Clinical Immunology 200 (2019) 1–9

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Type I and II interferons commit to abnormal expression of chemokine receptor on B cells in patients with systemic lupus erythematosus

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ARTICLE INFO

Keywords:
Systemic lupus erythematosus
B cells
CXCR3
CXCR5
Type I interferon
Type II interferon

ABSTRACT

Memory B cells are increased in systemic lupus erythematosus (SLE) cases, but the qualitative abnormalities and induction mechanism of these cells are unclear. Here, we subclassified B cells by their chemokine receptor expression and investigated their induction mechanism. The peripheral blood of patients with SLE showed higher levels of $CXCR5^-$ and $CXCR3^+$ B cells. $CXCR5^-CXCR3^+$ B cell levels were elevated in patients with active SLE, which decreased with improving disease conditions. Interferon (IFN)-γ stimulation increased CXCR3 expression, whereas IFN-β stimulation reduced CXCR5 expression in B cells. Furthermore, $CXCR5^-CXCR3^+$ B cells were induced by a combination of IFN-β and IFN-γ stimulation. Renal tissue examination of patients with active lupus nephritis confirmed the presence of $CD19^+CXCR3^+$ B cells. Collectively, the results revealed qualitative abnormalities accompanying reduced CXCR5 expression via type I IFN and enhanced CXCR3 expression via type II IFN in SLE, suggesting their involvement in B cell infiltration into tissues and inflammatory pathogenesis.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that manifests as organ dysfunction due to inflammation mediated by various autoantibodies and immune complexes arising from compromised self-tolerance [1,2]. The etiology of SLE involves natural immunity involving Toll-like receptor and type I interferon (IFN) as well as acquired immunity involving the activation of T and B cells. Many disease-susceptibility genes associated with these factors have been identified [3,4]. Our group and others previously reported that in SLE pathogenesis, autoantigen binding affinity and autoreactive B cell differentiation occur during immunoglobulin gene rearrangement [5,6]. For the differentiation and maturation of B cells, antigen stimulation via B cell receptors (BCR) along with stimulation by IL-21, a cytokine released by T follicular helper T (Tfh) cells to induce B cell differentiation, results in the overproduction of autoantibodies [7]. Additionally, SLE B cells exhibit enhanced surface expression of co-stimulators, such as CD40 and CD80, which strongly potentiate antigen presentation and cytokine production. This engages in interactive activation with Tfh cells. Therefore, B cells play a central role as stimulators of antigen presentation and in the response to autoantibody production during SLE onset and pathogenesis and thus are an attractive therapeutic target in SLE [8–10].

Patients with SLE have increased levels of peripheral blood memory B cells and plasma cells, which is associated with disease activity [11,12], and the importance of B cell abnormalities, particularly memory B cells, in SLE has been duly recognized. In recent years, researchers have performed genome-wide analyses of B cells from patients with SLE to determine the mechanism of the B cell abnormalities [13,14]. However, many aspects of essential B cell abnormalities remain unclear. In studies of the properties of B cells, T cell biology may provide useful information given the various similarities between these cell types. In contrast to B cells, T cells have been subclassified based on the differentiation and function of effector cells, with each subset characterized by the master transcription factor and chemokine receptor expression patterns [15,16]. Chemokines, which are ligands of

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https://doi.org/10.1016/j.clim.2018.12.017
Received 2 August 2018; Received in revised form 16 November 2018; Accepted 18 December 2018
Available online 19 December 2018
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chemokine receptors, act as chemotactic factors in cells and are involved in the onset and prolongation of inflammatory disease conditions [17]. For example, in Th1 cells, the master transcription factor T-bet induces CXCR3 expression and CXCL 9–11 serve as ligands for the cell to infiltrate inflammation sites, where IFN-γ is produced to induce cellular immunity. In Tfh cells, Bcl-6 is the master transcription factor that induces CXCR5, thereby enabling localization to the germinal center where CXCL13 is abundant. Interleukin (IL)-21 production by Tfh cells induces the differentiation and maturation of B cells to facilitate antibody production [7]. Recently, the plasticity of differentiation of both cell types has received attention in the field of T cell research [18–20]. Tfh cells share the same differentiation pathway as Th1 cells and produce not only IL-21 but also IFN-γ [21]. A recent study using a lupus mouse model demonstrated that IFN-γ-mediated STAT1 activation in B cells induced autoantibody production and Tfh cell differentiation [22]. B cells, which share functions with T cells, are thought to act through a similar mechanism, but their function in human SLE remains unknown.

In the present study, we examined the functional abnormalities of B cells that play an important role in the pathology of SLE. We analyzed B cell phenotypes by focusing on chemokine receptors used for subset classification of T cells to explore the mechanism by which qualitative abnormalities in B cells are induced upon cytokine stimulation, as well as the potential correlations with pathological conditions.

2. Material and methods

2.1. Patients

Patients with SLE who fulfilled the classification criteria for SLE (ACR 1997 [23,24] or SLICC 2012 [25]) and patients with rheumatoid arthritis (RA) who fulfilled the RA classification criteria (ACR/EULAR 2010 [26]) were enrolled in this study. We included age- and sex-matched healthy individuals as control subjects. The Human Ethics Review Committee for the university reviewed and approved our study, including the collection of peripheral blood samples from healthy adults and patients with SLE and RA. Each subject provided a signed participation consent form.

2.2. Treatment and clinical features

Background factors investigated were age, sex, duration of SLE, and treatment history (corticosteroids and immunosuppressants). The attending physician evaluated disease activity based on the SLEDAI [27] and BILAG scores [28]. Laboratory tests included those for lymphocyte cell count, anti-ds-DNA antibodies, and serum complement (CH50) levels.

2.3. Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) from 20 normal volunteers, 71 patients with SLE, and 31 patients with RA were immediately separated from the peripheral blood using lymphocyte separation medium (ICN, Cappel Pharmaceuticals, Aurora, OH, USA). After washing, PBMCs were incubated in 100 μL of FACS buffer (0.25% human globulin, 0.5% human albumin, and 0.1% NaN_3 in phosphate-buffered saline) in a FACS tube at 4 °C for 15 min. The cells were treated with fluorescein isothiocyanate-conjugated CD19 (BD Pharmingen, Franklin Lakes, NJ, USA), phycoerythrin-conjugated CXCR5 (R&D Systems, Minneapolis, MN, USA), PerCP-Cy5.5 conjugated CXCR3 (BD Pharmingen), allophycocyanin-conjugated CD27 (BioLegend, San Diego, CA, USA), allophycocyanin-H7-conjugated CD20 (BD Pharmingen), V450-conjugated CD3 (BD Pharmingen), and V500-conjugated IgD (BD Pharmingen) for 30 min at 4 °C. The cells were washed with phosphate-buffered saline and analyzed with a FACSVerse (BD Pharmingen) and FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Pan B cell and naïve B cell isolation from healthy donors

After withdrawing blood from healthy donors ($n = 3$), PBMCs were immediately separated using lymphocyte separation medium (ICN, Cappel Pharmaceuticals) and incubated with CD19 Dynabeads (Dynal, Oslo, Norway) as described previously [29]. Pan B cell purity was > 90% according to flow cytometric analysis. Naïve B cells were isolated from PBMCs using a MojoSort™ Human Naïve B Cell Isolation Kit (BioLegend) according to the manufacturer's instructions. Naïve B cell purity was > 90% according to flow cytometric analysis.

2.5. In vitro culture of B cells

Purified $CD19^+$ B cells or naïve B cells (2×10^5 cells/200 μL) were cultured either alone or with 1 μg/mL anti-BCR mAbs (anti-Igλ and anti-Igκ, BD Pharmingen), 2 μg/mL human sCD40L (PeproTech, Rocky Hill, NJ, USA), and 100 ng/mL IL-21 (Miltenyi Biotec, Bergisch Gladbach, Germany), 1, 10, or 100 U/mL IFN-β (Miltenyi Biotec), or 1, 10 or 100 ng/mL IFN-γ (R&D Systems) for 2 or 4 days at 37 °C.

2.6. Quantitative PCR

BCL-6 and XBP-1 gene expression levels were measured by quantitative reverse transcription-PCR (Applied Biosystems, Foster City, CA, USA). mRNA concentrations were normalized to 100 pg/reaction. All reactions were standardized to the expression of 18S ribosomal RNA. The relative quantity was calculated using the quantification comparative cycle threshold formula-referenced sample.

2.7. Immunofluorescence staining

The formalin-fixed paraffin embedded sections (3-μm-thick) of renal biopsy tissues (from 5 patients with lupus nephritis class III or IV) were dewaxed in xylene and rehydrated in a graded series of ethanol. Formalin-fixed paraffin embedded sections were treated with protease K (Dako, Copemhagen, Denmark) for 1 h for immunofluorescence analysis. Endogenous biotin was blocked using DAKO® Protein Block Serum-Free (Dako Corporation, Carpinteria, CA, USA). The sections were then incubated for 1 h at room temperature with rabbit anti-human CD19 antibody (Abcam, Cambridge, UK) and mouse anti-human CXCR3 antibody (Abcam), followed by incubation with the secondary antibodies Alexa Fluor 546 goat anti-mouse IgG (H + L) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) four 30 min. The nuclei were counterstained with DAPI.

2.8. Statistical analysis

Data are expressed as percentages of patients or means as appropriate. Statistical significance was tested using a Steel-Dwass test as shown in Figs. 1, 2, and 3A and Wilcoxon rank sum test as shown in Fig. 3B and Supplemental Figs. 1 and 2. The baseline and post-treatment values within each sample were compared using the Wilcoxon signed-rank test (Fig. 3C–E). Statistical significance was determined using a Student's *t*-test as shown in Fig. 4 and Supplemental Fig. 3. A *p*-value < .05 was considered to indicate statistically significant differences between groups. All statistical analyses were performed with JMP statistical software version 9.0.2 (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Patients' background

Representative baseline characteristics of the 71 patients with SLE are shown in Table 1. The study population consisted of 71 patients with SLE with a mean age of 42 years (range, 15–76 years), including 64 females (90.1%) with the disease for a mean duration of 93 months

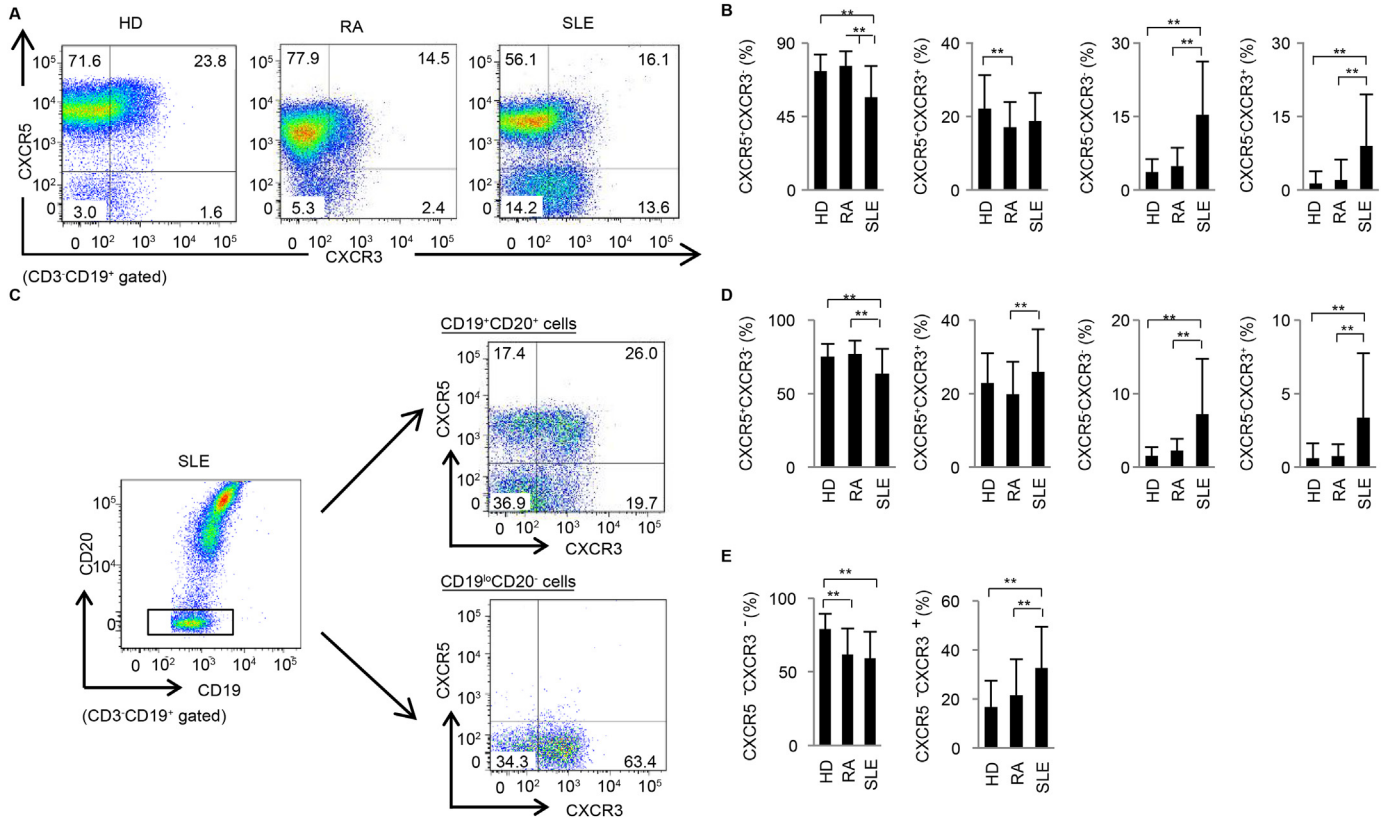


Fig. 1. CXCR3 and CXCR5 expression levels in peripheral blood B cells. (A) and (B); CXCR3 and CXCR5 expression levels were evaluated in $CD3^-CD19^+$ cells. (C) and (D); CXCR3 and CXCR5 expression levels were evaluated in $CD3^-CD19^+CD20^+$ B cells. (C) and (E); CXCR3 and CXCR5 expression levels were evaluated in $CD3^-CD19^{low}CD20^-$ cells. Healthy donor (HD); $n = 20$, RA; $n = 31$, SLE; $n = 71$. $^{**}p < .01$.

(range, 1–384 months). Twenty-five patients (35.2%) had new onset SLE and were treatment-naïve. Disease activity showed a wide distribution. An anti-double-stranded DNA antibody reacted positively in 34 patients (51.5%). The mean SLEDAI score was 9 points (range, 0–37 points) and the mean BILAG score was 10 points (range, 0–45 points, calculated by assigning 9 points for A, 3 points for B, and 1 point for C). Twenty patients (28.2%) had active lupus nephritis and 16 patients (22.5%) had active central nervous system lupus (Table 1). Twenty-five patients (35.2%) were treatment-naive, while 38 patients (53.5%) were given steroids at a mean dose of 7.6 ± 9.0 mg. Thirty-one patients had RA, with a mean age of 60 years (range, 33–86 years), including 24 females (77.4%). RA showed a mean duration of 69 months (range, 3–288 months). Twenty-three patients (74.2%) were being administered MTX, while 15 patients (48.4%) were bio-naïve. For disease activity, simplified disease activity index was 23.6 ± 11.3, clinical disease activity index was 21.7 ± 10.0, and disease activity score-28-erythrocyte sedimentation rate was 5.1 ± 1.1. The control subjects included 20 staff members of our hospital (age, 40 ± 11 years; 80% female). The healthy donors and patients with SLE were age-matched, but the mean age of patients with RA was higher than those of patients in the other two groups ($p < .01$). The sex was matched between the three groups.

3.2. $CXCR5^-$ and $CXCR3^+$ memory B cell levels were elevated in the peripheral blood of patients with SLE

To evaluate the expression level of chemokine receptors in peripheral blood B cells in patients with SLE, PBMCs were separated from the peripheral blood of healthy individuals, patients with RA, and patients with SLE. PBMCs were analyzed for B cell surface antigens, differentiation markers, and chemokine receptors. The $CD3^-CD19^+$ cell fraction from patients with SLE showed a lower proportion of $CXCR5^+CXCR3^-$ cells and higher proportion of $CXCR5^-CXCR3^-$ and $CXCR5^-CXCR3^+$ cells compared to that in healthy individuals and patients with RA ($p < .01$ for both, Fig. 1A, B). Next, the $CD3^-CD19^+$ cell fraction was gated for CD19 and CD20, as $CD19^+CD20^-$ and $CD19^+CD20^+$ B cells contaminated the $CD3^-CD19^+$ cells, and we wished to eliminate the effects of these cell populations. The $CD19^+CD20^+$ B cell fraction from patients with SLE also showed a lower proportion of the $CXCR5^+CXCR3^-$ population and higher proportion of the $CXCR5^-CXCR3^-$ and $CXCR5^-CXCR3^+$ populations compared to that in healthy individuals and patients with RA ($p < .01$ for both, Fig. 1C, D). The $CD19^{low}CD20^-$ cell fraction exhibited negligible CXCR5 expression in healthy individuals, patients with RA, and patients with SLE, and the proportion of the $CXCR5^-CXCR3^+$ population in patients with SLE was higher than that in healthy individuals and patients with RA ($p < .01$ for both, Fig. 1C, E). A similar trend was observed for the absolute number of B cells (Supplemental Fig. 1).

We next analyzed the stage of differentiation at which $CXCR5^-CXCR3^-$ B and $CXCR5^-CXCR3^+$ B cells had expanded. First, when the $CD19^+CD20^+$ B cell fraction was classified by B cell differentiation markers (IgD and CD27), the cell fraction from patients with SLE contained a lower proportion of IgD^+CD27^+ B cells compared to that in healthy individuals ($p < .01$) and a significantly higher proportion of IgD^-CD27^- B cells compared to that in healthy individuals or patients with RA ($p < .05$) (Fig. 2A, B). When chemokine receptor expression was analyzed at each differentiation stage, higher proportions of $CXCR5^-CXCR3^-$ and $CXCR5^-CXCR3^+$ populations were found at all differentiation stages ($^*p < .05$, $^{**}p < .01$, Fig. 2C and Supplemental Fig. 2).

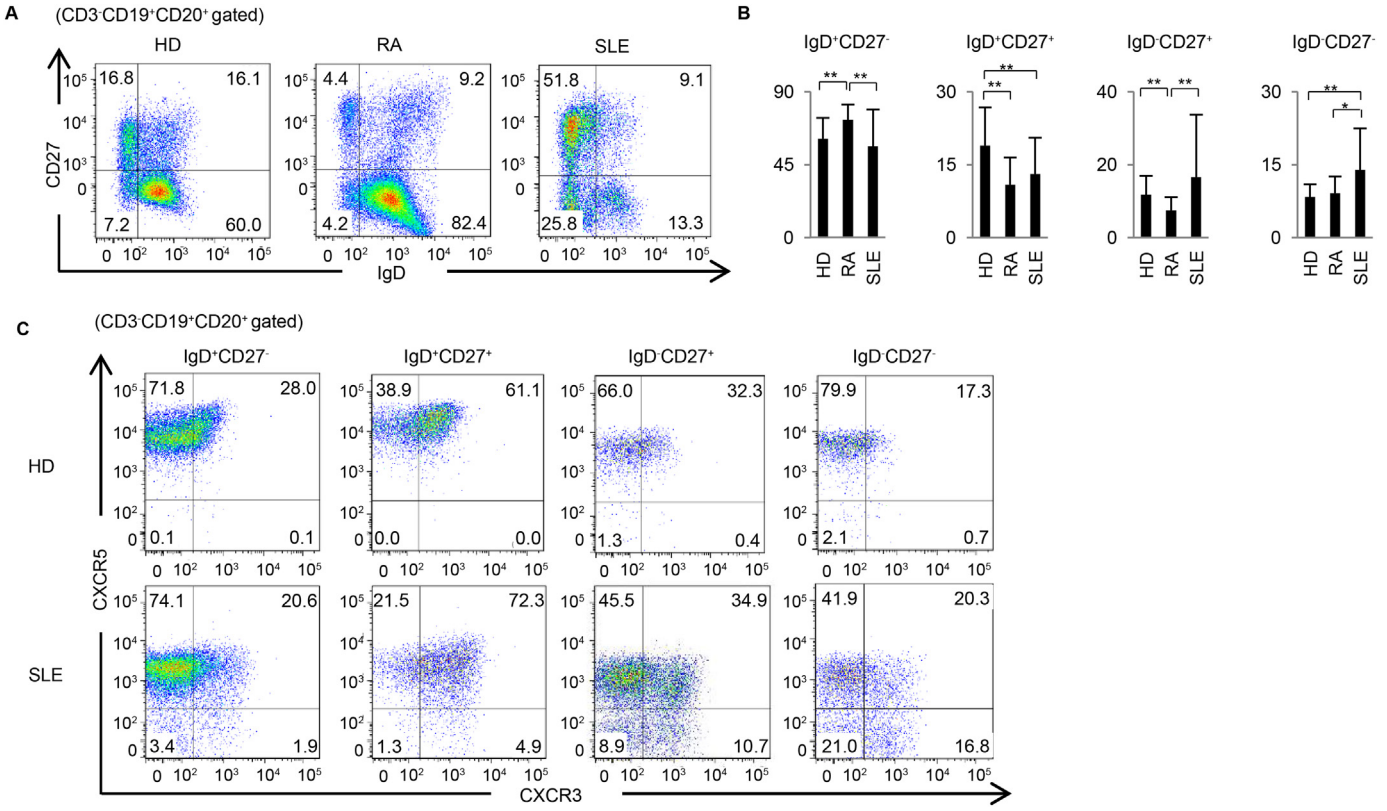


Fig. 2. CXCR3 and CXCR5 expression levels at each differentiation stage of B cells. (A) and (B); IgD and CD27 expression levels were evaluated in $CD3^-CD19^+CD20^+$ B cells. Healthy donor (HD); n = 20, RA; n = 31, SLE; n = 71. *$p < .05$, **$p < .01$. (C) CXCR3 and CXCR5 expression levels were evaluated at each differentiation stage of $CD3^-CD19^+CD20^+$ B cells in HD and patients with SLE.

3.3. $CXCR5^-CXCR3^+$ B cell levels were elevated in patients with active SLE, which decreased with improvement in disease condition

$CXCR5^-CXCR3^-$ and $CXCR5^-CXCR3^+$ B cells, which were found at increased levels in the peripheral blood of patients with SLE, were investigated for their potential correlation with clinical conditions. Analysis of the overall population of patients with SLE ($n = 71$) revealed no correlation between chemokine receptor-based B cell classification and disease activity or organ dysfunction (data not shown). However, subgroup analysis of treatment-naïve patients with SLE ($n = 25$) indicated a higher proportion of $CXCR5^-CXCR3^-$ and $CXCR5^-CXCR3^+$ cells in the $CD19^+CD20^+$ B cell fraction from patients with active SLE (SLEDAI ≥ 10, $n = 11$) compared to that in healthy individuals ($n = 20$, $p < .01$, Fig. 3A). A higher proportion of $CXCR5^-CXCR3^+$ cells was observed in the $CD19^+CD20^+$ B cell fraction from patients with active SLE (SLEDAI ≥ 10, n = 11) compared to those in inactive SLE (SLEDAI < 10, $n = 14$; $p < .05$, Fig. 3A). Evaluation of the actual cell numbers indicated a lower proportion of $CD19^+CD20^+CXCR5^+CXCR3^-$ B cells and higher proportion of $CD19^+CD20^+CXCR5^-CXCR3^+$ B cells in patients with active SLE ($p < .05$ for both, Fig. 3B). We also analyzed patients treated with a high-dose of steroids ($n = 9$) to detect changes in subsets before and after treatment. High-dose steroid treatment significantly improved the disease condition as assessed by SLEDAI and BILAG scores ($p < .05$ for both, Fig. 3C). The proportions of $CXCR5^-CXCR3^-$ and $CXCR5^-CXCR3^+$ cells in the $CD19^+CD20^+$ B cell fraction did not change before and after treatment (Fig. 3D), while the number of $CD19^+CD20^+CXCR5^-CXCR3^+$ B cells was significantly reduced ($p < .05$, Fig. 3E).

3.4. IFN-γ stimulation increased CXCR3 expression and IFN-β stimulation decreased CXCR5 expression in human naïve B cells

Based on the above results, we examined the mechanism by which $CXCR5^-$ and $CXCR3^+$ B cells are induced. Specifically, naïve B cells isolated from the peripheral blood of healthy individuals ($n = 3$) were stimulated with various cytokines (IL-21, IFN-γ, and IFN-β) in combination with BCR and CD40L, and chemokine receptor expression was evaluated after 4 days of incubation at 37 °C. Stimulation with IFN-γ in combination with BCR and CD40L significantly increased the proportion of $CXCR3^+$ B cells compared to BCR and CD40L stimulation alone ($p < .05$, Fig. 4A, B). Furthermore, induction of $CXCR3^+$ B cells by stimulation with IFN-γ was concentration-dependent. In contrast, stimulation with IFN-β in combination with BCR and CD40L reduced the expression of CXCR5 and significantly increased the proportion of $CXCR5^-$ B cells compared to that in non-stimulated cells ($p < .05$, Fig. 4A, C). We confirmed that the largest increase in the proportion of $CXCR5^-$ B cells occurred at the highest concentration of IFN-β (100 U/mL), although we could not confirm the concentration-dependence of IFN-β stimulation in inducing $CXCR5^-$ B cells. A similar trend was observed for IFN-γ stimulation, but the difference was not significant. The largest increases in the proportions of $CXCR5^-$ B cells and $CXCR3^+$ B cells were induced by stimulation with 100 U/mL IFN-β and 100 ng/mL IFN-γ in combination with BCR and CD40L. Furthermore, $CXCR5^-CXCR3^+$ B cells were induced by stimulation with 100 U/mL IFN-β and 100 ng/mL IFN-γ in combination with BCR and CD40L ($p < .05$, Fig. 4A, D).

3.5. IFN-γ stimulation induced T-bet expression in human pan B cells

Pan B cells isolated from the peripheral blood of healthy individuals were stimulated with various cytokines (IL-21, IFN-γ, and IFN-β) in

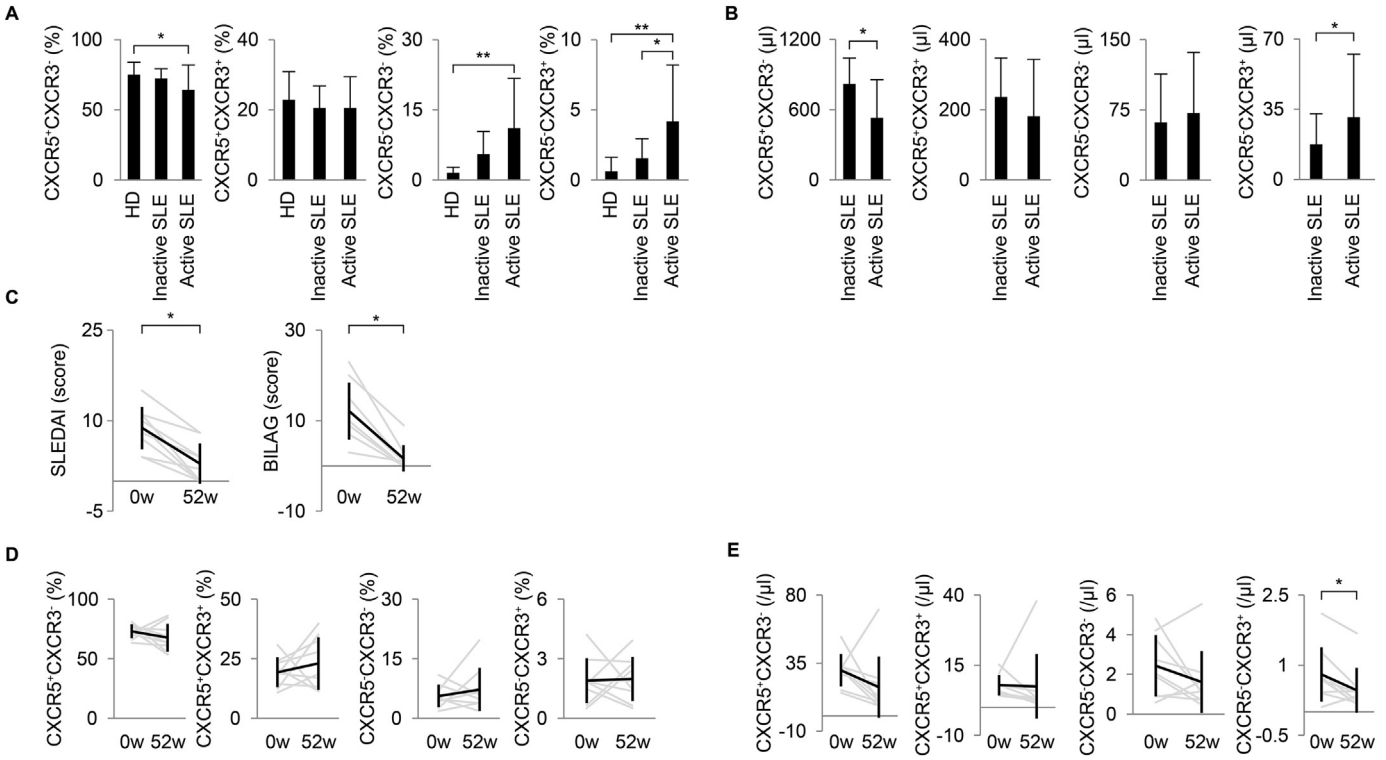
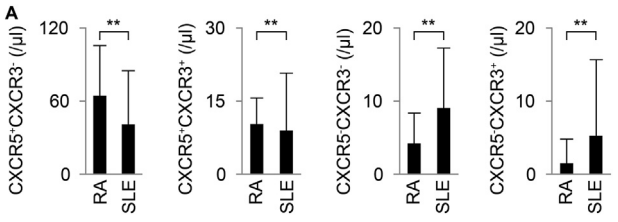


Fig. 3. Correlations between CXCR3, CXCR5 expression levels, and clinical conditions in patients with SLE. (A); CXCR3 and CXCR5 expression levels were evaluated in HD (n = 20) and treatment-naïve patients with SLE (n = 25). Proportion of each subset in the $CD3^-CD19^+CD20^+$ B cell fraction. (B) CXCR3 and CXCR5 expression levels were evaluated in treatment-naïve patients with SLE (n = 25). The absolute number of cells in each subset. (A) and (B); Active SLE was defined as SLEDAI ≥10. Eleven patients had active SLE and 14 patients had inactive SLE. (C) The change in the disease condition before and after treatment was evaluated in patients treated with a high-dose of steroids (n = 9). (D) and (E); The change in the proportion (D) and in the actual cell number (E) of each subset in B cells before and after treatment (at 52 weeks) was evaluated in patients treated with a high-dose of steroids (n = 9). *p < .05.



Supplemental Fig. 1. Absolute number of cells of $CXCR5^{-/+}CXCR3^{-/+}$ population. (A) Absolute number of cells of each subset in $CD3^-CD19^+$ cells. (B) Absolute number of cells of each subset in $CD3^-CD19^+CD20^+$ B cells. (C) Absolute number of cells of each subset in $CD3^-CD19^{low}CD20^-$ cells. **p < .01.

combination with BCR and CD40L, and the expression of the transcription factor T-bet was evaluated after 4 days of incubation at 37 °C. Stimulation with IFN-γ in combination with BCR and CD40L significantly promoted the expression of T-bet in $CD19^+CD20^+$ B cells compared to that in unstimulated controls (p < .01, Supplemental Fig. 3A). Additionally, changes in T-bet expression were evaluated by qPCR after 2 days of incubation under the same conditions, and the results showed the same trend (p < .01, Supplemental Fig. 3B). Furthermore, the correlation between T-bet and CXCR3 was evaluated by flow cytometry. In $CD19^+CD20^+$ B cells stimulated with IFN-γ in combination with BCR and CD40L, the T-bet-high population was also positive for CXCR3, indicating that both were induced in a similar manner (Supplemental Fig. 3C).

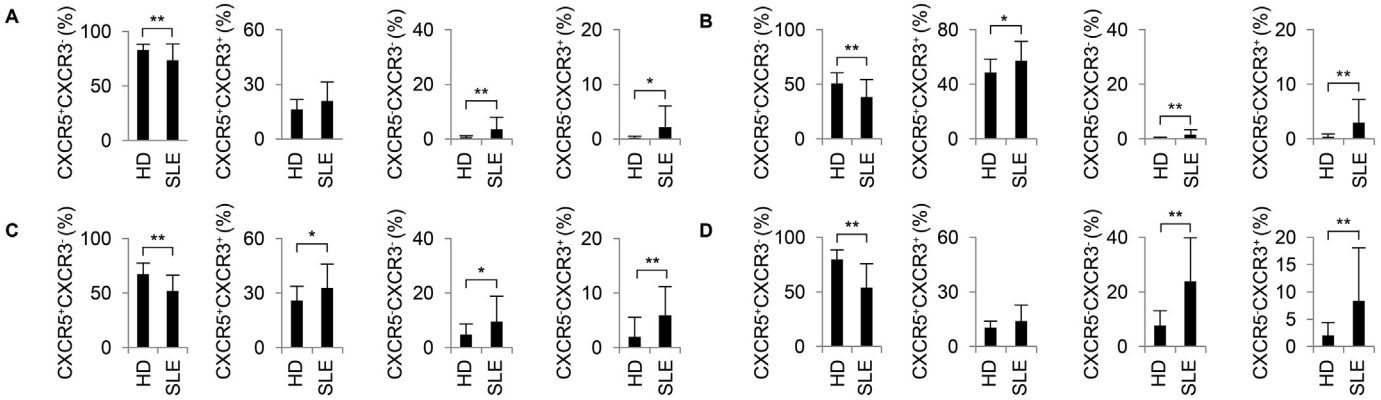
3.6. Renal tissues from patients with active lupus nephritis contained $CD19^+CXCR3^+$ B cells

To evaluate $CD19^+CXCR3^+$ B cell infiltration into tissues, renal biopsy tissues from patients with active lupus nephritis (n = 5) were examined by immunofluorescence staining. Renal biopsy tissues from patients with active lupus nephritis confirmed the infiltration of $CD19^+CXCR3^+$ B cells into the periglomerular interstitium (Fig. 5A–D). The specimen shown is from a 28-year-old man with high disease activity and an SLEDAI of 16 points. He was classified as IV-G (A/C) with lupus vasculopathy. Additionally, we quantified $CXCR3^+$ and $CXCR3^-$ B cells in renal biopsy tissues from patients with active lupus nephritis (lupus nephritis class III or IV). The ratio of $CXCR3^+$ B cells among total B cells was 30.8 ± 14.9%, while no infiltration of B cells was observed in the renal biopsy tissues from a normal control (a patient with microscopic hematuria) and a patient with lupus nephritis class II (data not shown).

4. Discussion

In this study, patients with SLE showed not only increased levels of effector B cells but also reduced CXCR5 expression mediated by type I IFN, as well as qualitative abnormalities accompanied by induced T-bet expression and enhanced CXCR3 expression mediated by type II IFN. Additionally, $CXCR5^-CXCR3^+$ B cells were induced by a combination of type I IFN and type II IFN stimulation, indicating their potential involvement in B cell infiltration into diseased tissues and inflammatory pathogenesis.

We examined the expression of chemokine receptors in B cells that are involved in SLE pathogenesis. The results revealed increased levels of $CXCR5^-CXCR3^-$ B cells and $CXCR5^-CXCR3^+$ B cells in the



Supplemental Fig. 2. Proportion of CXCR5$^{-/+}$CXCR3$^{-/+}$ population at each differentiation stage of B cells. (A)–(D) Proportion of each subset in IgD$^+$CD27$^-$ B cells (A), IgD$^+$CD27$^+$ B cells (B), IgD$^-$CD27$^+$ B cells (C), and IgD$^-$CD27$^-$ B cells (D) in HD and patients with SLE. (E) Proportion of CXCR5$^-$CXCR3$^{-/+}$ B cells at each differentiation stage in patients with SLE. *$p < .05$, **p < .01.

peripheral blood of patients with SLE. Our findings partially support those of a previous study showing increased CXCR3 expression in memory B cells and plasma cells of patients with SLE [30] as well as another study that reported reduced CXCR5 expression in peripheral blood B cells from patients with either RA or SLE [31]. In agreement with previous reports, patients with SLE contained fewer IgD$^+$CD27$^+$ B cells and more IgD$^-$CD27$^-$ B cells. However, the IgD$^-$CD27$^-$ B cell fraction, which was elevated in patients with SLE, accounted for greater proportions of CXCR5$^-$CXCR3$^-$ and CXCR5$^-$CXCR3$^+$ B cells ($p < .05$). Indeed, we confirmed that the active SLE subgroup of treatment-naïve patients with SLE contained a higher proportion of CXCR5$^-$CXCR3$^+$ B cells and that the number of CXCR5$^-$CXCR3$^+$ B cells significantly decreased as the disease condition improved. These findings further support the involvement of the same subset in the inflammatory condition of SLE. Analysis of the overall patient population revealed no significant difference based on the presence of active disease. This suggests that treatment-related modifications and/or heterogeneity occurred in SLE conditions.

Based on these results, memory B cells that expand in the peripheral blood of patients with SLE can be characterized by migration out of the germinal center because of reduced CXCR5 expression and migration into the inflammatory focus resulting from enhanced CXCR3 expression. We confirmed the presence of CD19$^+$CXCR3$^+$ B cells in the renal tissue of patients with active SLE. In support of this phenomenon, a study of SLE model mice (NZB/W) revealed higher CXCR3 expression in CD138^{high}MHC II$^+$ plasma cells along with infiltration of CD138$^+$CXCR3$^+$IgG$^+$ cells in the inflammatory site within the renal tissue [32].

Next, we investigated the mechanism by which these subsets were induced. A previous study showed that T-bet was expressed in the IgG2a$^+$ memory B cells of mice upon IFN-γ stimulation [33] and another study observed elevated T-bet expression in the peripheral B cells of patients with SLE compared to that in healthy individuals [34]. Thus, we investigated the changes in the expression of transcription factors

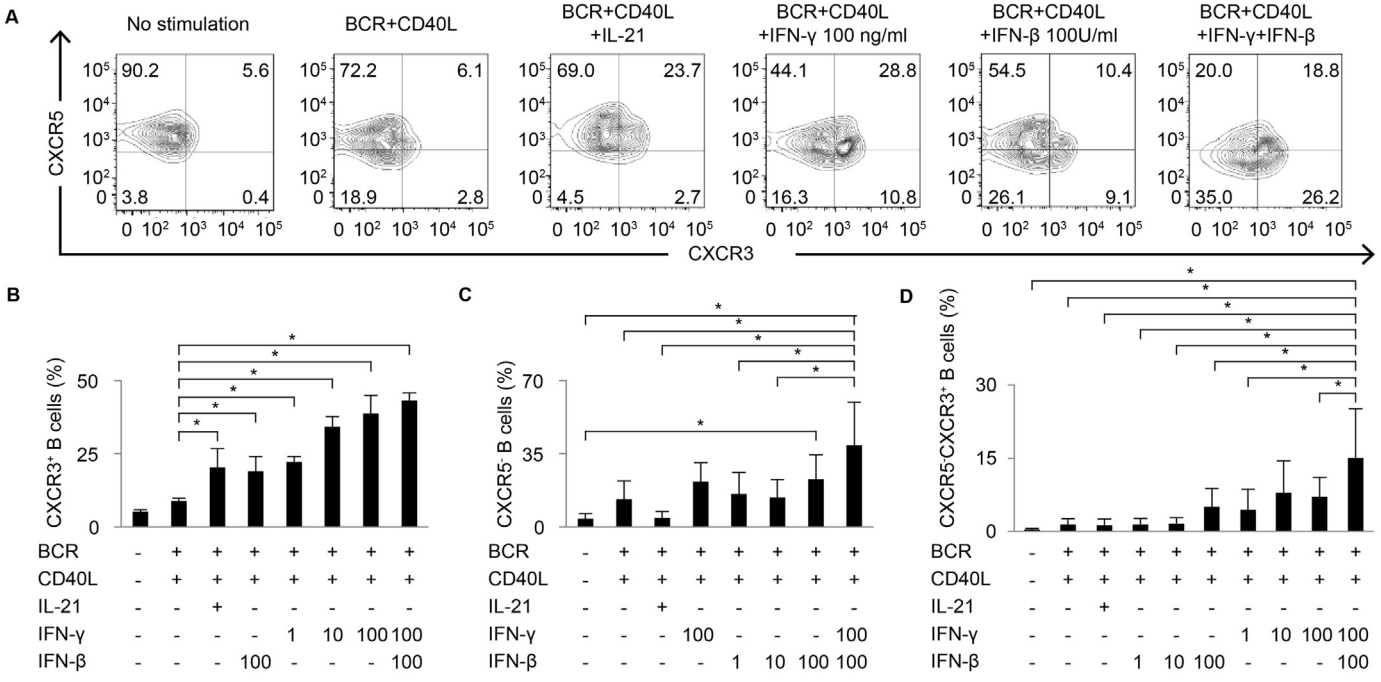
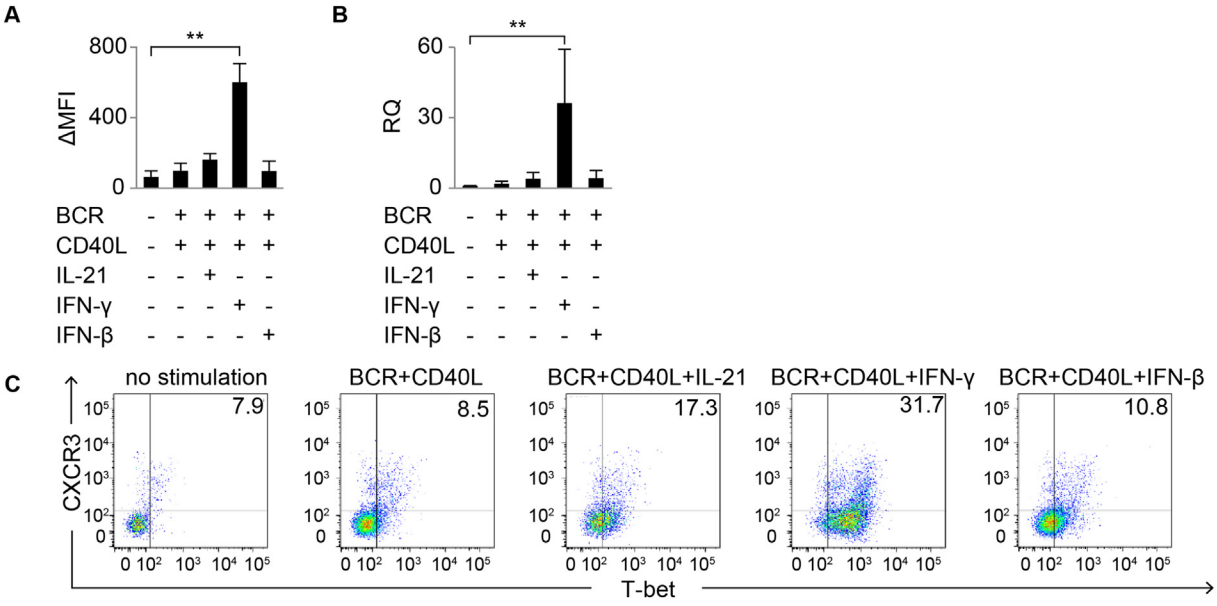


Fig. 4. Changes in CXCR3 and CXCR5 expression in human naïve B cells following stimulation with various cytokines. Naïve B cells isolated from the peripheral blood of healthy individuals (n = 3) were stimulated with various cytokines (IL-21, IFN-γ, and IFN-β) in combination with BCR and CD40L and incubated at 37 °C for 4 days. (A) In naïve B cells, changes in the expression of CXCR3 (X-axis) and CXCR5 (Y-axis) were evaluated by 8-color flow cytometry and are presented as contour plots. (B)–(D) CXCR3 and CXCR5 expression levels were evaluated in naïve B cells. *p < .05.



Supplemental Fig. 3. Changes in T-bet expression in human pan B cells following stimulation with various cytokines. Pan B cells isolated from the peripheral blood of healthy individuals (n = 3) were stimulated with various cytokines (IL-21, IFN-γ, and IFN-β) in combination with BCR and CD40L, and then incubated at 37 °C for 2 days. (A) In pan B cells, changes in the expression of T-bet were evaluated by ΔMFI using 8-color flow cytometry. (B) In pan B cells, changes in the expression of T-bet were evaluated by relative quantification using qPCR. (C) In $CD19^+CD20^+$ B cells, the correlation of T-bet (X-axis) and CXCR3 (Y-axis) was evaluated by 8-color flow cytometry and presented as dot plots.

Table 1
Patients' Background.

	SLE	RA	HD
	(n = 71)	(n = 31)	(n = 20)
Age (years), mean (range)	42 (15–76)	60 (33–86)	40 (27–60)
Sex (female), n (%)	64 (90.1)	24 (77.4)	16 (80.0)
Disease duration (months), mean (range)	93 (1–384)	69 (3–288)	
Naïve to treatment, n (%)	25 (35.2)	2 (6.5)	
Prednisolone use at baseline, n (%)	38 (53.5)		
Dose of prednisolone (mg/day), mean ± SD	7.6 ± 9.0		
Immunosuppressant use at baseline			
Azathioprine, n (%)	10 (14.1)		
Tacrolimus, n (%)	7 (9.9)		
Methotrexate, n (%)	6 (8.5)		
Cyclosporine, n (%)	2 (2.8)		
Mizoribine, n (%)	2 (2.8)		
Combination therapy at baseline, n (%)	23 (32.4)		
Lymphocyte cell count (cells/μl), mean ± SD	993 ± 604		
Anti-ds-DNA antibodies (positivity), n/N (%)	34/66 (51.5)		
Hypocomplementemia, n/N (%)	22/65 (33.8)		
SLEDAI score, mean (range)	9 (0–37)		
BILAG score, mean (range)	10 (0–45)		
one or more category A and/or two or more category B, n (%)	38 (53.5)		
Active lupus nephritis, n (%)	20 (28.2)		
Active NPSLE, n (%)	16 (22.5)		

SLEDAI; systemic lupus erythematosus disease activity index, BILAG; British Isles Lupus Assessment Group Index.

and chemokine receptors in B cells with various stimuli. In the present study, we evaluated the stimulatory effect of various cytokines in addition to BCR stimulation and co-stimulation (CD40L stimulation), which are essential for B cell activation. This is because cytokines, including interferons, are deeply involved in the pathological conditions of SLE. IL-21 is produced by Tfh cells, which play an important role in the T cell-B cell interaction. IL-21 leads to the induction of the class-switch of B cells. Additionally, 40–50% of patients with SLE showed elevated levels of IFN-α in their serum and at least 90% of patients with SLE had higher levels of type I IFN-related genes. Furthermore, some studies showed that patients with SLE had a higher level of IFN-γ in the serum compared to their healthy counterparts and that patients with lupus nephritis had elevated levels of IFN-γ in their serum compared to patients with SLE without lupus nephritis [35,36]. In another study, IFN-γ was detected in renal tissues from patients with lupus nephritis [37]. Finally, a study demonstrated that T cells from the peripheral blood of patients with SLE produced a higher level of IFN-γ upon CD3/28 stimulation and consequently produced a significantly higher level of BLyS compared to T cells from healthy individuals [38]. In the present study, IFN-γ stimulation in addition to BCR/CD40L stimulation promoted CXCR3 and T-bet expression in B cells. Additional data demonstrating that CXCR3 and T-bet are expressed in a correlated manner in B cells support the potential involvement of T-bet as a transcription factor for CXCR3, as observed in T cells. As described above, IFN-γ appears to play a key role in the pathogenic condition of SLE, and our study revealed the importance of IFN-γ in B cell function. However, we confirmed that CXCR5 expression was reduced following stimulation with IFN-β, a type I IFN. We also investigated the expression of Bcl-6, a primary transcription factor in Tfh cells, and confirmed its expression under all examined culture conditions without detectable changes upon stimulation with BCR/CD40L and IFN-β. This is in contrast to the reduced CXCR5 expression observed under the same stimulatory conditions (data not shown). Since we examined the expression of T-bet and Bcl-6 but no other transcription factor, further studies are needed to determine the involvement of other transcription factors.

5. Conclusion

In summary, patients with SLE showed not only increased levels of effector B cells but also reduced CXCR5 expression mediated by type I IFN, as well as qualitative abnormalities accompanied by induction of T-bet expression and enhancement of CXCR3 expression mediated by type II IFN, indicating their potential involvement in B cell infiltration into diseased tissues and inflammatory pathogenesis. Clinical trials

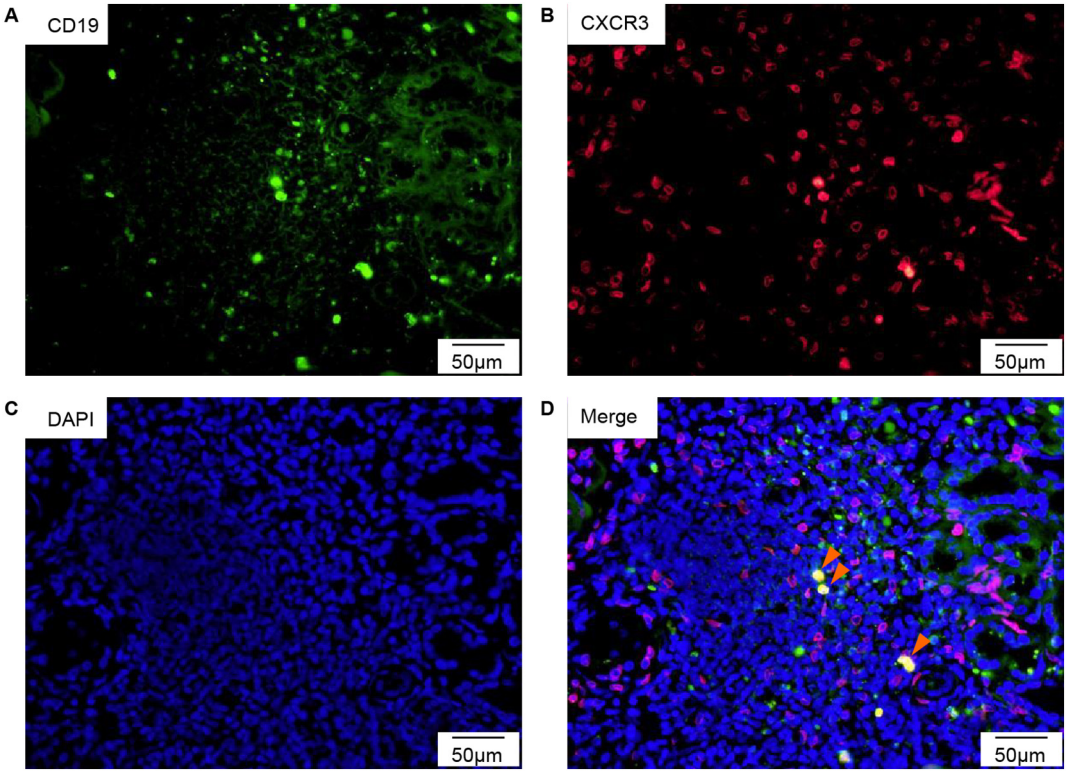


Fig. 5. Renal tissues from patients with active lupus nephritis. Renal biopsy tissues from patients with lupus nephritis were stained for CD19 and CXCR3. (A) CD19 (green). (B) CXCR3 (red). (C) DAPI (blue). (D) merge (yellow). This case is a representative specimen of the five cases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

have demonstrated the efficacy of anti-IFNAR antibodies in patients with SLE, and studies using anti-IFN-γ antibodies are currently underway [39–42]. The results of the present study provide insight into the importance of IFN in SLE conditions and suggest a strategy for improving pathological conditions by using IFN-induced transcription factors, such as T-bet, and chemokine receptors, such as CXCR3.

The following are the supplementary data related to this article.

Declaration of conflicting interests.

S. Nakayamada has received speaking fees from Bristol-Myers, UCB, Astellas, Abbvie, Eisai, Pfizer, and Takeda and research grants from Mitsubishi-Tanabe, Novartis, and MSD. K. Nakano has received speaking fees from UCB, Astellas, and Mitsubishi-Tanabe and research grants from Mitsubishi-Tanabe and Eisai. Y. Tanaka has received speaking fees and/or honoraria from Daiichi-Sankyo, Astellas, Pfizer, Mitsubishi-Tanabe, Bristol-Myers, Chugai, YL Biologics, Eli Lilly, Sanofi, Janssen, and UCB and has received research grants from Mitsubishi-Tanabe, Takeda, Bristol-Myers, Chugai, Astellas, Abbvie, MSD, Daiichi-Sankyo, Pfizer, Kyowa-Kirin, Eisai, and Ono.

Funding

This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan; Ministry of Education, Culture, Sports, Science, and Technology of Japan; Japan Agency for Medical Research and Development [grant number 17 K16219] and University of Occupational and Environmental Health, Japan through the UOEH Grant for Advanced Research.

Acknowledgements

The authors thank Ms. N. Sakaguchi for providing excellent technical assistance.

References

[1] G.C. Tsokos, et al., Systemic lupus erythematosus, N. Engl. J. Med. 365 (22) (2011) 2110–2121.
[2] G.C. Tsokos, et al., New insights into the immunopathogenesis of systemic lupus erythematosus, Nat. Rev. Rheumatol. 12 (12) (2016) 716–730.
[3] Y. Cui, et al., Genetic susceptibility to SLE: recent progress from GWAS, J. Autoimmun. 41 (2013) 25–33.
[4] I.T. Harley, et al., Genetic susceptibility to Lupus: New Insights from fine mapping and genome-wide association studies, Nat Rev Genet. 10 (5) (2009) 285–290.
[5] R. Übelhart, et al., Autoreactivity and the positive selection of B cells, Eur. J. Immunol. 45 (11) (2015) 2971–2977.
[6] S.P. Wang, et al., Amplification of IL-21 signaling pathway through Bruton's tyrosine kinase in human B cell activation, Rheumatology (Oxford) 54 (2015) 1488–1497.
[7] N. Gensous, et al., T follicular helper cells, interleukin-21 and systemic lupus erythematosus, Rheumatology (Oxford) 56 (4) (2017) 516–523.
[8] Y. Tanaka, Treatment of inflammatory immunologic disease 4. B cell targeting therapy using the anti-CD20 antibody rituximab in inflammatory autoimmune diseases, Intern. Med. 46 (16) (2007) 1313–1315.
[9] S. Iwata, Y. Tanaka, B cell subsets, signaling and their roles in secretion of autoantibodies, Lupus 25 (2016) 850–856.
[10] S. Nakayamada, Y. Tanaka, Relevance of lymphocyte subsets to B cell-targeted therapy in systemic lupus erythematosus, Int. J. Rheum. Dis. 18 (2015) 208–218.
[11] A.M. Jacobi, et al., Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95, Arthritis Rheum. 58 (2008) 1762–1773.
[12] A.M. Jacobi, et al., Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus, Arthritis Rheum. 48 (2003) 1332–1342.
[13] I. Sanz, et al., B cells as therapeutic targets in SLE, Nat. Rev. Rheumatol. 6 (2010) 326–337.
[14] A.M. Becker, et al., SLE peripheral blood B cell, T cell and myeloid cell transcriptomes display unique profiles and each subset contributes to the interferon signature, PLoS One 8 (2013) e67003.
[15] J.J. O'Shea, et al., Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells, Science 327 (5969) (2010) 1098–1102.
[16] J. Zhu, et al., Differentiation of effector CD4 T cell populations, Annu. Rev. Immunol. 28 (2010) 445–489.
[17] Y. Tanaka, et al., T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-Iβ, Nature 361 (1993) 79–82.
[18] J.A. Bluestone, et al., The functional plasticity of T cell subsets, Nat Rev Immunol. 9 (2009) 811–816.

[19] S. Nakayamada, et al., Helper T cell diversity and plasticity, Curr. Opin. Immunol. 24 (2012) 297–302.
[20] J.L. Cannons, et al., T follicular helper cell diversity and plasticity, Trends Immunol. 34 (2013) 200–207.
[21] S. Nakayamada, et al., Early Th1 cell differentiation is marked by a Tfh cell-like transition, Immunity 35 (2011) 919–931.
[22] P.P. Domeier, et al., IFN-γ receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity, J. Exp. Med. 213 (2016) 715–732.
[23] E. M. Tan, et al., The 1982 revised criteria for the classification of systemic lupus erythematosus, Arthritis Rheum. 25 (1982) 1271–1277.
[24] M.C. Hochberg, Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus, Arthritis Rheum. 40 (1997) 1725.
[25] M. Petri, et al., Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus, Arthritis Rheum. 64 (2012) 2677–2686.
[26] D. Aletaha, et al., Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League against Rheumatism collaborative initiative, Arthritis Rheum. 62 (2010) (2010) 2569–2581.
[27] C. Bombardier, et al., Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE, Arthritis Rheum. 35 (6) (1992) 630–640.
[28] D.P. Symmons, et al., Development and assessment of a computerized index of clinical disease activity in systemic lupus erythematosus. Members of the British Isles Lupus Assessment Group (BILAG), Q. J. Med. 69 (259) (1998) 927–937.
[29] A.M. Rasmussen, et al., A new method for detachment of Dynabeads from positively selected B lymphocytes, J. Immunol. Methods 146 (1992) 195–202.
[30] M.W. Nicholas, et al., A novel subset of memory B cells is enriched in autoreactivity and correlates with adverse outcomes in SLE, Clin. Immunol. 126 (2008) 189–201.
[31] M. Henneken, et al., Differential expression of chemokine receptors on peripheral blood B cells from patients with rheumatoid arthritis and systemic lupus erythematosus, Arthritis Res Ther. 7 (2005) R1001–R1013.
[32] S. Lacotte, et al., Early differentiated CD138(high) MHCII$^+$ IgG$^+$ plasma cells express CXCR3 and localize into inflamed kidneys of lupus mice, PLoS One 8 (2013) e58140.
[33] N.S. Wang, et al., Differential Transcriptional programming of Class-specific B Cell memory by T-bet and RORα, Nat. Immunol. 13 (6) (2012) 604–611.
[34] O. Wu, et al., The expressions of Toll-like receptor 9 and T-bet in circulating B and T cells in newly diagnosed, untreated systemic lupus erythematosus and correlations with disease activity and laboratory data in a Chinese population, Immunobiology 214 (5) (2009) 392–402.
[35] A. Zickert, et al., IL-17 and IL-23 in lupus nephritis - association to histopathology and response to treatment, BMC Immunol. 16 (2015) 7, https://doi.org/10.1186/s12865-015-0070-7.
[36] N. Calvani, et al., Up-regulation of IL-18 and predominance of a Th1 immune response is a hallmark of lupus nephritis, Clin. Exp. Immunol. 138 (1) (2004) 171–178.
[37] S. Wen, et al., IFN-γ, CXCL16, uPAR: potential biomarkers for systemic lupus erythematosus, Clin. Exp. Rheumatol. 36 (1) (2018) 36–43.
[38] M. Harigai, et al., Excessive production of IFN-gamma in patients with systemic lupus erythematosus and its contribution to induction of B lymphocyte stimulator/B cell-activating factor/TNF ligand superfamily-13B, J. Immunol. 181 (3) (2008) 2211–2219.
[39] S. Oon, et al., Targeted therapeutics in SLE: emerging strategies to modulate the interferon pathway, Clin Transl Immunology. 5 (2016) e79.
[40] R. Furie, et al., Anifrolumab, an Anti-Interferin-α Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus, Arthritis Rheumatol. 69 (2017) 376–386.
[41] A.W. Andrew, et al., Blockade of Interferon-γ Normalizes Interferon-Regulated Gene Expression and Serum CXCL10 Levels in Patient with Systemic Lupus, Arthritis Rheumatol. 67 (2015) 2713–2722.
[42] V.P. Werth, et al., Brief Report: Pharmacodynamics, Safety, and Clinical Efficacy of AMG 811, a Human Anti-Interferon-γ Antibody, in patients with Discoid Lupus Erythematosus, Arthritis Rheumatol. 69 (2017) 1028–1034.