

Role of Fractalkine in the Pathogenesis of Primary Sjögren Syndrome: Increased Serum Levels of Fractalkine, Its Expression in Labial Salivary Glands, and the Association with Clinical Manifestations

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ABSTRACT. Objective. To investigate the expression of fractalkine and identify the clinical effects of fractalkine and its receptor (CX3CR1) in patients with primary Sjögren syndrome (pSS).

Methods. Serum fractalkine levels were determined by ELISA. Immunohistochemical staining was done to compare the expression of fractalkine and CX3CR1 between salivary glands (SG) of patients with SS and controls. The cells to be merged with fractalkine were evaluated by confocal microscopy. Type of CX3CR1-expressing cells among infiltrating lymphocytes in SG was analyzed by confocal microscopy. Further, associations among fractalkine, proinflammatory cytokines, and clinical profiles were investigated.

Results. Serum fractalkine levels in patients with pSS were higher than those in the control group ($p = 0.026$). SG expression of fractalkine and its receptor was upregulated in patients with pSS compared to that in the controls by immunohistochemistry. Higher histological grade was associated with more fractalkine-positive cells per total epithelial cells. Epithelial cells were the main fractalkine-expressing cell type in the SG. Serum fractalkine levels were significantly correlated with proinflammatory cytokines levels (interleukin 17: $r = 0.685$, $p = 0.029$; tumor necrosis factor- α : $r = 0.444$, $p = 0.003$), antinuclear antibody ($r = 0.349$, $p = 0.022$), and immunoglobulin G levels ($r = 0.325$, $p = 0.044$). Serum fractalkine levels in patients with extraglandular manifestations of pSS were significantly higher than in those without extraglandular manifestations ($p = 0.026$).

Conclusion. Fractalkine and CX3CR1 may play a role in the pathogenesis of pSS, including extraglandular manifestations. (First Release Oct 15 2014; J Rheumatol 2014;41:2425–38; doi:10.3899/jrheum.130892)

Key Indexing Terms:

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Primary Sjögren syndrome (pSS) is a chronic, slowly progressive autoimmune disease characterized by lymphocytic infiltration of the exocrine glands resulting in xerostomia and keratoconjunctivitis sicca¹. About one-third of patients present with extraglandular manifestations (i.e., arthritis, interstitial lung disease, vasculitis, tubulointerstitial nephritis, peripheral and central neuropathies, and gastrointestinal tract disorders). Disruption of acinar and ductal structures is hallmark in lacrimal and salivary glands (SG) of patients with pSS. Primary SS is also characterized by periductal mononuclear cell infiltration and immune hyperactivity². Although the etiology of pSS remains unknown, the roles of various proinflammatory cytokines have long been thought to be important in disease development^{3,4}. The involved cytokines are diverse, ranging from typical proinflammatory cytokines such as interleukin 17 (IL-17) and tumor necrosis factor- α (TNF- α), to novel cytokines such as IL-21. Moreover, B cell activating factor and a proliferation-inducing ligand contribute to auto-antibody production in patients with pSS. Diverse immune

cells also contribute to the development of pSS, including Th17 cells, B cells, and antigen-presenting cells (APC)^{4,5,6}. Follicular helper T cells, which are important for the production of IL-21 related to autoantibody formation, play a role in the pathogenesis of pSS⁷.

Recruitment of autoreactive lymphocytes to inflamed tissues is a key feature of organ-specific autoimmune conditions. Chemokines induce accumulation of leukocytes at inflammatory sites and modulate inflammatory activities through the recruited cells. In the field of SS, the first report about various chemokine mRNA distributions in SG was in 2001⁸. The chemokine fractalkine (CX3CL1) is synthesized as a type 1 transmembrane molecule by endothelial cells. Soluble fractalkine is a potent chemoattractant of T cells and monocytes, whereas the cell-bound chemokine promotes strong adhesion of leukocytes to activated endothelial cells where it is primarily expressed⁹. The fractalkine receptor (CX3CR1) is widely expressed in various cell types such as T cells, natural killer cells, monocytes, dendritic cells, and neuronal cells¹⁰. Fractalkine has a proinflammatory role in autoimmune diseases. It upregulates matrix metalloproteinase 2 production in rheumatoid arthritis¹¹, and its serum level is higher in patients with active systemic lupus erythematosus (SLE) than in controls¹². Fractalkine plays a role as an accelerating factor in the autoimmune exocrinopathy of thymectomized NFS/sld mice¹³. In a previous study, SG-specific fractalkine transgenic mice showed higher expression of 25-kDa fragmented fractalkine in SG¹³. Moreover, B cells and CD4+ T cells were mainly infiltrated in SG of the transgenic mice. However, the precise roles of fractalkine and its receptor in pSS are still poorly understood.

To investigate whether fractalkine participates in the pathogenesis of pSS, we examined the serum levels of fractalkine, its expression in SG, and its expression of the fractalkine receptor (CX3CR1) in peripheral blood mononuclear cells (PBMC) in patients with pSS. In addition, we examined whether the level of fractalkine is associated with clinical laboratory variables, extraglandular manifestations, or the levels of other inflammatory cytokines.

MATERIALS AND METHODS

Patients and controls. Forty-three patients fulfilling American College of Rheumatology/European League Against Rheumatism consensus criteria for pSS (1 man, 42 women) and 31 age-matched and sex-matched healthy controls (1 man, 30 women) were included in our study. All subjects were recruited from the rheumatology outpatient clinic at Seoul St. Mary's Hospital, Seoul, South Korea, from October 2009 to December 2010. We included male and female patients over 18 years old. Patients were permitted to take any immunosuppressive or immunomodulating medications related to pSS. Patients with an ongoing or recent infection during our study were excluded. The mean \pm SD ages of the pSS group and the control group were 53.63 ± 12.29 years and 58.03 ± 6.35 years, respectively. Twenty labial SG (LSG) samples were obtained from the group of patients with pSS for histological evaluation. LSG control samples were obtained from 5 subjects who presented to the rheumatology clinic with sicca symptoms, but neither autoantibody (anti-Ro or anti-La) nor results of LSG biopsies fulfilled the classification criteria for SS. Eleven PBMC samples

from patients with pSS and 9 PBMC samples from healthy controls were obtained to evaluate CX3CR1 expression. Written informed consent was obtained from all subjects. This study was approved by the ethics committee of Seoul St. Mary's Hospital (KC13SISE0293). All patients underwent physical examinations and serological evaluations, including measurement of antinuclear antibody (ANA), rheumatoid factor (RF), immunoglobulins, complement factors 3 (C3), C4, extractable nuclear antigen antibody, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP).

Tissue samples and cells. Labial minor SG biopsies were obtained with informed consent from 25 patients who underwent diagnostic evaluation of sicca symptoms indicative of SS. Among them, 20 were diagnosed with pSS and the other 5 were disease controls. Five or 6 minor SG lobules were carefully harvested and placed in formalin fixative. Standard paraffin preparations were prepared and these were sectioned at 5 μ m thickness and then stained with H&E. The slides were examined for the presence of lymphocytic infiltrates and/or foci by 3 observers (using standardized criteria). A focus was defined as an aggregate of ≥ 50 lymphocytes with a few plasma cells. The focus score is reported as the number of foci per 4 mm² tissue¹⁴.

Human head and neck squamous cell carcinoma A253 cells (ATCC) were cultured in 10% fetal bovine serum (Gibco) containing McCoy's 5a medium (ATCC). They were stimulated with recombinant IL-1 β , TNF- α , and interferon (IFN)- γ (R&D Systems) for 1–3 days to check fractalkine mRNA expression and protein levels.

Measurement of serum fractalkine levels. Serum fractalkine concentrations in 43 patients with pSS and 31 controls were measured by Luminex Bead-based Multiplex Assay (R&D Systems). Fractalkine protein levels were determined by sandwich ELISA. The culture supernatant was collected from A253 cells stimulated with or without IL-1 β , TNF- α , and IFN- γ for 3 days. Monoclonal mouse antihuman fractalkine (2 μ g/ml, R&D Systems) and biotinylated detection antibodies (0.5 μ g/ml, R&D Systems) served as the primary and secondary antibodies, respectively. The fractalkine concentration was used to normalize for variations in cell number. This ELISA detected the chemokine domain of human fractalkine, and the sensitivity limit was about 150 pg/ml.

Immunohistochemical staining for fractalkine and CX3CR1. SG tissue was snap-frozen in liquid nitrogen and stored at -80°C . Tissue sections were blocked with 1% normal goat serum, followed by staining with antibodies to fractalkine (1 μ g/ml, R&D Systems), CX3CR1 (2 μ g/ml, Biolegend), or isotype control antibody (1 μ g/ml, R&D Systems). The sections were then incubated with the appropriate biotinylated secondary antibodies (Santa Cruz Biotechnology), followed by an avidin-enzyme complex. Phosphate buffered saline (PBS) containing 0.05% Tween 20 was used for washing after each step. Chromogenic reactions were visualized with 3, 3'-diaminobenzidine (Sigma), and the nuclei were counterstained with hematoxylin. Slides were mounted in a permanent mounting media (Dako). In additional immunohistochemical staining for other chemokine receptors, tissue sections were stained with antibodies to CXCR2, CXCR3 (Abcam), and CXCR5 (Biolegend).

Confocal microscopic analysis. Tissue sections (7 μ m) were fixed in 4% paraformaldehyde and stained using biotin-conjugated antihuman fractalkine antibody (R&D Systems), purified antihuman cytokeratin antibody, APC conjugated antihuman CD31 antibody, Alexa647-conjugated antihuman CX3CR1 antibody, Alexa488-conjugated antimouse immunoglobulin G (IgG) antibody, Dylight594-conjugated antirabbit IgG antibody, streptavidin-FITC (Biolegend), purified antihuman CD3 antibody, purified antihuman CD4 antibody, purified antihuman CD14 antibody (Santa Cruz Biotechnology), and purified antihuman aquaporin 5 antibody (Abcam). The analysis was performed using a LSM 510 Meta confocal microscopy system (Zeiss). Quantification of fractalkine and cytokeratin double-positive population in SG tissue sections was derived from patients with SS (6 slides for grade 1 and 7 slides for grade 4) using TissueFAX (Tissue Gnostics).

Quantitative reverse transcription-PCR (RT-PCR). Trizol (Invitrogen) was used to isolate mRNA, according to the manufacturer's instructions. Total RNA (2 g) was reverse transcribed for 10 min at 25°C, 30 min at 55°C, and finally for 5 min at 85°C using a Transcriptor First Strand cDNA Synthesis kit (Roche). Real-time PCR amplification was performed in a StepOne plus machine (Life Technology) using FastStart Universal SYBR Green Master (Roche) according to the manufacturer's guidelines. The following sense and antisense primers for each molecule were used for fractalkine (forward) 5'-TCT GCC ATC TGA CTG TCC TG-3' (reverse) 5'-CCA CAG ACT CGT CCA TTC C-3'; CX3CR1 (forward) 5'-GGG ACT GTG TTC CTG TCC AT-3' (reverse) 5'-GAC ACT CTT GGG CTT CTT GC-3'; and β -actin (forward) 5'-GGA CTT CGA GCA AGA GAT GG-3' (reverse) 5'-TGT GTT GGG GTA CAG GCT TTG-3'. The PCR cycling conditions were as follows: 10 min at 95°C, 45 cycles of 15 s at 95°C, and 45 s at 60°C. β -actin was PCR-amplified in each sample to verify that equivalent amounts of RNA were added to each PCR reaction. Relative fold induction was calculated using the equation $2^{-(\Delta C_t)}$, where ΔC_t is $C_{t(\text{target})} - C_{t(\beta\text{-actin})}$, and C_t is the cycle at which the threshold is crossed. PCR product quality was monitored using post-PCR melting curve analysis.

Flow cytometry. We obtained the single-cell suspensions of PBMC from healthy volunteers ($n = 9$) or patients with pSS ($n = 11$) by standard Ficoll-Hypaque method. Cells were washed with FACS buffer (0.5% bovine serum albumin, 0.02 N sodium azide in PBS, pH 7.4) and stained with the following antibodies: purified anti-CX3CR1 antibody, APC conjugated antirat IgG antibody, PE-conjugated anti-CD14 antibody, PerCP conjugated anti-CD3 antibody, V450 conjugated antiCD4 antibody, and V500 conjugated antiCD8 antibody (BD Bioscience). The FACS analysis was performed using an LSRII Fortessa machine (BD Bioscience), and data were analyzed with Flow Jo software version 7.6 (Treestar). The lymphocyte or monocyte group was gated on the whole cell region using forward/side scatter properties.

Statistical analysis. Statistical analyses were performed using SAS software version 9 (SAS Institute Inc.) and GraphPad Prism version 5.01 software (GraphPad Prism). Experimental values are presented as mean \pm standard error of triplicate cultures and representative of experiments performed on 3 separate occasions. Differences in mean fractalkine levels of patients with pSS and controls were analyzed using Student t test. Statistical significance of the confocal microscopy and A253 cell culture results was determined by the Mann-Whitney U test or ANOVA with Bonferroni's posthoc analysis. Pearson's and Spearman's rank tests were used to assess the correlation between fractalkine levels and other laboratory data. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Increased levels of serum fractalkine and its expression in LSG of patients with pSS. Mean \pm SD of serum fractalkine levels in patients with pSS ($n = 43$) and controls ($n = 31$) by ELISA were 431.86 ± 105.74 and 184.73 ± 19 pg/ml, respectively (Figure 1A). The fractalkine levels of patients with pSS were significantly higher than those of the controls ($p = 0.026$). Immunohistochemical staining was performed to determine whether fractalkine and its receptor were expressed by the LSG of the 20 patients with pSS and 5 controls. All 20 pSS samples exhibited significant expression of fractalkine and its receptor, whereas the 5 control samples rarely expressed fractalkine and its receptor (Figure 1B).

The patients with pSS were categorized according to their grade of lymphocytic infiltration (grades 1–4). Five samples were included per grade. No increase was observed in fractalkine-positive cells per field or its receptor-positive

cells per field in either the grade 1 or 2 groups, but the grade 3 and 4 groups had a higher number of positively stained cells. Overall, higher tissue grade was associated with more fractalkine-positive and its receptor-positive cells (Figure 1C). Classification of focus score included 10 cases (Focus 1: 2, Focus 2: 3, Focus 3: 3, and Focus 4: 2). The analysis by focus score group showed similar tendency, but statistical significance was revealed in only comparison value of Focus 1 group with those of Focus 4 group (fractalkine-positive cells, $p = 0.036$; CX3CR1-positive cells, $p = 0.037$). To quantify fractalkine-positive and CX3CR1-positive cells per total epithelial cells and infiltrating lymphocytes, TissueFAX was done. Fractalkine-positive epithelial cells in tissue grade 4 showed higher percent than the cells in grade 1 ($p = 0.004$). However, we could not quantify CX3CR1-positive cells per infiltrating lymphocytes because there were too few lymphocytes in tissue grade 1, and CX3CR1-positive lymphocytes population was too small to detect by TissueFAX in tissue grade 4.

Epithelial cells in SG express fractalkine, not endothelial cells, and IFN- γ stimulates fractalkine production in SG epithelial cell lines. Prior studies using mice with SS models have reported that fractalkine expression increases in lacrimal glands and SG^{13,15}. Therefore, we investigated the cell type expressing fractalkine and its receptor by confocal microscopy in SG of patients with pSS. Confocal microscopy revealed that fractalkine expression in LSG from patients with pSS merged with expression of cytokeratin, an epithelial cell marker (Figure 2A). Expression of aquaporin 5, a marker of acinar epithelial cells, merged with fractalkine expression (Figure 2B). A portion of aquaporin 5 was expressed in ductal epithelial cell. However, fractalkine expression merged with CD31 expression (Figure 2C). CD31 is a marker of endothelial cells. Consequently, based on the immunohistochemical staining and confocal microscopy results, ductal epithelial cells may mainly express fractalkine in LSG of patients with pSS.

To compare interaction of fractalkine-CX3CR1 with those of other chemokine-chemokine receptors, we additionally investigated other chemokine receptors that are known to be associated with pSS by immunohistochemical staining. Five SG samples (grade 4) were stained by CXCR2 (interaction with CXCL1), CXCR3 (interaction with IP-10), and CXCR5 (interaction with CXCL13). Expressions of CXCR2 and CXCR3 were increased in ductal and acinar epithelial cells of inflamed tissue (Figure 2D). However, increased expression of CXCR5 was not found. Based on these results, it is possible that there are potentially different types of regulation patterns in interactions between fractalkine with its receptor.

Various cytokines, including IL-1 β , TNF- α , and IFN- γ , were administered to A253 cells, a representative SG epithelial cell line, to verify whether ductal epithelial cells produce fractalkine. After a 24-h stimulation, the

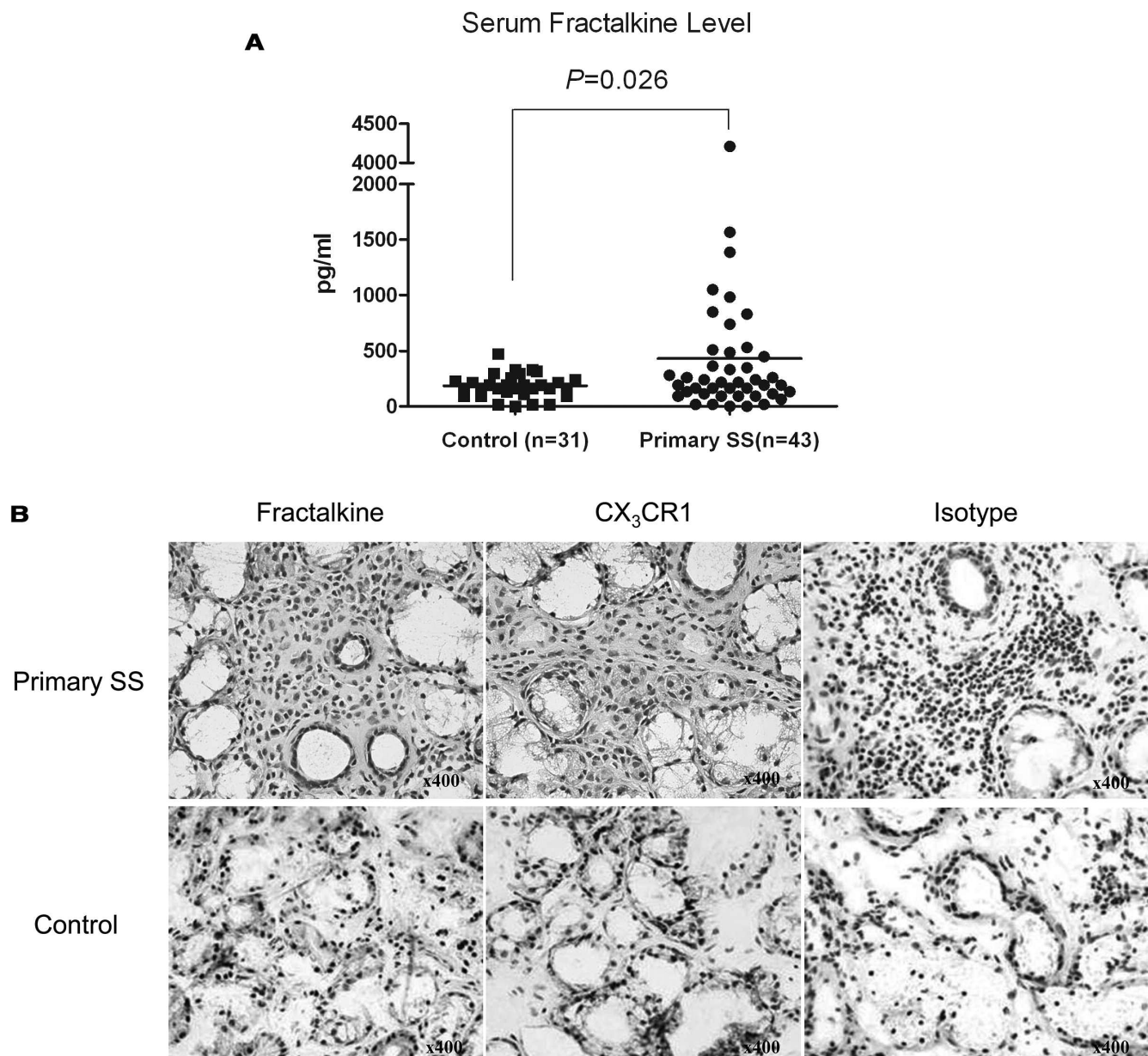


Figure 1. Serum fractalkine levels. **A.** Serum fractalkine levels in patients with pSS ($n = 43$) and controls ($n = 31$) were measured by ELISA. The patients with pSS had significantly higher serum fractalkine levels than those of controls ($p = 0.026$). Horizontal bars represent the median of all samples within a group. **B.** The LSG of patients with pSS exhibited increased fractalkine and receptor expression. The periductal areas of the patients with SS exhibited intense fractalkine and receptor staining, whereas controls were rarely stained. **C.** The higher the LSG focus score grade, the more positive cell stained by fractalkine and its receptors. There was a similar tendency in an analysis according to focus score. Confocal microscopy and TissueFAX shows fractalkine- and CX3CR1-positive cells per total epithelial cells and infiltrating lymphocytes. pSS: primary Sjögren syndrome; LSG: labial salivary gland.

IFN- γ -treated group showed increased fractalkine mRNA expression (Figure 2E; $p < 0.001$). Similarly, fractalkine levels by ELISA increased significantly after 72 h of IFN- γ stimulation ($p < 0.001$), suggesting that SG epithelial cells produce fractalkine in patients with pSS.

CX3CR1 expression in PBMC and LSG of patients with pSS according to cell types. We evaluated CX3CR1 mRNA expression in PBMC from patients with pSS ($n = 11$) and

controls ($n = 9$) by quantitative RT-PCR, but no difference was observed (Figure 3A). The percentages of CX3CR1-positive cells in CD4 T cells, CD8 T cells, and monocytes were examined by flow cytometry to investigate CX3CR1-expressing cell types in PBMC. The evaluation revealed that the percentage of CX3CR1-positive cells in patients with pSS was not significantly different from that of the controls in all cell types (Figure 3B). CX3CR1 expression of LSG was

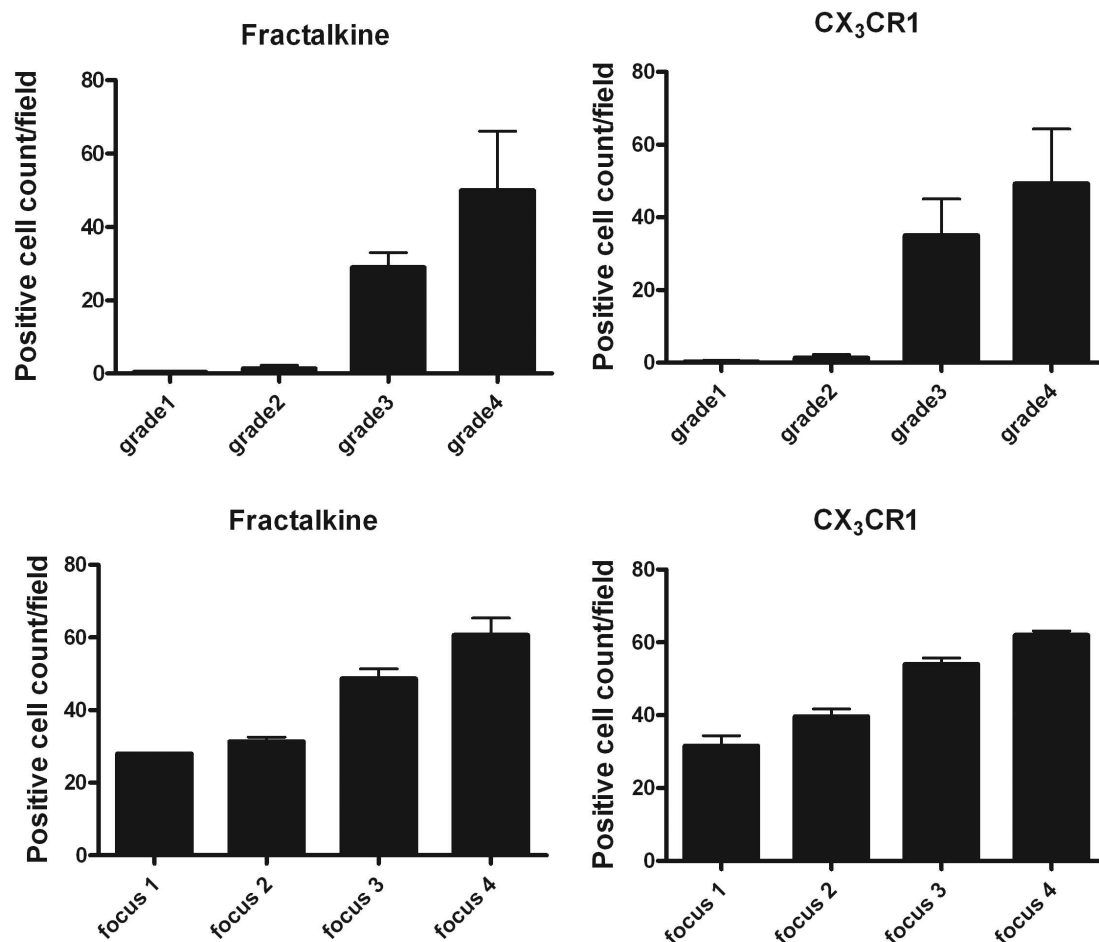


Figure 1C. Part 1.

further evaluated according to cell types of CD3 lymphocytes, CD4 T cells, CD8 T cells, and monocytes by confocal microscopic analysis. CD3 and CD4 were merged with CX3CR1 (Figure 3C). There were few cells expressing CD8 and CD14. These markers were not merged with CX3CR1.

Serum fractalkine levels in patients with pSS are correlated with proinflammatory cytokines, ANA, and IgG levels. The cytokine profiles and autoantibodies of the 43 patients with pSS were examined to identify a possible correlation between serum fractalkine levels and other serological data of patients with pSS. Because IL-1 β and IL-17 were detected in only 10 patients with pSS, Spearman's rank test was applied to the patient data. Other cytokine profiles were analyzed by Pearson's rank test. Serum fractalkine levels in the patients did not correlate with IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-8, or IL-33, but did correlate with IL-17, TNF- α , and IFN- γ levels (IL-17: $r = 0.685$, $p = 0.029$; TNF- α : $r = 0.444$, $p = 0.003$; IFN- γ : $r = 0.65$, $p < 0.001$). The regression lines for these correlations are shown in Figures 4A, 4B, and 4C. Further, we investigated which variables correlated with serum fractalkine levels; ESR, CRP, and RF titer were not

correlated, but serum fractalkine levels were correlated with ANA and IgG levels (ANA: $r = 0.349$, $p = 0.022$; IgG: $r = 0.325$, $p = 0.044$). The regression lines for these correlations are shown in Figures 4E and 4F. However, the patients with or without anti-Ro/SSA antibody did not differ significantly in terms of serum fractalkine levels ($p = 0.48$, data not shown). Anti-La/SSA antibody, in common with anti-Ro/SSA antibody, did not show statistical significance ($p = 0.462$, data not shown).

Association between serum fractalkine levels and extraglandular manifestations in patients with pSS. We also examined the relationship between serum fractalkine levels and extraglandular manifestations (EGM). Based on the presence or absence of EGM, we divided all patients with pSS ($n = 43$) into 2 subgroups. Among them, 19 patients had EGM, whereas the remaining 24 patients had only glandular symptoms. The distribution of EGM in patients with pSS were as follows: leukopenia ($n = 6$), Raynaud phenomenon ($n = 5$), polyarthritis ($n = 4$), interstitial lung disease ($n = 1$), vasculitis ($n = 1$), lymphadenopathy ($n = 1$), and skin manifestation ($n = 1$). Measurement of serum fractalkine

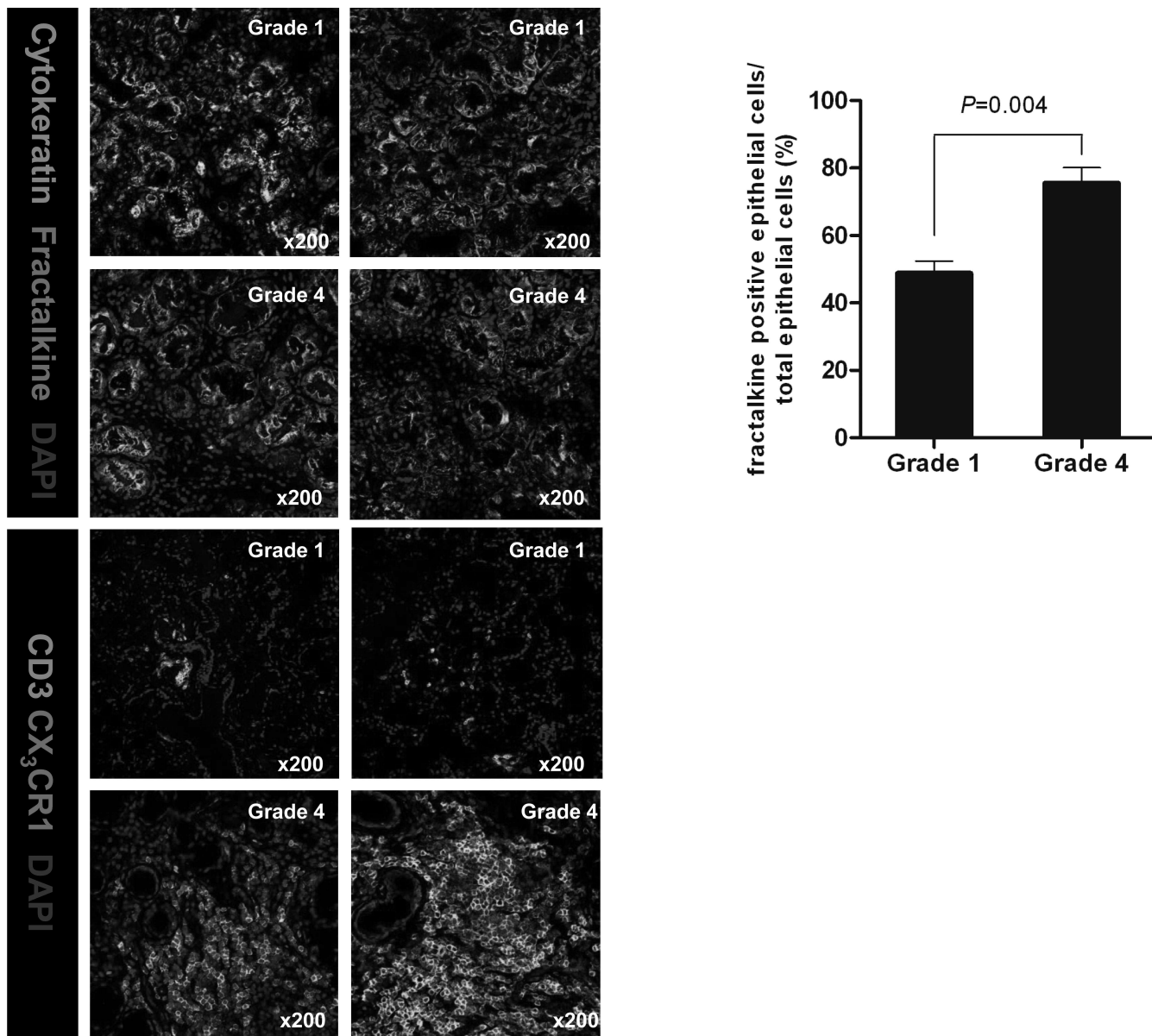


Figure 1C. Part 2.

levels in patients with EGM ($n = 19$) and without EGM ($n = 24$) revealed mean fractalkine levels of 483.31 ± 94.84 and 391.12 ± 175.62 pg/ml, respectively. Serum fractalkine levels of the subgroup with EGM were higher than those without EGM by the Mann-Whitney U test ($p = 0.026$). Figure 5 shows the differences in serum fractalkine levels between the subgroups.

DISCUSSION

We examined whether fractalkine participates in the pathogenesis of pSS, and demonstrated that fractalkine and its receptor were upregulated in LSG from patients with pSS. The fractalkine-expressing cells in LSG of patients with pSS were mainly epithelial cells. Serum fractalkine levels corre-

lated with proinflammatory cytokines, ANA, and IgG. Additionally, serum fractalkine levels in patients with EGM were higher than those without EGM. However, the populations of CX₃CR1-expressing cells among various cell types did not show notable differences in patients with pSS and controls in our study. A previous study based on SLE showed that the percentages of CX₃CR1-expressing cells increase in the PBMC of patients with SLE compared to controls¹². Moreover, serum CX₃CR1+ CD4+ cells increased in patients with inflammatory bowel disease, but not in controls¹⁶. Therefore, we investigated the phenotype of CX₃CR1-expressing cells in PBMC of patients with pSS and compared it with controls. Unexpectedly, the expression profile in CX₃CR1-expressing cells was not different from

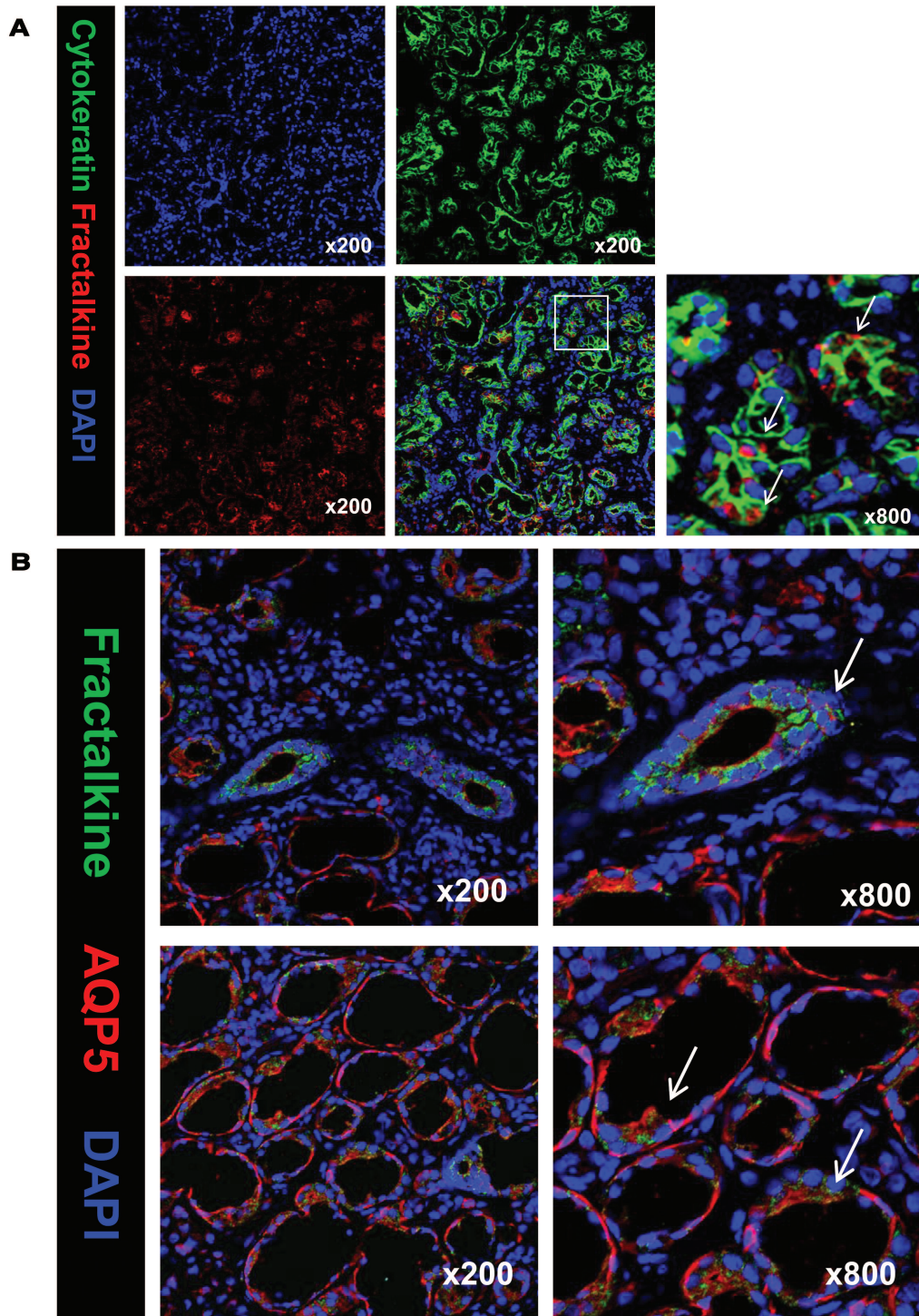


Figure 2. Confocal microscopic analysis of fractalkine-expressing cells in the LSG of patients with pSS. **A.** Immunofluorescent double-staining of fractalkine-expressing cells (red) and cytokeratin (green). **B.** Immunofluorescent double-staining of fractalkine-expressing cells (green) and aquaporin 5 (red). **C.** Immunofluorescent double-staining of fractalkine-expressing cells (green) and CD31 (white). The fractalkine-expressing cells merged with cytokeratin-positive cells and aquaporin 5 positive cells. **D.** Immunohistochemical staining using antibodies against CXCR2, CXCR3, and CXCR5. **E.** Fractalkine mRNA expression and protein levels in A253 cells after stimulation of IL-1 β , TNF- α , and IFN- γ . Fractalkine mRNA and protein levels increased after IFN- γ stimulation. LSG: labial salivary gland; pSS: primary Sjögren syndrome; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon.

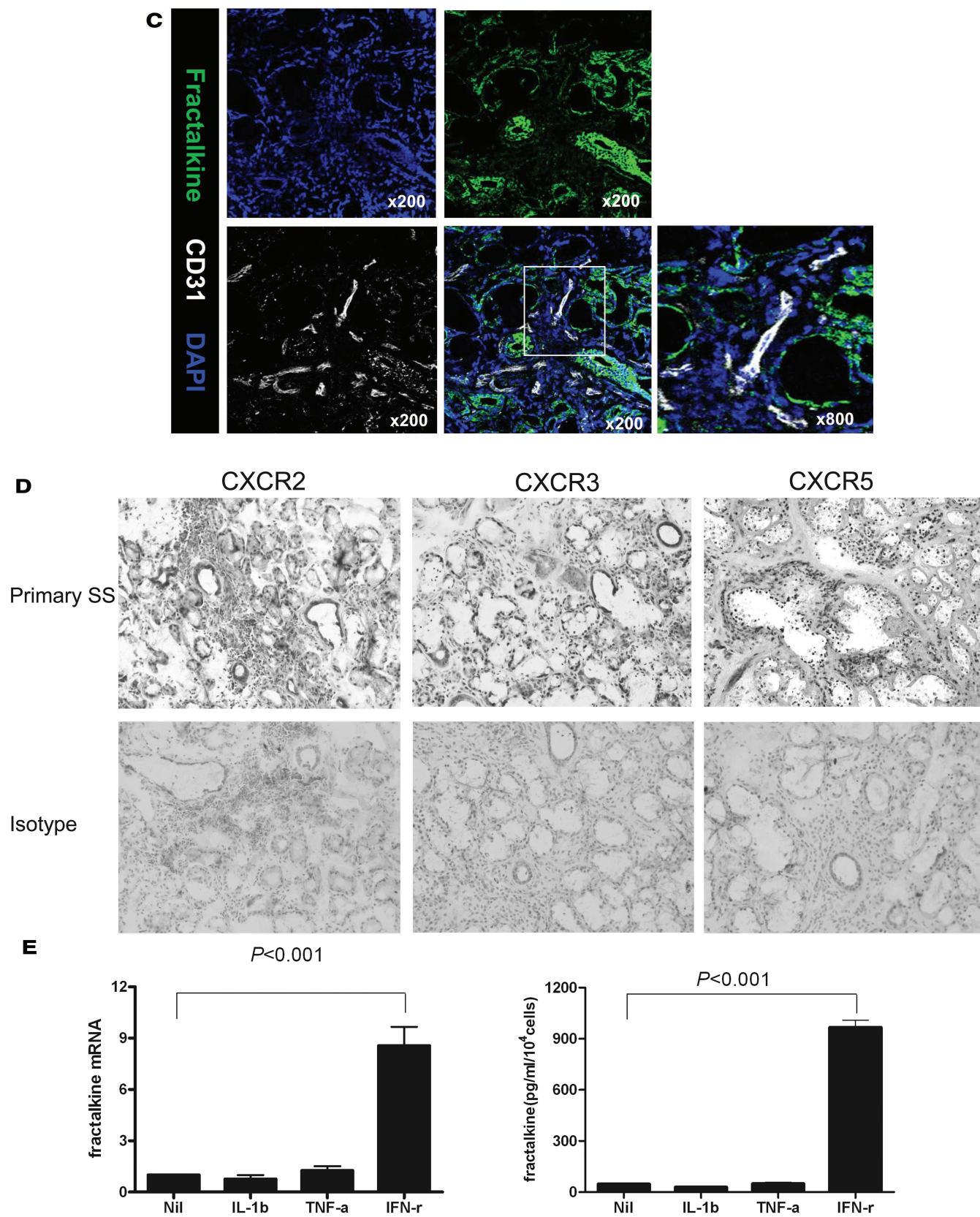


Figure 2C–E.

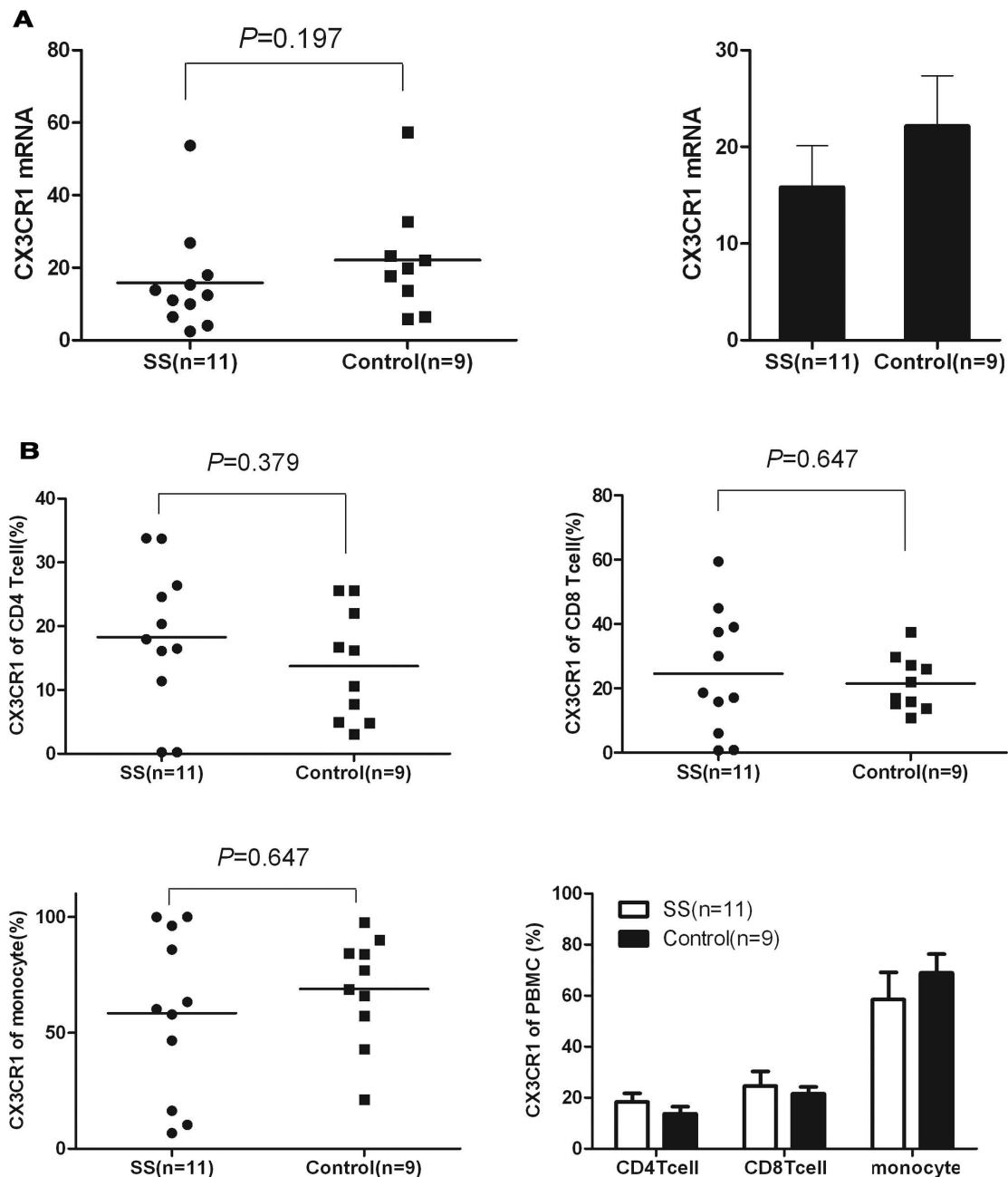


Figure 3. Fractalkine receptor expression in PBMC of patients with pSS. **A.** The patients with pSS ($n = 11$) did not show higher CX3CR1 mRNA expression in PBMC than did the controls ($n = 9$). **B.** The percentages of CX3CR1-positive cells in CD4 T cells, CD8 T cells, and monocytes of PBMC were not different between patients with pSS and controls. Horizontal bars represent median of all samples within a group. **C.** Immunofluorescent double-staining of CD3, CD4, CD8, CD14, and CX3CR1. CD3 and CD4 were merged with CX3CR1, but CD8 and CD14 were not merged with CX3CR1. PBMC: peripheral blood mononuclear cells; pSS: primary Sjögren syndrome.

that in controls. Unlike the PBMC results, expression of fractalkine/CX3CR1 in LSG of patients with pSS was higher than in controls.

Fractalkine is mainly expressed on endothelial cells, intestinal epithelial cells, and synovocyte-like fibro-

blasts^{9,17,18}. Several studies have reported that fractalkine regulates monocytes, lymphocytes, dendritic cells, and natural killer cells^{19,20}. Fractalkine is present on mucin-like stalk structures²¹. Cleavage at the base of this stalk by metalloproteinases generates a soluble chemokine that

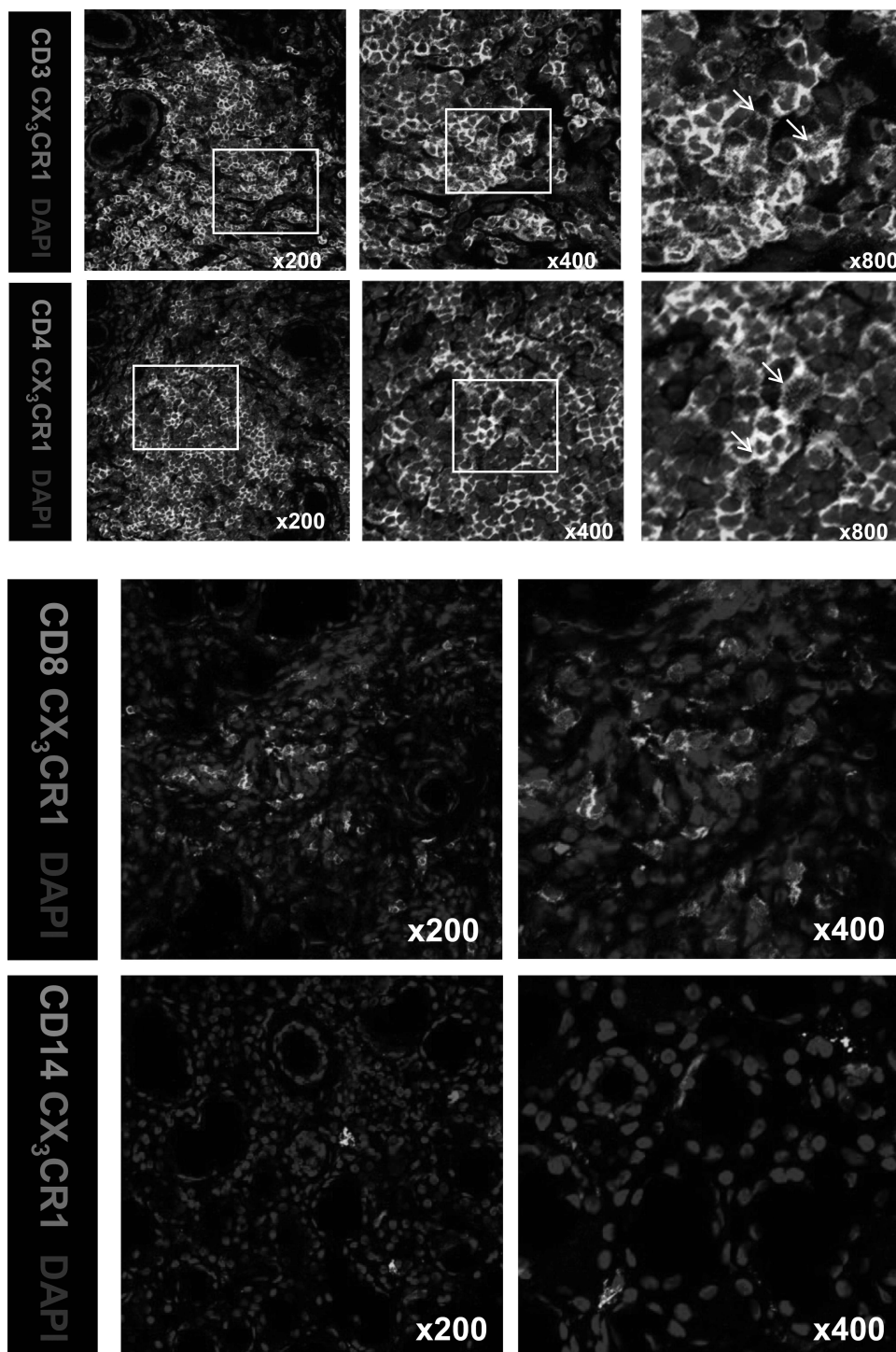


Figure 3C.

functions as a classic chemoattractant²². Fractalkine production is closely associated with proinflammatory cytokines. Juel, *et al*²³ has reported that the proinflammatory cytokine IFN- γ from T cells induces fractalkine expression in a retinal pigment epithelial cell line

(ARPE-19). These data are similar to our results. Fractalkine expression may be markedly induced by various cytokines such as TNF- α , IL-1 β , and IFN- γ from human endothelial cells⁹. These results imply that fractalkine expression might be induced in epithelial and endothelial

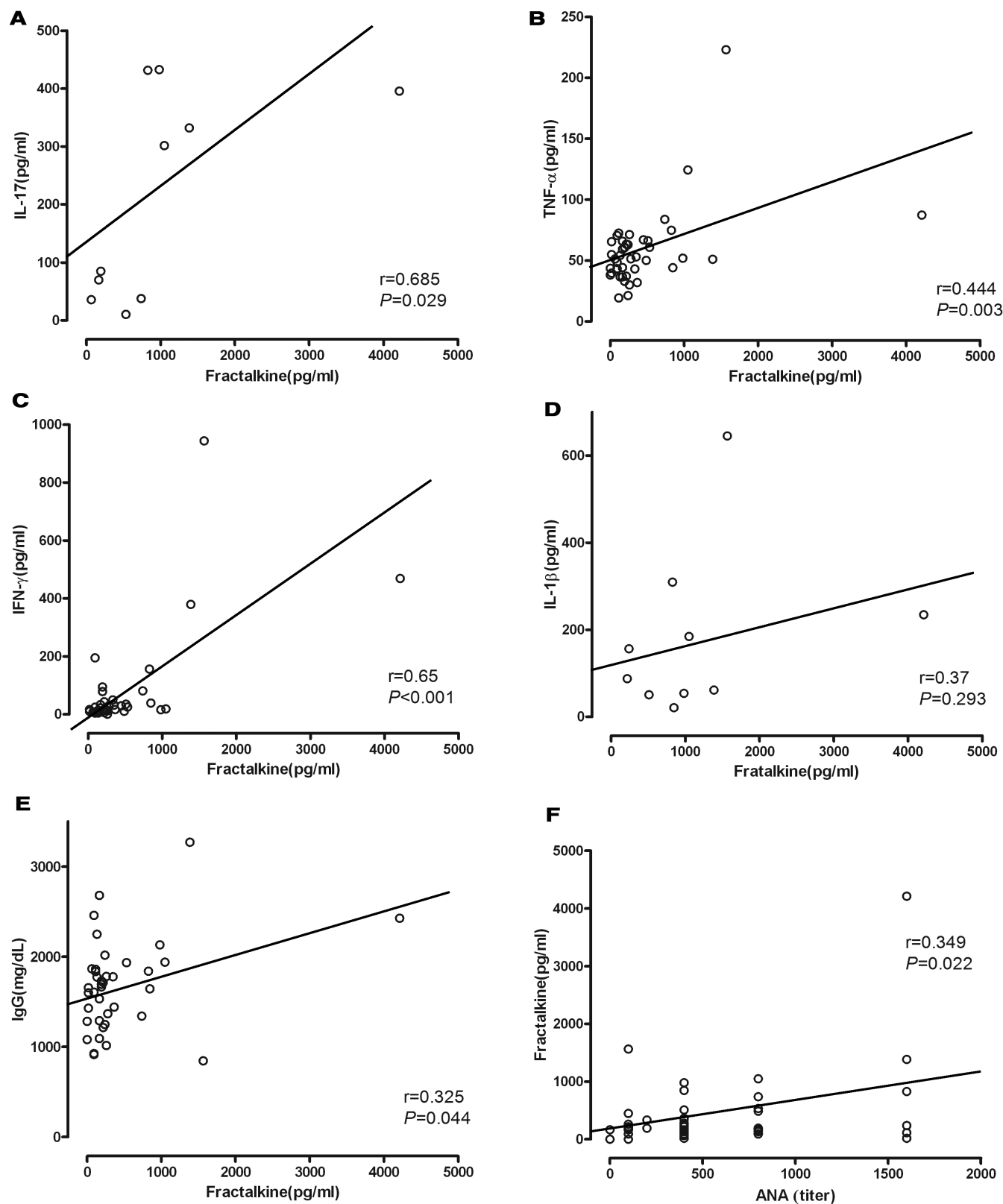


Figure 4. Correlation analysis among serum fractalkine levels, inflammatory cytokines, and autoantibodies in patients with pSS. A. Serum fractalkine levels in patients with pSS ($n = 43$) correlated significantly with serum IL-17 levels. Serum levels of TNF- α and IFN- γ were significantly correlated with serum fractalkine levels (B, C). Serum levels of IL-1 β were not correlated with serum fractalkine levels (D). Serum fractalkine levels were correlated with IgG and ANA titers (E, F). The correlation coefficients (r) and p values are shown. pSS: primary Sjögren syndrome; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; IgG: immunoglobulin G; ANA: antinuclear antibody.

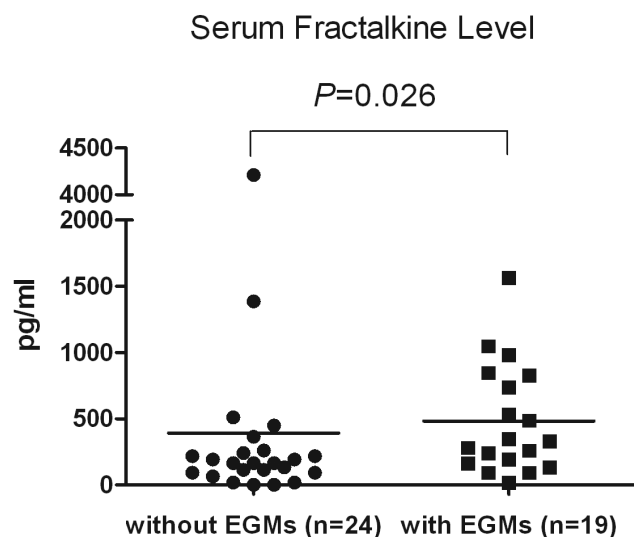


Figure 5. Increased serum fractalkine levels in patients with pSS with EGM. Each data point represents an individual subject; horizontal lines show the median values. pSS: primary Sjögren syndrome; EGM: extraglandular manifestation.

cells after stimulation by proinflammatory cytokines. However, we did not examine the mechanism responsible for the elevated fractalkine expression in pSS in our present study. Because fractalkine is expressed in secondary lymphoid follicles²⁴, this chemokine could be involved in local B cell trafficking. In a study based on the major B cell subset from tonsils, PI3K and Src family kinases were involved in signal transduction initiated by the fractalkine-CX3CR1 pathway²⁵. Considering the chemotactic function of fractalkine, these results suggest that interactions between fractalkine-expressing epithelial cells and CX3CR1-expressing immune cells in local LSG might be more important than elevated serum fractalkine levels in the pathogenesis of pSS. However, our study is not enough to support the importance of local interaction between fractalkine and CX3CR1.

In our current study, serum fractalkine levels were positively correlated with ANA and IgG levels in patients with pSS. It is unclear whether fractalkine directly affects autoantibody formation in autoimmune diseases. Tarrant, *et al*²⁶ reported that CX3CR1^{-/-} mice have a 50% decrease in antitype II collagen autoantibody formation in a collagen-induced arthritis model compared to wild-type mice. Moreover, they showed decreased Th17 intraarticular cytokine expression (IL-17 and IL-23) and a decreased total number of Th17 cells in inflamed joints of CX3CR1^{-/-} mice. Corcione, *et al*²⁵ reported that ovalbumin immunized CX3CR1^{-/-} or CX3CL1^{-/-} mice show significantly decreased specific IgG production compared to that in wild-type mice. Additionally, CX3CR1^{-/-} mice had the lowest serum antigen-specific IgG titer, followed by CX3CL1^{-/-} mice. Plasmacytoid dendritic cells (PDC) are important for their

association with autoantibody production and fractalkine. PDC are stimulated by CpG oligonucleotides by the Toll-like receptor 9, and drive purified naive B cells into IgM-producing plasma cells and trigger IgG synthesis in memory B cells in the absence of T cell assistance²⁷. A subset of CX3CR1+ CD8β+ dendritic cells shares gene signature overlap with PDC²⁸. Because PDC regulate B cell differentiation and antibody production, we considered the possibility that the subset of CX3CR1+ CD8β+ affects antibody production. Therefore, fractalkine might affect autoantibody formation indirectly in patients with autoimmune diseases.

The EGM in patients with pSS can be divided into 2 categories in which periepipithelial organ involvement is the result of lymphocytic invasion beyond the exocrine glands. The extraepithelial manifestations are produced from an immune complex deposit attributable to ongoing B cell hyperreactivity²⁹. A previous report showed that hypergammaglobulinemia correlates significantly with the presence of EGM such as palpable purpura, lymphadenopathy, and splenomegaly³⁰. When we divided patients into 2 subgroups based on the presence or absence of EGM, we found that fractalkine levels were elevated in patients with EGM rather than those without EGM. Considering that fractalkine might affect autoantibody formation, this finding was predictable. Several reports have indicated that a T cell subset is associated with autoantibody formation and EGM in patients with pSS^{4,7,31}. Follicular helper T (TFH) cells, defined as CD4+CXCR5+ICOS+PD-1+ T cells, mediate activation of antigen-specific naive or memory B cells that trigger germinal center formation through secretion of IL-21³². The percentage of peripheral TFH cells increases significantly in patients with EGM⁷. However, there are insufficient reports indicating that TFH cells express fractalkine.

Germinal centers are composed of centrocytes, centroblasts, follicular dendritic cells (FDC), and TFH cells. They are important sites for immunoglobulin class switching. B cells can be selected to terminally differentiate into long-lived memory B cells and memory plasma cells in germinal centers^{33,34}. FDC have a pivotal role in selecting centroblasts for maturation. Foussat, *et al*³⁵ reported that FDC express fractalkine in hyperplastic lymph nodes by immunohistochemistry. The fractalkine gene is also expressed by the FDC-like HK cell line, B cell tumor of germinal center origin, and CD40-activated B cells *in vitro*, but not by normal B cells. Considering that fractalkine is produced in germinal centers and that activated Th cells express CX3CR1, fractalkine might have a role in the migration of T cells to germinal centers. Moreover, fractalkine expression by CD40-activated B cells may participate in the cognate T-B interaction for maturation of the B cell response. However, the precise roles of fractalkine in the germinal center are still poorly understood.

We cautiously suggest use of fractalkine in the future. Fractalkine is associated with disease activity in SLE^{12,36}. Moreover, there are several reports that the crosstalk between the fractalkine/CX3CR1 system and tumor microenvironment affect mature B cell malignancies³⁷. Therefore, fractalkine may be a candidate representing disease activity and be a predictor for lymphoma development in pSS. However, further studies are needed to evaluate fractalkine and its mechanisms of signal pathway action in autoimmune diseases, which may lead to a novel therapeutic strategy for treating pSS.

Fractalkine and CX3CR1 may play a role in the pathogenesis of pSS, including EGM. Fractalkine showed a good correlation with proinflammatory cytokines, ANA, and IgG.

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