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Inhibition of Fractalkine Ameliorates Murine Collagen-Induced Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with massive infiltration of inflammatory cells in the synovium of multiple joints. We and others have shown that fractalkine (FKN/CX3CL1), a chemokine expressed on fibroblast-like synoviocytes and endothelial cells in RA synovium, may contribute to the accumulation of T cells, macrophages, and dendritic cells, which express CX3CR1, the receptor for FKN. This interaction might be involved in adhesion of the inflammatory cells to endothelial cells, migration into the synovium, and cytokine production. In this study, we examined the effect of FKN inhibition on murine collagen-induced arthritis. Anti-FKN mAb significantly lowered clinical arthritis score compared with control Ab, and reduced infiltration of inflammatory cells and bone erosion in the synovium. However, anti-FKN mAb did not affect the production of either serum anti-collagen type II (CII) IgG or IFN-γ by CII-stimulated splenic T cells. Furthermore, treatment with anti-FKN mAb inhibited migration of adoptively transferred splenic macrophages into the inflamed synovium. Our results suggest that anti-FKN mAb ameliorates arthritis by inhibiting infiltration of inflammatory cells into the synovium. Thus, FKN can be a new target molecule for the treatment of RA.

Treatment with anti-mouse FKN mAb

Five hundred micrograms of hamster anti-mouse FKN mAb or control Ab (hamster IgG; ICN Pharmaceuticals, Aurora, OH) was injected into the mouse peritoneal cavity three times per week from day 0 for 14 days. The anti-mouse FKN mAb inhibited migration of mouse CX3CR1-transfected B30.1/19 pre-B cells induced by mouse FKN (data not shown). Mice were observed for signs of arthritis. The disease severity was recorded for each limb as follows: score 0, normal; 1, erythema and mild swelling confined to the mid-foot (tarsals) or ankle joint; 2, erythema and mild swelling extending from the ankle to the mid-foot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joint; 4, erythema and severe swelling of the ankle, foot, and digits. The clinical arthritis score was defined as the sum of the scores of all four paws of each mouse. At day 15, ankle joints were harvested and histologically examined after H&E staining.

Serum anti-CII IgG levels

At day 15, serum anti-mouse CII Ab (all IgG) levels in the CIA mice were measured using an ELISA kit (Chondrex, Redmond, WA) according to the protocol provided by the manufacturer. IFN-γ production by CII-stimulated splenic T cells

Thy1.2-positive splenic T cells of CIA mice treated with anti-FKN mAb or control Ab and normal mice at day 7 were purified using MACS microbead-coupled mAbs and magnetic cell separation columns (Miltenyi Biotec, Auburn, CA). An APC-enriched population was prepared from normal spleenocytes depleted of Thy1.2-positive T cells and B220 (CD45R)-positive B cells using MACS. Purified Thy1.2-positive splenic T cells (4 × 10⁴) and 1 × 10⁵ APC-enriched splenocytes were cocultured in 96-well culture plates in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) with 10% FCS (Sigma-Aldrich) supplemented where indicated with 50 μg/ml bovine CII. After 3-day incubation, the concentration of IFN-γ in the culture supernatant was measured using an ELISA kit (BioSource International, Camarillo, CA).

Migration of Mac-1-positive splenocytes into the synovium

Mac-1-positive splenocytes from CIA mice were purified using MACS. The purified Mac-1⁺ splenocytes were labeled with CellTracker Orange 5-(and-6)iodoacetamido-(4-chloromethyl)benzoylaminotetramethylrhodamine (CMTMR; Molecular Probes, Eugene, OR) according to the protocol supplied by the manufacturer. The CMTMR-labeled 1.2 × 10⁵ cells were i.v. injected into the tail vein of CIA mice at day 7. The recipient mice were injected with 500 μg of anti-FKN mAb or control Ab i.p. 24 and 2 h before the transfer. After 24 h, ankle joints were harvested, embedded in glycol methacrylate, and sagittal 3-μm-thick microtome sections were prepared (SRL, Tokyo, Japan). The numbers of CMTMR-labeled cells that migrated into the synovium between tibiotalar and tarsometatarsal joints were counted under fluorescent microscopy (Fluoview300; Olympus, Tokyo, Japan). Four slides from each joint were independently evaluated by two observers, and the mean cell count was calculated. An additional two slides from each joint were dried at 37°C for 2 h, incubated with 0.1% trypsin at 37°C for 20 min, and then washed with water for 5 min. The sections were incubated for 12 h at 4°C with rat anti-F4/80 mAb (Cl:3–1; Serotec, Raleigh, NC) or normal rat IgG as a control Ab at 45 μg/ml in the presence of 1% BSA in PBS. The samples were then washed twice for 5 min in PBS and stained with Alexa Fluor 488-conjugated goat anti-rat IgG (Molecular Probes) at 20 μg/ml for 1 h at room temperature. The number of Alexa Fluor 488-labeled cells in CMTMR-labeled cells that migrated into the synovium was counted under fluorescent microscopy.

FACS analysis

PBMC and spleen cells from normal mice or CIA mice were adjusted to 1 × 10⁹ cells and incubated with hamster anti-CX3CR1 mAb raised against a peptide corresponding to the C-terminal portion of mouse CX3CR1 for 30 min, rinsed with PBS-3% FCS. PE-conjugated rabbit anti-hamster IgG (Southern Biotechnology Associates, Birmingham, AL) was used as a second Ab. Then, the cells were stained with FITC-conjugated anti-CD4 mAb (H129.19; Becton Dickinson, San Jose, CA), FITC-conjugated anti-CD8 mAb (KT15; Beckman Coulter, Fullerton, CA), or FITC-conjugated anti-Mac-1 mAb (M1/70; Beckman Coulter) and analyzed with FACS Calibur (Becton Dickinson).

RT-PCR

At day 15, inflamed ankle joints and the surrounding skin were harvested. Tissues around the ankle joint were cut into small pieces. Total RNA was prepared from the tissue using Isogen (Qiagen, Valencia, CA) and treated with DNAse I (Life Technologies, Gaithersburg, MD). First-strand cDNA was synthesized using oligo(dT)₁₅–₁₈ primers (Pharmacia Biotech, Piscataway, NJ) and SuperScript II reverse transcriptase (Life Technologies). The amount of cDNA for amplification was adjusted to the amount of RNA measured by OD meter and also β₂-microglobulin (β₂m) PCR products using serially diluted cDNA. The cDNA was amplified with primers for β₂m (5’-CTG ACC GGC CTT CTG TAT GCT ATC 3’, 5’-CAT GTC CGC CACA GGA GAC GC 3’), CD4 (5’-TTG ACC TCC TAC CCC AGA GC 3’, 5’-GGG GGA CAG GAG TGA TAA GC 3’), and GAPDH (5’-TG TGT GGA TGT GAG TAA GGC 3’, 5’-GAG GTC CTG GAT CCA GTC GAC G 3’). The PCR products were described previously (27). The PCR products were then separated by electrophoresis through 1.5% agarose.

Immunohistochemistry

Ankle joints of CIA mice were dissected at day 15. Immunohistochemistry was conducted on carboxymethyl cellulose-embedded sections of frozen synovial samples. Briefly, 5-μm-thick cryostat sections were prepared using CryoMold Transfer kit (Finsyte, Tokyo, Japan) according to the protocol provided by the manufacturer. The sections were fixed in 4% paraformaldehyde for 10 min, and then the samples were rehydrated in PBS for 5 min three times. Nonspecific binding was blocked with 1.5% H₂O₂ in methanol for 15 min, and then with 5% normal donkey serum in PBS for 40 min. Serial sections were then incubated for 2 h at room temperature with the primary Abs: rabbit anti-mouse CX3CR1 Ab raised against a peptide corresponding C-terminal portion of mouse CX3CR1 or normal rabbit IgG as a control Ab at 5 μg/ml in the presence of 5% normal donkey serum in PBS. The samples were then washed twice for 5 min in PBS, and expression was detected using the Envision+ kit (Dako, Carpinteria, CA). For staining with F4/80, CD4, and CD8, sections were incubated with 10 μg/ml rat anti-mouse F4/80 mAb (Cl:A3–1), 2 μg/ml rat anti-mouse CD4 mAb (GK1.5), Santa Cruz Biotechnology, Santa Cruz, CA), 5 μg/ml anti-mouse CD8 mAb (53-6.7; Becton Dickinson), or normal rat IgG. Subsequently, the samples were washed twice for 5 min in PBS and incubated with biotin-conjugated rabbit anti-rat IgG (Dako) for 30 min at room temperature. After washing twice in PBS for 5 min, sections were incubated with peroxidase-conjugated streptavidin (Dako) for 30 min at room temperature. Diaminobenzidine chromogen and buffered substrate (Dako) were used for visualization. Sections were counterstained with hematoxylin for 5 s and washed in tap water for 10 min.

For double staining with F4/80, CD4 or CD8, and CX3CR1, the sections were incubated for 2 h at room temperature with rabbit anti-mouse CX3CR1 Ab or normal rabbit IgG as a control Ab at 10 μg/ml in PBS-1% BSA. The samples were then washed twice for 5 min in PBS and incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes) at 20 μg/ml for 1 h at room temperature. For staining with F4/80, after washing twice in PBS for 5 min, sections were incubated with rat anti-mouse F4/80 mAb (Cl:A3–1) or normal rat IgG at 10 μg/ml. Subsequently, the samples were washed twice for 5 min in PBS and incubated with Alexa Fluor 488-conjugated goat anti-rat IgG (Molecular Probes) at 20 μg/ml for 1 h at room temperature. For staining with CD4 or CD8, sections were incubated with 2 μg/ml rat anti-mouse CD4 mAb (GK1.5), 5 μg/ml anti-mouse CD8 mAb (53-6.7), or normal rat IgG. Subsequently, the samples were washed twice for 5 min in PBS and incubated with biotin-conjugated rabbit anti-rat IgG. After washing in PBS twice for 5 min, the samples were incubated with Alexa Fluor 488-conjugated streptavidin (Molecular Probes). The numbers of Alexa Fluor 568-labeled CX3CR1-positive cells in Alexa Fluor 488-labeled F4/80-, CD4-, or CD8-positive cells were counted under fluorescent microscopy.

Statistical analysis

Differences in arthritis score, anti-CII IgG production, IFN-γ production, and numbers of migrated Mac-1- or F4/80-positive splenocytes between control Ab and anti-mouse FKN mAb-treated mice were examined for statistical significance using the Mann-Whitney’s U test. The incidence of arthritis was examined using the χ² test. All data were expressed as mean ± SEM. Values of p < 0.05 were considered statistically significant.

Results

Effect of anti-mouse FKN mAb on CIA mice

To evaluate the effect of inhibition of FKN and CX3CR1 interaction on CIA mice, 500 μg of anti-mouse FKN mAb or control Ab was injected into the peritoneal cavity three times per week after the second booster immunization (day 0). Clinical scores of arthritis were recorded for 2 wk. Treatment with anti-FKN mAb significantly reduced the clinical arthritis score compared with treatment with control Ab (Fig. 1, p < 0.05 from day 8 to day 15). A similar
of arthritis (73%) treatment with anti-FKN mAb significantly reduced the incidence at day 15. Furthermore, mice treated with control Ab (A) showed mononuclear cell infiltration and synovial hyperplasia (Fig. 2, A and B). There was also significant pannus formation and bone erosion of the joint. In comparison, mice treated with anti-FKN mAb showed milder histological changes (Fig. 2, C and D). These results showed that treatment with anti-FKN mAb reduced clinical arthritis score, incidence of arthritis, and histological changes in CIA mice.

Effect of FKN inhibition on humoral and cellular immunity against CII

We also examined alternative possible mechanisms of the protective effect of anti-FKN mAb on CIA by studying the humoral and cellular immunity against CII. To evaluate the effect on humoral immunity, serum anti-CII Ab (total IgG) levels were measured by ELISA at day 15. Although production of serum anti-CII IgG was not detected in normal mice (<5 U/ml, n = 3), serum anti-CII IgG was produced in CIA mice. However, the levels of serum anti-CII IgG in mice treated with anti-FKN mAb were not significantly different from those treated with control Ab (Fig. 3).

To evaluate the effect of FKN inhibition on cellular immunity, we measured IFN-γ production by splenic T cells stimulated with CII. Splenic T cells from normal mice with or without CII stimulation did not produce IFN-γ (<6 pg/ml, n = 3). Although IFN-γ was produced by nonstimulated splenic T cells of CIA mice, treatment with anti-FKN mAb did not change the levels of IFN-γ production. Moreover, stimulation with CII up-regulated IFN-γ production by the splenic T cells from CIA mice; however, the level of production was not significantly different between mice treated with control Ab and anti-FKN mAb (Fig. 3). These results suggest that treatment with anti-FKN mAb does not affect humoral and cellular immunity against CII in CIA mice.

Inhibition of macrophage migration into the synovium by anti-FKN mAb

Next, we examined the effect of anti-FKN mAb treatment on inflammatory cell migration into the synovium. Mac-1 (CD11b)-positive splenocytes from CIA mice at day 7 without Ab treatment were purified. The purity of the cells was >98%. The purified Mac-1+ splenocytes were labeled with CMTMR. Subsequently, the labeled splenocytes, which were probably a mixture of macrophages, neutrophils, and dendritic cells, were i.v. transferred to normal or CIA mice. The recipient mice were not treated with either anti-FKN mAb or control Ab until day 5, and were injected with 500 μg of anti-FKN mAb or control Ab i.p. 24 and 2 h before the transfer. This double Ab injection did not reduce arthritis score (data not shown), indicating the transient effect on, but not depletion of, arthritis-promoting environment of recipient mice. Twenty-four hours later, the number of cells that migrated into the synovium was counted. Very few labeled Mac-1+ splenocytes migrated into the synovium of normal mice (8.0 ± 2.0, n = 3). In

FIGURE 1. Inhibition of clinical arthritis score in CIA mice by treatment with anti-FKN mAb. Five hundred micrograms of hamster anti-mouse FKN mAb or control Ab was injected into the peritoneal cavity three times per week from second booster immunization (day 0) for 2 wk. The disease severity was recorded as an arthritis score. Each point represents the mean ± SEM value of eight animals. *, p < 0.05.

FIGURE 2. Histological changes induced by treatment with anti-FKN mAb. Ankle joints harvested at day 15 were stained with H&E. Representative photomicrographs of histology from experiment in Fig. 1 are shown. Mice treated with control Ab (A and B) showed mononuclear cell infiltration (MC), synovial hyperplasia (SH), pannus formation (P), and bone erosion (BE). Mice treated with anti-FKN mAb exhibited reduction of these changes (C and D). T, Talus; N, navicular; C, cuneiform; M, metatarsal. Original magnification, ×40 in A and C; ×200 in B and D.

FIGURE 3. Effect of anti-FKN mAb on serum anti-CII IgG levels. Serum anti-CII Ab (total IgG) levels in CIA mice treated with control Ab or anti-FKN mAb at day 15 were measured using ELISA. Data represent the mean ± SEM values of eight animals. N.S., Not significant.
contrast, a significant number of labeled Mac-1+ splenocytes migrated into the CIA synovium (Fig. 5A). Treatment with anti-FKN mAb significantly reduced the migration of Mac-1-positive splenocytes into the synovium compared with that with control Ab (Fig. 5B). Frequencies of F4/80-positive cells, the other macrophage marker, in the migrated Mac-1+ cells were 77.4 ± 1.7% and 73.7 ± 2.7% from the mice treated with control Ab and anti-FKN mAb, respectively. Calculated number of migrated F4/80-positive cells (number of migrated CMTMR-positive cells) × (frequency of F4/80-positive cells in the CMTMR-positive cells)) was also significantly reduced by treatment with anti-FKN mAb (252.6 ± 19.4 and 142.1 ± 23.2, p < 0.005, treated with control Ab and anti-FKN mAb, respectively). These results suggest that a majority of migrated Mac-1-positive splenocytes is F4/80-positive macrophages and that the migration of splenic macrophages into the synovium is reduced by treatment with anti-FKN mAb. In contrast, migration of Thy1.2 (CD90)-positive splenic T cells into the synovium of CIA mice was not detected by this method.

**CX3CR1 expression in the CIA mice**

Expression of CX3CR1 on monocytes/macrophages and T cells in the peripheral blood and the spleen of CIA mice was analyzed by flow cytometry. More than half of Mac-1-positive monocytes or macrophages expressed CX3CR1 (peripheral blood, 61.0 ± 11.3%, n = 3; spleen, 54.9 ± 7.5%, n = 5). In contrast, the expression level of CX3CR1 on peripheral blood and splenic T cells was low (CD4, 0.7 ± 0.2% and 0.6 ± 0.1%; CD8, 0.5 ± 0.3% and 2.1 ± 0.2%, peripheral blood and spleen, respectively, n = 3).

CX3CR1 expression in synovial tissue was analyzed by immunohistochemistry. CX3CR1 was expressed on synoviocytes in CIA mice (Fig. 6A), but not in normal mice (Fig. 6B). Abundant F4/80-positive macrophages, moderate number of CD4-positive T cells, and a few CD8-positive T cells were detected in synovial tissue in CIA mice (Fig. 6, C–E). Double staining with F4/80 and CX3CR1 showed that most of CIA synovial macrophages expressed CX3CR1 (Fig. 6, F–H). Frequency of CX3CR1-positive cells in F4/80-positive macrophages was 79.4 ± 1.8% (n = 3). Significant number of CD4 and CD8 T cells also expressed CX3CR1 (35.4 ± 1.5% (n = 3) and 16.4 ± 1.9% (n = 4), respectively) (Fig. 6, I–N).

**FKN expression in the synovium of CIA mice**

FKN expression in CIA synovial tissue was analyzed using RT-PCR. FKN mRNA was expressed in normal synovium, but was up-regulated in the synovial tissues of CIA mice compared with that of normal mice (Fig. 7).

**Discussion**

The major findings of the present study were: 1) inhibition of FKN reduced clinical symptoms and incidence of arthritis, with amelioration of histopathological features of chronic arthritis in CIA mice; and 2) inhibition of FKN reduced macrophage migration into the synovium, although the treatment did not affect humoral and cellular immunity against CII. These results suggest that abrogation of FKN and CX3CR1 interaction ameliorates murine CIA most likely by inhibition of inflammatory cell infiltration into the synovium.

We and other groups reported previously that FKN was expressed by endothelial cells and FLS in human RA synovium, and synovial T cells, macrophages, and dendritic cells expressed CX3CR1, the receptor for FKN, suggesting that these inflammatory cells may migrate into the synovium by interaction of FKN and CX3CR1 (24–26). In this study, we have clearly shown that inhibition of FKN activity in vivo reduces macrophage migration into the CIA synovium. It has been thought that macrophages in RA synovium produce proinflammatory cytokines and chemokines, which up-regulate expression of chemokines expression by FLS (28). These chemokines provoke migration of inflammatory cells, such as T cells, B cells, macrophages, and dendritic cells. It was also reported that synovial macrophages could differentiate into osteoclasts (29), which cause bone resorption in the joint,
resulting in joint destruction. Thus, reduction of macrophage infiltration could suppress the inflammatory cytokine production, inflammatory cell migration, and osteoclastogenesis. Therefore, inhibition of macrophage migration might be useful for RA treatment. In fact, recent study has shown that a CCR1 antagonist, which reduces macrophage accumulation in the RA synovium, is effective for RA (30).

Treatment with anti-FKN mAb prevented macrophage migration into the synovium up to 40%, but not completely (Fig. 5). Other chemokine and chemokine receptor interactions such as MCP-1/CCL2 and CCR2 and IL-8/CXCL8 and CXCR2 may also contribute to the macrophage migration (31). In this regard, each of the gene deletions of MCP-1 (32, 33), CCR2 (34, 35), IL-8 receptor (36), or CX3CR1 (18, 19) resulted in ~50% decrease in atherosclerotic lesion formation or numbers of infiltrated macrophages in the lesion in experimental atherosclerosis mice. These three chemokines might be involved in macrophage migration.

We have already shown that peripheral blood CX3CR1-positive T cells expressing type-1 cytokines and cytotoxic molecules are increased in RA patients and migrate into RA synovium, suggesting that migrated CX3CR1-positive T cells might contribute to the pathogenesis of RA (23, 24). As similar to RA, significant number of CD4- and CD8-positive T cells expressed CX3CR1 in synovial tissue of CIA. Moreover, the frequencies of CX3CR1 expression of CD4- and CD8-positive T cells in the CIA synovial tissue were much higher than those of peripheral blood or spleen. These results suggest that FKN and CX3CR1 interaction might induce T cell migration as well as macrophage into the synovium of CIA. It is also possible that T cells and macrophages acquire CX3CR1 expression after migrating in the synovial tissue and are retained in the tissue by FKN.

Serum anti-CII IgG production in CIA mice and IFN-γ production by CII-stimulated splenic T cells was not changed by anti-FKN mAb. These results suggest that FKN inhibition does not affect humoral and cellular immunity against CII. Furthermore, our preliminary data indicated that stimulation with FKN did not up-regulate IL-6 or matrix metalloproteinase-2 production by FLS, IL-1β production by splenic macrophages, and IFN-γ production.

**FIGURE 6.** CX3CR1 expression in CIA synovium. CX3CR1 expression in synovial tissue samples from three CIA mice (A) and three normal mice (B) were examined by immunohistochemistry. Sections of CIA synovial tissues were stained with F4/80 (C), CD4 (D), and CD8 (E). All sections were counterstained with hematoxylin. Synovial tissues from CIA mice were double stained with F4/80, CD4 or CD8, and CX3CR1 and analyzed with fluorescent microscopy (F, F4/80; G, CX3CR1; H, merged F with G; I, CD4; J, CX3CR1; K, merged I with J; L, CD8; M, CX3CR1; N, merged L with M). Arrows indicate double-positive cells. Original magnification, ×200.

**FIGURE 7.** FKN expression in CIA synovium. FKN mRNA expression in the synovium was analyzed in three normal mice and three CIA mice using RT-PCR. PCR products were separated by electrophoresis through 1.5% agarose.
By splenic T cells in CIA mice. Taken together, it is tempting to speculate that FKN inhibition reduces arthritis probably by hampering inflammatory cell infiltration.

It was reported that inhibition of MIP-1α or RANTES, the ligands for CCR1 and CCR5, suppressed arthritis of murine CIA (37, 38), although the severity of arthritis was not changed in the CCR5-deficient mice (39). It is indicating that CCR1 may be an important chemokine receptor for the arthritis. Matthys et al. showed that CXC4 antagonists reduced CIA in the IFN-γ receptor-deficient mice, but there is no data in the intact IFN-γ mice (40). Blockade of CCR2 was only beneficial during the initiation phase in the CIA mice, while in the progression phase it aggravated the arthritis (41). Interference of CCR2 on the regulatory T cells might be responsible for the increased arthritis in the progression phase. Moreover, CIA was worsened in the CCR2-deficient mice (39). These results suggest that CCR2 blockade may not be good for RA treatment. In contrast, inhibition of FKN during the progression phase reduced arthritis, suggesting that the FKN might be an additional target for RA treatment.

We have established the method to analyze migration of CMTMR-labeled cells into the synovium of CIA mice in vivo. Using this method, we could clearly show the role of FKN on macrophage migration into the synovium. It has been reported that several kinds of inhibitors for chemokines and chemokine receptors can ameliorate animal models of arthritis (37, 38, 40–47). However, as far as we know, this is the first report that directly shows the prevention of inflammatory cell migration into the synovium by a chemokine inhibitor. This method might allow us to analyze the migration of other cell types such as dendritic cells and B cells into the synovium, as well as the role of molecules involved in leukocyte trafficking such as chemokines and adhesion molecules, on intraarticular cell migration in vivo.

In conclusion, we have demonstrated in the present study that inhibition of FKN significantly improved clinical arthritic score and histopathology of CIA mice most likely by the suppression of inflammatory cell migration into the synovium. Thus, blockade of FKN and CX3CR1 interaction might be useful for RA treatment.

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References


