Proinflammatory Role of Fractalkine (CX3CL1) in Rheumatoid Arthritis

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Abstract. ***Objective.*** Fractalkine (CX3CL1) represents the sole member of the so-called CX3C chemokines. In rheumatoid arthritis (RA), functional studies suggest a role for this chemokine in monocyte chemotaxis and angiogenesis in the rheumatoid synovium. We analyzed the expression of fractalkine within different T cell subsets of the peripheral blood and expression of its receptor CX3CR1 within the rheumatoid synovium to further characterize its pathogenic role in RA.

Methods. Peripheral blood mononuclear cells (PBMC) were isolated from 17 patients with RA and analyzed by flow cytometry in comparison to healthy blood donors. To identify the T helper cell cytokine profile of fractalkine-expressing cells, flow cytometric analysis of PBMC was performed after stimulation with PMA and ionomycin. Expression of fractalkine and its receptor was characterized in RA synovium by immunohistochemistry and laser capture microdissection microscopy.

Results. Flow cytometric analysis of fractalkine-expressing T cell subsets revealed a low proportion of fractalkine-expressing CD4+ and CD8+ T cells in both RA patients and controls. In addition, fractalkine was predominantly expressed in CD4+ T cells with a Th1-type cytokine expression profile. In RA synovium, fractalkine was detected in synovial macrophages, dendritic cells, endothelial cells, and a small proportion of T cells. The fractalkine receptor CX3CR1 was found in synovial macrophages, dendritic cells, and T cells as well as in synovial fibroblasts. Fractalkine stimulation of cultured synovial fibroblasts resulted in a marked upregulation of matrix metalloproteinase-2 (MMP-2) production.

Conclusion. The results suggest that fractalkine may represent a Th1-type chemokine. Upregulation of MMP-2 production in synovial fibroblasts upon fractalkine stimulation *in vitro* supports the hypothesis of a proinflammatory role of this chemokine in RA. (J Rheumatol 2003;30:1918–27)

Key Indexing Terms:
CHEMOKINES FRACTALKINE RHEUMATOID ARTHRITIS T CELLS

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease of unknown etiology leading to progressive joint destruction by chronic synovial inflammation. Histopathologic changes of the rheumatoid synovium are characterized by a marked hyperplasia of synovial fibroblasts and an infiltrate of monocytes/macrophages as well as an accumulation of CD4+ T lymphocytes[1]. While synovial fibroblasts and synovial macrophages promote the inflammatory process by production of tissue-destroying matrix metalloproteinases (MMP) and proinflammatory cytokines [interleukin 1ß (IL-1ß), IL-6, tumor necrosis factor-α (TNF-α)], the functional role of CD4+ T cells in the pathogenesis of RA remains to be clarified[2]. Several factors that may promote monocyte and lymphocyte infiltration in RA have been analyzed, including cell adhesion molecules, cytokines, and chemokines.

Chemokines constitute a superfamily of small, secreted proteins involved in transendothelial leukocyte migration and activation along chemoattractant gradients during inflammation[3]. These molecules are structurally related and have been classified according to the arrangement of the N-terminal conserved cysteine (C) motif into 4 groups, designated CC, CXC, CX3C, and C chemokines[4]. Whereas the CXC and CC groups both include several members, the C and CX3C chemokine subfamilies are represented so far by only one member, i.e., lymphotactin (Lptn, XCL1) and fractalkine (neurotactin, CX3CL1), respectively. Chemokines mediate their activities by binding to cell surface receptors that belong to the large family of G-protein coupled, 7 transmembrane domain receptors.

Fractalkine (CX3CL1), the sole member of the CX3C chemokines, has been identified by bioinformatics[5,6]. It was found to represent a type 1, 397aa residue membrane-bound protein containing a chemokine domain at the amino

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terminus tethered on a long mucin-like stalk. The extracellular domain of fractalkine is released as a 95 kDa glycoprotein[5]. The cleavage and shedding of fractalkine has recently been shown to be mediated by the TNF-α-converting enzyme (TACE, ADAM 17)[7]. Fractalkine was found to be expressed on TNF-α and IL-1ß activated endothelial cells[8], dendritic cells[9], neuronal cells[10-12], astrocytes[13], lymphocytes[14], and epithelial cells[15,16] as well as smooth muscle cells[17]. It acts via a G-protein coupled, 7 transmembrane receptor[18] that has been detected on natural killer (NK) cells[18,19], microglia[11,12], T cells[20], and mast cells[21]. Functionally, membrane-bound fractalkine has been shown to chemoattract monocytes and lymphocytes, but not neutrophils *in vitro*[5].

In RA, expression of fractalkine has been detected in CD3+ T cells and monocytes of the peripheral blood as well as in synovial macrophages, fibroblasts, and endothelial cells in RA synovium[14]. Functional studies suggested a proinflammatory role for this chemokine in monocyte chemotaxis[14] and angiogenesis[22] in the pathogenesis of RA.

We analyzed the expression of fractalkine within different T cell subsets of the peripheral blood and expression of the receptor within the rheumatoid synovium to further characterize its functional role in RA. Because fractalkine stimulates MMP expression in cultured microglial cells[23] *in vitro,* we hypothesized that fractalkine may also stimulate MMP expression in synovial fibroblasts.

MATERIALS AND METHODS

Patients and samples. Peripheral blood mononuclear cells (PBMC) were obtained for flow cytometric analysis from 17 female RA patients (mean age 41 yrs) and 12 healthy age and sex matched blood donors as controls. The study was approved by the local Institutional Review Board. All patients with RA met the American College of Rheumatology 1987 criteria for the diagnosis of RA[24]. The mean disease duration was 7.3 years (range 1–28 yrs). Most of the patients (12/17, 70.5%) were rheumatoid factor (RF) positive and received standard disease modifying antirheumatic drug therapy (methotrexate, leflunomide, or both) and low dose prednisone (< 10 mg/day). Patients had active disease, defined as at least 3 swollen and tender peripheral joints and morning stiffness for > 1 h, with or without an elevated erythrocyte sedimentation rate (ESR > 28 mm/h) or C-reactive protein (CRP > 8 mg/l). The mean clinical disease activity assessed according to the method defined by van der Heijde, *et al*[25] was 3.18 (range 2.29–4.44).

Synovial tissues were obtained at the time of surgery from RA patients who underwent synovectomy or total joint replacement surgery. After surgery, tissue specimens were snap-frozen in liquid nitrogen and embedded in Tissue-Tek OCT freezing medium (Sakura Finetek, Torrance, CA, USA) for cryosections. In addition, paraffin embedded tissue was used for immunohistochemistry and double-staining experiments. Synovial fibroblast cultures were generated from synovial tissue samples as described[26]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Eggenstein, Germany) containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (BioWhittaker Europe, Apen, Germany). The medium was changed twice weekly and cells were split in half at confluency. For *in vitro* stimulation experiments, cultured synovial fibroblasts were used between passages 3 and 8 to ensure exclusion of macrophages from culture. The following cell lines were used for analysis of fractalkine and fractalkine receptor expression by reverse transcription-polymerase chain reaction (RT-PCR): *T Jurkat*, a T cell acute lymphoblastic leukemia cell line (TIB-152) obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), *HOS*, an osteosarcoma cell line (CRL-1543; ATCC), and 2 renal fibroblast cell lines, *TK 173* and *TK 188* (kindly provided by Prof. C.A. Müller, Section for Transplantation Immunology and Immunohematology, Tuebingen, Germany). In addition, primary osteoclasts and chondrocytes were kindly provided by Dr. V. Viereck, Department of Gynecology and Obstetrics, University of Goettingen, Germany.

Flow cytometric analysis of PBMC. Flow cytometry of PBMC was performed as described[27]. In brief, PBMC were isolated from heparinized blood samples by Ficoll-Hypaque density centrifugation (Biochrom, Berlin, Germany). For fractalkine staining, cells were blocked with blocking buffer [phosphate buffered saline (PBS)/1% bovine serum albumin (BSA)/10% normal goat serum/0.1% NaN_3] for 20 min and subsequently resuspended in PBS/0.1% BSA containing 0.5% saponin (Sigma, Deisenhofen, Germany) and incubated with a primary monoclonal mouse anti-human fractalkine antibody (1:100; clone 51637.11, R&D Systems, Wiesbaden, Germany) for 1 h at 4°C. After washing in PBS/0.1% BSA, specifically bound antibody was detected by addition of R-phycoerythrin (PE) conjugated goat $F(ab')_2$ fragments of anti-mouse IgG antibody (Coulter-Immunotech, Krefeld, Germany). After washing in PBS/0.1% BSA, cells were stained for cell surface antigens with fluorophore-labeled antibodies for 30 min at 4°C. Antibodies anti-CD3-PE, anti-CD8-PerCP, anti-CD4-PerCP, and anti-CD56-FITC were purchased from Becton Dickinson (Heidelberg, Germany) and anti-CD3-FITC, anti-CD14-FITC, and the isotype control IgG1-FITC/IgG2a-PE were from Coulter-Immunotech. After washing in PBS/0.1% BSA, cells were resuspended in PBS and subjected to flow cytometric analysis on a FACSCalibur (Becton Dickinson), using CellQuest® software. Bivariate dot plot analysis was generated upon data reanalysis to display the frequencies of individual cells coexpressing certain levels of cell surface antigens and fractalkine. As a control, an isotype control of the same species was used in each case. In addition, cells were incubated for 2 min with ≥ 100-fold excess of unlabeled monoclonal antibody prior to the addition of the fluorophore-labeled antibody in blocking experiments.

To analyze cytokine coexpression within the CD4+ and CD8+ T cell subset, isolated PBMC were stimulated in Iscove's medium (GibcoBRL)/10% heat inactivated fetal calf serum/100 units/ml penicillin/100 µg/ml streptomycin with phorbol 12-myristate 13-acetate (PMA; 20 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) in the presence of Brefeldin A (5 µg/ml; Sigma). After 6 h total incubation time, cells were centrifuged at 550 g for 5 min, washed in PBS/0.1% BSA, and resuspended in PBS/1% BSA/2% Endobulin (Immuno GmbH, Hamburg, Germany). Stimulated and unstimulated PBMC were subsequently stained for fractalkine and cell surface antigens as described above. For detection of intracellular cytokines the following fluorophore labeled antibodies were used: PE conjugated anti-human IL-4 (Th2-type cytokine) and anti-human interferon-γ (IFN-γ; Th1-type cytokine) antibody (both Becton Dickinson). In addition, the anti-fractalkine antibody was detected with FITC conjugated rabbit $F(ab')_2$ fragments of anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA, USA).

Immunohistochemistry for fractalkine (CX3CL1) and its receptor (CX3CR1). Paraffin sections of synovial tissue samples (4–6 µm) were used for detection of fractalkine and fractalkine receptor expression within the rheumatoid synovium. For double-staining experiments sections were first stained for cell surface antigens as described[28]. First primary antibodies included anti-human CD4 (1:25, clone 1F6; Novocastra, Dossenheim, Germany), anti-human CD8 (1:25, clone 4B11; Novocastra), anti-CD68 (1:50, clone KP1; Dako Hamburg, Germany), and anti-human fibroblast antibody (1:50, clone D-7FIB; DPC Biermann, Bad Nauheim, Germany). Primary antibodies were detected with $F(ab')_2$ fragments of a rabbit anti-mouse IgG antibody conjugated with Rhodamine Red-X (1:100; Dianova, Hamburg, Germany). Slides were washed in Tris buffered saline

(TBS; 0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl, pH 7.4) containing 0.05% Tween 20 between each step. Sections were then incubated with a monoclonal mouse anti-human fractalkine antibody (1:100, clone; R&D Systems). Isotype controls of irrelevant specificity were always included as negative control. The second primary antibody was detected with a FITC conjugated goat anti-mouse antibody (1:500; Jackson Immuno Research). Sections were then mounted in fluorescent mounting medium (Dako) and subjected to fluorescence microscopy on a Leitz fluorescence microscope.

To detect expression of the fractalkine receptor, a polyclonal rabbit anti-human CX3CR1 antibody (1:100; Torrey Pines Biolabs, Houston, TX, USA) was used. Reactive sites were detected with a FITC conjugated goat anti-rabbit antibody (1:500; Dianova).

For detection of CX3CR1 expression in cultured synovial fibroblasts, cells were grown on 2-well chamber slides (Nalge Nunc International, Naperville, IL, USA), fixed in acetone at –20°C for 20 min, and processed as described above.

Laser capture microdissection microscopy (LCM). For analysis of distinct cell types of the rheumatoid synovium, LCM was performed. Cryostat sections were cut at 4–6 µm, air dried, and fixed in 70% ethanol for 5 min. After washing in TBS, sections were stained for cell surface antigens as described above. Slides were finally dehydrated for 30 s in a graded alcohol series (70% EtOH, 90% EtOH, 100% EtOH), rinsed in xylene (3 min), air dried, and immediately subjected to LCM on a PALM laser capture microscope (PALM Microlaser Technologies, Bernried, Germany). Cells (10–20) were collected by laser microbeam microdissection and captured by laser pressure catapulting. RNA was extracted from isolated cells and from extracellular matrix as negative control using the Purescript® RNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The RNA samples were subsequently subjected to the following RT-PCR analysis.

RT-PCR. To detect fractalkine and fractalkine receptor transcription in different cell lines (*T Jurkat*, *HOS*, *TK178*, *TK188*), primary osteoclasts and chondrocytes, cultured synovial fibroblasts, and laser microbeam microdissected cells, a method of RT-PCR was established.

RNA isolation and reverse transcription was performed as described[29]. ß-Actin RT-PCR was performed in each case to control the quality of cDNA. To begin, 2 µl (5 µl in case of LCM samples) of cDNA were amplified by PCR in a final volume of 50 µl containing 1 µl of each dNTP (10 mM), 1.5 mM $MgCl_2$, 5 µl PCR buffer, 0.5 µl primer (10 nmol/µl), and 2.5 U of *Taq* polymerase (Gibco-BRL). For RT-PCR, the following oligonucleotide primers were designed: fractalkine (GenBank accession number NM002996): fractalkine-F 5'->3' ACCGCAGACAGGACCAGGGATTTC, fractalkine-R 5'->3' GGCAGGGCAGCACAGCGTCTTG; PCR product: 376 bp; fractalkine receptor (GenBank accession number NM001337) fractalkine receptor-F 5'-> 3' TGGCCTTGTCTGATCTGCTGTTTG; fractalkine receptor-R 5'->3' CAGGGGGAGTAGGAAGCCAAGAA; PCR product: 395 bp. Cycle conditions included 94°C, 3 min denaturation, followed by 35 (60 in LCM samples) cycles of 94°C, 45 s denaturation, 61.7°C, 45 s annealing, 72°C, 1.30 min elongation, and a final elongation step at 72°C for 10 min. Negative controls included distilled water instead of cDNA and positive controls included the cloned PCR product in each case. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. Gels were read on a Fluor-S™ Multiimager (BioRad, Hercules, CA, USA) and analyzed by densitometry using Multi-Analyst™ software (BioRad). To control the specificity of the PCR assay, PCR products were sequenced in both directions by cycle sequencing with dye-dideoxy terminator dNTP on an automated DNA sequencer (ABI Prism®, Applied Biosystems, Weiterstadt, Germany).

Immunoblot analysis for CX3CR1. For immunoblot analysis, total protein was isolated from cultured synovial fibroblasts using the TriZol© method according to the manufacturer's instructions (Gibco-BRL). Total protein was finally resuspended in detergent based lysis buffer containing 0.4% sodium desoxycholate, 1% NP-40, 1.9% EGTA, and 10 mmol/l Tris (pH 7.4). Immunoblot analysis was performed as described[30]. In brief, 200 µg total protein were loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, gels were electroblotted to nitrocellulose membranes (Hybond© ECL; Amersham, Braunschweig, Germany). To control the protein transfer, membranes were stained with Ponceau Red. Membranes were blocked and then incubated with the anti-human CX3CR1 antibody (1:100) for 1 h at room temperature. After washing, specifically bound antibodies were detected by a horseradish peroxidase conjugated goat anti-rabbit antibody (1:3000; Amersham). The substrate reaction was visualized by chemiluminescence (ECL©, Amersham) according to the manufacturer's instructions. Positive controls included proteins isolated from PBMC.

Stimulation of fibroblast cultures with recombinant human fractalkine. For stimulation experiments, fibroblasts were cultured at a density of 2×10^4/ml on 24 well tissue culture plates in DMEM containing 10% FCS and 100 units/ml penicillin and 100 µg/ml streptomycin. At confluency, medium was removed, cells were washed once in PBS, and medium was changed to DMEM supplemented with 1% insulin-transferrin-selenium (ITS; Gibco-BRL), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were subsequently stimulated with human recombinant fractalkine (R&D Systems) at a concentration of 50 ng/ml for 48 h. To control the specificity of fractalkine action, 5 µg/ml anti-human fractalkine antibody (R&D Systems) was added to the control wells. After 24 h and 48 h cell culture supernatants were collected.

MMP-2 concentrations were measured in culture supernatants by ELISA (Amersham) according to the manufacturer's instructions. In addition, MMP-2 and MMP–9 were analyzed by gelatin zymography in culture supernatants as described[31]: cell supernatants were loaded on SDS-10% polyacrylamide gels (Bio-Rad) containing 1.0 mg/ml gelatin. After electrophoresis, gels were incubated in renaturation buffer (2.5% Triton X-100) for 30 min, followed by incubation in 5 mmol/l $CaCl_2$, 50 mmol/l Tris-HCl, pH 7.5, at 37°C overnight and subsequent Coomassie Brilliant Blue staining (0.5% Coomassie Blue in 40% methanol/10% acetic acid) for 1 h at room temperature. After destaining of the gels in 40% methanol/10% acetic acid for 15 min, gels were scanned with a Fluor-S Multiimager and analyzed by densitometry using the Multi-Analyst software. Molecular sizes of the bands displaying enzymatic activity were determined by comparison to standard proteins and to purified MMP (Calbiochem-Novabiochem, San Diego, CA, USA).

Statistical analysis. All values are expressed as the mean ± SEM. Statistical differences between groups were evaluated by Student t test. P values were determined by a 2-sided calculation. Results were considered to be statistically significant if p values were < 0.05.

RESULTS

Expression of fractalkine (CX3CL1) in Th1-type CD4+ T cells in peripheral blood. PBMC isolated from 17 patients with RA and 12 healthy blood donors were analyzed for the expression of fractalkine by flow cytometry (Figure 1a). Results revealed that fractalkine could be detected at low frequency within the CD4+ and the CD8+ T cell subset in both groups (CD4+: 3.36 ± 0.48 vs 2.49 ± 0.42%, p = 0.04; CD8+: 3.82 ± 0.66 vs 4.35 ± 0.46%, p = 0.31). In contrast, the proportion of CD14+ fractalkine positive cells was significantly lower in the patient group (9.85 ± 3.11%, $p < 0.001$), whereas the proportion of fractalkine-expressing NK cells (CD56+) was again similar to the control group (15.28 ± 2.24 vs 11.66 ± 1.76, p = 0.18).

To further characterize the cytokine expression profile of fractalkine-expressing CD4+ and CD8+ T cells, PBMC were stimulated *in vitro* with PMA and ionomycin for 6 h. As illustrated in Figure 1b, the proportion of fractalkine

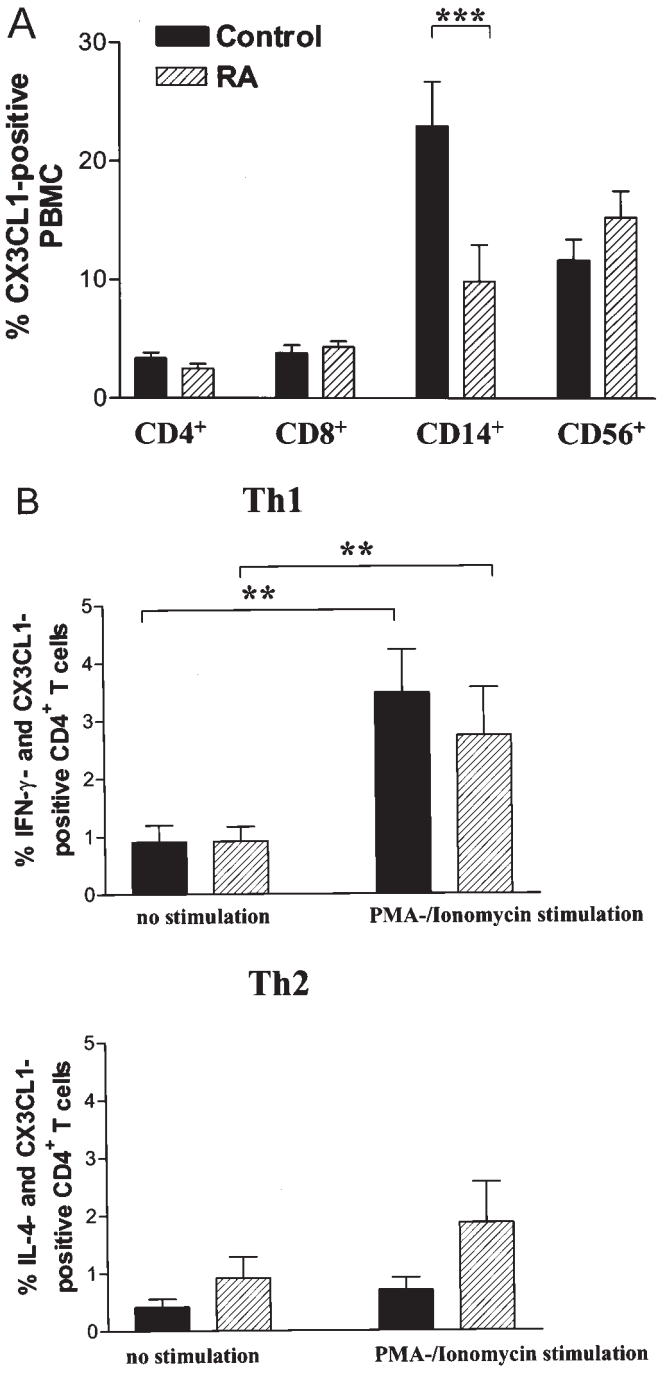

Figure 1. Expression of fractalkine (CX3CL1) in PBMC of 17 patients with RA and 12 controls. A. Flow cytometric analysis of fractalkine (CX3CL1) expression in different subsets of PBMC revealed that fractalkine was expressed in a low proportion of CD4+ and CD8+ T cells in both groups. The frequency of CX3CL1-expressing CD14+ cells was significantly lower in the patient group ($p < 0.001$), whereas CD56+ NK cells were detected at similar frequencies in patient and control groups. B. After PMA/ionomycin stimulation of PBMC, the proportion of fractalkine-expressing Th1-type CD4+ T cells characterized by cosecretion of IFN-γ was significantly enhanced in both patients ($p = 0.009$) and controls ($p = 0.008$). In contrast, there was no significant difference for the proportion of Th2-type CD4+ T cells (IL-4+) between unstimulated and PMA/ionomycin stimulated PBMC. **$p < 0.01$; ***$p < 0.001$.

positive Th1-type CD4+ T cells (IFN-γ+) was significantly enhanced in both RA patients and healthy controls ($p = 0.009$, $p = 0.008$, respectively). This effect could not be detected in Th2-type CD4+ T cells (IL-4+). There was no significant difference between patient and control groups for the proportion of fractalkine-expressing Th1-type CD4+ T cells after PMA/ionomycin stimulation ($p = 0.52$). Similar results were obtained by stimulation of PBMC with TNF-α (25 ng/ml) instead of PMA and ionomycin (data not shown).

Expression of fractalkine and its receptor in the rheumatoid synovium. To analyze expression of fractalkine and its receptor within the synovial tissue, immunohistochemistry and double-staining for cell surface markers were performed. Prominent expression of fractalkine could be observed within CD68+ synovial macrophages (Figure 2a), CD1a+ dendritic cells (Figure 2b), and endothelial cells (data not shown), and in a small proportion of CD4+ (Figure 2c) and CD8+ T cells in the sublining layer (Figure 2d).

The fractalkine receptor was also detected in synovial macrophages (Figure 2e), dendritic cells (Figure 2f), and CD3+ T cells (Figure 2g). In addition, synovial fibroblasts were found to stain positive for CX3CR1 expression (Figure 2h). Immunohistochemistry for fractalkine in osteoarthritis (Figure 2i), and normal synovium (Figure 2j) revealed only weak staining signals in a small number of large cells scattered throughout the synovium.

These immunohistochemical findings could subsequently be confirmed by laser capture microdissection of the different cell types. Cells were microdissected from cryostat sections of RA synovial tissue specimens after immunohistochemical staining for cell surface antigens and subsequently subjected to RT-PCR analysis. As shown in Figure 3, transcription of fractalkine receptor could be observed in synovial macrophages (CD68+), dendritic cells (CD1a+), T cells (CD3+), and synovial fibroblasts. By semiquantitative analysis, the ratio of CX3CR1/ß-actin was lower in synovial fibroblasts than in macrophages, dendritic cells, and T cells. Microdissected extracellular matrix and water instead of cDNA were used as negative controls in each of these experiments.

Detection of CX3CR1 in cultured synovial fibroblasts. To further analyze CX3CR1 expression in different cell types, RT-PCR analysis was performed with RNA isolated from the cell lines *HOS*, *T Jurkat*, *TK178*, *TK188,* primary chondrocytes, and osteoclasts as well as cultured synovial fibroblasts generated from RA synovial tissues. As shown in Figure 4a, CX3CR1 transcription could be observed in primary osteoclasts, cell lines *T Jurkat*, *HOS*, *TK 173*, and *TK 188*, as well as in all cultured synovial fibroblasts, but not in primary chondrocytes. To confirm this finding, immunoblot analysis was performed with total protein isolated from cultured synovial fibroblasts at passage > 3 to exclude macrophage contamination of the culture. The immunoblot (Figure 4b) revealed a specific band at 50 kDa.

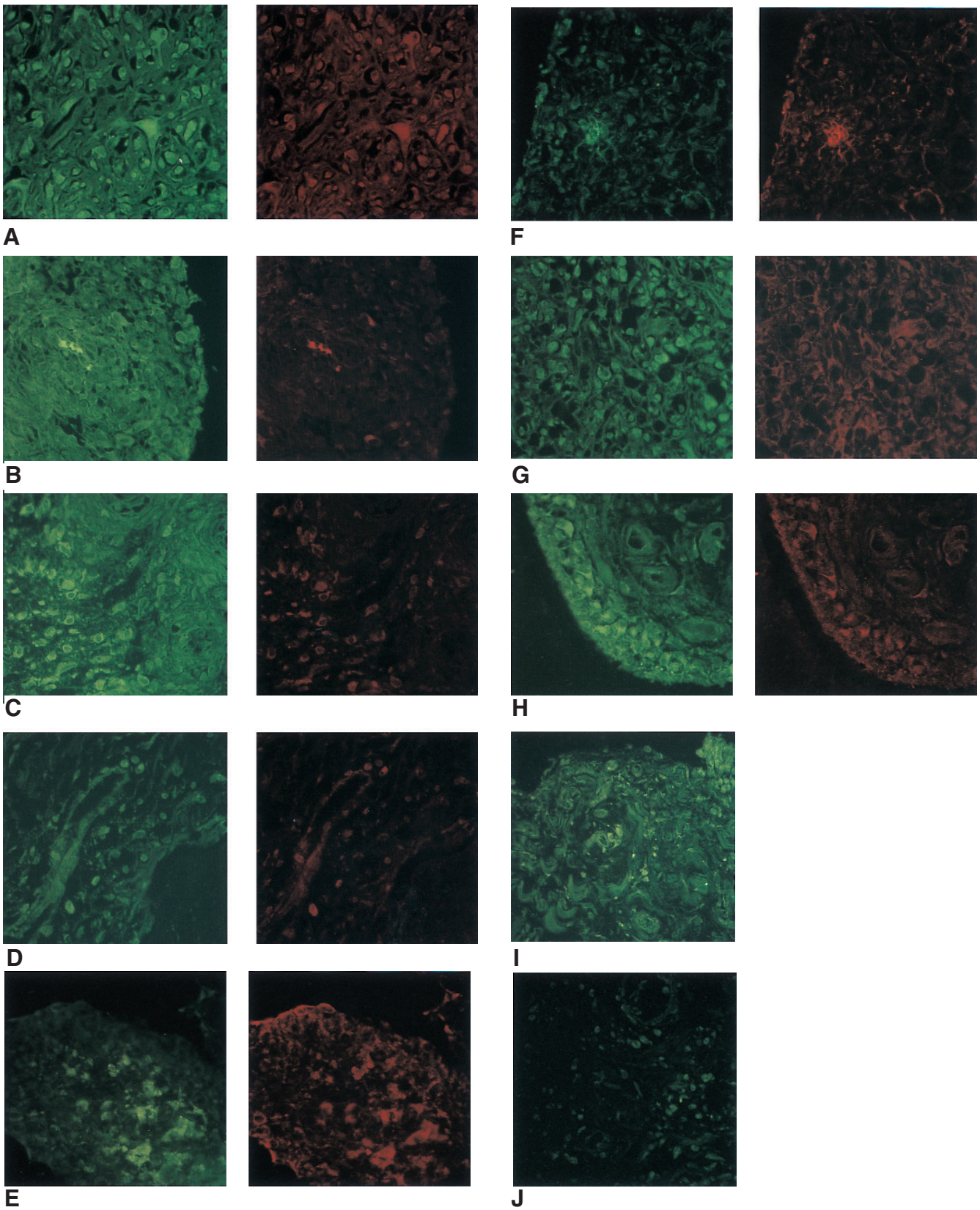

Figure 2. Expression of fractalkine and its receptor in RA synovium. Using immunohistochemistry for fractalkine (FITC) and double-staining for cell surface markers (Rhodamine Red-X), fractalkine expression could be observed within CD68+ synovial macrophages (A), CD1a+ dendritic cells (B), and in a small proportion of CD4+ (C) and CD8+ T cells in the sublining layer (D). The fractalkine receptor was detected in synovial macrophages (E), dendritic cells (F), CD3+ T cells (G), and in synovial fibroblasts (H). Only weak staining signals for fractalkine were detected in osteoarthritis samples (I) and normal synovium (J).

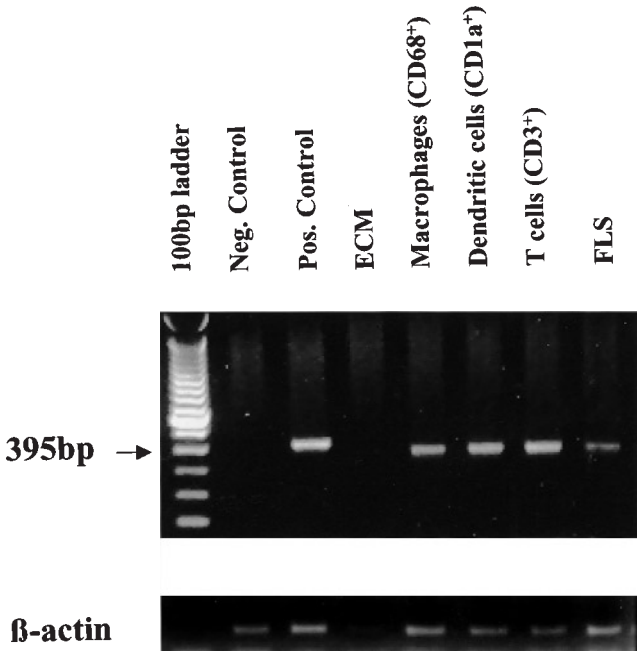

Figure 3. Laser capture microdissection analysis of CX3CR1 transcription in different cell types of RA synovium. Distinct cell types of RA synovium were microdissected from cryostat sections of RA synovial tissue specimens after immunohistochemical staining for cell surface antigens. After RNA isolation, reverse transcription was performed and cDNA quality was checked by ß-actin PCR. Transcription of the fractalkine receptor CX3CR1 could be observed in synovial macrophages (CD68+), dendritic cells (CD1a+), T cells (CD3+), and synovial fibroblasts by RT-PCR with CX3CR1-specific primers. Microdissected extracellular matrix (ECM) and water instead of cDNA were used as negative controls.

Interestingly, renal fibroblasts (*TK 461*) were also found to be positive for fractalkine receptor expression (data not shown). These results could also be seen by immunofluorescence analysis of cultured synovial fibroblasts (Figure 4c): synovial fibroblasts revealed strong fluorescent staining predominantly localized at the cell surface. No staining was observed in the case of the isotype control (Figure 4d).

Enhancement of MMP-2 production in fractalkine stimulated synovial fibroblasts. To analyze the functional role of fractalkine receptor expression on synovial fibroblasts, stimulation experiments were performed with cultured synovial fibroblasts. Cells were stimulated with fractalkine for a period of 48 h. Cell culture supernatants were subsequently analyzed for MMP-2 concentration by ELISA and zymography. Results revealed that MMP-2 could be significantly increased by stimulation with recombinant fractalkine ($p < 0.005$). MMP-2 production was shown to be upregulated in fractalkine stimulated synovial fibroblasts in a dose-dependent manner by MMP-2 zymography (Figure 5a). After 48 h of fractalkine stimulation at a concentration of 50 ng/ml, MMP-2 concentrations were found to be up to 2.5-fold higher in stimulated fibroblast cultures in comparison to unstimulated controls by ELISA ($p < 0.005$) (Figure 5b). This effect could be completely abrogated by addition of the fractalkine-specific antibody (5 µg/ml).

DISCUSSION

The functional role of fractalkine in RA was characterized by analysis of fractalkine expression in different T cell subsets in the peripheral blood and by assessment of fractalkine receptor expression in RA synovium.

Flow cytometric studies of PBMC revealed a low frequency of fractalkine positive CD4+ and CD8+ T cells in both RA patients and healthy controls. In support of these findings, Ruth, *et al*[14] detected only a small proportion of fractalkine-expressing CD3+ T cells (3%) in RA peripheral blood and synovial fluid (SF) samples. Since RA patients enrolled in both studies were receiving immunosuppressive drug therapy and a significant correlation has been detected between clinical disease variables and fractalkine receptor expression[14], a downregulation of fractalkine expression in our study population due to immunosuppressive drug therapy could not be excluded.

In contrast, both CD14+ monocytes and CD56+ NK cells were shown to express membrane associated fractalkine at a higher frequency in comparison to T cells in peripheral blood in this study. The finding of a significantly reduced proportion of fractalkine positive CD14+ monocytes in RA patients suggests that a great proportion of these cells may infiltrate into the rheumatoid synovium. Functional studies investigating fractalkine mediated chemoattraction of monocytes[5,32] as well as flow cytometric detection of a great percentage of CX3CR1 positive monocytes in RA peripheral blood and synovial fluid[14] may support this hypothesis.

To further characterize the cytokine expression profile of fractalkine positive T cells, PBMC were isolated from peripheral blood and stimulated *in vitro* with PMA and ionomycin. Results show that fractalkine expression could be predominantly induced in CD4+ T cells with a Th1-type cytokine expression profile, demonstrated by cosecretion of IFN-γ in both RA patients and healthy controls. That other proinflammatory signals such as lipopolysaccharide, IL-1, TNF-α, CD40L, and IFN-γ induce fractalkine expression in endothelial cells in contrast to antiinflammatory signals (IL-4, IL-13) indicates a tight regulation of fractalkine expression by pro- and antiinflammatory mediators. Fraticelli, *et al*[33] observed that the Th1-type T cells preferentially express the fractalkine receptor CX3CR1 and respond to fractalkine, in contrast to Th2-type T cells. In RA, Nanki, *et al*[34] recently analyzed fractalkine receptor (CX3CR1) expression by peripheral blood and synovial T cells and reported that CX3CR1 positive T cells expressing both type 1 cytokines (IFN-γ, TNF-α) and cytotoxic molecules (granzyme A, perforin) are upregulated in RA patients, and may migrate into RA synovium in response to CX3CL1 found to be expressed on endothelial cells and fibroblast-like synoviocytes. These findings further suggest that Th1-type T cells

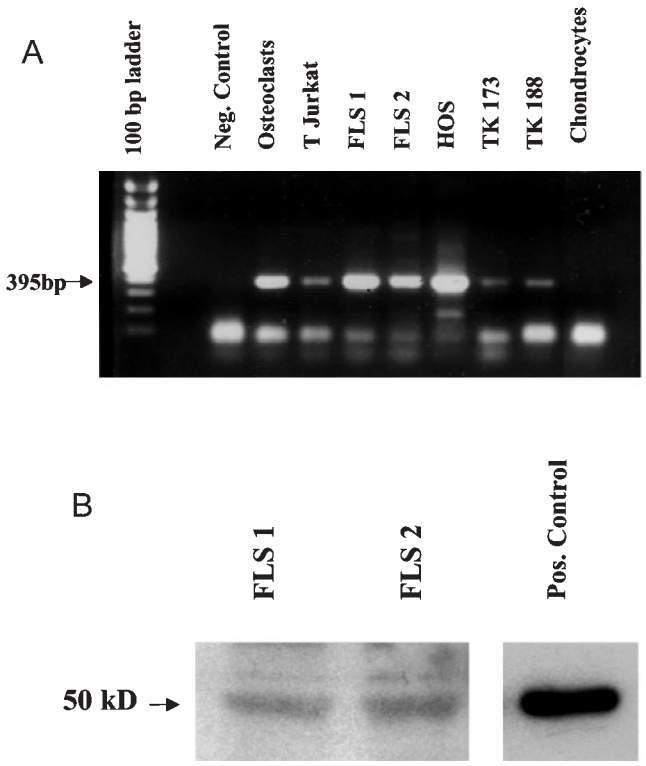

Figure 4. Detection of the fractalkine receptor (CX3CR1) in cultured synovial fibroblasts. RT-PCR analysis for CX3CR1 transcription was performed with RNA isolated from cell lines *HOS*, *T Jurkat*, *TK178*, *TK188*, primary chondrocytes, and osteoclasts as well as cultured synovial fibroblasts (FLS) generated from RA synovial tissues. A. CX3CR1 transcription was detected in primary osteoclasts, cell lines *T Jurkat*, *HOS*, *TK173*, and *TK188*, and all cultured synovial fibroblasts, but not in primary chondrocytes. B. Immunoblot analysis of cultured fibroblasts (FLS) revealed a CX3CR1-specific band at 50 kDa. C. In addition, fractalkine receptor expression could be detected in cultured fibroblasts by immunofluorescence assay. D. No staining was observed in the isotype control.

may play an important proinflammatory role in RA pathogenesis. Our findings in RA peripheral blood T cell subsets suggest that fractalkine itself may represent a Th1-type chemokine that plays a crucial role in the pathogenesis of RA characterized as a Th1 associated disease[35,27]: analysis of the cytokine expression of T cells in RA has revealed a marked Th1/Th2 cytokine imbalance with a predominance of Th1-type cytokines (IFN-γ, IL-2) in T cells of both RA peripheral blood and synovial fluid. That a prominent expression of fractalkine could also be observed in other Th1 associated diseases, such as Crohn's disease[15], cardiac allograft rejection[36], and psoriasis[37], supports the hypothesis that fractalkine may represent a Th1-type chemokine.

In RA synovium, immunohistochemical staining of cryostat sections and double-staining of cell surface markers revealed a prominent expression of fractalkine in endothelial cells, monocytes/macrophages, dendritic cells, and in a minor percentage of CD4+ and CD8+ T cells, whereas only a few cells scattered throughout the synovial membrane were immunoreactive in osteoarthritis samples. Our results correspond well with immunohistochemical data in RA synovium and rat adjuvant induced arthritis[14], whereas Nanki, *et al*[34] could not determine fractalkine expression by RT-PCR analysis in CD14+ macrophages in RA synovium. Our findings correspond with results of flow cytometric studies describing a low frequency of fractalkine positive T cells in the peripheral blood. In correlation with previous results[14], the fractalkine receptor CX3CR1 could be detected

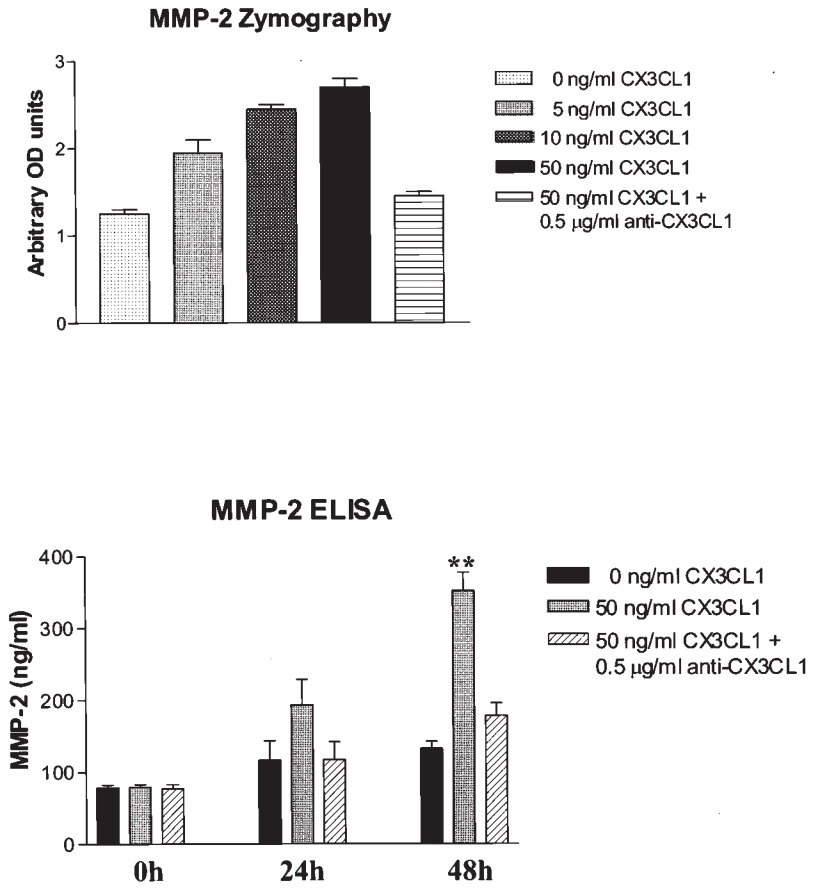

Figure 5. Enhancement of MMP-2 production in cultured synovial fibroblasts during fractalkine stimulation. To analyze the functional role of fractalkine, fibroblasts were stimulated with human recombinant fractalkine for a period of 48 h and MMP-2 concentrations were measured in cell culture supernatants by zymography and ELISA. A. MMP-2 production was shown to be upregulated in a dose-dependent manner by MMP-2 zymography. B. After 48 h of fractalkine stimulation (50 ng/ml), MMP-2 concentrations were up to 2.5-fold higher in stimulated cultures in comparison to unstimulated controls by ELISA ($p < 0.005$). This effect could be completely abrogated by addition of fractalkine-specific antibody (5 µg/ml). ** $p < 0.005$.

in monocytes/macrophages, dendritic cells, and some CD3+ T cells in RA synovium. In addition, we observed for the first time that synovial fibroblasts also express the fractalkine receptor at both the protein and the RNA level using immunohistochemistry and laser capture microdissection microscopy. This novel finding was confirmed by RT-PCR and Western blot analysis of cultured synovial fibroblasts generated from RA synovial tissue. Fractalkine receptor expression has only been described in NK cells, microglial cells, T cells, and mast cells to date. This is the first report of CX3CR1 expression in synovial fibroblasts.

To further characterize its functional role, cultured synovial fibroblasts were stimulated with human recombinant fractalkine. Stimulation revealed a marked upregulation of MMP-2 production within these cells, an effect that could efficiently be blocked by addition of a fractalkine-specific antibody. RA synovial fibroblasts have previously been shown to secrete a large amount of different matrix metalloproteinases responsible for articular tissue damage by proteolytic degradation of extracellular matrix: MMP-1, 2, 3, 8, and 9 have been detected in RA synovial fluid specimens[38]. MMP-2 has been purified from human rheumatoid synovial fibroblasts[39] and was shown to be activated by TNF-α[40]. MMP-2 thus represents one of the most important matrix-degrading metalloproteinases in the pathogenesis of joint destruction in RA. Functional studies detected a chemotactic activity of shed fractalkine for T lymphocytes[20], monocytes[32], and IL-2 activated NK cells[19]. In RA, soluble fractalkine-depleted synovial fluids revealed a significantly decreased chemotactic activity for monocytes compared with nondepleted samples[14]. In addition, significantly elevated concentrations of soluble fractalkine were detected in RA synovial fluid specimens in comparison with osteoarthritis controls, and enhanced fractalkine expression

was detected in rat adjuvant induced arthritis at a time of prominent joint inflammation in the rat joint. As well, in a recent study[22], recombinant human fractalkine was shown to induce blood vessel growth in an *in vivo* matrigel plug assay. Studies thus have demonstrated a functional role for this chemokine in monocyte chemotaxis and angiogenesis in RA. In addition to these findings, our observations of a marked upregulation of MMP-2 production in fractalkine-stimulated cultured synovial fibroblasts support the hypothesis of a proinflammatory role for this chemokine in RA.

The functional role of T cells for the pathogenesis of RA has still to be elucidated: synovial T cell hyporesponsiveness[41] indicated by a reduced response to mitogenic stimulation[42] and impaired T cell receptor-mediated signaling[43], low levels of T cell-derived cytokines in RA synovial fluids and synovial tissue cells[44], and the limited beneficial effects of T cell-directed therapies[45] argue against a central role of T cells in the pathogenesis of RA. However, association of disease susceptibility with HLA-DR4 antigens[46] and prominent accumulation of CD4+ T cells expressing several activation markers within the rheumatoid synovium[47] suggest a proinflammatory function for T cells in RA. In addition, recent studies provide accumulating evidence that Th1-type T cells were able to contribute to the chronic destructive process by production of the proinflammatory cytokine interleukin 17[48,49] that synergistically interacts with monocyte-derived cytokines IL-1 and TNF-α. Our results further suggest that certain subsets of Th1-type T cells may interact with synovial fibroblasts through cell-surface signaling by membrane-bound fractalkine or soluble fractalkine and stimulate them to produce and secrete MMP. These Th1-type T cells may thus exert a proinflammatory function in RA.

Predominant expression of fractalkine in T cells with a Th1-type cytokine expression profile and MMP-2 upregulation in fractalkine-stimulated cultured synovial fibroblasts suggest a proinflammatory role for this chemokine in the pathogenesis of RA. Further studies are needed to evaluate whether inhibition of fractalkine activity or blocking its signal transduction pathway may represent a novel therapeutic strategy for the treatment of RA.

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