The levels of CXCL12 and its receptor, CXCR4, as a biomarker of disease activity and cutaneous manifestation in adult-onset Still's disease

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ABSTRACT

Objective. *This study evaluated the SDF-1/CXCL12 and soluble CXCR4 (sCXCR4) levels, and investigated their clinical relevance in adult-onset Still's disease (AOSD).*

Methods. *Forty-two AOSD patients and 30 healthy controls (HC) were enrolled for serum sampling. Expression levels of CXCL12 and CXCR4 in skin biopsy materials of 40 AOSD patients, 10 patients with eczema, or 10 psoriasis, and 10 HC skin were evaluated with immunohistochemistry.*

Results. *The serum CXCL12 levels in patients with AOSD (2,452±1,531 pg/mL) were higher than those in HC (1,708±1,322 pg/mL, p=0.017). The serum sCXCR4 levels in patients with AOSD (14,449±16,627 pg/mL) were higher than those in HC (3,046±2,554 pg/mL, p<0.001). Serum CXCL12 levels correlated positively with counts of leukocytes and neutrophils, erythrocyte sedimentation rate, ferritin, and C-reactive protein (CRP). Serum sCXCR4 levels correlated positively with systemic scores, platelet counts, and CRP levels. The serum levels of CXCL12 and sCXCR4 were decreased significantly in the patients with AOSD followed after resolution of disease activity. On immunohistochemical stain, the mean percentage of CXCR4-positive inflammatory cells was 51.4±27.5% and that of CXCL12-positive inflammatory cells was 16.7±13.3% in AOSD patients. CXCR4 was more frequently expressed in inflammatory cells from AOSD patients than in those with eczema or psoriasis and HC skin.*

Conclusion. *These results demonstrate that sCXCR4 could be a clinical biomarker of evaluation for disease activity in AOSD, and show that CXCR4/CXCL12 may influence the inflammatory condition and skin manifestations of AOSD.*

Introduction

Adult-onset Still's disease (AOSD) is an uncommon autoinflammatory disorder involving several organs including skin, joints, liver, heart, and spleen. It has a variety of clinical features, such as a high fever, myalgia, sore throat, maculopapular erythema and organomegaly, and complications may arise due to macrophage activation syndrome and haepatic failure (1). Although pathogenesis of AOSD is unknown, certain aetiologic causes including genetic predisposition and environmental factors, such as viral or bacterial infections, have been considered to play a part in developing the disease. In genetically vulnerable individuals, environmental factors can trigger a systemic autoinflammatory condition, such as inflammasome activation or immune dysregulation with overproduction of proinflammatory cytokines (1-5).

Chemokines were introduced to small-sized (8-14 kDa) chemotactic cytokines that provide directional guidance for the migration of leukocytes (6). They play important roles in cell movement, proliferation, and differentiation by binding to their receptors (7). Several chemokines have been reported in patients with AOSD and may serve as biomarkers for predicting disease activity or the persistence of arthritis (8-11). Interleukin-8 (IL-8) levels, also known as CXC motif chemokine 8 (CXCL8), have been suggested as a clinical marker of the persistence with arthritis in patients with AOSD (11, 12). Recent studies reported that the serum and skin levels of CXCL9, CXCL11, and CXCL13 of patients with AOSD were increased in comparison with those of healthy controls (HC), and were associated with certain laboratory markers for disease activity, suggesting that they could be pathogenic and clinical markers (9, 10). However, studies on the

various chemokines involved in AOSD are extremely limited.

The chemokine CXCL12 is an important member of the CXC chemokine family known as stromal-derived factor (SDF)-1α, and has two receptors (CXCR7 and CXCR4) (13). The SDF-1/CXCL12-CXCR4 axis is known to have roles in cell migration, survival, and proliferation, and also influences the leukocyte count in bone marrow (14). A study described that the synovial fluid level of CXCL12 was increased in patients with rheumatoid arthritis (RA) in comparison with those of osteoarthritis (OA) (15). Moreover, a recent study revealed higher serum CXCL12 levels in systemic lupus erythematosus (SLE) than HC (16). This chemokine with its receptor appears to be associated in a few rheumatic disorders including SLE, Sjögren's syndrome, systemic sclerosis and RA (4, 15-18). Although the CXCR4 ligand could play a role in AOSD, no study has investigated its involvement in AOSD.

Therefore, in this study, we investigated the serum CXCL12 and soluble CXCR4 (sCXCR4) levels in AOSD patients, and evaluated their clinical relevance to the disease. To determine the cutaneous manifestation of CXC12 and CXCR4 in AOSD, we confirmed immunohistochemical staining with biopsy specimens of the skin materials from 40 patients with untreated active AOSD for CXCL12 and its receptor, CXCR4.

Materials and methods

Subjects

Forty-two AOSD patients and thirty HC were recruited for the present study. The diagnosis of all AOSD patients was confirmed according to the Yamaguchi's criteria with the exclusion of several diseases that may accompany fever, such as infectious, neoplastic, or other autoimmune disorders (19). HC without any history of rheumatic disease were recruited by advertisements placed in our hospital. Serum samples were collected from the participants, and stored in a -70°C freezer for enzyme-linked immunosorbent assay (ELISA). Among the 42 AOSD patients, 29 patients had initial active condition before initiation of treatment. Ten AOSD patients were in flares during follow-up, and the other 3 patients were in the remission stages at the time of sampling. Medical past and current histories, clinical signs and symptoms, and the physical examination findings were collected into a database with results of laboratory test. Disease activities of AOSD were evaluated according to systemic score described previously (20). The Institutional Review Board (IRB) in Ajou University Hospital approved the present study (AJIRB-MED-CT3-16-112), and written informed consent was received from the participants.

Measurement of serum CXCR4 and CXCL12 levels

Serum sCXCR4 levels of participants were assessed with a commercial ELISA kit (LSBio, Seattle, WA, USA) by the manufacturer's instructions. Also, serum CXCL12 levels were assessed with a kit for ELISA (R&D Systems, Minneapolis, MN, USA).

Histopathologic evaluation of skin specimens

Skin biopsy materials were retrospectively collected from 40 active AOSD patients for evaluation of skin rash between 2000 and 2017. For this evaluation, informed consent was waived by the IRB because of retrospective nature. Three pathologists (HY, JEK, and JHH) reviewed independently all slides of haematoxylin and eosin-stained sections for evaluation of skin histological parameters, such as epidermal changes including vacuolisation of basal keratinocyte, epidermal necrosis and parakeratosis, and the presence of macrophage infiltration, inflammatory cell infiltration, interstitial mucin deposition, and karyorrhexis.

Immunohistochemical evaluation of skin specimens

Expression levels of CXCL12 and CXCR4 in skin biopsies were measured with 40 patients with active AOSD, 10 eczema, 10 psoriasis, and 10 HC were investigated via immunohistochemistry. We used the lymphoid cell staining pattern in paracortical zone of tonsil as a positive control. Immunohistochemistry was performed as previously described (10). The primary antibodies were used according to the each described concentration: 200 for CD68 (Novocastra Laboratories Ltd, Newcastle, UK); 1:10 for CD4 and 1:50 for CD8 (Thermo Fisher Scientific, Fremont, CA, USA); 1:50 for CXCL9, CXCL10, and CXCL11 (R&D Systems); 1:20 for CXCR3 (R&D Systems); 1:50 for CXCL12 and 1:20 for CXCR4 (Abcam, Cambridge, UK). Detection was confirmed by a Ventana Optiview DAB kit (Ventana Medical Systems Inc.). Granular cytoplasmic staining pattern was considered positive (Fig. 2E). Scores of the chemokines were calculated as percentages of the stained inflammatory cell number per the all infiltrating inflammatory cell number in whole biopsied tissues.

Statistical analyses

The shown data are described as frequency (%) or means ± standard deviation. Group difference of the serum levels of CXCL12 and sCXCR4 was performed with the Mann-Whitney U-test. Difference of the serial serum chemokine levels in patients who were followed was performed with the Wilcoxon signed-rank test. Associations between disease activity markers and the serum chemokine levels were evaluated with the Pearson's correlation. Group differences in skin immunohistochemistry levels of CXCL12 and CXCR4 was performed with the Mann-Whitney U-test. Statistical analyses were assessed by SPSS software (v. 23.0; SPSS Inc., Chicago, IL, USA). A p-value <0.05 was regarded as a statistically significant value.

Results

Clinical characteristics of patients with AOSD and HC

The clinical characteristics and laboratory findings of the participants were shown in Table I. The female of AOSD patients was 88.1%. The mean age of patients with AOSD was 43.8±15.3 years, and that of HC was 39.0±12.6 years. The age and gender between the two groups were not different significantly. The main clinical symptoms were skin rash (n=32; 76.2%), high spiking fever (n=36; 85.7%), arthri-

Table I. Clinical characteristics of the patients.

	AOSD (n=42)	HC (n=30)	*p*-value
Age (years)	43.8 ± 15.3	39.0 ± 12.6	0.167
Gender (F/M)	37/5	26/4	1.000
Fever	36 (85.7)		
Sore throat	19 (45.2)		
Skin rash	32 (87.2)		
Lymphadenopathy	10 (23.8)		
Splenomegaly	6 (14.3)		
Hepatomegaly	4 (9.5)		
Pericarditis	5 (11.9)		
Pleuritis	6 (14.3)		
Arthralgia	39 (92.9)		
Arthritis	24 (57.1)		
Haemoglobin, g/dL	11.3 ± 1.7		
Leukocyte, /μL	13,279 ± 9,227		
Neutrophil, /μL	11,210 ± 9229.9		
Eosinophil, /μL	198 ± 301		
Platelet, $\times 10^3$/μL	282.8 ± 111.7		
Ferritin, ng/mL	3,753.3 ± 5,347.3		
ESR, mm/h	54.6 ± 24.9		
CRP, mg/dL	7.00 ± 6.35		
AST, mg/dL	60.5 ± 63.2		
ALT, mg/dL	68.6 ± 94.1		
Bilirubin	0.54 ± 0.34		
Albumin	3.90 ± 0.54		
ANA positivity	2 (4.8)		
RF positivity	2 (4.8)		
Systemic score	4.57 ± 1.61		
Glucocorticoid dose (mg/d, prednisolone equivalent)	10.2 ± 9.3		

AOSD: adult onset Still's disease; HC: healthy controls; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; AST: aspartate transaminase; ALT: alanine transaminase; ANA: antinuclear antibody; RF: rheumatoid factor. All values are presented as numbers (with percentages) or means ± SD. The systemic scoring system of Pouchot *et al.*[4] assigns a score from 0 to 12, with 1 point for each of the following manifestations: fever, typical rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function test data, splenomegaly, lymphadenopathy, leukocytosis ≥15,000/mm^2, sore throat, myalgia, and abdominal pain.

tis (n=24; 57.1%), sore throat (n=19; 45.2%), and hepatomegaly (n=4; 9.5%) in the patients with AOSD.

Serum CXCL12 and sCXCR4 levels

The serum sCXCR4 and CXCL12 levels of the AOSD patients and HC were shown in Figure 1. The serum sCXCR4 levels of the AOSD patients (14,449±16,627 pg/mL) were increased in comparison with those of the HC (3,046±2,554 pg/mL, $p<0.001$) (Fig. 1A). Moreover, the CXCL12 levels of the AOSD patients (2,452±1,531 pg/mL) were increased in comparison with those of the HC (1,708±1,322 pg/mL, $p=0.017$) (Fig. 1B).

The serum sCXCR4 and CXCL12 levels were not different according to clinical pattern of AOSD (monocyclic, polycyclic and chronic articular) in 29 patients with initial active disease activity before treatment (data not shown). The sCXCR4 and CXCL12 levels were compared among patients with initially active condition, those in flare and in remission. The serum levels of sCXCR4 were increased in 29 initially active AOSD patients (15,477±17,842 pg/mL) and 10 flare patients (15,245±14,551 pg/mL) compared to those of the 3 remission patients (1,859±1,579 pg/mL, $p=0.031$). Furthermore, we evaluated that CXCL-12 levels were affected by glucocorticoid treatment. However, the CXCL-12 levels were not associated with glucocorticoid treatment (r=-0.140, $p=0.680$).

Follow-up blood sampling was done at 9.2±7.8 months after the first serum samplings in 13 patients with AOSD. The follow-up mean systemic score was 0.31±0.63 after treatment with corticosteroid and disease modifying antirheumatic drugs. The mean ESR was 8.3±10.3 mm/hr, and the mean CRP was 0.30±0.62 mg/dL. The follow-up serum levels of sCXCR4 and CXCL12 were 7,910± 3,666 and 1,838 ±913 pg/mL, respectively. The follow-up serum levels of sCXCR4 and CXCL12 fell significantly in comparison with those of the initial serum samples ($p=0.006$ and 0.028, respectively; Fig. 1C-D).

Association between serum CXCL12 and CXCR4 levels and several disease activity markers

Table II shows the associations between the serum sCXCR4 and CXCL12 levels and several known disease activity markers in AOSD patients. Serum levels of sCXCR4 were associated with systemic scores (r=0.429, $p=0.005$), platelet counts (r=0.442, $p=0.003$), and CRP levels (r=0.391, $p=0.010$). Furthermore, serum levels of CXCL12 were associated with ESR (r=0.310, $p=0.046$), and levels of CRP (r=0.449, $p=0.003$) and ferritin (r=0.343, $p=0.026$), and with counts of leukocyte (r=0.338, $p=0.029$) or neutrophil (r=346, $p=0.025$).

Immunohistochemical data

The pattern of immunohistochemical stain in inflammatory cells of skin materials was similar to that of positive controls (Fig. 2-3). Table III shows the results of immunohistochemical stain for CXCR4 and CXCL12. The mean CXCR4-positive inflammatory cell percentage was 51.4±27.5% (range from 5 to 90%) and mean CXCL12-positive inflammatory cell percentage was 16.7±13.3% (range from 1 to 50%). The mean CXCR4-positive inflammatory cell percentage in skin materials of patients with AOSD was significantly higher than that of HC ($p=0.001$), and patients with eczema ($p=0.003$) or psoriasis ($p=0.001$). However, the mean CXCL12-positive inflammatory cell percentage in skin materials from patients with AOSD was significantly higher than that from HC ($p=0.034$), but was not different from that in psoriasis ($p=0.465$) or eczema ($p=0.729$). Inflammatory cells expressing CXCL12 and CXCR4 were not different according to the presence of macrophage infiltration, keratinocyte vacuolisation, or interstitial mucin deposition (Supplementary Table S1).

The associations between the CXCR4- or CXCL12-positive inflammatory cell

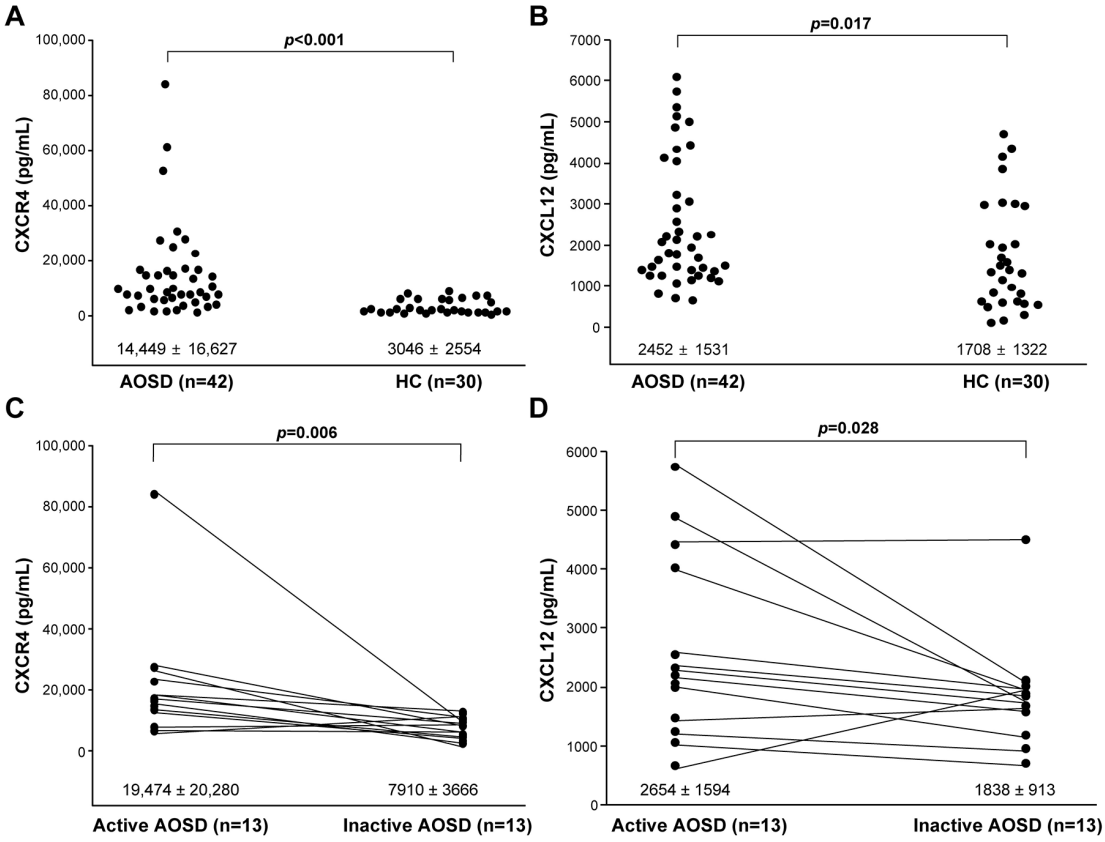

Fig. 1. The serum levels of CXC motif chemokine 12 (CXCL12) (**A**) and CXCR4 (**B**) in 42 adult-onset Still's disease (AOSD) patients and 30 healthy controls (HC). The Mann-Whitney U-test was performed for this data. The follow-up serum levels of CXCL12 (**C**) and CXCR4 (**D**) in 13 AOSD. The data was evaluated with the Wilcoxon signed-rank test. All data are expressed as means ± SD.

Table II. Correlations of CXCL12 and CXCR4 levels with disease activity markers and between each other in 42 AOSD patients.

Disease activity markers	Correlation coefficient, r (*p*-value)	
	CXCR4	CXCL12
Systemic score	**0.429 (0.005)**	0.192 (0.224)
Leukocyte	0.221 (0.160)	**0.338 (0.029)**
Neutrophil	0.214 (0.174)	**0.346 (0.025)**
Eosinophil	-0.148 (0.350)	-0.253 (0.106)
Haemoglobin	-0.031 (0.844)	0.173 (0.272)
Platelet	**0.442 (0.003)**	0.046 (0.773)
ESR	0.290 (0.062)	**0.310 (0.046)**
CRP	**0.391 (0.010)**	**0.449 (0.003)**
Ferritin	0.085 (0.594)	**0.343 (0.026)**
LDH	-0.085 (0.593)	0.216 (0.170)
Albumin	-0.157 (0.320)	-0.285 (0.068)
Bilirubin	-0.188 (0.233)	-0.099 (0.534)
AST	-0.131 (0.410)	0.188 (0.233)
ALT	-0.115 (0.470)	0.058 (0.713)
CXCL12	0.266 (0.088)	

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; LDH: lactate dehydrogenase; AST: aspartate transaminase; ALT: alanine transaminase. Pearson's correlation coefficients were calculated. The systemic scoring system of Pouchot *et al.*[4] assigns a score from 0 to 12, with 1 point for each of the following manifestations: fever, typical rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function test data, splenomegaly, lymphadenopathy, leukocytosis ≥15,000/mm^2, sore throat, myalgia, and abdominal pain. These data were assessed by Pearson's correlation.

percentage and the inflammatory cell percentage expressing another markers, such as CD4, CD8, CXCR3, CXCL9, and CXCL10, in the AOSD skin materials are shown in Supplementary Table S2. CXCR4 and CXCL12 levels were not correlated with any grade of another markers, and only CXCL12 and CXCL10 levels correlated each other (r=0.36, *p*=0.04).

Discussion

This study evaluated serum levels of CXCL12 and its receptor, CXCR4, to confirm the clinical association of these markers in patients with AOSD, and these levels were compared to those of HC. Furthermore, we demonstrated that CXCL12 and sCXCR4 serum levels were associated with those of several inflammatory markers, as well as with systemic scores, in patients with AOSD. The serum sCXCR4 and CXCL12 levels were decreased significantly in follow-up sampling of AOSD patients after treatment. Moreover, we confirmed that CXCL12 and CXCR4 expression in skin lesions taken from patients with AOSD.

Several studies reported high levels of CXCL12 in synovial joints in RA compared to OA and HC (21-23). CXCL12 and CXCR4 have been suggested to stimulate angiogenesis and mononuclear cell infiltration into the joints in the RA synovium (21-23). One study found that mean plasma CXCL12 level in RA patients was elevated in comparison with that in HC, but also that CXCL12 levels were independent of

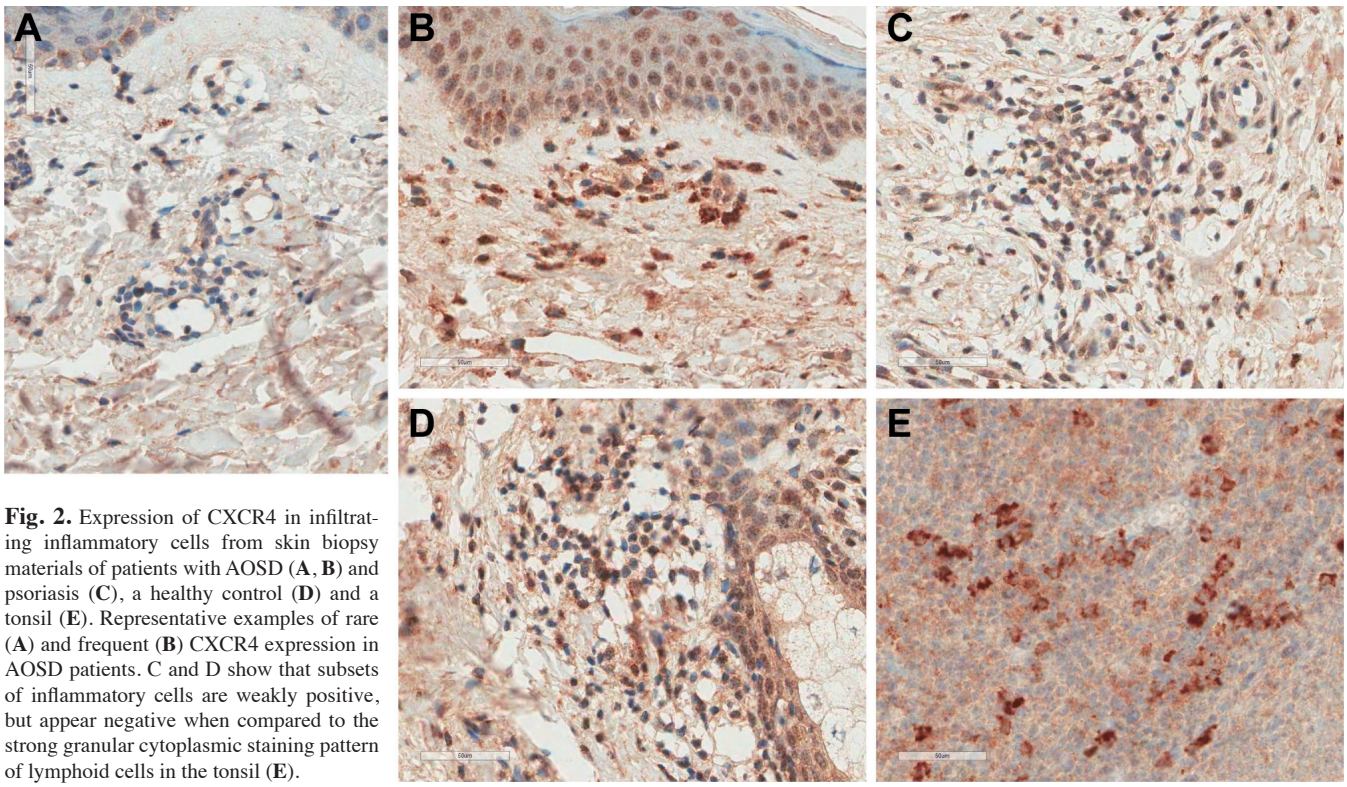

Fig. 2. Expression of CXCR4 in infiltrating inflammatory cells from skin biopsy materials of patients with AOSD (**A**, **B**) and psoriasis (**C**), a healthy control (**D**) and a tonsil (**E**). Representative examples of rare (**A**) and frequent (**B**) CXCR4 expression in AOSD patients. C and D show that subsets of inflammatory cells are weakly positive, but appear negative when compared to the strong granular cytoplasmic staining pattern of lymphoid cells in the tonsil (**E**).

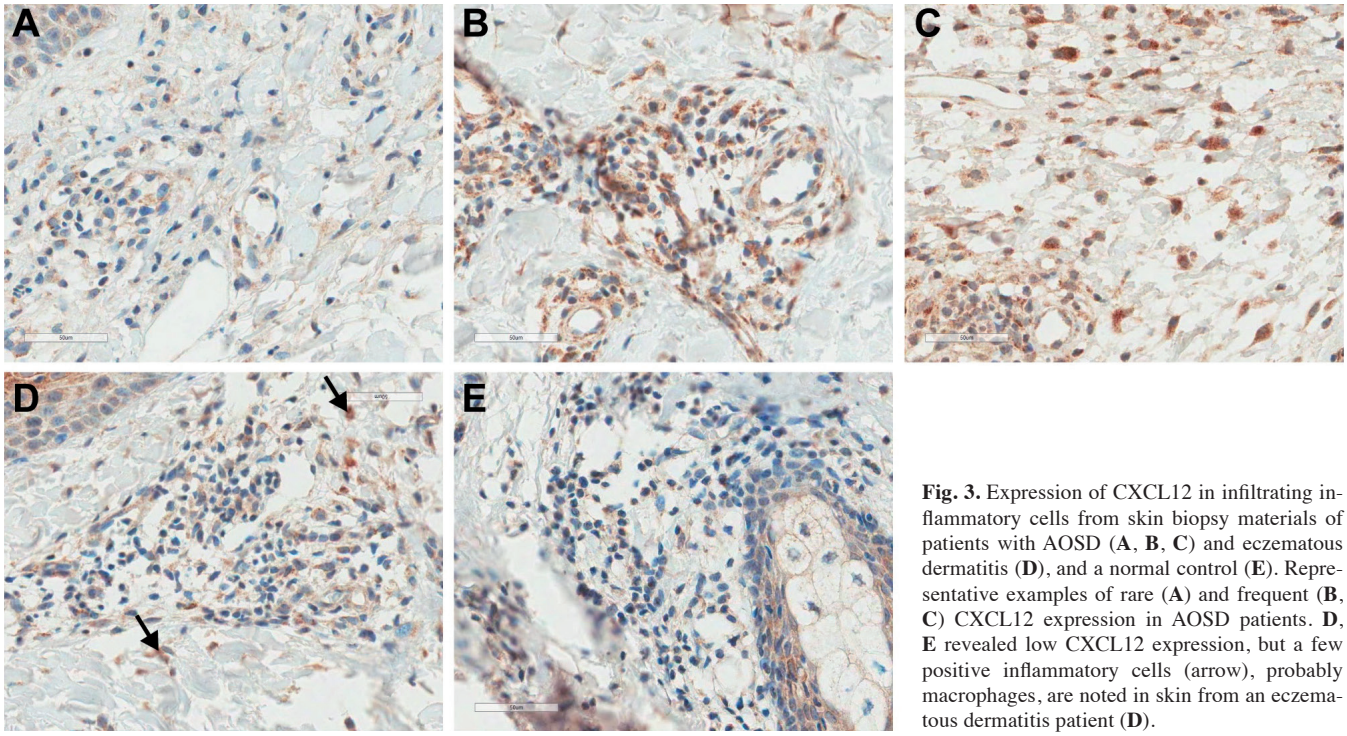

Fig. 3. Expression of CXCL12 in infiltrating inflammatory cells from skin biopsy materials of patients with AOSD (**A**, **B**, **C**) and eczematous dermatitis (**D**), and a normal control (**E**). Representative examples of rare (**A**) and frequent (**B**, **C**) CXCL12 expression in AOSD patients. **D**, **E** revealed low CXCL12 expression, but a few positive inflammatory cells (arrow), probably macrophages, are noted in skin from an eczematous dermatitis patient (**D**).

disease activity markers in RA, in addition to the response to methotrexate treatment (24). A recent study evaluated whether the CXCL12 and CXCR4 levels in synovial tissue correlated with the joint destruction and prognosis of enrolled RA patients treated by golimumab. In addition, they showed that synovial CXCL12 levels were associated with some clinical markers for disease activity, and that CXCR4 levels were associated with joint destruction (25). In this study, we found that the serum expression of CXCL12 and sCXCR4 in AOSD patients was significantly increased than that in HC. In addition, the serum CXCL12 levels were associated with a few markers for disease activity including counts of leukocytes and neutrophils, ESR, CRP, and ferritin, but not the levels of systemic score. Moreover,

Table III. CXCR4 and CXCL12 immunostaining results in skin biopsies from 40 AOSD patients, 10 psoriasis patients, 10 eczema patients, and 10 healthy controls (HC).

	Staining cell percent AOSD	Staining cell percent psoriasis	*p*-value (AOSD vs. psoriasis)	Staining cell percent eczema	*p*-value (AOSD *vs*. eczema)	Staining cell percent HC	*p*-value (AOSD *vs*. HC)
CXCR4	51.4 ± 27.5	15.5 ± 6.8	0.001	20.5 ± 6.9	0.003	18.0 ± 7.5	0.001
CXCL12	16.7 ± 13.3	22.7 ± 19.5	0.465	13.0 ± 6.7	0.729	8.0 ± 2.6	0.034

P-values determined using the Mann-Whitney U test. *P*-values were <0.001 and 0.100 for CXCR4 and CXCL12, respectively, in one-way analysis of variance.

the serum sCXR4 levels were associated with the CRP levels, platelet counts and systemic scores. Furthermore, the follow-up serum sCXCR4 and CXCL12 levels were decreased significantly after treatment. These data suggest that the chemokine CXCL12-CXCR4 axis is activated in AOSD, and that its axis could contribute to the inflammation and disease activity of AOSD. In other respects, it is possible to speculate a non-specific increase of sCXCR4 due to disease activity as a decoy receptor (26). Therefore, further *in vitro* studies are needed to understand why sCXCR4 is increased in AOSD. Also, serum CXCL12 and sCXCR4 levels were not different among patients with different AOSD patterns, including chronic articular AOSD. Elevated CXCL12 and sCXCR4 levels could affect synovial inflammation similar to RA synovial inflammation in AOSD, but we did not evaluate levels of CXCL12 or CXCR4 in synovium of patients with AOSD. Thus, further larger sample size studies with synovial fluid are needed for confirming the influence of the CXCL12 and CXCR4 on the inflammation of chronic articular pattern in AOSD.

CXCL12-CXCR4 axis signaling has been reported to have an important role for the angiogenesis and vascular remodeling associated with some inflammatory skin diseases (27, 28). In particular, the CXCL12 and CXCR4 expression was increased on inflamed skins, including imiquimod-induced skin inflammation and a psoriasis mouse model (27). Furthermore, skin inflammation in both models was inhibited after treatment with the CXCR4 antagonist, with an association seen between reduced inflammatory angiogenesis and inflammatory cell accumulation. In this study, we evaluated the CXCL12 and CXCR4 expression in skin materials taken from patients with AOSD and compared these chemokine levels to those of HC and the skin material of psoriasis and eczema patients. Interestingly, the mean CXCR4-positive inflammatory cell number in the skin lesions from patients with AOSD was higher than that of HC, and psoriasis or eczema patients. However, the mean CXCL12-positive inflammatory cell number on the skin materials of AOSD patients was higher than that of HC alone, but did not differ from the numbers in the lesions of patients with eczema or psoriasis. We showed the different profiles in expression of CXCL12 and CXCR4, among several inflammatory skin diseases, *i.e*., eczema, psoriasis, and AOSD skin rash. The diverse profiles of the CXCL12-CXCR4 in skin lesions could be explained by differences of the inducer and producer cells present according to the inflammatory environment of these diseases. Furthermore, these chemokines are suggested to associate with angiogenesis and fibrosis, therefore, chronic skin lesions, such as eczema and psoriasis, could be associated with these chemokine axis. However, AOSD skin lesions are almost acute lesions and not related with fibrosis, except atypical skin lesions. Therefore, further study should be evaluated for difference of the chemokine expressions according to typical acute and atypical chronic skin lesions in AOSD.

This study had some limitations, including a lack of patients with other febrile disorders, malignant diseases or rheumatic diseases associated with an increased inflammatory cell expression of CXCL12, as controls. In addition, we were unable to measure the expression of CXCL12 and its receptors in follow-up skin materials of patients with AOSD, due to the absence of such biopsies. However, this study is the first to investigate CXCL12 and CXCR4 levels from serum and skin biopsies of patients with AOSD.

In conclusion, this study confirmed significantly increased CXCL12 and CXCR4 levels in serum and skin lesions taken from AOSD patients. These data show that CXCL12 and CXCR4 may play a role in inflammatory condition and skin manifestations in AOSD.

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