Overexpression of CXCR4 on circulating B cells in patients with active systemic lupus erythematosus

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Abstract

Objective

To evaluate the roles of circulating B cells in the pathogenic process of systemic lupus erythematosus (SLE) by measuring the expression of chemokines and their receptors.

Methods

Peripheral-blood mononuclear cells were obtained from 17 active, 21 inactive SLE patients, and 13 healthy controls. The expression of CXCR4, CXCR5, and CCR7 on CD19$^+$ B cells was determined by flow cytometry, serum concentration of CXCL12 was measured by enzyme-linked immunosorbent assay, and the chemotactic responsiveness of B cells toward CXCL12 was evaluated. B or plasma cells expressing CXCR4 in renal biopsy specimens were detected using immnofluorescent staining.

Results

Flow cytometric analysis revealed that expression level of CXCR4 on circulating B cells was significantly higher in patients with active disease than in those with inactive disease or controls. Serum CXCL12 concentration was not different between these groups. In addition, the migratory ability of B cells toward CXCL12 was enhanced in active SLE patients. Finally, CXCR4-expressing B cells were more frequently observed in the renal biopsy specimens of lupus nephritis.

Conclusion

Up-regulated CXCR4 expression on circulating B cells in active SLE may enhance their chemotactic response toward CXCL12, which may promote infiltration of these cells into inflamed renal tissue and contribute to the development of SLE.

Key words
B cells, chemokine, CXCR4, systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterised by the production of autoantibodies to various cellular components (1). Antibodies are produced by antibody-secreting cells, such as B cells, plasmablasts, and plasma cells, in the bone marrow and the secondary lymphoid organs (2, 3). In the process of systemic immune responses, these antibody-secreting cells are released into the peripheral blood from secondary lymphoid organs (4). The pathogenic role of B cells in SLE has been investigated previously (5, 6), and the importance of autoantibody-independent functions, including the activation of auto-reactive T cells, production of proinflammatory cytokines, and organisation of ectopic lymphoid tissue have been demonstrated (6). In addition, anti-double stranded DNA (dsDNA) antibody is detected specifically in patients with SLE and involved in disease pathogenesis (7, 8). We previously investigated anti-dsDNA antibody-secreting cells with regard to their contribution to the pathogenesis of SLE and found that they are recruited into the circulation during the active disease phase in SLE patients (9). However, the mechanisms by which these cells emerge in the circulation remain unknown. Chemokines and their corresponding chemokine receptors play important roles in lymphopoiesis; lymphocyte differentiation, homing, and recirculation; and immune responses of lymphocyte subsets under physiological and pathological conditions (10, 11). Among many chemokine receptors, CXCR5, CCR7, and CXCR4 are classical chemokine receptors on B cells that are reported to be involved in their development (12). During B-cell maturation, which mainly occurs in secondary lymphoid organs, the up-regulation of CXCR5 and CCR7 on these cells is important for follicle organisation and migration toward the T-cell zone (13-15). These cells undergo proliferation and isotype switching; finally, mature B cells migrate to the venous sinus in a CXCR4-dependent manner and emerge in the circulation within other secondary lymphoid organs, inflamed tissues, or bone marrow. Some reports have already reported an association between SLE and CXCR4 expression on peripheral B cells (16, 17). However, there have been no reports focusing on both aberrant expression levels of CXCR4 on B cells in a targeted organ and their functional abnormality. The present study was undertaken to evaluate the mechanisms that contribute to the emergence of B cells in the circulation by focusing on the roles of chemokines in patients with active and inactive SLE.

Materials and methods

Patients and treatment

We studied 38 SLE patients who visited Keio University Hospital from 2010 to 2012 and fulfilled the American College of Rheumatology classification criteria (18). Thirteen sex-matched healthy controls were enrolled. Renal biopsy samples were obtained from seven SLE patients and all diagnosed according to International Society of Nephrology/Renal Pathology Society classification (19). These patients were classified as IV-G (A/C) +V (n=6) and IV-S (A/C) +V (n=1). Five patients with IgA nephropathy were enrolled as disease controls for renal histological analysis. All samples were obtained after the patients provided written informed consent, and the study was approved by the institutional review board.

Clinical features

Clinical information at blood examination was obtained from all SLE patients. Data collected included demographic features, treatment regimens, and individual items of the classification criteria for SLE and SLE Disease Activity Index (SLEDAI) (20). We also recorded laboratory findings, including serum albumin level, leukocyte count, lymphocyte count, haemoglobin level, platelet count, CH50 level, and serum anti-dsDNA antibody titer. Persistent proteinuria was defined by >0.5 g/g Cr for a urine protein:creatinine ratio. Treatment including dosage of corticosteroids was evaluated. SLEDAI was calculated for each patient. Active disease was defined as SLEDAI ≥5 whereas inactive disease was considered as

SLEDAI <5, according to a previous study (21). Serum anti-dsDNA antibodies were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (MESACUP DNA-II test, MBL, Nagoya, Japan), according to the manufacturer's instructions.

Flow cytometric analysis of chemokine receptors

Human peripheral-blood mononuclear cells (PBMCs) were isolated from heparinised venous blood using Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density-gradient centrifugation. PBMCs were analysed using flow cytometry (FACS Calibur flow cytometer using CellQuest™ software) for the expression or mean fluorescence intensity (MFI) of CXCR4, CXCR5, and CCR7 on $CD19^+$ B cells. MFI of the anti-chemokine receptor staining was calculated according to statistical thresholds set in reference to staining with negative control antibodies. Cells were stained with anti-CXCR4 (BD PharMingen, San Diego, CA, USA), CXCR5 (BD PharMinigen), CCR7 (BD PharMingen), and CD19 (Beckman Coulter, Fullerton, CA, USA) monoclonal antibodies.

Serum CXCL12 measurement

Serum was obtained from all patients and controls at the same time that heparinised venous blood samples were taken. The level of serum CXCL12, a ligand for CXCR4, was measured by an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The assay range was 156–10,000 pg/mL.

Chemotaxis assay

PBMCs were counted and resuspended in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2-mM L-glutamine, 50-U/mL penicillin, and 50-µg/mL streptomycin. A chemotaxis assay was performed to test the chemotactic responsiveness of B cells toward CXCL12 (4). $CD19^+$ B cells were enriched from PBMCs using a magnetic-activated cell sorting (MACS®) system (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs were treated with anti-CD19 magnetic bead-coupled monoclonal antibody (Miltenyi Biotech, Bergisch Gladbach, Germany) and then separated on a MACS column. Flow cytometric analysis revealed that the sorted fraction consistently showed >95% $CD19^+$ cells. Next 5×10^5 B cells were added to the upper chamber of Transwell (Corning, Tewksbury, MA, USA) inserts, and the lower Transwell was filled with 0 or 300 ng/mL CXCL12 (R&D Systems). After incubation for 90 min at 37°C in 5% CO_2, the cells in the lower chamber were collected and the total number was counted. Finally, the migration rate was calculated by dividing the total number of cells in the lower chamber by the number of cells originally applied to the upper chamber.

Immunohistochemistry

Paraffin sections (5-µm thickness) of renal biopsy samples were boiled in 1.0-mM ethylenediaminetetraacetic acid (pH 8.0) and blocked with 5% bovine albumin solution. These sections were separately incubated with mouse monoclonal antihuman CD20 (1:1, Dako, Glostrup, Denmark) or mouse monoclonal antihuman CD138 (1:25, Dako) for 30 min at room temperature. They were then incubated with Alexa Fluor-568 conjugated goat antimouse IgG (1:250, Invitrogen, Carlsbad, CA, USA) for 60 min at room temperature. We used the biotin-blocking system (Dako) to inhibit non-specific staining due to the presence of endogenous biotin. Each section was incubated overnight at 4°C with biotin-conjugated mouse antihuman CXCR4 (1:5, R&D Systems). Finally, these sections were stained with Alexa Fluor-488 conjugated Streptavidin (Invitrogen). TO-PRO3 (Invitrogen) was used to counterstain nuclei. Images were taken using a Fluoview FV1000 confocal laser fluorescence microscope (Olympus, Tokyo, Japan). The proportions of $CD20^+$ or $CD138^+$ cells that expressed CXCR4 were determined by counting of at least 100 cells of interest by two independent observers (H.H. and Y.O.).

Statistical analysis

Continuous values are shown as means ± standard deviations. The differences between results for two groups were compared using the nonparametric Mann-Whitney U-test. Differences in results for active SLE patients, inactive SLE patients, and normal healthy

Table I. Clinical features of patients with active and inactive SLE.

Demographic and clinical features	Disease activity		Healthy controls (n=13)	*p*-value
	Active (n=17)	Inactive (n=21)		
Gender (% female)	76.5	85.7	76.9	0.5
Age (years)	41.9 ± 10.7	43.8 ± 17.5	40.6 ± 12.3	0.4
Persistent proteinuria (%)	70.6	9.5	-	<0.001
Serum albumin (g/dL)	3.0 ± 0.7	4.2 ± 0.3	-	<0.001
Leukocyte counts (10^3/µL)	5.3 ± 3.2	5.4 ± 2.6	-	0.4
Lymphocyte counts (10^3/ µL)	1.2 ± 1.7	1.3 ± 4.2	-	0.5
Haemoglobin (g/dL)	10.9 ± 2.5	12.8 ± 1.8	-	0.2
Platelet counts (10^4/µL)	10.3 ± 7.7	17.2 ± 10.3	-	0.02
SLEDAI	6.9 ± 1.9	2.1 ± 1.5	-	<0.001
C3 (mg/dl)	66.2 ± 11.5	112.1 ± 32.7	-	0.01
C4 (mg/dl)	18.2 ± 9.3	42.3 ± 10.7	-	0.02
CH50 (U/mL)	21.8 ± 10.1	35.9 ± 9.8	-	0.02
Serum anti-dsDNA antibody (IU/mL)	214.1 ± 144.3	69.4 ± 96.3	-	<0.001
Dosage of prednisolone (mg/day)	7.1 ± 9.1	6.5 ± 8.1	-	0.4
Immunosuppressants		-		
Tacrolimus (%)	4 (23.5)	5 (23.8)	-	0.9
Cyclosporine (%)	1 (5.8)	2 (15.4)	-	0.7
Azathioprine (%)	3 (17.6)	2 (15.4)	-	0.5
Mizoribine (%)	0 (0)	1 (7.2)	-	0.4
Mycophenolate mofetil (%)	0 (0)	0 (0)	-	-

SLEDAI, systemic lupus erythematosus disease activity index; CH50, 50% complement haemolytic activity.

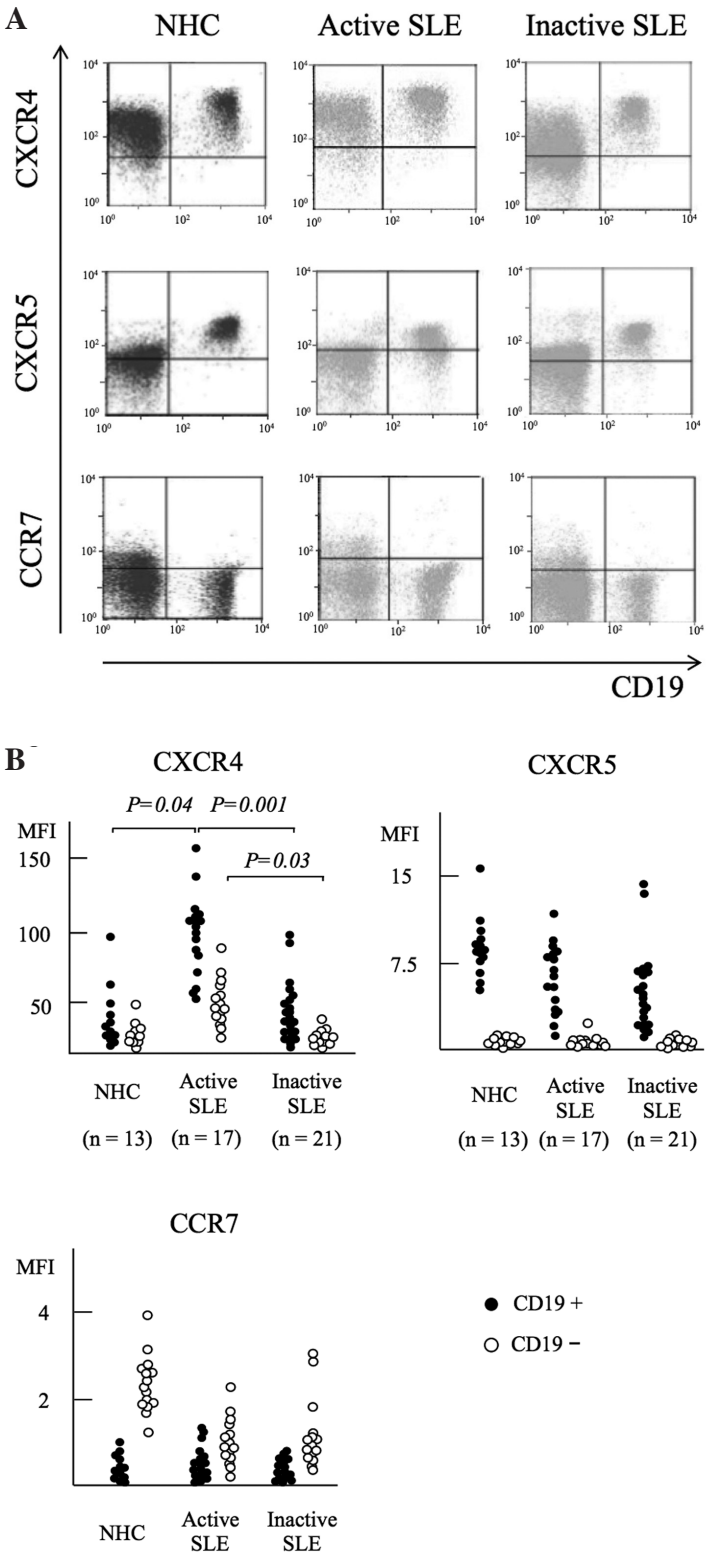

Fig. 1. A-B. Chemokine receptor expression on peripheral CD19⁺ or CD19⁻ cells in normal healthy controls and patients with active or inactive SLE.
A: Representative dot plots for the CD19⁺ population.
B: Expression levels of CXCR4, CXCR5, and CCR7. Significant differences were seen in all comparisons between CD19⁺ and CD19⁻ cells (p<0.01) for CXCR4, CXCR5, and CCR7 expression. CXCR4 expression on CD19⁺ cells of active disease patients was significantly higher than that of both inactive disease patients and normal healthy controls (p=0.001 and 0.04, respectively). There were no significant differences in CXCR5 or CCR7 expression on CD19⁺ cells between SLE patients and normal healthy controls.

controls were tested by non-repeated analyses of variance.

Results

Patient characteristics

Demographic and clinical features were compared between 17 patients with active SLE and 21 with inactive SLE (Table I). Patients in the active phase showed a higher frequency of persistent proteinuria (p<0.001), lower serum albumin levels (p<0.001), higher anti-dsDNA antibody titers (p<0.001), lower C3, C4 (p=0.01, p=0.02), and lower 50% complement haemolytic activity (CH50; p=0.02). Furthermore, all the patients with active disease were manifested by nephritis based on the presence of either persistent proteinuria or active sediment. At the time of sampling, most patients were receiving <5 mg/day of corticosteroids, and the immunosuppressants did not differ between patients with active and inactive disease. Because mycophenolate mofetil has not been approved as an SLE treatment in Japan, none of the patients received this medication.

Analysis of chemokine receptor expression on peripheral-blood B cells

We first analysed the expression of the chemokine receptors CXCR4, CXCR5, and CCR7 on CD19⁺ B cells of patients and healthy controls. Representative dot plots are shown in Figure 1A. CXCR4 expression on CD19⁺ cells of active disease patients was significantly higher than that in both inactive disease patients and normal healthy controls (p=0.001 and 0.04, respectively; Fig. 1B). There were no significant differences in CXCR5 or CCR7 expression on CD19⁺ cells of SLE patients and normal healthy controls (Fig. 1B). No differences in CXCR5 and CCR7 expression on CD19⁺ cells and CD19⁻ cells were observed between normal healthy controls and patients with active or inactive SLE. CXCR4 expression on CD19⁻ cells was significantly higher in active disease patients than in inactive disease patients (p=0.03). Taken together, these results demonstrate that patients with active disease tend to have a higher CXCR4 expression on both CD19⁺ and CD19⁻ popu-

lations and the expression levels were higher on CD19+ cells than on CD19– cells. Therefore, we further analysed the association between the expression of CXCR4 and its ligand, CXCL12.

CXCL12 level in serum of SLE patients and normal healthy controls

We next quantified the level of CXCL12 in the serum of SLE patients and normal healthy controls. As shown in Figure 2A, there were no significant differences in the level of CXCL12 among the normal healthy controls and the active or inactive disease patients.

Enhanced migratory ability of B cells toward CXCL12 in patients with active SLE

We investigated the migratory response of CD19+ B cells toward CXCL12 using a Transwell-based chemotaxis assay. The addition of CXCL12 resulted in chemotactic responses in patients with active or inactive SLE and in normal healthy controls (Fig. 2B). The migratory ability toward CXCL12 was significantly enhanced in SLE patients compared with normal healthy controls (33.9% *vs*. 16.0%, p=0.001). We then compared the chemotactic responses of CD19+ B cells from either active or inactive disease patients. Remarkably, the circulating B cells from patients in the active phase shared an enhanced CXCL12-promoted chemotaxis, with a significant increase in migration (52.9% *vs*. 27.6%, p=0.004). In addition, we calculated the fold change from baseline and compared between migration rate with 0 and 300 ng/ml CXCL12 (Fig. 2C). A significantly higher fold increase was seen in active SLE patients compared with both inactive SLE patients and normal healthy controls (6.7±3.1 *vs*. 3.8±2.3 *vs*. 3.8±2.0, respectively; p=0.04).

Increased proportion of CXCR4-expressing B cells in renal biopsy samples of lupus nephritis

Representative images of periodic acid–Schiff (PAS) staining and immunohistochemistry are shown in Figure 3. Figure 3A shows PAS staining of samples from both patients with IgA nephropathy and lupus nephritis. In IgA nephropathy, PAS staining showed focal segmental mesangial proliferation and mild cell infiltration in tubulointerstitial lesions. In contrast, diffuse globally affected glomeruli were observed and tubulointerstitial inflammatory cells formed multiple clusters in lupus nephritis. Immunohistochemical staining of samples of IgA nephropathy (Fig. 3B) and lupus nephritis (Fig. 3C) are also shown. Previously mentioned the focally organised tubulointerstitial inflammatory cells are mostly CD20+ cells. Aggregates of CD20+ cells generated lymphoid-like structure in tubulointerstitial lesion. As shown in Figure 4A, B cells were more likely to be found in renal biopsy samples from lupus nephritis cases than in those from IgA nephropathy cases (p=0.005). Next, we found a higher frequency of CXCR4+ B cells in the interstitial lesions of lupus nephritis compared with IgA nephropathy (p<0.001; Fig. 4B). Figure 4C shows that the percentage of CXCR4+ plasma cells was not different between these two groups (p=0.1).

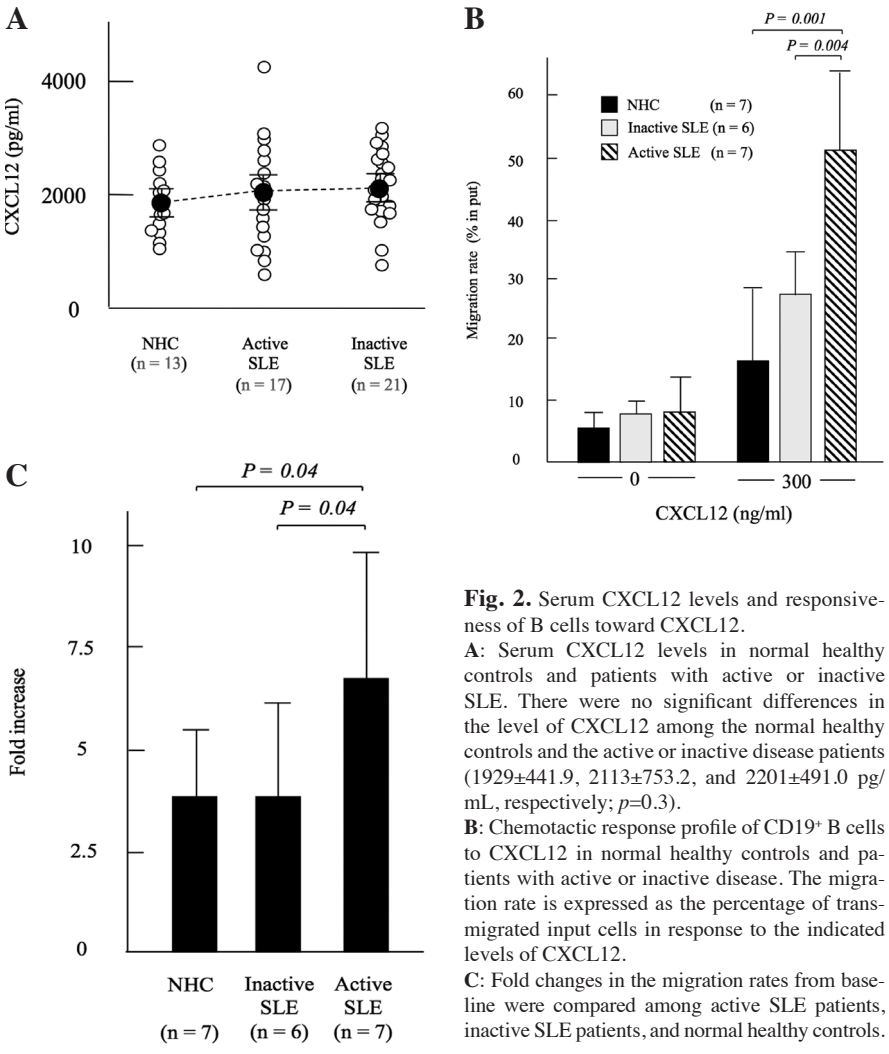

Fig. 2. Serum CXCL12 levels and responsiveness of B cells toward CXCL12.
A: Serum CXCL12 levels in normal healthy controls and patients with active or inactive SLE. There were no significant differences in the level of CXCL12 among the normal healthy controls and the active or inactive disease patients (1929±441.9, 2113±753.2, and 2201±491.0 pg/mL, respectively; p=0.3).
B: Chemotactic response profile of CD19+ B cells to CXCL12 in normal healthy controls and patients with active or inactive disease. The migration rate is expressed as the percentage of transmigrated input cells in response to the indicated levels of CXCL12.
C: Fold changes in the migration rates from baseline were compared among active SLE patients, inactive SLE patients, and normal healthy controls.

Discussion

In this study, we evaluated the mechanisms contributing to the emergence of B cells in circulation by focusing on the roles of relevant chemokines and their receptors. CXCR4 expression on peripheral-blood B cells and their migration ability of B cells towards CXCL12 were enhanced in active SLE patients compared with both inactive SLE patients or normal healthy controls, whereas no significant difference in the serum level of CXCL12 was observed irrespective of disease activity status. Furthermore, the percentage of CXCR4+ B cells in the interstitial legions was significantly higher in kidney biopsy samples from lupus nephritis pa-

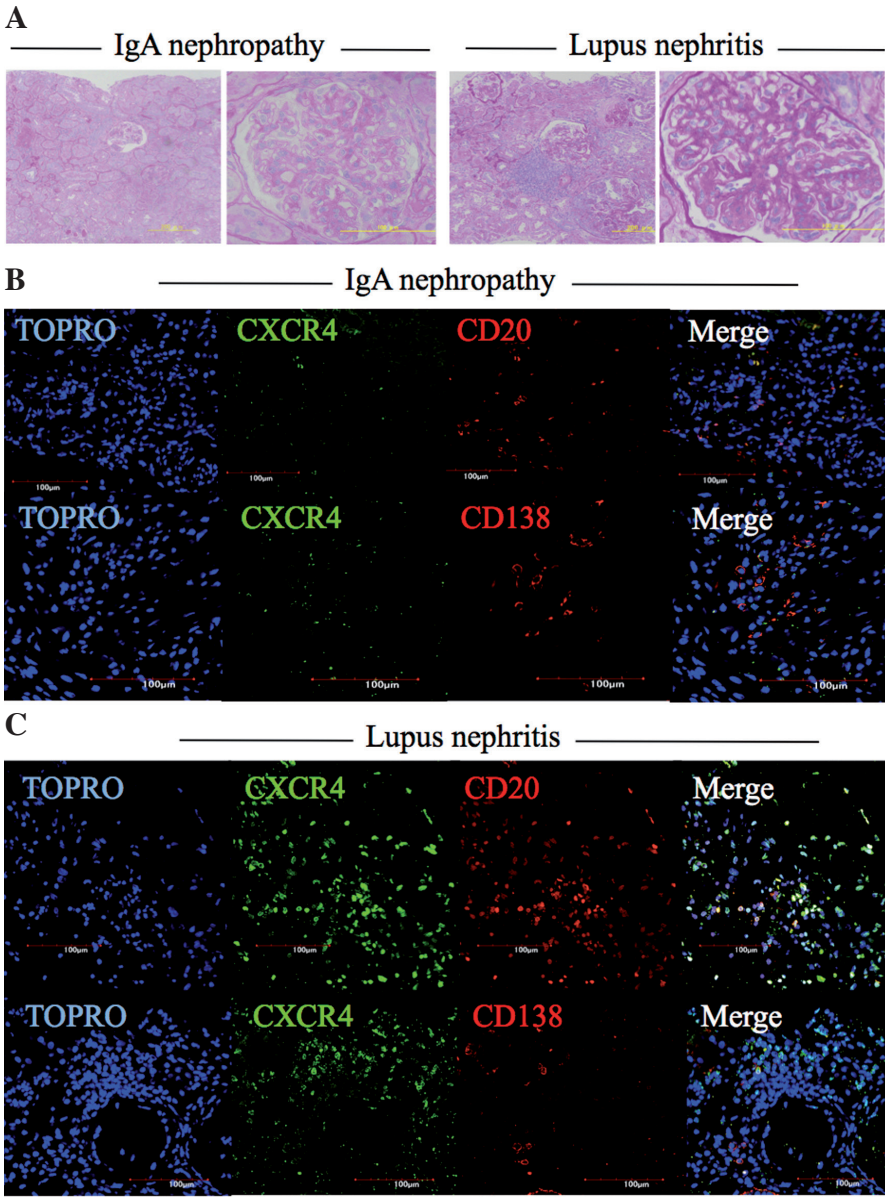

Fig. 3. Representative images of renal PAS staining and immunohistochemical staining of samples from patients with lupus nephritis and IgA nephropathy.
A: PAS staining of samples from patients with lupus nephritis and IgA nephropathy. Renal immunohistochemistry examination of samples from a patient with IgA nephropathy.
B: and a patient with lupus nephritis.
C: Renal CD20$^+$ cell infiltration was higher in lupus nephritis than in IgA nephropathy.

tients than in those from IgA nephropathy patients. These findings together indicate that up-regulated expression of CXCR4 may be associated with B-cell migration from circulation into the lesions and contribute to the development of lupus nephritis or SLE.

CXCR4-CXCL12 axis is involved in B-cell differentiation and migration in the secondary lymphoid organs (22, 23). Also, CXCR4-expressing mature B cells can emerge into circulation from secondary lymphoid organs based on CXCL12 expression in the splenic red pulp (24). Thus, B cells with up-regulated expression of CXCR4 may effectively migrate toward circulation after maturation. In some pathological conditions, including inflammation and infection, these B cells can generate ectopic lymphoid tissue in targeted organs after emergence in the circulation (25). Furthermore it has been reported that higher expression levels of CXCL12 was observed in renal samples of lupus nephritis (16), and we found that infiltrating CXCR4$^+$ B cells were increased in the lesion. Taken together, these results suggest that B cells with up-regulated expression of CXCR4 have an important role in the pathogenesis of lupus nephritis and associated with local expression of pathogenic autoantibody such as anti-dsDNA antibody.

Accumulating data indicate that B cells likely contribute to the development of SLE through both autoantibody-dependent and antibody-independent mechanisms (6). Via their autoantibody-independent functions, B cells may be deleterious through the activation of autoreactive T cells, production of proinflammatory cytokines, and organisation of ectopic lymphoid tissue. Ectopic lymphoid tissues often develop in autoimmune diseases, including in the joints in rheumatoid arthritis (26), the salivary glands in Sjögren's syndrome (27), the thyroid gland in Hashimoto's disease (28), and interstitial lesions of kidney in SLE (29). In many cases, the formation of well-developed ectopic lymphoid tissue correlates with increased severity of disease. B cells within these lymphoid structures secrete autoantibodies and are required to locally maintain activated autoimmune cells (30). In SLE, autoreactive B cells that infiltrate the kidney may contribute to maintaining activated autoimmunity. Chang *et al*. demonstrated aggregates of T, B cells, and plasmablasts generated germinal center-like structure in tubulointerstitial lesion (29). We also found multiple focal clusters of B cells in tubulointerstitial lesions of kidney samples from patients with lupus nephritis. These lymphoid structures may be functional and associated with B-cell clonal expansion and somatic hypermutations. The association between CXCL12 and CXCR4 on B cells may be one of the key factors contributing to organising this process. Therefore, the kidney is not only an autoimmune target but also a site where the pathological processes of SLE is promoted.

There have been some reports investigating the association between SLE and expression levels of chemokine receptors on B cells. Wang *et al*. demonstrated that CXCR4 is likely to be up-regulated in various leukocytes, in-

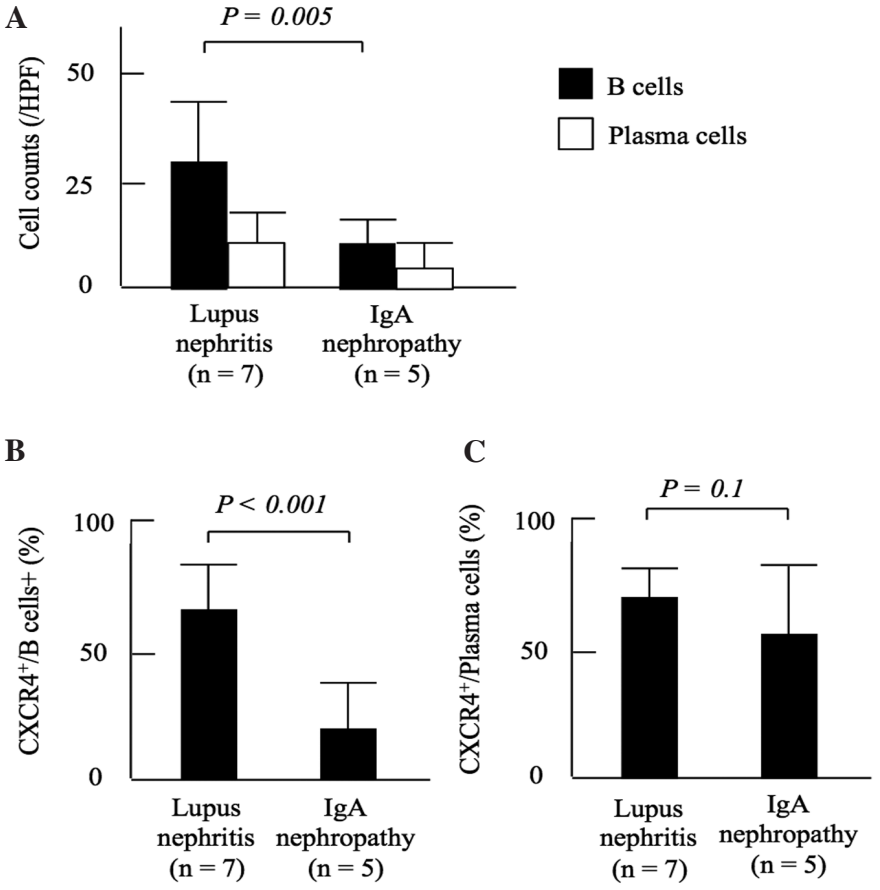

Fig. 4. Comparison of cell phenotypes between lupus nephritis and IgA nephropathy that infiltrated the kidney.
A: B cells were found more frequently in samples of lupus nephritis than in those of IgA nephropathy.
B and **C**: Calculated percentages of cells expressing CXCR4 among $CD20^+$ or $CD138^+$ cells.

cluding $CD19^+$ B cells in SLE patients (16). They enrolled 45 SLE patients and divided them into two groups according to the SLEDAI score (<10 or ≥10). They concluded that SLE patients with a high SLEDAI score had higher expression levels of CXCR4 on $CD19^+$ cells. As previously mentioned, they also reported that severe lupus nephritis was associated with higher expression levels of CXCL12 based on renal immunohistochemistry. This report supports our findings and concept of the role of CXCR4-CXCL12 axis in the pathogenesis. On the other hand, Biajoux *et al*. showed opposite results, such as reduced levels of CXCR4 mRNA in leukocytes and decreased activity of migration toward CXCL12 (17). Some reasons for this difference may be that their definition of activity of SLE. Their threshold of activity was defined as SLEDAI = 3 which was much lower than the general standard (31). Thus, population of active SLE included very mild patients in their study. This may cause the difference of the results compared with ours and Wang's findings.

There are some limitations to our study. Since the sample size is smaller and access to renal biopsied samples was limited, further studies with a larger sample size are needed to confirm our findings. Although CXCR4 is the only chemokine receptor which we focused on in this study, it is possible that other chemokine-chemokine receptor pairs may regulate $CXCR4^+$ B cell infiltration in the pathogenesis. Thus our conclusions need careful consideration at this point. However, infiltrating CXCR4-expressing B cells are associated with pathogenesis at least, in part.

In summary, up-regulated expression of CXCR4 on circulating B cells and enhanced chemotactic responsiveness toward CXCL12 were observed in patients with active SLE. The infiltration of these cells into inflamed tissues, including the kidneys, may contribute to the development of SLE pathogenesis.

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