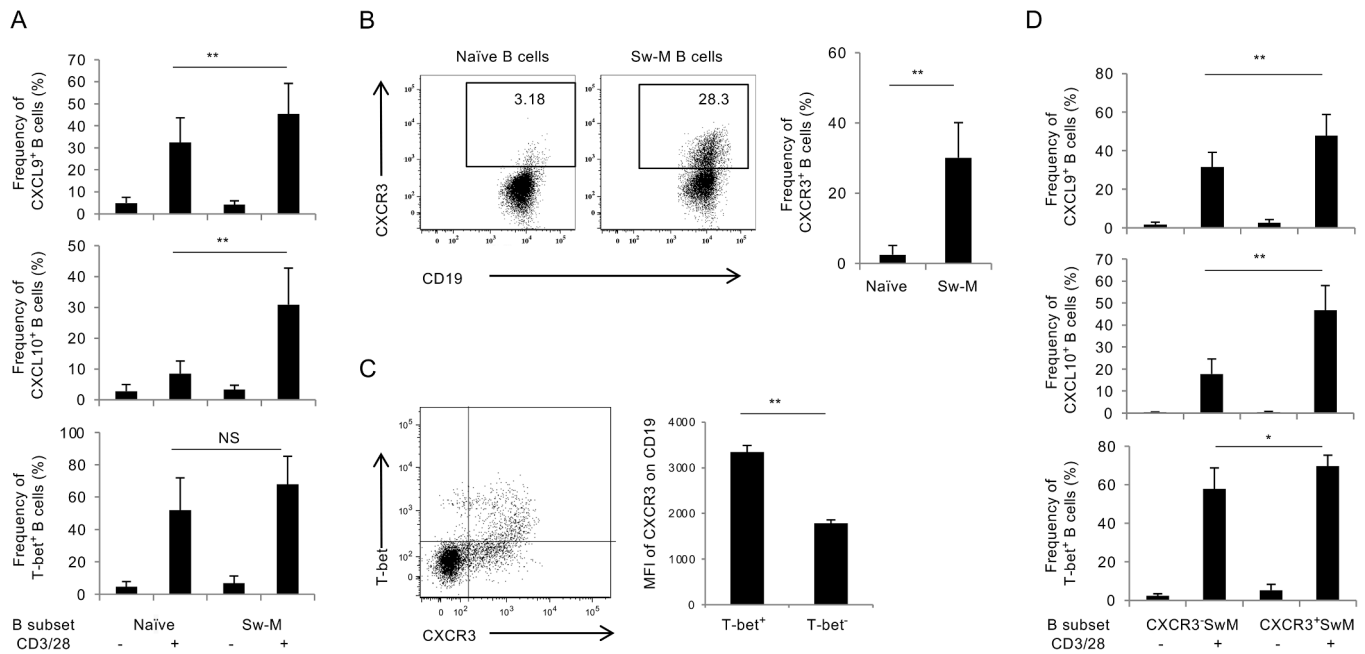


Fig. 3. CXCR3⁺ switched memory B cells have high potential to give rise to T-bet⁺CXCL9/10-producing cells. (A) Purified naïve (IgD⁺CD27⁻) and switched memory (Sw-m, IgD⁺CD27⁺) B cell subsets from HDs were sorted by flow cytometry and cocultured with Th1 in the presence or absence of T cell activator for 48 h. Frequency of CXCL9, CXCL10, and T-bet in B cells was detected by flow cytometry (n = 5, **P < 0.01). (B) Analysis of CXCR3 in B cell subsets from HDs by flow cytometry (n = 5, **P < 0.01). (C) Mean fluorescence intensity (MFI) of CXCR3 in T-bet⁺ B cells vs T-bet⁻ B cells (n = 5, **P < 0.01). (D) CXCR3⁺ or CXCR3⁻ Sw-m B cells from HDs were cocultured with Th1 in the presence or absence of T cell activator for 48 h. Frequency of CXCL9, CXCL10, and T-bet in B cells was determined by flow cytometry (n = 5, *P < 0.05, ** P < 0.01).

inactive RA (PBiRA) and active RA (PBAra). As shown in Fig. 4A, levels of transcripts of *CXCL9/10* and *TBX21* in these cells were undetected and only marginal, respectively. Given that SFRA enriches IFN- γ -producing T-cells [8] we next determined mRNA expression of these genes in B cells from SFRA. Consistent with our hypothesis, B cells from SFRA significantly expressed *CXCL9/10* and *TBX21* mRNA (Fig. 4A). We also compared the expression levels of *CXCL9/10* mRNA in CD4⁺ T cells, CD8⁺ T cells and B cells in SFRA and found that B cells remarkably expressed *CXCL9/10* mRNA (Fig. 4B). To obtain further evidence for our hypothesis, we determined a relationship between T-bet⁺ B cells and Th1 cells in SFRA. Indeed, the frequency of T-bet⁺ B cells positively correlated with that of activated Th1 and whole Th1 but not Th17 in SFRA (Fig. 4C–E). T-bet⁺ B cells are often defined as CD11c⁺CD21⁻CD19⁺ cells in humans [17,37]. As shown in Fig. 4F, CD11c⁺CD21⁻ B cells were slightly but significantly detected in B cells from PBAra. Notably, CD11c⁺CD21⁻ B cells were remarkably abundant in SFRA. These findings suggest that B cells in SFRA exhibit spontaneous expression of CXCL9/10 and T-bet probably via stimulation by activated Th1 cells in vivo.

3.5. Anti-IFN- γ antibody and JAK inhibitors abrogate the generation of CXCL9/10-producing T-bet⁺ B cells

To test whether IFN- γ produced by Th1 cells plays a critical role in CXCL9/10 production in B cells, Th1 cells were activated first and cocultured with B cells in the presence or absence of Th1 cell supernatant. CXCL9/10 expression was significantly enhanced with Th1 cell supernatant, and this enhancement was almost completely abrogated by anti-IFN- γ antibody (Fig. 5A). JAK inhibitors are currently available and used for the treatment for RA in clinical practice [38]. Given that IFN- γ utilizes the JAK-STAT signaling pathway [39] we determined whether the generation of CXCL9/10-producing T-bet⁺ B cells in coculture with activated Th1 could be affected by the JAK inhibitors, tofacitinib and baricitinib, which effectively suppress activation of JAK1/3 and JAK1/2, respectively. Both inhibitors inhibited the frequency of CXCL9/10-

producing and T-bet⁺ B cells in a dose-dependent manner, although suppression appears to be more remarkable in baricitinib than in tofacitinib (Fig. 5B). Interestingly, the frequency of CD80⁺ B cells in coculture with activated Th1 was not altered in the presence of both inhibitors (Fig. 5B), suggesting a JAK-independent pathway involved in this process. These results suggest that IFN- γ produced by Th1 cells utilizes the JAK-STAT signaling pathway to induce CXCL9/10 production in B cells.

3.6. B cell derived CXCL9/10 can attract CD4⁺ T cells

The central function of CXCL9/10 is to induce the migration of CXCR3⁺ cells into inflammatory sites [40]. We thus determined whether B cell derived CXCL9/10 can attract CD4⁺ T cells that include CXCR3⁺ Th1 cells. To this end, in vitro chemotaxis assays were performed. CD4⁺ T cells were added to the upper chamber, and the supernatant of coculture of B cells and Th1 cells were placed in the lower chamber. As a result, the supernatant of coculture of B cells and stimulated Th1 cells significantly induced the migration of CD4⁺ T cells, which was then inhibited by anti-CXCR3 antibody (Fig. 6). These findings suggest that B cell derived CXCL9/10 play a critical role in the recruitment of CXCR3⁺ Th1 cells into inflammatory sites.

4. Discussion

In this study, we demonstrate that Th1 milieu induces CXCL9/10 expression at both mRNA and protein levels in human B cells. Notably, CXCL9/10-producing B cells expressed T-bet and were enriched in the fraction of CXCR3⁺ switched memory B cells. Besides, SFRA showed the coexistence of highly activated Th1 cells and CXCL9/10-producing T-bet⁺ B cells.

CXCL9/10 are both ligands for CXCR3 and thus efficiently recruit CXCR3⁺ subpopulations of T cells, B cells or other cells at inflammatory sites [22,41,42]. We found that B cell derived CXCL9/10 significantly facilitated the migration of CD4⁺ T cells that include CXCR3⁺ T cells

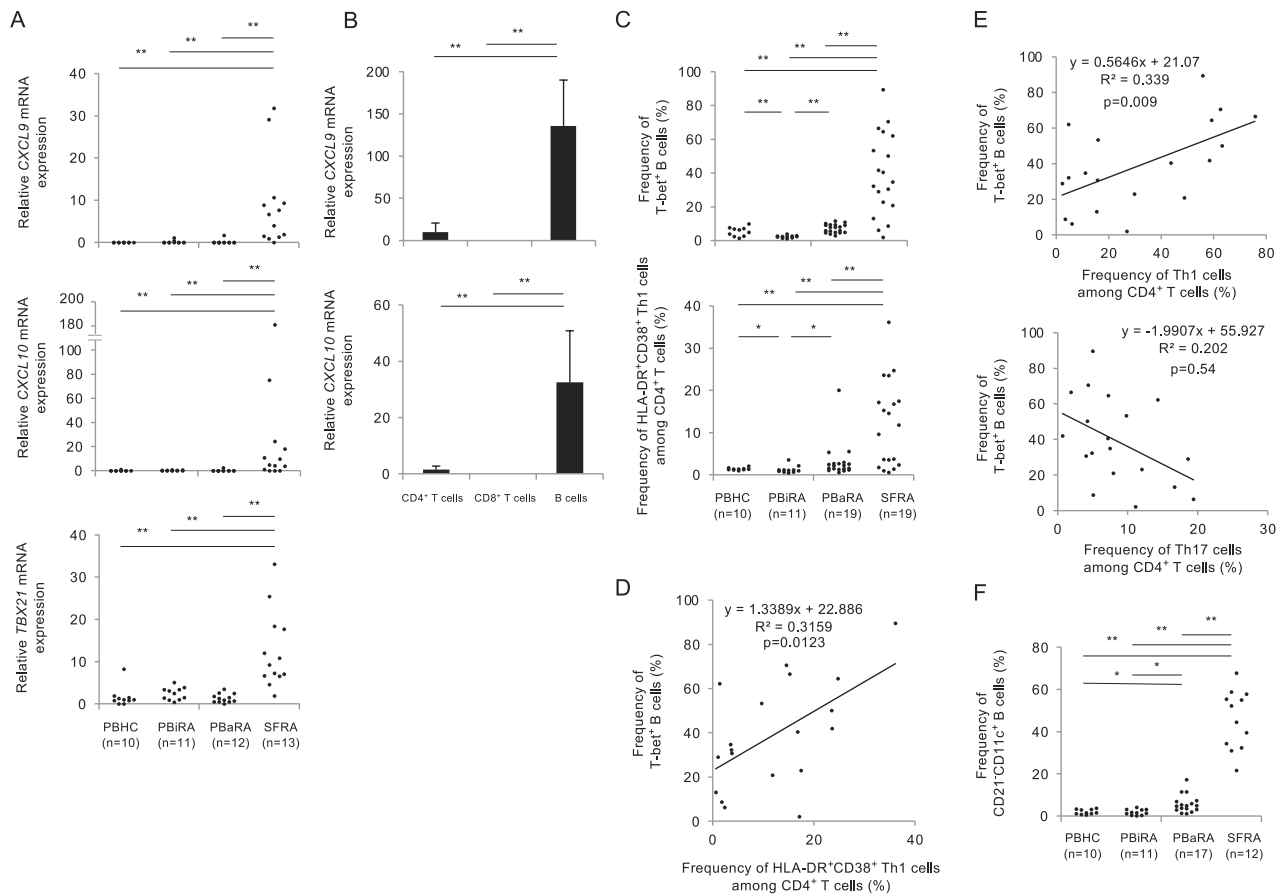


Fig. 4. B cells in SFRA exhibit spontaneous expression of CXCR9/10 and T-bet. (A) mRNA expression of *CXCL9*, *CXCL10*, and *TBX21* in whole B cells sorted from PBHC, PBiRA, PBaRA, and SFRA was analyzed (** $P < 0.01$). (B) mRNA expression of *CXCL9* and *CXCL10* in CD4⁺ T cells, CD8⁺ T cells and whole B cells in SFRA ($n = 6$, ** $P < 0.01$). (C) Frequency of T-bet⁺ B cells and activated Th1 (HLA-DR⁺CD38⁺) cells was analyzed in PBHC, PBiRA, PBaRA, and SFRA (* $P < 0.05$, ** $P < 0.01$). (D) Correlation between the frequency of T-bet⁺ B cells and activated Th1 cells in SFRA ($n = 19$). (E) Correlation between the frequency of T-bet⁺ B cells and whole Th1 (CD4⁺CXCR3⁺CCR6⁻) and Th17 (CD4⁺CXCR3⁻CCR6⁺) cells in SFRA ($n = 19$). (F) Frequency of CD21⁺CD11c⁺ B cells was analyzed in PBHC, PBiRA, PBaRA, and SFRA (* $P < 0.05$, ** $P < 0.01$).

(Fig. 6). CXCL9/10 would probably promote further recruitment of CXCR3⁺ B cells that have high potential for CXCL9/10 production (Fig. 3). Recruited CXCR3⁺ T cells, in turn, would activate CXCR3⁺ B cells to produce proinflammatory mediators including CXCL9/10 and express a variety of costimulatory molecules. T cell-B cell interaction, however, is not just a one-way street, and T cells can be fully activated by antigens presented by B cells along with costimulatory molecules and proinflammatory mediators, a positive feedback loop frequently involved in autoimmune diseases [43].

The function of CXCL9/10 is not solely to recruit CXCR3⁺ cells to inflammatory sites. These chemokines bind to G protein-coupled receptors to activate various downstream signaling pathways [44]. A previous study showed that CXCL10 induces osteoclast differentiation leading to bone destructions [45]. CXCL10 induces the expression of RANKL, a key cytokine for osteoclastogenesis, in CD4⁺ T cells [27,45]. This is also the case with B cells. We previously showed that B cells can induce RANKL expression upon BCR/CD40 stimulation with IFN- γ , and this induction is further augmented by CXCL10 [14]. These findings suggest that CXCL9/10 production by B cells also contributes to bone destruction in RA. Although anti-CXCL10 mAb showed clinical efficacy in RA patients who respond inadequately to MTX [31] an impact of this reagent on bone damage in vivo remains to be elucidated.

A majority of CXCL9/10-producing B cells express T-bet (Figs. 2–4). It is well established that T-bet is the master TF for Th1 differentiation [15]. Over the past decade, however, a novel B cell subset expressing T-bet has gained much attention due to its pivotal roles in infection,

autoimmunity and aging [16]. T-bet⁺ B cells are often defined as CD11c⁺CD21^{lo/-}CD19⁺ cells in humans [46]. CD11c⁺ B cells are abundantly found in autoimmune diseases such as RA, systemic lupus erythematosus and scleroderma [17,47]. T-bet plays a critical role in IgG2a class switching in mice models [48] however its role in human B cells remains somewhat elusive. At present this novel subset is proposed as not only a precursor of antibody-secreting cells but also a potent antigen-presenting cell [16]. Our current study showed remarkable increase of T-bet⁺ B cells and CD11c⁺CD21⁻ B cells in SFRA compared with PB from active RA (Fig. 4).

What, then, is a critical cytokine to generate T-bet⁺ B cells? IFN- γ is considered as a key cytokine for the generation of this subset [19]. Consistent with this idea, we found that Th1 cell-derived IFN- γ is indispensable for the generation of T-bet⁺ B cells. This is also supported by our data that anti-IFN- γ antibody and two JAK inhibitors capable of blocking IFN- γ signaling significantly abrogated its generation (Fig. 5). Recent findings, however, suggest that another cytokine IL-21 and TLR7/9 are also very crucial for the generation of T-bet⁺ B cells [49,50]. Whether these cells reported by others and T-bet⁺ B cells in our study have similar characteristics needs further investigation in the future. Our current model is depicted in Supplementary Fig. 2.

Our current study has several limitations. First, in addition to CXCL9 and CXCL10, CXCL11 is another ligand of CXCR3, however CXCL11 was not detected by microarray analysis. Regulation of CXCL11 expression in human B cells needs further interrogation. Second, recent evidence suggests that CXCR3⁺CCR6⁻ T cells used in this study include bona fide

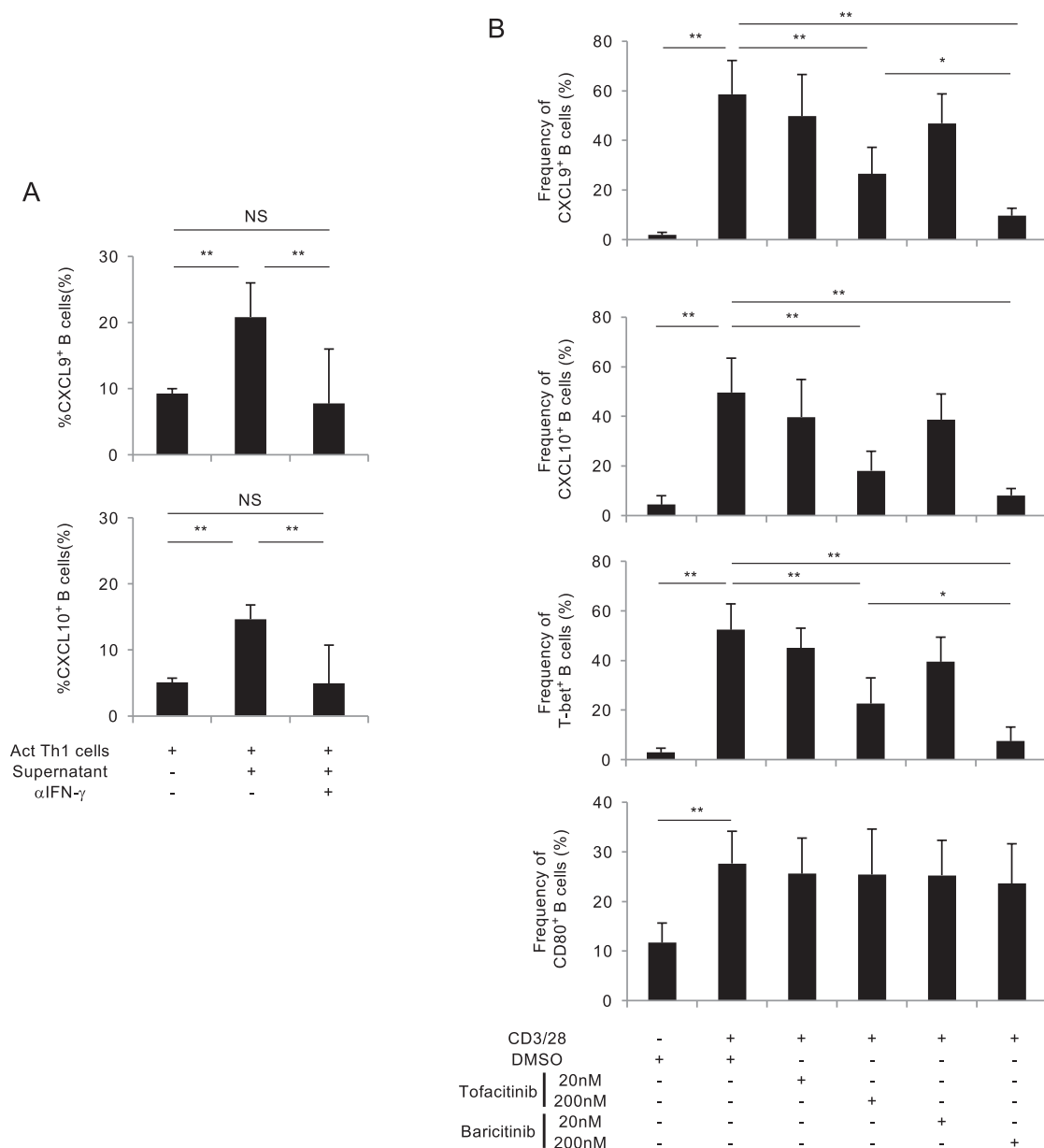


Fig. 5. Anti-IFN- γ antibody and JAK inhibitors abrogate the generation of CXCL9/10-producing T-bet $^{+}$ B cells. (A) After culture with T cell activator for 48 h, Th1 cells and supernatants were separated. B cells were cultured with stimulated Th1 cells with 2-fold diluted supernatants in the presence or absence of anti-IFN- γ antibody for another 48 h. The frequency of CXCL9 $^{+}$ and CXCL10 $^{+}$ B cells was determined by flow cytometry ($n = 4$, $**P < 0.01$). (B) Whole B cells from HDs were cocultured with Th1 in the presence of T cell activator with DMSO (0.05%) or the indicated doses of tofacitinib or baricitinib for 48 h. Frequency of CXCL9, CXCL10, T-bet, and CD80 in B cells was determined by flow cytometry ($n = 5$, $*P < 0.05$, $**P < 0.01$).

Th1 cells and CXCR3 $^{+}$ T follicular helper (Tfh) cells [51]. Our preliminary experiments, however, showed that CXCR3 $^{+}$ Tfh cells have a low potential to induce CXCL9/10 production in B cells as compared with bona fide Th1 cells (data not shown). Third, we showed CXCL9/10 and T-bet were both up-regulated in Th1 milieu, however we did not reveal the association of T-bet and CXCL9/10. Fourth, it is well known that synovial fibroblasts are the main producer of CXCL10 in RA, and thus the impact of B cell derived CXCL10 on RA pathogenesis needs to be carefully evaluated. Given that B cell depletion therapy exhibits a comparable efficacy toward RA as compared with other biologics, B cells would be able to regulate synovial inflammation by novel Ab-independent mechanisms.

In conclusion, we demonstrate that B cells give rise to a novel T-bet $^{+}$ B cell subset producing CXCL9/10 in the presence with INF- γ producing Th1 cells. Highly activated Th1 cells and CXCL9/10-producing T-bet $^{+}$ B

cells significantly coexist in SFRA. CXCL9/10 might contribute to RA progression in a direct and indirect manner, such as chemo-attraction of CXCR3 $^{+}$ T/B cells, T cell activation by antigen presentation, differentiation of B cells, and bone destruction. The novel CXCL9/10-producing T-bet $^{+}$ effector B cells might be an ideal pathogenic B cell target for RA therapy in the future.

CRediT authorship contribution statement

Tsuyoshi Nakayama: Methodology, Software, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Motoki Yoshimura:** Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Kazuhiko Higashioka:** Methodology, Validation, Formal analysis, Investigation, Writing - review & editing. **Kohta Miyawaki:**

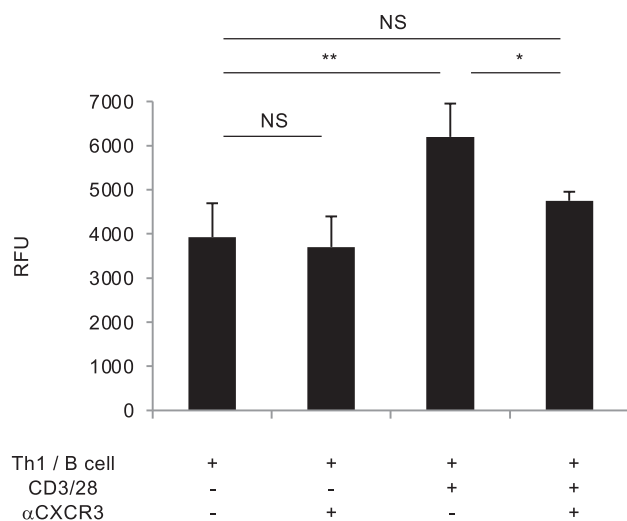


Fig. 6. B cell derived CXCL9/10 attract CD4⁺ T cells. Migration of CD4⁺ T cells in response to the supernatant of coculture of B cells and either unstimulated or stimulated Th1 cells in the presence or absence of anti-CXCR3 antibody was determined by in vitro chemotaxis assay. Four independent experiments were performed. *P < 0.05; RFU, relative fluorescence unit.

Methodology, Software, Validation, Formal analysis, Resources, Writing - original draft, Writing - review & editing. **Yuri Ota:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Masa-hiro Ayano:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Yasutaka Kimoto:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Hiroki Mitoma:** Methodology, Validation, Resources, Writing - original draft, Writing - review & editing. **Nobuyuki Ono:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Yojiro Arinobu:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Makoto Kikukawa:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Hisakata Yamada:** Validation, Resources, Writing - original draft, Writing - review & editing. **Koichi Akashi:** Methodology, Validation, Resources, Writing - original draft, Writing - review & editing. **Takahiko Horiuchi:** Methodology, Validation, Writing - original draft, Writing - review & editing. **Hiroaki Niino:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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