Acta Medica Okayama

Recruitment of CD16+ monocytes into synovial tissues is mediated by fractalkine and CX3CR1 in rheumatoid arthritis patients

Ryusuke Yano[*]   Masahiro Yamanura[†]   Katsue Sunahori[‡]

Kouji Takasugi[**]   Jiro Yamana[††]

Masanori Kawashima[‡‡]   Hirofumi Makino[§]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama University,
[**]Okayama University,
[††]Okayama University,
[‡‡]Okayama University,
[§]Okayama University,

Recruitment of CD16+ monocytes into synovial tissues is mediated by fractalkine and CX3CR1 in rheumatoid arthritis patients[*]

Ryusuke Yano, Masahiro Yamanura, Katsue Sunahori, Kouji Takasugi, Jiro Yamana, Masanori Kawashima, and Hirofumi Makino

Abstract

CD16+ monocytes, identified as a minor population of monocytes in human peripheral blood, have been implicated in several inflammatory diseases, including rheumatoid arthritis (RA). Fractalkine (FKN, CX3CL1), a member of the CX3 C subfamily, is induced by pro-inflammatory cytokines, while a receptor for FKN, CX3CR1, is capable of mediating both leukocyte migration and firm adhesion. Here, we investigated the role of FKN and CX3CR1 in activation of CD16+ monocytes and their recruitment into synovial tissues in RA patients. High levels of soluble FKN were detected in the synovial fluid and sera of RA patients. Circulating CD16+ monocytes showed a higher level of CX3CR1 expression than CD16- monocytes in both RA patients and healthy subjects. High level expression of CX3CR1 was also seen in CD16+ monocytes localized to the lining layer in RA synovial tissue. In the in vitro culture experiments, IL-10 induced CX3CR1 expression on the surface of monocytes, and TNFalpha induced membrane-bound FKN as well as soluble FKN expression in synovial fibroblasts. Moreover, soluble FKN was capable of inducing IL-1beta and IL-6 by activated monocytes. These results suggest that FKN might preferentially mediate migration and recruitment of CD16+ monocytes, and might contribute to synovial tissue inflammation.

KEYWORDS: CD16, monocytes, fractalkine, CX3CR1, rheumatoid arthritis

_____

Original Article

Acta Medica
Okayama

http://www.lib.okayama-u.ac.jp/www/acta/

Recruitment of CD16⁺ Monocytes into Synovial Tissues Is Mediated by Fractalkine and CX3CR1 in Rheumatoid Arthritis Patients

Ryusuke Yano, Masahiro Yamamura*, Katsue Sunahori, Kouji Takasugi, Jiro Yamana, Masanori Kawashima, and Hirofumi Makino

Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

CD16⁺ monocytes, identified as a minor population of monocytes in human peripheral blood, have been implicated in several inflammatory diseases, including rheumatoid arthritis (RA). Fractalkine (FKN, CX3CL1), a member of the CX3 C subfamily, is induced by pro-inflammatory cytokines, while a receptor for FKN, CX3CR1, is capable of mediating both leukocyte migration and firm adhesion. Here, we investigated the role of FKN and CX3CR1 in activation of CD16⁺ monocytes and their recruitment into synovial tissues in RA patients. High levels of soluble FKN were detected in the synovial fluid and sera of RA patients. Circulating CD16⁺ monocytes showed a higher level of CX3CR1 expression than CD16⁻ monocytes in both RA patients and healthy subjects. High level expression of CX3CR1 was also seen in CD16⁺ monocytes localized to the lining layer in RA synovial tissue. In the *in vitro* culture experiments, IL-10 induced CX3CR1 expression on the surface of monocytes, and TNFα induced membrane-bound FKN as well as soluble FKN expression in synovial fibroblasts. Moreover, soluble FKN was capable of inducing IL-1β and IL-6 by activated monocytes. These results suggest that FKN might preferentially mediate migration and recruitment of CD16⁺ monocytes, and might contribute to synovial tissue inflammation.

Key words: CD16, monocytes, fractalkine, CX3CR1, rheumatoid arthritis

Fractalkine (FKN, CX3CL1), a member of the CX3 C subfamily, is a unique chemokine in which the first 2 conserved cysteine residues are separated by 3 non-conserved amino acids. Unlike other chemokines, FKN exists in 2 forms: a membrane-anchored and a soluble form. Soluble FKN acts as a chemoattractant, whereas the membrane-anchored molecule functions as an adhesion molecule.

The membrane-anchored form of FKN comprises a chemokine domain tethered to the cell surface via a heavily glycosylated mucin-like stalk, followed by a single transmembrane and a single cytoplasmic domain [1, 2]. The full-length molecule can be cleaved from the cell membrane by tumor necrosis factor (TNF) α-converting enzyme (TACE), a member of the ADAM (disintegrin and metalloprotease) family, and ADAM10, to produce a soluble form comprising the chemokine domain and most of the stalk region [1]. FKN is found to be expressed on the surface of endothelial cells, epithelial cells [3, 4],

dendritic cells [5, 6] and neurons [7] and is induced by pro-inflammatory cytokines such as interleukin (IL)-1 and TNFα [8, 9]. The receptor for FKN, CX3CR1, is capable of mediating both leukocyte migration and firm adhesion. CX3CR1 is expressed on a number of leukocytes, including monocytes, T cell subsets, and NK cells [10, 11].

Rheumatoid arthritis (RA) is a chronic disorder of unknown etiology that leads to progressive joint destruction. RA is characterized by a massive synovial infiltration of lymphocytes and macrophages [12], and extensive proliferation of fibroblast-like synoviocytes [13]. In association with joint inflammation, blood monocytes are functionally activated in RA [14]. We previously demonstrated that CD16$^+$ monocytes with low CD14 expression are increased according to disease activity [15]. CD14$^+$/CD16$^+$ cells were defined as a subset of monocytes that accounts for $\sim$ 10% of all peripheral monocytes in healthy individuals, and an increase in these cells has been demonstrated in other inflammatory conditions, including sepsis, human immunodeficiency virus 1 infection and cancer [16]. Compared with CD14^{++}/CD16$^-$ conventional monocytes, CD16$^+$ monocytes express higher levels of major histocompatibility complex (MHC) class II antigens, adhesion molecules, chemokine receptors, and proinflammatory cytokines such as TNFα, but lower levels of the anti-inflammatory cytokine, *i.e.*, IL-10 [15, 17]. Cytokines such as macrophage colony-stimulating factor (M-CSF), IL-10, and transforming growth factor β (TGFβ) have been implicated in induction of cell surface CD16 [17], and a spillover of these cytokines from the inflamed joints may be important in the pathogenesis of RA [15].

Interactions of FKN and CX3CR1 might contribute to the accumulation of CX3CR1$^+$ T cells expressing type 1 cytokines and possessing cytotoxic granules in the RA synovium [18]. Such a scenario could be applicable to the mechanism of macrophage infiltration into the synovial tissues, and we hypothesized that the FKN/CX3CR1 pathway might mediate the recruitment of CD16 monocytes in RA patients. In the current study, we investigated the expressions of FKN and its receptor CX3CR1 on the circulating CD16$^+$ monocytes and infiltrated CD16$^+$ macrophages in RA patients. Furthermore, we examined the induction of FKN and CX3CR1 expression by

various pro-inflammatory cytokines.

Patients and Methods

Patients and samples.   Subjects consisted of 22 patients with RA (19 women and 3 men; mean $\pm$ SD age, 62.4 ± 2.5 years), 16 patients with osteoarthritis (OA) (11 women and 5 men; mean $\pm$ SD age, 66.3 ± 1.5 years), and 12 healthy age-matched volunteers (8 women and 4 men; 50.9 ± 9.3 years) who served as controls. The presence of RA was diagnosed according to the revised 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) [19], and all patients provided written informed consent to participate in the study. Paired samples of serum and synovial fluid were obtained from the RA and OA patients. Most RA patients received nonsteroidal anti-inflammatory drugs, prednisolone (≤ 7.5 mg/ day), and disease-modifying antirheumatic drugs. Clinical parameters in the study RA patients were as follows (mean $\pm$ SD): serum C-reactive protein level, 45.7 ± 10.8 mg/liter; IgM class rheumatoid factor (RF) titer, 129 ± 38.9 units/ml. Synovial tissue samples were obtained from RA patients at the time of surgical treatment.

Two-color immunofluorescence labeling. Cryostat sections (4 μm) from RA synovial tissues were fixed in acetone, and blocked with 10% goat serum for 30 min. Double immunofluorescence was performed by serially incubating sections with 10 μg/ml of mouse IgG1 anti-CD16 monoclonal antibody (mAb) (Sigma, St. Louis, MO, USA), rat IgG2b anti-CX3CR1 mAb (2A9-1; Medical & Biological Laboratories, Nagoya, Japan), and isotype control mAbs at 4 °C overnight, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG1 mAb (AbD Serotec, Oxford, UK) and Texas red-conjugated goat anti-rat IgG mAb (Rockland, Philadelphia, PA, USA) for 30 min at room temperature. Double immunofluorescence of sections was examined with an LSM510 inverted confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Flow cytometry.   Peripheral blood mononuclear cells (PBMC) from patients with RA and healthy controls (HC) were prepared from heparinized blood samples by centrifugation over Ficoll-

Hypaque density gradients (Pharmacia, Uppsala, Sweden). Cells were washed well with RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA), and were resuspended in phosphate buffered saline (PBS) with 1% heat-inactivated fetal calf serum (FCS; Life Technologies). Cell surface expressions of CD14, CD16, and CX3CR1 were analyzed by cell surface staining and flow cytometric analysis, as described previously [15]. PBMC suspensions were incubated with FITC-conjugated anti-CX3CR1 mAb (2A9-1; Medical & Biological Laboratories), or isotype-matched control mAb (Immunotech, Marseille, France). Cells were washed and then incubated with phycoerythrin (PE)-conjugated anti-CD14 mAb (61D3; eBioscience, San Diego, CA, USA) and phycoerythrin-cyanin 5.1 (PC5)-conjugated anti-CD16 mAb (3G8; Immunotech). After washing, cells were resuspended in 1% FCS/PBS. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Isolation and culture of blood monocytes. PBMC from HC were resuspended at a density of 5×10^6 cells in culture medium (RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 2% nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin) with 10% FCS. The cell suspensions were incubated in 6-well plates (Corning, Corning, NY, USA) at 37 °C for 2 h in a humidified atmosphere containing 5% CO$_2$. After nonadherent cells were removed, adherent monocytes were gently harvested using a rubber scraper. The purity was confirmed to be > 95% CD14$^+$ by flow cytometry. The monocyte suspensions were stimulated in fresh 10% FCS/culture medium with 10 ng/ml of TNFα (Dainippon, Osaka, Japan), IL-1β (Otsuka, Tokushima, Japan), interferon γ (IFNγ) (PeproTech, London, UK), LPS (*Escherichia coli* 055: B5; Sigma) and IL-10 (Becton Dickinson). Total RNA was isolated from monocytes collected after 3 h of culture.

To examine the effects of FKN on IL-1β and IL-6 production, monocytes were treated for 3 h with 10 mM Dibutyryl-cAMP(DBcAMP) (BIOMOL, Plymouth Meeting, PA, USA) prior to addition of recombinant human FKN (R & D systems, Minneapolis, MN, USA). The culture supernatants were harvested after 36 h of FKN treatment and measured for IL-1β and IL-6 concentrations.

Isolation and culture of synovial fibroblasts. Fresh synovial tissues from RA patients were minced with scissors and digested for 1 h with collagenase (WAKO, Osaka, Japan) and DNase (Sigma) in RPMI-1640 at 37 °C. After removing tissue debris through a cell strainer, the cells were washed twice with medium. The resultant single cell suspensions were dispensed into the wells of a 24-well microtiter plate (Costar, Cambridge, MA, USA) at a density of 2×10^6/ml in 2 ml of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% FCS, 25 mM of HEPES (Life Technologies), 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO$_2$. The culture supernatants were harvested 72 h later and stored at -30 °C until chemokine assay. For the detection of chemokine mRNA expression, total RNA was isolated from fresh synovial tissue cells. Synovial fibroblasts were obtained by allowing synovial tissue cells to adhere to tissue culture plates, followed by removal of nonadherent cells, as described previously [20]. These cells were split weekly, once primary cultures had reached confluence, and experiments were performed using synovial fibroblast cell lines after the third or fourth passage. The resultant cells were morphologically homogeneous fibroblast-like cells that had typical bipolar configuration under inverse microscopy, and there were < 2% of contaminating lymphocytes or macrophages, as assessed by flow cytometry and staining with antibodies against CD3, CD14, and CD19 (Becton Dickinson). Synovial fibroblasts were stimulated in fresh 10% FCS/culture medium with 10 ng/ml of TNFα, IL-1β, IFNγ and LPS. Total RNA was isolated from synovial fibroblasts collected after 3 h of culture.

In a similar manner, synovial fibroblasts were stimulated with 10 ng/ml of TNFα, IL-1β, IFNγ and LPS for 24 h. The culture supernatants and cell lysates were then treated with passive lysis buffer (Promega, Madison, WI, USA), collected, and subjected to measurement of membrane-anchored and soluble FKN concentrations.

Isolation of mRNA and real-time polymerase chain reaction (PCR). Total cellular RNA was extracted from monocytes and synovial fibroblasts

using an RNA isolation kit (RNeasy Mini kit; Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA with Molony murine leukemia virus reverse transcriptase (US Biochemical, Cleveland, OH, USA) and oligo-(dT)$_{15}$ primers (Promega). Real-time PCR was performed with a LightCycler Instrument (Roche Diagnostics, Penzberg, Germany) in glass capillaries.

The cDNA samples were denatured at 95 °C for 10 min, then amplified for 40–50 cycles of 95 °C for 10 sec, 65 °C for 15 sec, and 72 °C for 10 sec (β-actin and CX3CR1) or 95 °C for 10 sec, 70 °C for 15 sec, and 72 °C for 10 sec (FKN). Amplification curves of the fluorescence values versus cycle number were obtained, and a melting curve analysis was performed. The levels of FKN and CX3CR1 expression were determined by normalizing relative to β-actin expression. The forward and reverse primers were as follows: for β-actin, 5'-GCATGGGT CAGAAGGATTCCTATG-3' and 5'-CATCTCCA TCACTCGTGTGGTAAG-3'; for FKN, 5'-TTAT CACTCCTGTCCCTGACGC-3' and 5'-GCCATCTC TCCTGCCATCTTTC-3'; for CX3CR1, 5'-TTGC CTGAATCCTCTCATCTATGC-3' and 5'-CATC TCCATCACTCGTGTGGTAAG-3'.

Immunoassays for cytokines. The sera and synovial fluid were diluted 1:10 with PBS and subjected to quantitative sandwich enzyme-linked immunosorbent assay (ELISA). Concentrations of FKN, IL-1β, and IL-6 were measured in duplicate using cytokine-specific capture with biotinylated detection mAb and recombinant cytokine proteins, FKN, IL-1β, and IL-6 (Becton Dickinson), according to the manufacturer's instructions. The detection limits were 20 pg/ml for IL-1β and IL-6, and 80 pg/ml for FKN.

Statistical analysis. Samples with values below the detection limit for the assay were regarded as negative and assigned a value of 0. Data were expressed as the mean value $\pm$ SEM of the number of samples evaluated. The statistical significance of differences between 2 groups was determined by Mann-Whitney U test or Wilcoxon's signed rank test. *P* values less than 0.05 were considered statistically significant.

Results

Levels of soluble FKN protein in serum and

synovial fluid from RA patients and OA patients. We used ELISA to determine the levels of FKN in paired synovial fluid and serum samples obtained from 22 RA patients and 16 OA patients and 12 healthy controls (HC) (Fig. 1). FKN levels in both the synovial fluid (34.32 ± 15.36 ng/ml) and serum (53.33 ± 19.63 ng/ml) of RA patients were significantly greater than the levels in the synovial fluid (3.17 ± 2.48 ng/ml; $p < 0.05$) and serum (8.00 ± 4.46 ng/ml; $p < 0.05$) of OA patients and in the serum (0.56 ± 0.56 ng/ml) of HC. In addition, the level of FKN did not significantly correlate with the CRP level (data not shown).

CX3CR1 expression in vascular endothelial cells and synovial lining macrophages in RA synovial tissue. To determine the expression of CX3CR1 at the site of inflammation, synovial tissues from 5 patients with RA were analyzed by 2-color immunofluorescence labeling using anti-CD16 antibody and anti-CX3CR1 antibody. Fig. 2 shows representative staining patterns of CD16 and CX3CR1 expression in the synovial tissue. The CD16 and CX3CR1 double-positive cells were widely distributed throughout the synovial tissues, including the lining and sublining layers; while vascular endothe-
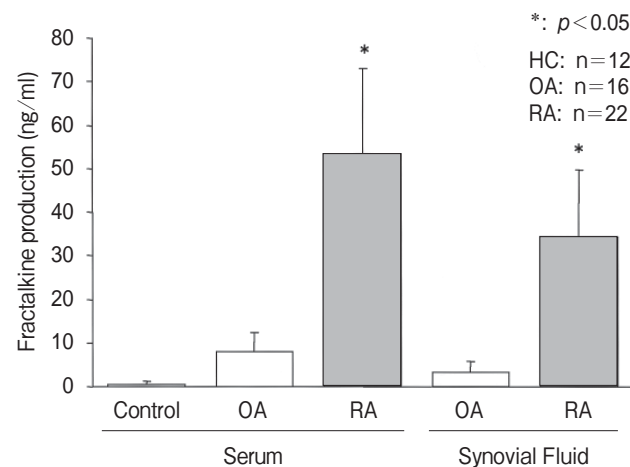

Fig. 1   Levels of soluble fractalkine (FKN) protein in both serum and synovial fluid from rheumatoid arthritis (RA) and osteoarthritis (OA) patients and healthy controls (HC). Concentrations of FKN were measured in duplicate by the quantitative sandwich enzyme-linked immunosorbent assay (ELISA). n = number of samples tested. Values are the mean $\pm$ SEM. *$p < 0.05$ versus OA samples.

lial cells were stained with CX3CR1 and lacked CD16 immunoreactivity.

Higher expression of CX3CR1 on CD14$^+$/ CD16$^+$ blood monocytes. The cell surface expression of CD14 and CD16 on peripheral blood monocytes from patients with RA and HC was determined by flow cytometric analysis. Fig. 3A shows the representative distribution of the CD14$^+$/CD16$^+$ or CD14$^+$/CD16$^-$ monocyte subpopulations and their CX3CR1 expressions in the patients with RA and HC. The population of CD14$^+$/CD16$^+$ blood monocytes was larger in patients with RA ($11.9 \pm 5.8\%$) than in the HC ($9.2 \pm 2.1\%$), confirming our previous observation [15]. We next compared the intensity of CX3CR1 on monocytes from patients with RA and HC. The mean fluorescence intensity of CX3CR1 on CD14$^+$/CD16$^+$ monocytes was significantly greater than that on CD14$^+$/CD16$^-$ monocytes both in patients with RA ($p < 0.05$) and in HC ($p < 0.05$). The mean fluorescence intensity of CX3CR1 on CD14$^+$/CD16$^+$ monocytes in patients with RA was not significantly different from that in HC (Fig. 3B).

IL-10-induced expression of CX3CR1 mRNA in peripheral blood monocytes. We compared the expression of CX3CR1 on monocytes stimulated by TNFα, IL-1β, IFNγ, LPS and IL-10. Peripheral blood monocytes were incubated for 3 h in culture medium with 10 ng/ml of TNFα, IL-1β, IFNγ, LPS or IL-10, and the total RNAs were iso-

lated and subjected to quantitative real-time PCR. IL-10 induced 4.5-fold higher CX3CR1 mRNA expression ($p < 0.05$), while the treatment with LPS significantly reduced CX3CR1 mRNA expression ($p < 0.05$) (Fig. 4).

TNFα- induced expression of FKN mRNA in synovial fibroblasts. We next compared the expression of FKN on fibroblasts in the presence of TNFα, IL-1β, IFNγ and LPS. Synovial fibroblasts were incubated for 3 h in culture medium with 10 ng/ ml of TNFα, IL-1β, IFNγ, or LPS, and total RNA was isolated to determine the expression of FKN by quantitative real-time PCR. Treatment with TNFα significantly increased FKN mRNA expression ($p < 0.05$) (Fig. 5).

Synovial fibroblasts synthesized FKN in response to the stimulation with TNFα. We next determined the concentration of soluble and membrane-bound FKN in the supernatant and cell lysates from the synovial fibroblasts in the presence of various cytokines. We detected the induction of soluble FKN in the supernatant from TNFα- and IL-1β-stimulated fibroblasts, although IFNγ and LPS did not affect the release of soluble FKN ($p < 0.05$) (Fig. 6A). Similarly, the amount of membrane-bound FKN in synovial fibroblasts was significantly up-regulated in the presence of TNFα ($p < 0.05$) (Fig. 6B).
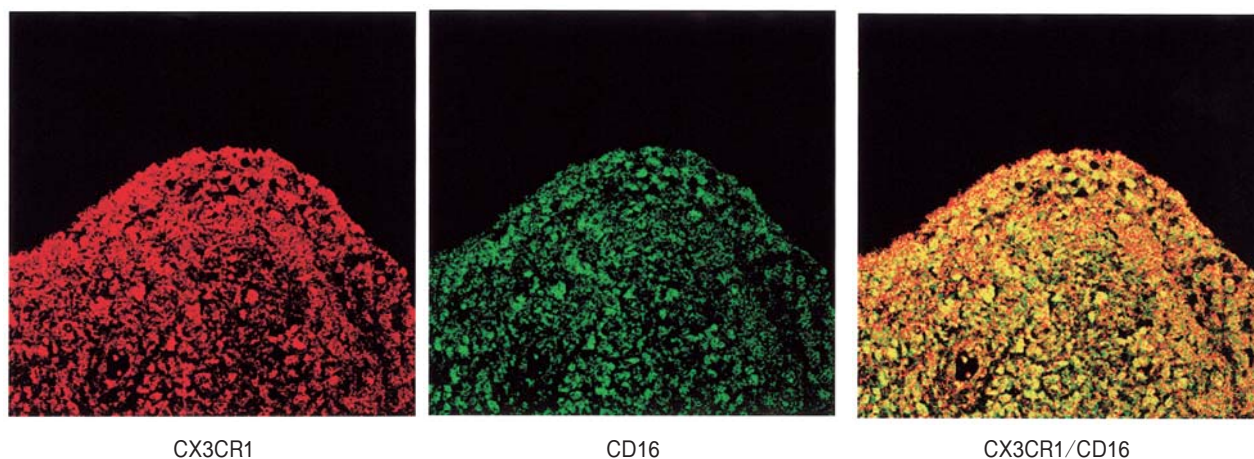

CX3CR1   CD16   CX3CR1/CD16

Fig. 2 Expression of CD16 and CX3CR1 in synovial tissues of rheumatoid arthritis (RA) patients. Synovial tissue sections from RA patients were stained with anti-CX3CR1 Ab and anti-CD16 Ab followed by incubation with isotype-specific Texas red- and FITC-conjugated secondary Abs (CX3CR1, red; CD16, green).

Effects of FKN on IL-1β and IL-6 produc-
tion by monocytes.   It has been reported that
immobilized and membrane-bound FKN markedly
induced IFNγ production by NK cells [21].
However, it remains to be determined whether FKN
induces cytokine production by monocytes.
Therefore, we examined whether soluble FKN
induced production of cytokines by monocytes.
Activated monocytes that had been pretreated with

DBcAMP were stimulated with recombinant human
FKN. The culture supernatants were harvested and
subjected to ELISA to determine their concentra-
tions of IL-1β and IL-6. The addition of recombinant
FKN increased the production of both IL-1β and
IL-6 by monocytes in a dose-dependent manner
($p < 0.05$) (Fig. 7A and B).
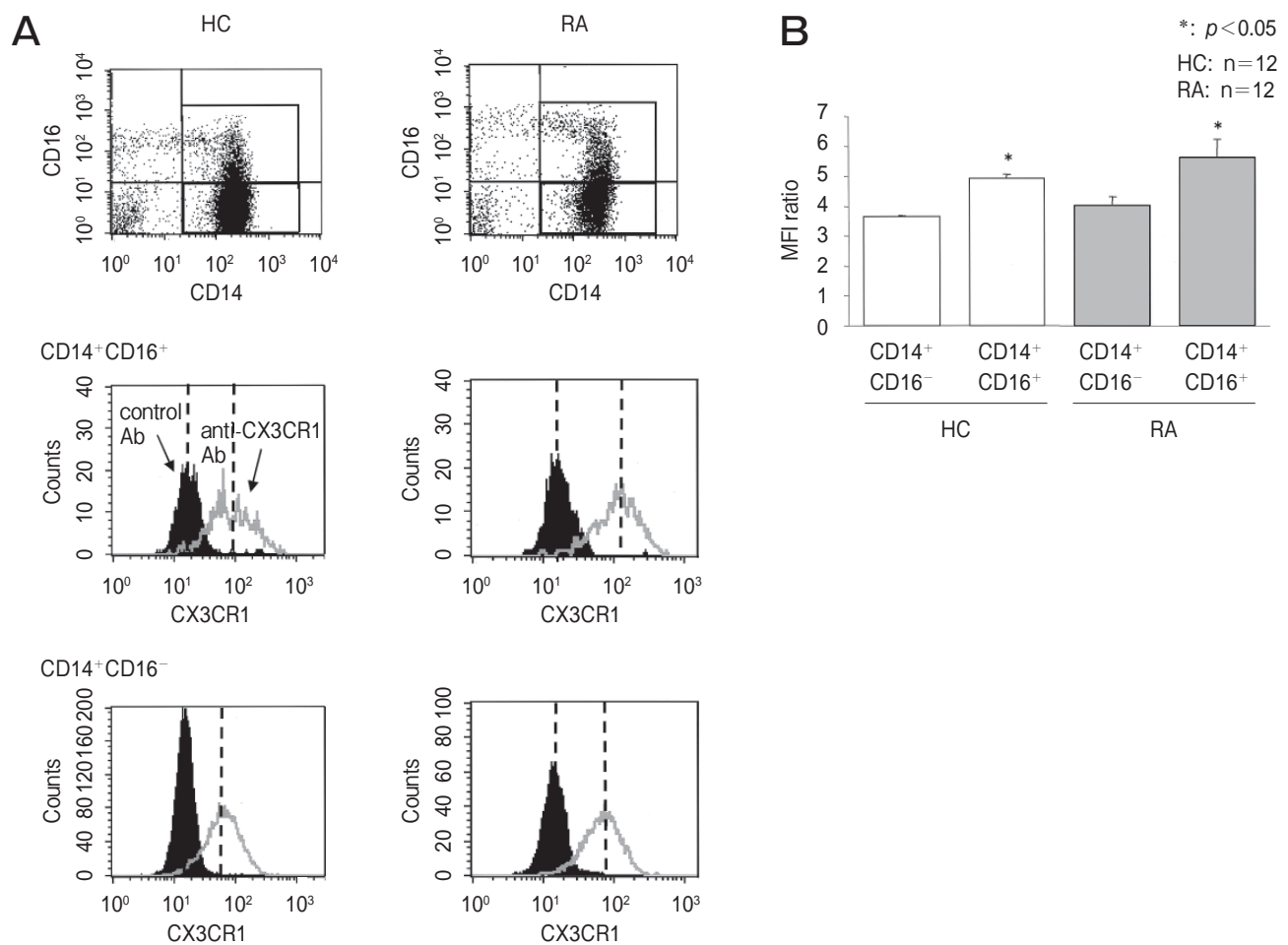
Fig. 3   Expression of CX3CR1 on $CD14^+/CD16^+$ blood monocytes. (**A**) Peripheral blood mononuclear cells from patients with RA and healthy controls (HC) were stained with FITC-conjugated anti-CX3CR1 mAb, phycoerythrin (PE)-conjugated anti-CD14 mAb, and phycoerythrin-cyanin 5.1 (PC5) -conjugated anti-CD16 mAb. Flow cytometric analysis of the cell surface expression of CD14, CD16, and CX3CR1 was performed by gating on monocytes according to size, granularity, and CD16 expression. Two subsets of monocytes were identified: $CD14^+/CD16^+$ and $CD14^+/CD16^-$. The $CD14^+/CD16^+$ monocytes in the upper right panel account for 8.8% and 13.8% of all CD14-expressing cells in the representative HC and RA patient, respectively. Representative histographic patterns of CX3CR1 expression on $CD14^+/CD16^+$ and $CD14^+/CD16^-$ blood monocytes from an HC and RA patient are shown. (**B**) The intensity of CX3CR1 on $CD14^+/CD16^+$ blood monocytes was expressed as the ratio of the mean fluorescence intensity (MFI) of staining with anti-CX3CR1 to that of staining with the control antibody. Values are the mean ± SEM. *$p < 0.05$ versus $CD14^+/CD16^-$ blood monocytes.
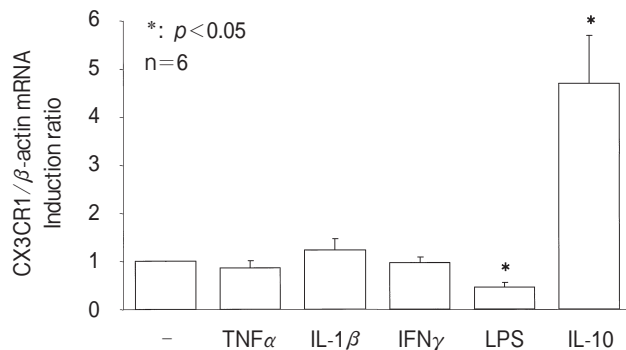
Discussion

The CD14$^+$/CD16$^+$ monocytes express higher levels of class II MHC molecules, adhesion molecules, and chemokine receptors and are able to more efficiently produce proinflammatory cytokines, as compared with the classic CD14^{++}/CD16$^-$ monocytes [17]. Thus, this minor population is consid-

Fig. 4 Expression of CX3CR1 mRNA in cultured monocytes. Monocyte suspensions from HC were stimulated in fresh 10% FCS/culture medium with 10 ng/ml of TNFα, IL-1β, IFNγ, LPS and IL-10. Total RNA was isolated from monocytes after 3 h of culture. CX3CR1 mRNA was detected by RT-PCR. Levels of CX3CR1 mRNA were normalized relative to β-actin expression. Values are the mean $\pm$ SEM. *$p < 0.05$ versus the control.
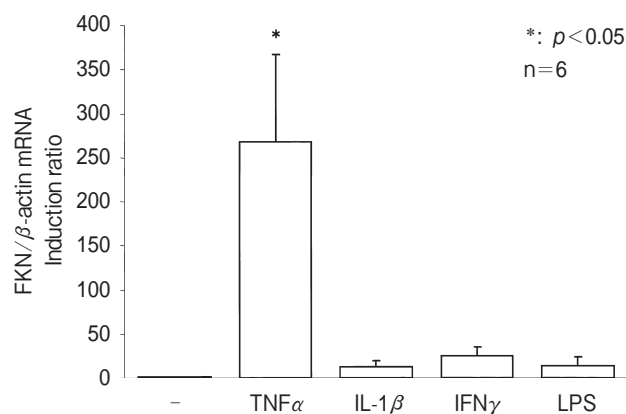
Fig. 5 Expression of FKN mRNA in cultured synovial fibroblasts. Synovial fibroblasts from RA patients were stimulated in fresh 10% FCS/culture medium with 10 ng/ml of TNFα, IL-1β, IFNγ and LPS. Total RNA was isolated from synovial fibroblasts after 3 h of culture. FKN mRNA was detected by RT-PCR. Levels of FKN mRNA were normalized relative to β-actin expression. Values are the mean $\pm$ SEM. *$p < 0.05$ versus the control.

ered to comprise proinflammatory monocytes that can rapidly migrate to the site of inflammation and readily differentiate into tissue macrophages. We previously demonstrated that CD14$^+$/CD16$^+$ blood monocytes are increased in active RA in association with a cytokine spillover from joints with inflammation [15]. Moreover, CD14$^+$/CD16$^+$ monocytes express high levels of Toll-like receptor 2 and are able to efficiently produce proinflammatory cytokines such as TNFα [22]. In agreement with previous studies [23, 24], we here found that CD16$^+$ monocytes expressed CX3CR1 more prominently than CD16$^-$ monocytes, both in RA patients and healthy controls.
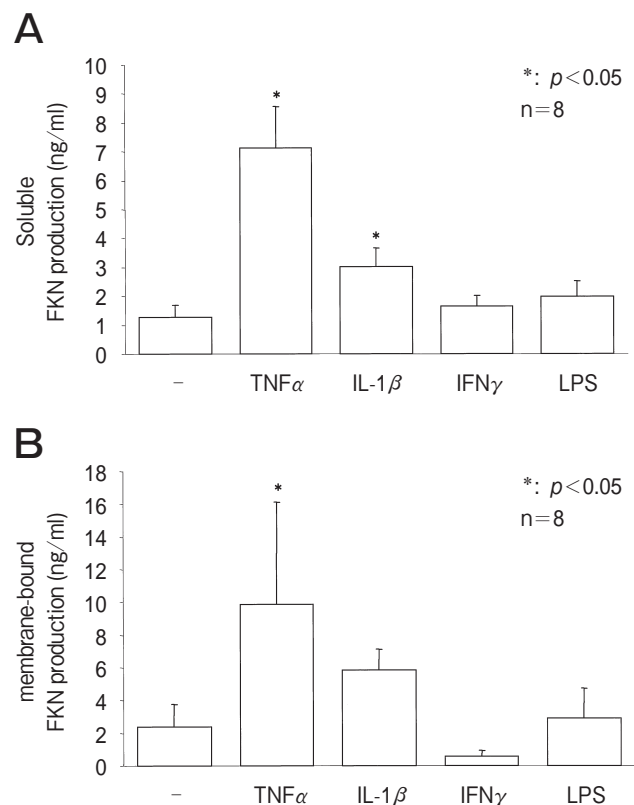
IL-10 plays a predominant role in limiting immune and inflammatory responses by regulating the func-

Fig. 6 Induction of soluble FKN in the culture supernatant and membrane-bound FKN in the cell lysate of synovial fibroblasts. Synovial fibroblasts from RA patients were stimulated with 10 ng/ml of TNFα, IL-1β, IFNγ, and LPS for 24 h. Then, culture supernatants (**A**) and cell lysates that were treated with passive lysis buffer (**B**) were collected and measured for soluble and membrane-anchored FKN concentrations by ELISA. Values are the mean $\pm$ SEM. *$p < 0.05$ versus the control.

A

B
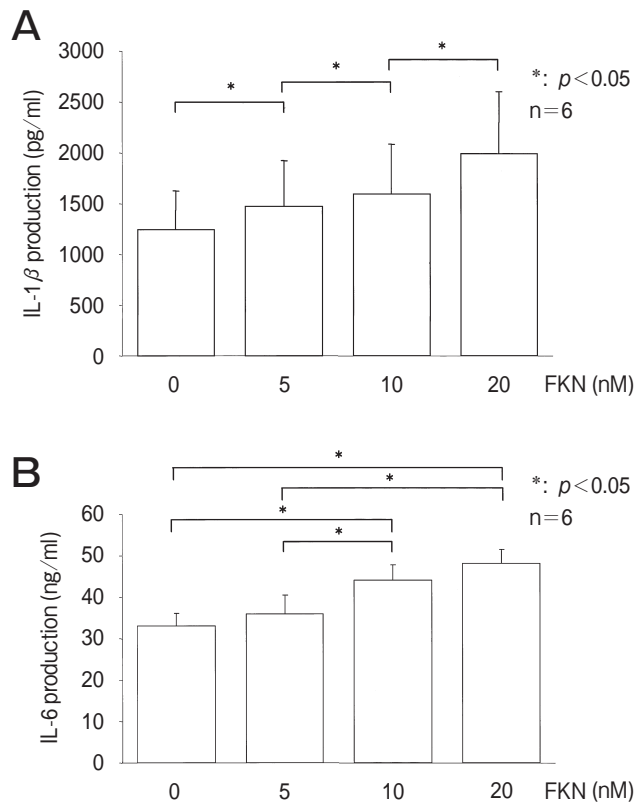
Fig. 7   Effects of FKN on IL-1β and IL-6 production by cultured monocytes. Monocytes from HC were treated for 3 h with DBcAMP (10 mM) prior to the addition of various concentrations of recombinant human FKN. The culture supernatants were harvested after 36 h of FKN treatment, and then IL-1β (**A**) and IL-6 (**B**) concentrations were measured by ELISA. Values are the mean ± SEM.

tion of both macrophages and Th1 cells [25]. IL-10 in combination with M-CSF and IL-4 contributes to the development of the rare population of CD14$^+$/CD16$^+$ cells derived from human monocytes [26]. Incubation of a microglial cell line with LPS down-regulated CX3CR1 mRNA expression to almost undetectable levels. However, in the presence of IL-10, LPS did not reduce CX3CR1 transcript production [27]. We confirmed by RT-PCR that IL-10 induced high levels of CX3CR1 mRNA expression on monocytes. These findings suggest that IL-10 induces the synchronous expression of both CD16 and CX3CR1 on the cell surface of monocytes. Consequently, the CD16$^+$ monocyte subpopulation revealed prominent expression of CX3CR1. The

ligand for CX3CR1, FKN, is also regulated by various cytokines, and it has been reported that FKN is up-regulated by treatment with TNFα, IL-1β, or IFNγ. In the present study, FKN was prominently induced in synovial fibroblasts by TNFα, and neither IL-1β nor IFNγ altered the FKN levels. Since Fraticelli et al. [28] reported that FKN expression was induced in human umbilical vein endothelial cells (HUVECs) by 24 h-incubation of IFNγ, the 3-h incubation time used in the present study may not have been sufficient for the induction of FKN in synovial cells.

Another important finding of our study is that high levels of soluble FKN were produced in the synovial fluid and detected in the sera of RA patients. Moreover, we confirmed by two-color immunofluorescence staining that high levels of CX3CR1 were expressed in CD16$^+$ monocytes localized to the lining and sublining layer. These results suggest that FKN may preferentially promote the migration of CD16$^+$ monocytes into the synovial tissues, and such recruitment of the proinflammatory monocyte subset may contribute to the tissue injury and destruction of the joints [24]. FKN stimulation of cultured synovial fibroblasts has been shown to result in a marked upregulation of matrix metalloproteinase-2 production [29]. In contrast, LPS-induced TNFα secretion by monocytes [30] and microglia [31, 32] were inhibited by FKN. These results suggest that FKN modulates immune responses to induce or inhibit the production of various cytokines. To assess the involvement of FKN in inflammatory processes, we examined the effect of soluble FKN on cytokine production by monocytes. We found that soluble FKN markedly induced IL-1β and IL-6 production by monocytes in a dose-dependent manner.

The mechanisms underlying the cytokine induction are unknown; however, Chandrasekar et al. [33] have shown that FKN induced its own expression in aortic smooth muscle cells via heterotrimeric Gi-proteins, phosphoinositide 3-kinase (PI3-kinase), phosphoinositide-dependent kinase1 (PDK1), Akt, NF-κB-inducing kinase (NIK), inhibitoryκB kinase (IKK) and NF-κB. In another study, FKN was shown to activate NF-κB and p53, resulting in IL-8 and FKN mRNA expression on intestinal epithelial cells [34]. These findings may suggest a role for FKN expressed on synovial fibroblasts through the

stimulation of IL-1β and IL-6 production by monocytes as well as FKN production by fibroblasts. Such soluble FKN-mediated paracrine and autocrine amplification loops would further activate the monocytes and lead to prominent cytokine production in the synovium of RA patients.

In summary, soluble FKN levels were significantly elevated in synovial fluid and sera, which induced the production of cytokines, such as IL-1β and IL-6, by monocytes in RA patients. The activated monocytes secreted IL-10 and up-regulated CX3CR1 and CD16$^+$ expression in an autocrine fashion. Interactions of FKN and CX3CR1 via chemotactic and adhesive processes might contribute to the accumulation of CD16$^+$ monocytes expressing CX3CR1 at the synovial tissue of RA patients and continuation of inflammation at the site. Recently, it has been reported that inhibition of FKN ameliorated collagen-induced arthritis in mice, most likely by suppressing inflammatory cell migration into the RA synovium [35]. Thus, FKN may become a new target molecule for the treatment of RA in the future.

References

1. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A and Schall TJ: A new class of membrane-bound chemokine with a CX3 C motif. Nature (1997) 385: 640–644.
2. Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, Vath J, Gosselin M, Ma J, Dussault B, Woolf E, Alperin G, Culpepper J, Gutierrez-Ramos JC and Gearing D: Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. Nature (1997) 387: 611–617.
3. Lucas AD, Chadwick N, Warren BF, Jewell DP, Gordon S, Powrie F and Greaves DR: The transmembrane form of the CX3CL1 chemokine fractalkine is expressed predominantly by epithelial cells in vivo. Am J Pathol (2001) 158: 855–866.
4. Muehlhoefer A, Saubermann LJ, Gu X, Luedtke-Heckenkamp K, Xavier R, Blumberg RS, Podolsky DK, MacDermott RP and Reinecker HC: Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa. J Immunol (2000) 164: 3368–3376.
5. Kanazawa N, Nakamura T, Tashiro K, Muramatsu M, Morita K, Yoneda K, Inaba K, Imamura S and Honjo T: Fractalkine and macrophage-derived chemokine: T cell-attracting chemokines expressed in T cell area dendritic cells. Eur J Immunol (1999) 29: 1925–1932.
6. Papadopoulos EJ, Sassetti C, Saeki H, Yamada N, Kawamura T, Fitzhugh DJ, Saraf MA, Schall T, Blauvelt A, Rosen SD and Hwang ST: Fractalkine, a CX3 C chemokine, is expressed by dendritic cells and is up-regulated upon dendritic cell maturation. Eur J Immunol (1999) 29: 2551–2559.
7. Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB and Feng L: Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proc Natl Acad Sci U S A (1998) 95: 10896–10901.
8. Yoshikawa M, Nakajima T, Matsumoto K, Okada N, Tsukidate T, Iida M, Otori N, Haruna S, Moriyama H, Imai T and Saito H: TNF-alpha and IL-4 regulate expression of fractalkine (CX3CL1) as a membrane-anchored proadhesive protein and soluble chemotactic peptide on human fibroblasts. FEBS Lett (2004) 561: 105–110.
9. Imaizumi T, Yoshida H and Satoh K: Regulation of CX3CL1/fractalkine expression in endothelial cells. J Atheroscler Thromb (2004) 11: 15–21.
10. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ and Yoshie O: Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell (1997) 91: 521–530.
11. Combadiere C, Salzwedel K, Smith ED, Tiffany HL, Berger EA and Murphy PM: Identification of CX3CR1. A chemotactic receptor for the human CX3 C chemokine fractalkine and a fusion coreceptor for HIV-1. J Biol Chem (1998) 273: 23799–23804.
12. Janossy G, Panayi G, Duke O, Bofill M, Poulter LW and Goldstein G: Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. Lancet (1981) 2: 839–842.
13. Qu Z, Garcia CH, O'Rourke LM, Planck SR, Kohli M and Rosenbaum JT: Local proliferation of fibroblast-like synoviocytes contributes to synovial hyperplasia. Results of proliferating cell nuclear antigen/cyclin, c-myc, and nucleolar organizer region staining. Arthritis Rheum (1994) 37: 212–220.
14. Burmester GR, Stuhlmuller B, Keyszer G and Kinne RW: Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? Arthritis Rheum (1997) 40: 5–18.
15. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, Iwahashi M, Ueno A, Ohmoto Y and Makino H: CD14$^+$, CD16$^+$ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum (2002) 46: 2578–2586.
16. Ziegler-Heitbrock HW, Fingerle G, Strobel M, Schraut W, Stelter F, Schutt C, Passlick B and Pforte A: The novel subset of CD14$^+$/CD16$^+$ blood monocytes exhibits features of tissue macrophages. Eur J Immunol (1993) 23: 2053–2058.
17. Ziegler-Heitbrock HW: Heterogeneity of human blood monocytes: the CD14$^+$ CD16$^+$ subpopulation. Immunol Today (1996) 17: 424–428.
18. Nanki T, Imai T, Nagasaka K, Urasaki Y, Nonomura Y, Taniguchi K, Hayashida K, Hasegawa J, Yoshie O and Miyasaka N: 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: 2878–2883.
19. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH and Luthra HS: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum (1988) 31: 315–324.
20. Okamoto H, Yamamura M, Morita Y, Harada S, Makino H and

Ota Z: The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. Arthritis Rheum (1997) 40: 1096–1105.

21. Yoneda O, Imai T, Nishimura M, Miyaji M, Mimori T, Okazaki T, Domae N, Fujimoto H, Minami Y, Kono T, Bloom ET and Umehara H: Membrane-bound form of fractalkine induces IFN-gamma production by NK cells. Eur J Immunol (2003) 33: 53–58.

22. Iwahashi M, Yamamura M, Aita T, Okamoto A, Ueno A, Ogawa N, Akashi S, Miyake K, Godowski PJ and Makino H: Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. Arthritis Rheum (2004) 50: 1457–1467.

23. Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW and Gabuzda D: Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. J Exp Med (2003) 197: 1701–1707.

24. Geissmann F, Jung S and Littman DR: Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity (2003) 19: 71–82.

25. Moore KW, de Waal Malefyt R, Coffman RL and O'Garra A: Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol (2001) 19: 683–765.

26. Li G, Hangoc G and Broxmeyer HE: Interleukin-10 in combination with M-CSF and IL-4 contributes to development of the rare population of CD14+ CD16++ cells derived from human monocytes. Biochem Biophys Res Commun (2004) 322: 637–643.

27. Kremlev SG and Palmer C: Interleukin-10 inhibits endotoxin-induced pro-inflammatory cytokines in microglial cell cultures. J Neuroimmunol (2005) 162: 71–80.

28. Fraticelli P, Sironi M, Bianchi G, D'Ambrosio D, Albanesi C, Stoppacciaro A, Chieppa M, Allavena P, Ruco L, Girolomoni G, Sinigaglia F, Vecchi A and Mantovani A: Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. J Clin Invest (2001) 107: 1173–1181.

29. Blaschke S, Koziolek M, Schwarz A, Benohr P, Middel P, Schwarz G, Hummel KM and Muller GA: Proinflammatory role of fractalkine (CX3CL1) in rheumatoid arthritis. J Rheumatol (2003) 30: 1918–1927.

30. Ollivier V, Faure S, Tarantino N, Chollet-Martin S, Deterre P, Combadiere C and de Prost D: Fractalkine/CX3CL1 production by human aortic smooth muscle cells impairs monocyte procoagulant and inflammatory responses. Cytokine (2003) 21: 303–311.

31. Mizuno T, Kawanokuchi J, Numata K and Suzumura A: Production and neuroprotective functions of fractalkine in the central nervous system. Brain Res (2003) 979: 65–70.

32. Zujovic V, Benavides J, Vige X, Carter C and Taupin V: Fractalkine modulates TNF-alpha secretion and neurotoxicity induced by microglial activation. Glia (2000) 29: 305–315.

33. Chandrasekar B, Mummidi S, Perla RP, Bysani S, Dulin NO, Liu F and Melby PC: Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. Biochem J (2003) 373: 547–558.

34. Brand S, Sakaguchi T, Gu X, Colgan SP and Reinecker HC: Fractalkine-mediated signals regulate cell-survival and immune-modulatory responses in intestinal epithelial cells. Gastroenterology (2002) 122: 166–177.

35. Nanki T, Urasaki Y, Imai T, Nishimura M, Muramoto K, Kubota T and Miyasaka N: Inhibition of fractalkine ameliorates murine collagen-induced arthritis. J Immunol (2004) 173: 7010–7016.