

# Expression of fractalkine and its receptor, CX3CR1, in atopic dermatitis: Possible contribution to skin inflammation

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**Expression of fractalkine and its receptor, CX<sub>3</sub>CR1,  
in atopic dermatitis: possible contribution to skin inflammation**

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## ABSTRACT

**Background:** Fractalkine (FKN) induces activation and adhesion of leukocytes expressing its receptor, CX<sub>3</sub>CR1. FKN is released from the cell surface by proteolytic cleavage as soluble FKN (sFKN).

5 **Objective:** To assess FKN and CX<sub>3</sub>CR1 expression in the skin, serum sFKN levels, and CX<sub>3</sub>CR1 expression on blood leukocytes in atopic dermatitis (AD) patients.

**Methods:** FKN and CX<sub>3</sub>CR1 expression in the skin was examined immunohistochemically. mRNA expression of FKN, thymus and activation regulated chemokine (TARC), and macrophage derived chemokine (MDC) in the skin was  
10 assessed by real-time RT-PCR. Serum sFKN levels were assessed by ELISA. Blood leukocytes were stained for CX<sub>3</sub>CR1 with flow cytometric analysis.

**Results:** FKN was strongly expressed on endothelial cells in skin lesions of AD and psoriasis, but not in normal skin. FKN mRNA levels in AD lesional skin increased to a similar extent to TARC and MDC mRNA levels. CX<sub>3</sub>CR1-expressing cells in the  
15 affected skin of patients with AD or psoriasis increased compared with normal skin. Serum sFKN levels were elevated in AD patients but not in psoriasis patients relative to normal controls. Serum sFKN levels were associated with the disease severity, and decreased with the improvement of skin lesion in AD patients. The frequency of CX<sub>3</sub>CR1<sup>+</sup> cells and CX<sub>3</sub>CR1 expression levels were decreased in CD8<sup>+</sup> T cells,  
20 monocytes, and NK cells from AD patients, which were not observed in psoriasis patients.

**Conclusions:** These results suggest that, through functions in both membrane-bound and soluble forms, FKN plays important roles in trafficking of CX<sub>3</sub>CR1<sup>+</sup> leukocytes during the inflammation of AD.

**Key words:** atopic dermatitis; chemokine; fractalkine; CX<sub>3</sub>CR1; endothelial cell; leukocyte activation; adhesion molecule; skin inflammation

*Abbreviations used*

FKN: fractalkine

sFKN: soluble fractalkine

AD: Atopic dermatitis

5 Th: T helper

IFN: interferon

IL: interleukin

NK: natural killer

Ab: antibody

10 TARC: Thymus and activation regulated chemokine

MDC: Macrophage derived chemokine

mAb: monoclonal antibody

CLA: cutaneous lymphocyte antigen

RT-PCR: reverse transcription-polymerase chain reaction

15 GAPDH: Glyceraldehyde-3-phosphate

TACE: tumor necrosis factor- $\alpha$ -converting enzyme

TIMP: tissue inhibitors of metalloproteinase

CTL: normal controls

Contact D: contact dermatitis

## INTRODUCTION

Atopic dermatitis (AD) is a chronic, highly pruritic, inflammatory skin disease that manifests as eczematous skin lesions <sup>1-3</sup>. A skin lesion in AD is characterized by preferential infiltration of activated T cells, monocytes/macrophages, and eosinophils.

5 Although it has been proposed that T helper (Th) 2-type cells play a key role in the pathogenesis of AD <sup>4-8</sup>, recent studies have revealed that Th1-type cytokines, including interferon (IFN)- $\gamma$  and interleukin (IL)-12, are predominantly expressed in chronic lesions of AD <sup>6,9,10</sup>. Furthermore, chronic activation of macrophages/monocytes with increased cytokine secretion is the other immune dysfunction that characterizes AD <sup>11</sup>.

10 The development of cutaneous lesions in AD is closely related to the accumulation of these leukocytes migrating from the blood into the affected skin through the endothelium. This process is highly dependent on expression of chemokines and chemokine receptors as well as adhesion molecules <sup>7</sup>.

Fractalkine (FKN) or CX<sub>3</sub>CL1 is a CX<sub>3</sub>C chemokine that is expressed on

15 activated vascular endothelial cells stimulated with IL-1, tumor necrosis factor- $\alpha$ , or IFN- $\gamma$ <sup>12,13</sup>. FKN is expressed in various organs including skin, tonsils, brain, and kidney <sup>12,14-16</sup> and interacts with its unique receptor, CX<sub>3</sub>CR1, expressed on monocytes, natural killer (NK) cells, and some T cells. FKN and CX<sub>3</sub>CR1 represent a novel type

20 of leukocyte trafficking molecule that regulates both adhesive and chemotactic functions <sup>17</sup>. The membrane-bound FKN is able to mediate firm adhesion of CX<sub>3</sub>CR1-expressing leukocytes without requiring selectin-mediated rolling or activation of integrins <sup>18,19</sup>. Furthermore, FKN is released from the cell surface by proteolytic cleavage as soluble FKN (sFKN) that has potent chemoattractant activity for CX<sub>3</sub>CR1<sup>+</sup>

25 cells compared to Th2 cells, cell infiltration via FKN-CX<sub>3</sub>CR1 interaction especially

promotes Th1 responses<sup>13</sup>. Recent studies have demonstrated that FKN-CX<sub>3</sub>CR1 interaction contributes to the development of various inflammatory diseases and vascular injury by recruiting inflammatory cells<sup>19</sup>.

5 These previous findings suggest the involvement of FKN-CX<sub>3</sub>CR1 interaction in the induction and development of inflammatory processes associated with AD. Therefore, we evaluated expression of FKN and CX<sub>3</sub>CR1 in the skin, serum sFKN levels, and CX<sub>3</sub>CR1 expression on circulating leukocytes in AD. The results of this study suggest that, through functions in both membrane-bound and soluble forms, FKN plays important roles in the trafficking of CX<sub>3</sub>CR1<sup>+</sup> leukocytes during the inflammation  
10 of AD.

## METHODS

### Patients and control subjects for sFKN measurement

Serum sFKN levels were examined in 32 patients with AD (13 females and 19 males,  $24.7 \pm 7.4$  years old). All patients fulfilled the criteria for AD proposed by Hannifin and Rajka<sup>1</sup> and they did not have any history of other atopic diseases such as bronchial asthma and allergic rhinitis. The clinical severity of AD was evaluated using the scoring system proposed by Rajka and Langeland<sup>21</sup>: 16 patients had moderate-type AD and 16 patients had severe-type AD. The clinical data and serum samples were obtained at the same time. All patients were treated with topical steroids in combination with oral anti-allergic drugs. However, none of the patients were treated with systemic steroids or immunosuppressive drugs. In addition, patients with contact dermatitis (n=15, 8 females and 7 males,  $24.3 \pm 2.5$  years old) or psoriasis vulgaris (n=23, 10 females and 13 males,  $30.5 \pm 5.9$  years old), both of which are considered Th1-mediated skin diseases, were examined as disease controls in this study. The control subjects were sex- and age-matched 30 healthy Japanese individuals (12 females and 18 males,  $26.7 \pm 4.5$  years old). To assess the effect of treatment on serum sFKN levels, we examined sFKN levels before and after treatment in 23 patients with severe-type AD (8 females and 15 males,  $22.8 \pm 7.2$  years old). These patients were intensively treated with topical steroids in combination with oral anti-allergic drugs during 2 weeks of hospitalization. None of these patients were treated with systemic steroids or immunosuppressive drugs. Serum specimens were aliquoted and kept frozen at  $-70^{\circ}\text{C}$  before use. The protocol was approved by the Committee at Kanazawa University Graduate School of Medical Science and informed consent was obtained from all patients and normal individuals.

### **ELISA for sFKN**

Unless indicated otherwise, reagents were obtained from R&D Systems (Minneapolis, MN). Human sFKN levels were measured in serum samples by specific ELISA. Briefly, 96-well polystyrene plates were coated overnight at 25°C with 2  
5  $\mu\text{g/ml}$  of purified goat IgG anti-human FKN antibody (Ab). After washing, plates were blocked for 1 hour at 20°C with a phosphate-buffered saline containing 1% bovine serum albumin and 5% sucrose. Recombinant human FKN and serum samples were added in triplicate and the plates were incubated for 2 hours at 20°C. After washing, the plates were incubated with biotinylated goat anti-human FKN Ab (250 ng/ml) for 2  
10 hours at 20°C and then with streptavidin-peroxidase for 1 hour at 20°C. Samples were developed with 0.1 ml/well of tetramethylbenzidine substrate diluted in a citrate-phosphate buffer. Reactions were stopped by adding 1M H<sub>2</sub>SO<sub>4</sub> and the plates were read at 450 nm. Thymus and activation regulated chemokine (TARC/CCL17) and Macrophage derived chemokine (MDC/CCL22), which are known as Th2-type  
15 chemokines<sup>22</sup>, were also examined by ELISA.

### **Flow cytometric analysis**

CX<sub>3</sub>CR1 expression levels by peripheral blood leukocytes were examined in patients with AD (n=16, 10 severe and 6 moderate AD patients, 7 females and 9 males,  
20  $25.5 \pm 5.8$  years old), those with psoriasis (n=8, 4 females and 4 males,  $33.2 \pm 7.8$  years old), and normal controls (n=10, 4 females and 6 males,  $28.3 \pm 6.2$  years old). Heparinized blood samples were collected and immediately placed on ice. Two-color analysis was performed with a combination of FITC-conjugated anti-CX<sub>3</sub>CR1 monoclonal Ab (mAb; Medical & Biological laboratories Corp., Nagoya, Japan) and  
25 phycoerythrin-conjugated anti-CD4 (Coulter Corp., Miami, FL), anti-CD8 (Coulter

Corp.), anti-CD14 (Coulter Corp.), or anti-CD16 (Coulter Corp.) mAbs. Three-color analysis was also conducted with a combination of FITC-conjugated anti-CX<sub>3</sub>CR1 (Medical & Biological laboratories Corp.), biotinylated anti-cutaneous lymphocyte antigen (CLA; BD PharMingen, San Diego, CA), and peridinin chlorophyll protein-conjugated anti-CD4 (BD PharMingen) mAbs. Phycoerythrin conjugated streptavidin (Southern Biotechnology Associates Inc, Birmingham, Ala) was used for reveal biotin-coupled Ab staining. The blood samples were stained at 4°C with a predetermined optimal concentration of the test mAb for 20 minutes, as previously described<sup>23</sup>. Blood erythrocytes were lysed with the Coulter Whole Blood Immuno-Lyse kit as instructed by the manufacturer (Coulter Corp.). Cells were washed and analyzed with a FACScan flow cytometer (BD PharMingen). The positive and negative population of cells was determined with the unreactive isotype-matched mAbs (Coulter Corp.) as a control for background staining.

### 15 **Immunohistochemical staining**

FKN and CX<sub>3</sub>CR1 expression in the skin was determined by the immunohistochemical staining in AD patients with chronic skin lesions (n=9, 4 females and 5 males, 28.0 ± 5.5 years old), patients with psoriasis (n=5, 2 females and 3 males, 34.1 ± 7.2 years old), and normal controls (n=4, 2 females and 2 males, 29.6 ± 4.8 years old), as previously described<sup>24</sup>. Briefly, formalin-fixed paraffin-embedded skin tissues were acetone-fixed and then incubated with 10% normal rat serum for 10 minutes at 37°C to block non-specific staining. Sections were stained with goat polyclonal IgG Ab specific for human FKN (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit polyclonal IgG Ab specific for human CX<sub>3</sub>CR1 (Chemicon International, Temecula, CA). Sections were incubated sequentially (20 minutes, 37°C) with biotinylated rabbit

anti-goat IgG secondary Ab for FKN staining or with biotinylated goat anti-rabbit IgG secondary Ab for CX<sub>3</sub>CR1 staining (Vectastatin avidin-biotin complex methods, Vector Laboratories, Burlingame, CA), then with horseradish peroxidase-conjugated avidin-biotin complexes (Vector Laboratories). Sections were finally developed with  
5 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and counter-stained with methyl green. In a similar way, the serial skin tissues were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD20, and anti-CD68 Abs (DakoCytomation Co. Ltd. Glostrup, Denmark) to identify leukocyte subsets of CX<sub>3</sub>CR1-expressing cells.

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#### **RNA isolation and real-time reverse transcription (RT)-polymerase chain reaction (PCR)**

Total RNA was isolated from frozen tissue of 5 chronic skin lesions of AD patients (3 females and 2 males,  $27.4 \pm 4.6$  years old), and 5 normal controls (3 females  
15 and 2 males,  $28.2 \pm 4.2$  years old) with QIAGEN RNeasy spin columns (QIAGEN Ltd., Crawley, UK). Total RNA from each sample was reversely transcribed into cDNA. Expression of FKN, TARC, and MDC was analyzed using a real-time PCR quantification method according to the manufacture's instructions (Applied Biosystems, Foster City, CA). Sequence-specific primers and probes were designed by  
20 Pre-Developed TaqMan® Assay Reagents or Assays-On-Demands™ (Applied Biosystems). Real-time PCR (40 cycles of denaturation at 92°C for 15 seconds, annealing at 60°C for 60 seconds) was performed on an ABI Prism 7000 Sequence Detector (Applied Biosystems). Glyceraldehyde-3-phosphate (GAPDH) was used to normalize mRNA. To compare target gene and housekeeping (GAPDH) gene mRNA

expression, relative expression of real-time PCR products was determined using the  $\Delta\Delta\text{Ct}$  method<sup>25</sup>. One of the control samples was chosen as a calibrator sample.

### **Statistical Analysis**

5           The Mann-Whitney U tests were used to compare variables between 2 groups and Bonferroni's test was used for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A p value  $<0.05$  was considered statistically significant. All data are shown as mean  $\pm$  SD.

## RESULTS

### Expression of FKN and CX<sub>3</sub>CR1 in the skin

We performed immunohistochemical analysis using specific Ab to assess FKN expression in the affected skin from patients with AD. In the skin from normal individuals, FKN was not expressed on endothelial cells. In contrast, vascular endothelial cells strongly expressed FKN in the affected skin, but not in the unaffected skin of patients with AD or psoriasis (Fig. 1A and data not shown). Then, we examined the distribution of CX<sub>3</sub>CR1<sup>+</sup> cells in the skin lesions from AD patients (Fig. 1B). CX<sub>3</sub>CR1<sup>+</sup> cells were sparsely detected by immunohistochemical staining in normal control skin tissues ( $1 \pm 3\%$  of infiltrating mononuclear cells). In contrast, a considerable rate of infiltrating cells expressed CX<sub>3</sub>CR1 in the affected skin tissues from AD patients ( $27 \pm 19\%$ ) and psoriasis patients ( $23 \pm 17\%$ ). Most of CX<sub>3</sub>CR1<sup>+</sup> cells were identified as CD3<sup>+</sup> T cells in AD patients ( $80 \pm 32\%$ ) and psoriasis patients ( $78 \pm 23\%$ ). The expression ratio of CD4/CD8 in infiltrating CD3<sup>+</sup> CX<sub>3</sub>CR1<sup>+</sup> cells was similar in AD patients ( $1.2 \pm 0.6$ ) and psoriasis patients ( $1.3 \pm 0.6$ ). Thus, the endothelial FKN expression was augmented with increased infiltration of CX<sub>3</sub>CR1<sup>+</sup> cells in the lesional skin from AD as well as psoriasis.

### Expression of FKN, TARC, and MDC mRNA in the skin

To determine the relative importance of FKN in comparison of TARC and MDC, Th2-type chemokines that have been shown to be involved in the AD skin inflammation<sup>26</sup>, we examined mRNA expression of FKN, TARC, and MDC in the AD lesional skin by real-time RT-PCR. FKN mRNA levels in AD patients were significantly 8.7-fold higher than those in normal controls ( $14.8 \pm 12.2$  vs.  $1.7 \pm 1.5$ ,  $p < 0.05$ ). Similarly, TARC mRNA levels in AD patients were significantly 7.6-fold

higher than those in normal controls ( $24.7 \pm 14.5$  vs.  $3.2 \pm 2.0$ ,  $p < 0.05$ ) while MDC mRNA levels in AD patients were significantly increased by 7.9-fold compared with normal controls ( $13.4 \pm 9.0$  vs.  $1.7 \pm 1.4$ ,  $p < 0.05$ ). Thus, mRNA expression levels of FKN in the skin of AD were increased to a similar extent to those of TARC and MDC.

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### Serum sFKN levels

Since FKN is released from the cell surface as a soluble form<sup>12</sup>, sFKN levels were assessed in serum samples from AD patients (Fig. 2). Serum sFKN levels in patients with AD were significantly 5.1-fold higher than those in normal controls ( $81 \pm 153$  vs.  $16 \pm 11$  pg/ml,  $p < 0.05$ ). By contrast, patients with psoriasis ( $34 \pm 51$  pg/ml) or contact dermatitis ( $22 \pm 20$  pg/ml) showed mean sFKN levels similar to those of normal controls. The affected skin area was similar in AD patients ( $52 \pm 23\%$  of total body) and psoriasis patients ( $49 \pm 26\%$ ), suggesting that sFKN levels were not simply determined by the extent of skin disease. Regarding the severity of AD, severe AD patients ( $141 \pm 201$  pg/ml) exhibited significantly increased sFKN levels compared with moderate AD patients ( $21 \pm 15$  pg/ml,  $p < 0.05$ ). Furthermore, sFKN levels were significantly elevated in severe AD patients relative to patients with psoriasis ( $p < 0.05$ ) or contact dermatitis ( $p < 0.05$ ) as well as normal controls ( $p < 0.005$ ). However, sFKN levels in moderate AD patients were comparable with those in normal controls. Blood eosinophil numbers and serum IgE levels did not correlate with the serum sFKN levels in AD patients (data not shown). We determined correlation of sFKN levels with serum levels of TARC and MDC that are increased in sera from AD patients<sup>27,28</sup>. Serum sFKN levels correlated positively with serum levels of TARC ( $r = 0.757$ ,  $p < 0.0001$ ) or MDC ( $r = 0.370$ ,  $p < 0.05$ ). Thus, serum sFKN levels were elevated in patients with AD,

especially severe AD, but not in other skin disorders, including psoriasis and contact dermatitis.

To determine the treatment effect on sFKN levels, 23 patients with severe AD were examined for serum sFKN levels before and after treatment. The skin lesions in all patients significantly improved (the score changed from  $7.6 \pm 1.2$  to  $4.7 \pm 1.3$ ,  $p < 0.0001$ ) by intensive therapy of topical steroids and oral anti-allergic drugs during 2 weeks of hospitalization. Similarly, serum sFKN levels in AD patients were significantly decreased by 31% after treatment compared with those before treatment ( $100 \pm 191$  vs.  $69 \pm 118$  pg/ml,  $p < 0.05$ ). Thus, serum sFKN levels correlated with the disease activity of AD.

#### **Frequency of CX<sub>3</sub>CR1-expressing cells in blood leukocytes and CX<sub>3</sub>CR1 expression levels on blood leukocytes**

CX<sub>3</sub>CR1 expression on peripheral blood CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD14<sup>+</sup> monocytes, and CD16<sup>+</sup> NK cells was assessed by flow cytometry with two-color analysis (Figs. 3 and 4). The frequency of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, monocytes, and NK cells was not significantly different among AD patients, psoriasis patients, and normal controls (data not shown). The frequency of CX<sub>3</sub>CR1-expressing cells in CD8<sup>+</sup> T cells was significantly decreased in AD patients compared with normal controls ( $p < 0.05$ ) and psoriasis patients ( $p < 0.05$ ). CX<sub>3</sub>CR1 expression levels in CX<sub>3</sub>CR1-expressing CD8<sup>+</sup> T cells were also significantly decreased in AD patients (mean fluorescence intensity,  $10 \pm 3$ ) compared with normal controls ( $16 \pm 3$ ,  $p < 0.0005$ ); however, they were not significantly different from those in psoriasis patients ( $13 \pm 4$ ). Similarly, AD patients had the significantly reduced frequency of CX<sub>3</sub>CR1<sup>+</sup> cells in monocytes relative to normal controls ( $p < 0.05$ ) and psoriasis patients

( $p < 0.05$ ), and CX<sub>3</sub>CR1 expression levels in CX<sub>3</sub>CR1-expressing monocytes were significantly lower in AD patients ( $11 \pm 6$ ) than those found in psoriasis patients ( $20 \pm 5$ ,  $p < 0.005$ ) as well as normal controls ( $23 \pm 5$ ,  $p < 0.0001$ ). The percentage of CX<sub>3</sub>CR1<sup>+</sup> cells in NK cells was also significantly lower in AD patients than in normal controls (5  $p < 0.05$ ) and psoriasis patients ( $p < 0.05$ ). AD patients also exhibited significantly reduced CX<sub>3</sub>CR1 expression levels in CX<sub>3</sub>CR1<sup>+</sup> NK cells relative to normal controls ( $25 \pm 11$  vs.  $36 \pm 6$ ,  $p < 0.05$ ) while there was no significant difference in CX<sub>3</sub>CR1 expression levels on CX<sub>3</sub>CR1<sup>+</sup> NK cells between AD patients and psoriasis patients ( $31 \pm 8$ ). However, the frequency of CX<sub>3</sub>CR1-expressing cells in CD4<sup>+</sup> T cells was similar 10 for AD patients, normal controls, and psoriasis patients, and expression levels of CX<sub>3</sub>CR1 in CX<sub>3</sub>CR1<sup>+</sup> CD4<sup>+</sup> T cells were also similar in AD patients ( $4.3 \pm 1.4$ ), psoriasis patients ( $5.4 \pm 1.4$ ), and normal controls ( $5.6 \pm 1.0$ ). Furthermore, the frequency of CX<sub>3</sub>CR1<sup>+</sup> cells among CLA<sup>+</sup> CD4<sup>+</sup> T cells in AD patients ( $18 \pm 5$  %) was not significantly different from that in normal controls ( $16 \pm 4$  %). In contrast, the 15 frequency of CX<sub>3</sub>CR1<sup>+</sup> cells in each leukocyte subset from psoriasis patients was comparable with that from normal controls. Thus, the frequency of CX<sub>3</sub>CR1-expressing cells and CX<sub>3</sub>CR1 expression levels were decreased in some peripheral leukocyte subsets of AD.

## DISCUSSION

The current study showed that FKN expression was augmented in the cutaneous vascular endothelial cells of AD patients. Consistent with the finding that up-regulated FKN expression on endothelial cells facilitates the recruitment of CX<sub>3</sub>CR1<sup>+</sup> cells to the skin <sup>17</sup>, CX<sub>3</sub>CR1-expressing leukocytes were increased in the affected skin of AD. Reflecting the augmented FKN expression in the skin, serum sFKN levels were elevated in AD patients and associated with the disease severity and activity. Released sFKN induces integrin activation and migration of CX<sub>3</sub>CR1-expressing cells similar to other soluble chemokines <sup>17,18,29</sup>. Collectively, the results of this study suggest that, through functions of both membrane-bound and soluble forms, FKN regulates the CX<sub>3</sub>CR1<sup>+</sup> leukocyte trafficking during the development of AD skin lesions and also suggest that the serum sFKN level is a useful clinical marker that reflects both the severity and activity of AD.

Chemokines and their receptors have the capability of regulating the selective migration of Th1 and Th2 cells to the target tissues and of controlling the Th1 and Th2 type responses <sup>30-32</sup>. Although the abnormal shift to Th2 cells in AD has been shown to be clear by analysis using peripheral blood <sup>33-35</sup>, direct assessment of infiltrating cells in AD skin lesions is less clear-cut. Specifically, previous studies using atopy patch tests with house dust mite allergens have demonstrated that a majority of T cells in the lesions express IFN- $\gamma$  mRNA and protein, alone or in combination with IL-4 <sup>10,36,37</sup>. Recent studies have revealed that CCR4 plays a critical role in the migration of Th2 cells from peripheral blood into the skin in patients with AD while Th1 cells are suggested to migrate from blood into the skin through CXCR3 <sup>38-40</sup>. Since FKN is induced by Th1 cytokines such as IFN- $\gamma$ , and CX<sub>3</sub>CR1 prefers expression on Th1 cells compared to Th2 cells <sup>13,41</sup>, the enhanced FKN expression on endothelial cells may

mediate Th1 cells into skin lesions and thereby participate in the amplification of the polarized Th1 response in AD. In this study, mRNA expression levels of FKN in the AD lesional skin were increased to a similar extent to those of Th2-type chemokines, TARC and MDC. Furthermore, serum sFKN levels were significantly associated with serum levels of TARC and MDC. These results suggest that FKN may contribute to the inflammation of AD in concert with TARC and MDC. Collectively, these findings supports the recent hypothesis that Th1 cytokines in addition to Th2 cytokines play important roles in the development of AD <sup>7,42</sup>.

Patients with AD exhibited elevated sFKN levels that significantly decreased with the improvement of skin lesions by treatment. Since sFKN enhances the chemotactic activity of CX<sub>3</sub>CR1-expressing cells <sup>12,43,44</sup>, sFKN may promote CX<sub>3</sub>CR1<sup>+</sup> cell infiltration into the affected tissue. However, recent studies have revealed that sFKN inhibits the adhesion of CX<sub>3</sub>CR1-expressing leukocytes to endothelial cells <sup>12,29,43,44</sup>. A similar function has been reported in other adhesion molecules including L-selectin <sup>45,46</sup>. Circulating L-selectin likely serves as a biologic buffer system to prevent leukocyte rolling at sites of subacute inflammation <sup>45,47</sup>. In a similar way, the increase in sFKN may reflect the defence system to avoid excessive inflammatory cell infiltration by inhibiting the adhesion of CX<sub>3</sub>CR1<sup>+</sup> leukocytes to endothelial cells in AD. Thus, although the biological significance of sFKN remains unclear, sFKN may be related to the inflammation associated with AD by interfering with cell to cell interaction.

The percentage of CX<sub>3</sub>CR1-expressing cells and CX<sub>3</sub>CR1 expression levels on blood CD8<sup>+</sup> T cells, monocytes, and NK cells were decreased in AD patients. This may be caused by leukocyte activation that sheds or down-regulates CX<sub>3</sub>CR1 as observed in other adhesion molecules, such as intercellular adhesion molecule-1 and

selectins <sup>46,48-51</sup>. Consistent with this possibility, the percentage of CD8<sup>+</sup> memory T cells expressing CX<sub>3</sub>CR1 is also decreased in patients with rheumatoid arthritis, suggesting that this receptor is shed or down-regulated upon lymphocyte activation <sup>52</sup>. Alternatively, since expression levels of adhesion molecules generally correlate with the capacity to bind their receptors <sup>53,54</sup>, leukocytes with high levels of CX<sub>3</sub>CR1 may selectively infiltrate the affected skin tissues through FKN-CX<sub>3</sub>CR1 interaction, which results in decreased blood leukocytes with elevated CX<sub>3</sub>CR1 levels and increased CX<sub>3</sub>CR1<sup>+</sup> cells in the lesional skin of AD. Furthermore, it is possible that circulating sFKN directly binds to blood CX<sub>3</sub>CR1<sup>+</sup> leukocytes and thereby down-regulates CX<sub>3</sub>CR1 expression. Although the mechanisms for decreased CX<sub>3</sub>CR1 expression on blood leukocytes of AD patients remain unknown, it may finally contribute to down-regulation of the inflammation by diminishing their migration capacity into the inflamed skin.

The endothelial FKN expression and frequency of infiltrating CX<sub>3</sub>CR1<sup>+</sup> cells in the lesional skin were increased in psoriasis as well as AD. By contrast, a previous study showed that FKN expression was enhanced in the skin from psoriasis, but not in AD <sup>13</sup>. Although the reason for this discrepancy is unclear, it may be due to the difference in AD populations studied since milder AD patients generally showed weaker FKN expression (data not shown). Despite the increase in FKN expression and skin CX<sub>3</sub>CR1<sup>+</sup> cell infiltration, serum sFKN levels and CX<sub>3</sub>CR1 expression on blood leukocytes in psoriasis patients were similar to normal controls, which was distinct from those of AD patients. Inducible FKN cleavage from the cell surface of endothelial cells depends on a tumor necrosis factor- $\alpha$ -converting enzyme (TACE), a member of a family of proteins containing a disintegrin and metalloprotease domain (ADAMS proteins) <sup>55,56</sup>. TACE activity is inhibited by tissue inhibitors of

metalloproteinase (TIMP)-3, but not by TIMP-1, -2, and -4<sup>57</sup>. Since TIMP-3 expression is augmented in psoriasis, but not in AD<sup>58,59</sup>, the enhanced TIMP-3 expression in psoriasis may inhibit TACE activity, perhaps resulting in reduced sFKN release and normal serum sFKN levels. These normal sFKN levels may not alter

5 CX<sub>3</sub>CR1 expression on circulating leukocytes from psoriasis patients. Although the mechanisms for the difference in sFKN levels and CX<sub>3</sub>CR1 expression on leukocytes between AD and psoriasis remain unknown, the results suggest that the contribution of CX<sub>3</sub>CR1-FKN interaction to the inflammation of AD is different from that of psoriasis. However, we cannot exclude the possibility that FKN is related to the development of

10 general skin inflammation, since local FKN expression was not significantly different between AD and psoriasis. Further studies will be needed to determine the relative importance of FKN in AD compared to other skin inflammatory diseases.

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**REFERENCES**

1. Hanifin JM, Rajika G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol* (Stockh) 1980;Suppl. 92:44-7.
2. Hanifin JM. Atopic dermatitis. *J Am Acad Dermatol* 1982;6:1-13.
- 5 3. Leung DY. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 2000;105:860-76.
4. Van der Heijden FL, Wierenga EA, Bos JD, Kapsenberg ML. High frequency of IL-4-producing CD4+ allergen specific T lymphocytes in atopic dermatitis lesional skin. *J Invest Dermatol* 1991;97:389-94.
- 10 5. Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J Clin Invest* 1994;94:870-6.
6. Hamid Q, Naseer T, Minshall EM, Song YL, Boguniewicz M, Leung DY. In vivo expression of IL-12 and IL-13 in atopic dermatitis. *J Allergy Clin Immunol* 1996;98:225-31.
- 15 7. Leung DY. Pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* 1999;104:S99-108.
8. Bos JD, Wierenga EA, Sillevius Smitt JH, van der Heijden FL, Kapsenberg ML. Immune dysregulation in atopic eczema. *Arch Dermatol* 1992;128:1509-12.
- 20 9. Grewe M, Gyufko K, Schopf E, Krutmann J. Lesional expression of interferon-gamma in atopic eczema. *Lancet* 1994;343:25-6.
10. Tsicopoulos A, Hamid Q, Haczku A, Jacobson MR, Durham SR, North J, et al. Kinetics of cell infiltration and cytokine messenger RNA expression after

intradermal challenge with allergen and tuberculin in the same atopic individuals. *J Allergy Clin Immunol* 1994;94:764-72.

11. Leung DYM. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol* 1995;96:302-18.
- 5 12. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 1997;385:640-4.
13. Fraticelli P, Sironi M, Bianchi G, D'Ambrosio D, Albanesi C, Stoppacciaro A, et al. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. *J Clin Invest* 2001;107:1173-81.
- 10 14. Hart DNJ, McKenzie JL. Isolation and characterization of human tonsil dendritic cells. *J Exp Med* 1988;168:157-70.
15. Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, et al. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc Natl Acad Sci U S A* 15 1998;95:10896-901.
16. Papadopoulos EJ, Fitzhugh DJ, Tkaczyk C, Gilfillan AM, Sasseti C, Metcalfe DD, et al. Mast cells migrate, but do not degranulate, in response to fractalkine, a membrane-bound chemokine expressed constitutively in diverse cells of the skin. *Eur J Immunol* 2000;30:2355-61.
- 20 17. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, et al. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 1997;91:521-30.
18. Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture,
- 25

- firm adhesion, and activation under physiologic flow. *J Exp Med* 1998;188:1413-9.
19. Umehara H, Bloom E, Okazaki T, Domae N, Imai T. Fractalkine and vascular injury. *Trends Immunol* 2001;22:602-7.
- 5 20. Yoneda O, Imai T, Goda S, Inoue H, Yamauchi A, Okazaki T, et al. Fractalkine-mediated endothelial cell injury by NK cells. *J Immunol* 2000;164:4055-62.
21. Rajka G, Langeland T. Grading of severity of atopic dermatitis. *Acta Derm Venerol* 1989;144:13-4.
- 10 22. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999;11:81-8.
- 15 23. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang MLK, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* 1996;5:551-62.
24. Nagaoka T, Kaburagi Y, Hamaguchi Y, Hasegawa M, Takehara K, Steeber DA, et al. Delayed wound healing in the absence of intercellular adhesion molecule-1 or L-selectin expression. *Am J Pathol* 2000;157:237-47.
- 20 25. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. *J Mol Diagn* 2001;3:55-61.

26. Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest* 1999;104:1097-105.
27. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al.  
5 Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001;107:535-41.
28. Galli G, Chantry D, Annunziato F, Romagnani P, Cosmi L, Lazzeri E, et al.  
10 Macrophage-derived chemokine production by activated human T cells in vitro and in vivo: preferential association with the production of type 2 cytokines. *Eur J Immunol* 2000;30:204-10.
29. Haskell CA, Cleary MD, Charo IF. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction. Rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation. *J Biol Chem* 1999;274:10053-8.  
15
30. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121-7.
31. Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, et al. International union of pharmacology. XXII. Nomenclature for chemokine  
20 receptors. *Pharmacol Rev* 2000;52:145-76.
32. D'Ambrosio D, Iellem A, Colantonio L, Clissi B, Pardi R, Sinigaglia F. Localization of Th-cell subsets in inflammation: differential thresholds for extravasation of Th1 and Th2 cells. *Immunol Today* 2000;21:183-6.

33. Nakazawa M, Sugi N, Kawaguchi H, Ishii N, Nakajima H, Minami M. Predominance of type 2 cytokine-producing CD4<sup>+</sup> and CD8<sup>+</sup> cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 1997;99:673-82.
34. Sato A, Tsuji K, Yamamura M, Morita Y, Kanzaki H, Tada J, et al. Increased type 2 cytokine expression by both CD4<sup>+</sup> CD45RO<sup>+</sup> T cells and CD8<sup>+</sup> CD45RO<sup>+</sup> T cells in blood circulation is associated with high serum IgE but not with atopic dermatitis. *J Invest Dermatol* 1998;111:1079-84.
35. Jujo K, Renz H, Abe J, Gelfand EW, Leung DY. Decreased interferon gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. *J Allergy Clin Immunol* 1992;90:323-31.
36. Thepen T, Langeveld-Wildschut EG, Bihari IC, van Wichen DF, van Reijsen FC, Mudde GC, et al. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial TH2 response to a TH1 response in situ: an immunocytochemical study. *J Allergy Clin Immunol* 1996;97:828-37.
37. Werfel T, Morita A, Grewe M, Renz H, Wahn U, Krutmann J, et al. Allergen specificity of skin-infiltrating T cells is not restricted to a type-2 cytokine pattern in chronic skin lesions of atopic dermatitis. *J Invest Dermatol* 1996;107:871-6.
38. Nakatani T, Kaburagi Y, Shimada Y, Inaoki M, Takehara K, Mukaida N, et al. CCR4 memory CD4<sup>+</sup> T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. *J Allergy Clin Immunol* 2001;107:353-8.
39. Sager N, Feldmann A, Schilling G, Kreitsch P, Neumann C. House dust mite-specific T cells in the skin of subjects with atopic dermatitis: frequency

- and lymphokine profile in the allergen patch test. *J Allergy Clin Immunol* 1992;89:801-10.
40. van Reijsen FC, Bruijnzeel-Koomen CA, Kalthoff FS, Maggi E, Romagnani S, Westland JK, et al. Skin-derived aeroallergen-specific T-cell clones of Th2 phenotype in patients with atopic dermatitis. *J Allergy Clin Immunol* 1992;90:184-93.
- 5
41. Nanki T, Imai T, Nagasaka K, Urasaki Y, Nonomura Y, Taniguchi K, et al. Migration of CX3CR1-positive T cells producing type 1 cytokines and cytotoxic molecules into the synovium of patients with rheumatoid arthritis. *Arthritis Rheum* 2002;46:2878-83.
- 10
42. Grewe M, Bruijnzeel-Koomen CAFM, Schopf E, Thepen T, Langeveld-Wildschut AG, Ruzicka T, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 1998;19:359-61.
- 15
43. Umehara H, Goda S, Imai T, Nagano Y, Minami Y, Tanaka Y, et al. Fractalkine, a CX3C-chemokine, functions predominantly as an adhesion molecule in monocytic cell line THP-1. *Immunol Cell Biol* 2001;79:298-302.
44. Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, et al. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* 1997;387:611-7.
- 20
45. Schleiffenbaum BE, Spertini O, Tedder TF. Soluble L-selectin is present in human plasma at high levels and retains functional activity. *J Cell Biol* 1992;119:229-38.
46. Gearing AJ, Newman W. Circulating adhesion molecules in disease. *Immunol Today* 1993;14:506-12.
- 25

47. Tedder TF, Steeber DA, Chen A, Engel P. The selectins: vascular adhesion molecules. *FASEB J* 1995;9:866-73.
48. Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, et al. GM-CSF and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursors. *J Immunol* 1990;145:576-84.
49. Tedder TF, Penta AC, Levine HB, Freedman AS. Expression of the human leukocyte adhesion molecule, LAM1. Identity with the TQ1 and Leu-8 differentiation antigens. *J Immunol* 1990;144:532-40.
- 10 50. Leeuwenberg JF, Smeets EF, Neefjes JJ, Shaffer MA, Cinek T, Jeunhomme TM, et al. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology* 1992;77:543-9.
51. Pigott R, Dillon LP, Hemingway IH, Gearing AJ. Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. *Biochem Biophys Res Commun* 1992;187:584-9.
- 15 52. Ruth JH, Rottman JB, Katschke KJ, Jr., Qin S, Wu L, LaRosa G, et al. Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum* 2001;44:2750-60.
53. Tang MLK, Steeber DA, Zhang XQ, Tedder TF. Intrinsic differences in L-selectin expression levels affect T and B lymphocyte subset-specific recirculation pathway. *J Immunol* 1998;160:5113-21.
- 20 54. Gearing AJ, Hemingway I, Pigott R, Hughes J, Rees AJ, Cashman SJ. Soluble forms of vascular adhesion molecules, E-selectin, ICAM-1, and VCAM-1: pathological significance. *Ann N Y Acad Sci* 1992;667:324-31.

55. Garton KJ, Gough PJ, Blobel CP, Murphy G, Greaves DR, Dempsey PJ, et al. Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J Biol Chem* 2001;276:37993-8001.
- 5 56. Tsou CL, Haskell CA, Charo IF. Tumor necrosis factor-alpha-converting enzyme mediates the inducible cleavage of fractalkine. *J Biol Chem* 2001;276:44622-6.
57. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett* 10 1998;435:39-44.
58. Suomela S, Kariniemi AL, Snellman E, Saarialho-Kere U. Metalloelastase (MMP-12) and 92-kDa gelatinase (MMP-9) as well as their inhibitors, TIMP-1 and -3, are expressed in psoriatic lesions. *Exp Dermatol* 2001;10:175-83.
- 15 59. Katoh N, Hirano S, Suehiro M, Ikenaga K, Yasuno H. Increased levels of serum tissue inhibitor of metalloproteinase-1 but not metalloproteinase-3 in atopic dermatitis. *Clin Exp Immunol* 2002;127:283-8.

## FIGURE LEGEND

**Fig 1.** Representative immunohistochemical expression of FKN (A) and CX<sub>3</sub>CR1 (B) in the lesional skin from AD patients, psoriasis patients, and normal control skin (CTL).

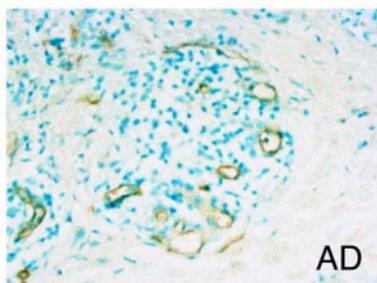
**Fig 2.** Serum levels of sFKN in patients with AD, those with psoriasis, those with contact dermatitis (Contact D), and normal controls (CTL). Patients with AD were grouped into patients with severe AD and those with moderate AD. Serum sFKN levels were determined by ELISA. The horizontal bars represent mean values with statistically significant differences between groups indicated. Note the logarithmic scale.

**Fig 3.** Representative expression of CX<sub>3</sub>CR1 on CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD14<sup>+</sup> monocytes, and CD16<sup>+</sup> NK cells in peripheral blood from patients with AD and normal controls (CTL). All samples were stained in parallel by two-color immunofluorescent staining of mononuclear cells and analyzed sequentially by flow cytometry with identical instrument settings. Quadrants were set according to the staining of control mAbs. The percentage represents the frequency of CX<sub>3</sub>CR1<sup>+</sup> cells in each leukocyte subset. Horizontal dashed lines in each histogram are provided for reference.

**Fig 4.** The frequency of CX<sub>3</sub>CR1<sup>+</sup> leukocyte subpopulations from patients with AD, those with psoriasis, and normal controls (CTL). The frequency of CX<sub>3</sub>CR1<sup>+</sup> cells was examined on CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD14<sup>+</sup> monocytes, and CD16<sup>+</sup> NK cells by two-color immunofluorescence staining with flow cytometric analysis, using the gates

shown in Fig. 3. All samples were stained and analyzed sequentially by flow cytometry in parallel using identical instrument settings. The horizontal bars represent mean values with statistically significant differences between groups indicated.

A. FKN



B. CX<sub>3</sub>CR1

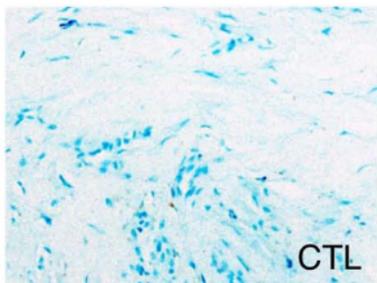
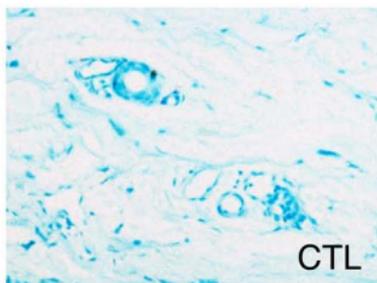
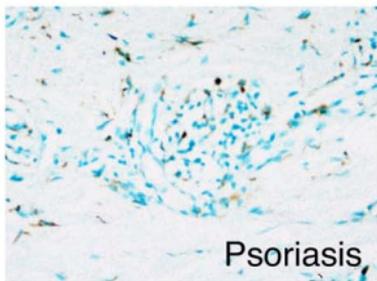
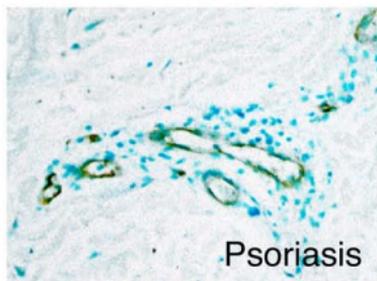
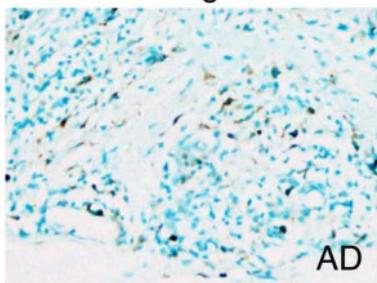


Figure 1  
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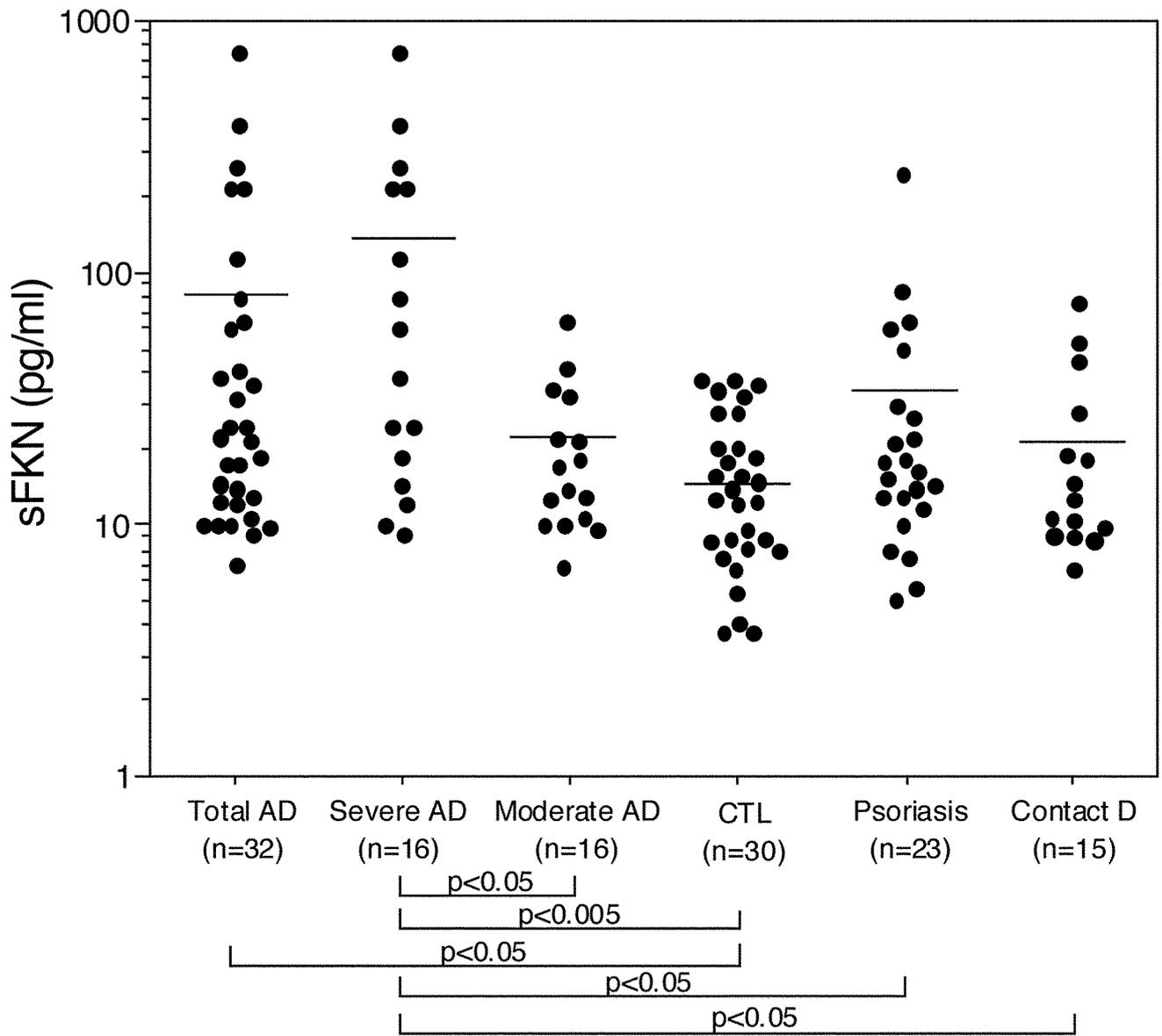


Figure 2  
Echigo T, et al.

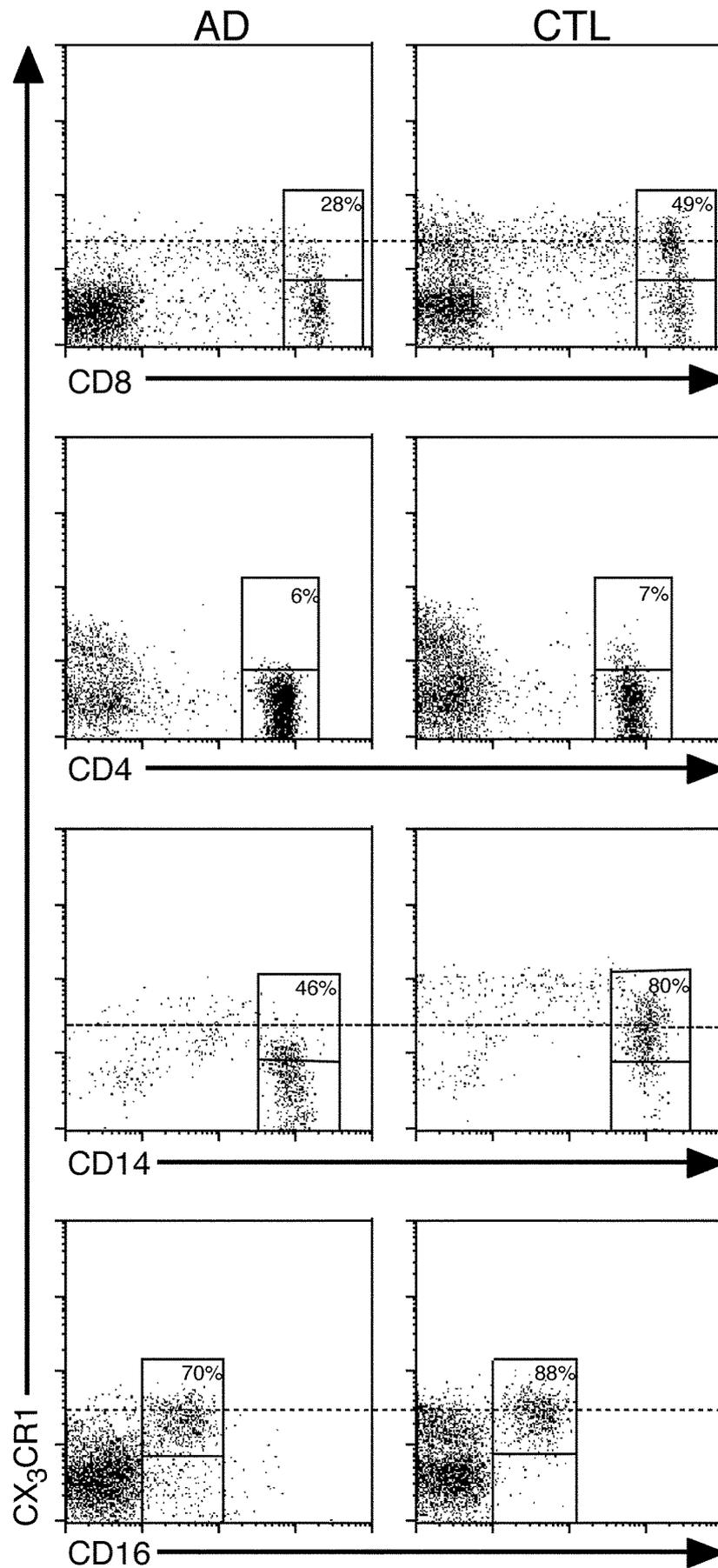


Figure 3  
Echigo T, et al.

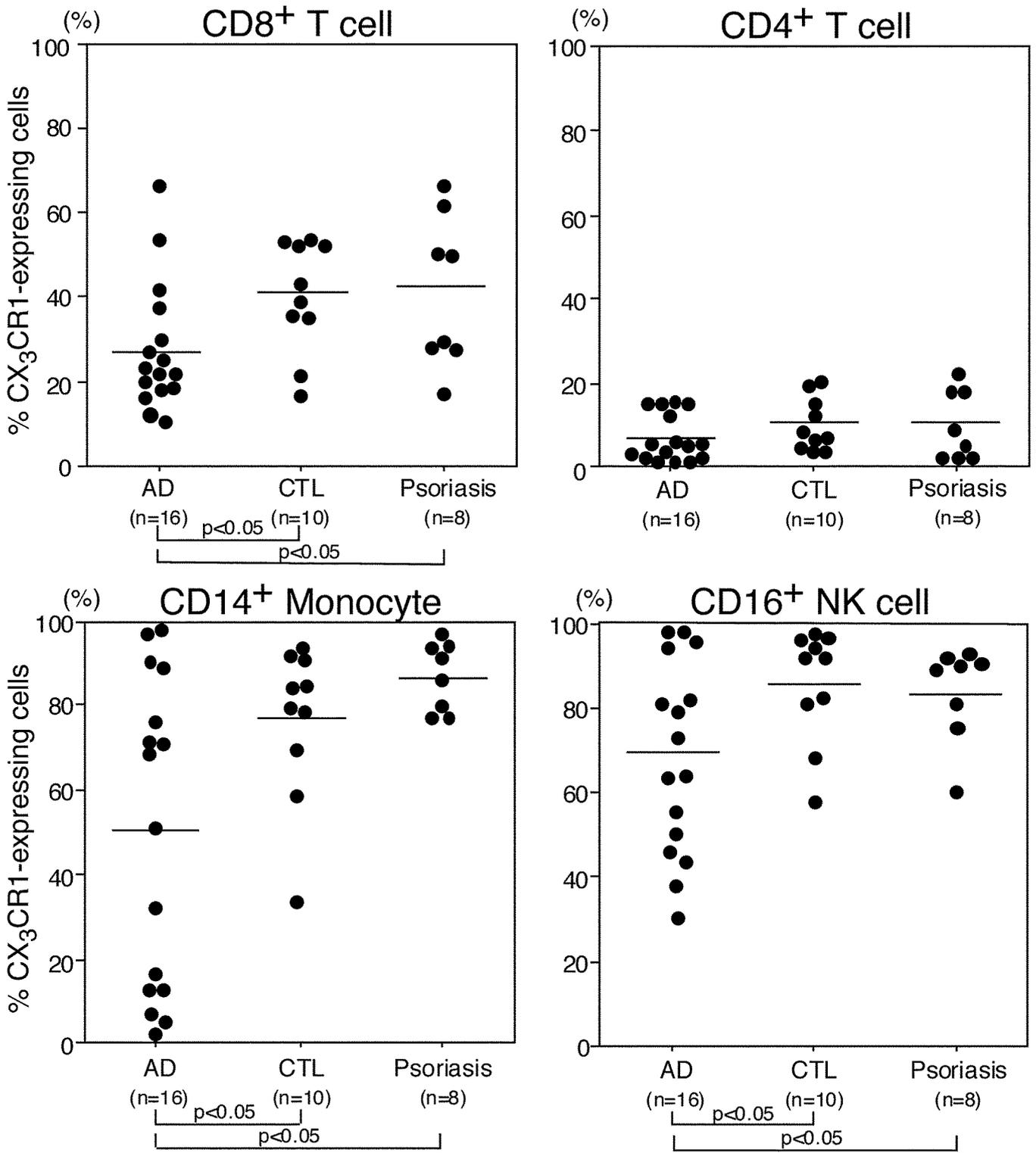


Figure 4  
Echigo T, et al.