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Stromal Cell-Derived Factor-1-CXC Chemokine Receptor 4 Interactions Play a Central Role in CD4$^+$ T Cell Accumulation in Rheumatoid Arthritis Synovium[1]

Toshihiro Nanki,[2]* Kenji Hayashida,*† Hani S. El-Gabalawy,‡ Sharon Suson,‡ Kenrin Shi,† Hermann J. Girschick,* Sule Yavuz,[2]* and Peter E. Lipsky[2,3]*‡

Rheumatoid arthritis (RA) is characterized by the accumulation of CD4$^+$ memory T cells in the inflamed synovium. To address the mechanism, we analyzed chemokine receptor expression and found that the frequency of CXC chemokine receptor (CXCR)4 expressing synovial tissue CD4$^+$ memory T cells was significantly elevated. CXCR4 expression could be enhanced by IL-15, whereas stromal cell-derived factor (SDF)-1, the ligand of CXCR4, was expressed in the RA synovium and could be increased by CD40 stimulation. SDF-1 stimulated migration of rheumatoid synovial T cells and also inhibited activation-induced apoptosis of T cells. These results indicate that SDF-1-CXCR4 interactions play important roles in CD4$^+$ memory T cell accumulation in the RA synovium, and emphasize the role of stromal cells in regulating rheumatoid inflammation. *The Journal of Immunology,* **2000, 165: 6590–6598.**

Rheumatoid arthritis (RA)[4] is characterized by chronic inflammation of multiple joints. Large numbers of CD4$^+$ T cells infiltrate the inflamed synovium. Most of the infiltrating CD4$^+$ T cells are memory T cells that express CD45RO (1–3). The mechanisms regulating the migration and accumulation of CD4$^+$CD45RO$^+$ T cells into the rheumatoid synovium have not been delineated. Lymphocyte migration is regulated by the activity of various adhesion molecules and the action of a number of chemokines and their specific receptors (4–8). Although the roles of adhesion molecules have been analyzed, the roles of chemokine and chemokine receptor interactions in the migration of CD4$^+$ CD45RO$^+$ T cells into the inflamed synovium have not been analyzed in detail.

Nine CC chemokine receptors (CCR1–9) (9–20), and five CXC chemokine receptors (CXCR1–5) (21–28) have been identified to date. Recently, it has been reported that expression of CCR5 and CXCR3 is up-regulated on synovial tissue or synovial fluid T cells (29–32). However, expression of other chemokine receptors by synovial T cells has not been analyzed; therefore, the role of specific chemokine receptors in the accumulation of CD4$^+$ T cells in the synovial tissue is still unclear.

To address this issue, we analyzed the expression of 11 different chemokine receptors (CCR1–3, CCR5–7, and CXCR1–5) by CD4$^+$ memory T cells from RA synovial tissue and peripheral blood, using single cell RT-PCR. The results indicate that CXCR4 is up-regulated on synovial tissue CD4$^+$ memory T cells and might play an important role in the accumulation of CD4$^+$ T cells within the inflamed RA synovium.

Materials and Methods

Specimens

Synovial tissues were obtained at surgery from RA and osteoarthritis (OA) patients. The synovial tissue was minced and incubated with 0.3 mg/ml collagenase (Sigma, St. Louis, MO) for 1 h at 37°C in RPMI 1640 medium (Life Technologies, Gaithersburg, MD). Partially digested pieces of the tissue were pressed through a metal screen to obtain single cell suspensions. Mononuclear cells were then isolated by Ficoll-Hypaque (Pharmacia Biotech; Piscataway, NJ) gradient centrifugation. Peripheral blood was obtained from RA patients and healthy donors. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. RA was diagnosed according to the American College of Rheumatology criteria (33). Informed consent was obtained from the patients, and the protocol was approved by the Institutional Review Board of The University of Texas Southwestern Medical Center at Dallas.

Single cell sorting and RT-PCR

The method for construction of cDNA libraries from single cells was similar to previously reported techniques (34, 35). The mononuclear cells were stained with FITC-conjugated anti-CD4 mAb (Q4120; Sigma) and PE-conjugated anti-CD45RO mAb (UCHL-1; Sigma), and individual CD4$^+$CD45RO$^+$ T cells were sorted into 96-well PCR plates (Robbins Scientific, Sunnyvale, CA) using the FACStar^{Plus} flow cytometer (Becton Dickinson, San Jose, CA).

Each well contained 4 μl of lysis buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 1 mM DTT; 10 μM dNTP (Sigma); 5 U/ml Prime RNase Inhibitor (5 Prime → 3 Prime, Boulder, CO); 300 U/ml RNAguard (Pharmacia Biotech); 200 ng/ml oligo$(dT)_{24}$ (Integrated DNA Technologies, Coralville, IA); and 0.5% Nonidet P-40). The samples were heated to 65°C for 1 min, cooled to 20°C for 3 min, and maintained on ice. Two units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and 50 U of Maloney murine leukemia virus reverse transcriptase (Life Technologies) were added, and the samples were incubated at 37°C for 15 min before heat inactivation at 65°C for 10 min. For polyadenylate

*Department of Internal Medicine and Harold C. Simmons Arthritis Research Center, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235; †Department of Orthopedic Surgery, Osaka University Medical School, Osaka, Japan; and ‡National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892

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[2] Current address: National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892.

[3] Address correspondence and reprint requests to Dr. Peter E. Lipsky, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Building 10, Room 9N228, 10 Center Drive, MSC 1820, Bethesda, MD 20892-1820. E-mail address: lipskyp@mail.nih.gov

[4] Abbreviations used in this paper: RA, rheumatoid arthritis; OA, osteoarthritis; SDF, stromal cell-derived factor; MIP, macrophage inflammatory protein; CXCR, CXC chemokine receptor; CCR, CC chemokine receptor; TCRBC, TCR Cβ.

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tailing at the 3′ end of the cDNA, 5 μl of tailing buffer (200 mM potassium cacodylate, pH 7.2; 4 mM $CoCl_2$; 0.4 mM DTT), 2 mM dATP (Boehringer Mannheim, Indianapolis, IN), and 10 U terminal transferase (Boehringer Mannheim) were added and incubated at 37°C for 20 min followed by heat inactivation at 65°C for 10 min. To amplify the cDNA nonspecifically, PCR was performed with 100 μl of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% Triton X-100, 1 mM dNTP, 10 U *Taq* DNA polymerase (Promega), and 2 μM X-$(dT)_{24}$ primer (ATG TCG TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC-dT_{24}) (Integrated DNA Technologies). Twenty-five cycles of amplification were performed with 1 min at 94°C, 2 min at 42°C, and 6 min at 72°C plus 10-s extension per cycle. Afterward, 5 U *Taq* DNA polymerase was added, followed by an additional 25 cycles of PCR.

For gene-specific amplification, 1 μl of nonspecifically amplified cDNA was amplified by PCR in 25 μl of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton X-100, 200 μM dNTP, and 0.625 U *Taq* DNA polymerase. The cycling program was 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles, followed by a final extension for 7 min. For nested amplification, 1 μl of amplified PCR mixture was added to a second PCR mixture (50 μl of 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% Triton X-100; 200 μM dNTP; and 1.25 U *Taq* DNA polymerase). The cycling program was 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles, followed by a final extension for 7 min. The primers were designed to be within 600 base pairs of the 3′ end of each mRNA. The primers used were TCR Cβ (TCRBC) 5′-TCA AGT CCA GTT CTA CGG GCT C, 3′-TCA TAG AGG ATG GTG GCA GAC A, 5′ (nested)-CTC TCG GAG AAT GAC GAG TGG AC; CCR1 5′-GAT TTG GGC TCT TGG AAT CCT G, 3′-GTG CTT AGC CCA CTC CCT GAA T, 3′ (nested)-AGG GCT TTC TTA GTT CCA CTG CC; CCR2B 5′-TGG GAG TTT TGG TGG AGT CCG AT, 3′-GGG GGA TGT GGC CTA AGA AGC AT, 3′ (nested)-GCC TAA GAA GCA TCT GAA CAA TGG; CCR3 5′-CTA AGG TCA TTA CCA CAG GCC AGG, 3′-AGC AGG GAA AGA ACT AGG CAC ATT, 5′ (nested)-GCA GCG TAC TCA TCA TCA ACC C; CCR5 5′-CTC AGG GAA TGA AGG TGT CAG A, 3′-TGC TAC TGT TGC ACT CTC CAC AAC T, 5′ (nested)-AGC CTC TGA ATA TGA ACG GTG AGC; CCR6 5′-CAT GGA ACT CAT GTT TTT AAA GGG C, 3′-CCA TGC CTA GCC CAT GAC AGT A, 3′ (nested)-CCC ATG ACA GTA CCT TCC TAA CA; CCR7 5′-AGC ACA CTC ATC CCC TCA CTT G, 3′-AGC CAA GAG CTG AGT GCA TGT C; 3′ (nested)-GAG TGC ATG TCA TCC CCA CTC T; CXCR1 5′-CAG ATC TAT GCC ACA AGA ACC CC, 3′-CTT TCT AGG GAT GCT GAT GCT GC, 3′ (nested)-CTG ATG CTG CAC GCC AGC CTG GA; CXCR2 5-ATT ACC AGG GAC TGA GGG GAG GG, 3′-GTG GCA TTA AGT CAC ATT GCG G, 3′ (nested)-GTC ACA TTG CGG TAC AAC TAT CAC; CXCR3 5′-CAC TGC CCT TCT CAT TTG GAA ACT, 3′-GCA AAT ATA GAG GTC TTG GGG AC, 5′ (nested)-AGT ACA AGG CAT GGC GTA GAG GG; CXCR4 5′-GGA CCT GTG GCC AAG TTC TTA GTT, 3′-ACT GTA GGT GCT GAA ATC AAC CCA, 3′ (nested)-CAG CTG GGG ATC ATT TCT AGC TTT; and CXCR5 5′-CAG GAC AAC GAG AAA GCC CTA AG, 3′-GGT CTC TGT GCT GCC TGT ACT GT, 5′ (nested)-GTA TCT CCT CGC AAG CTG GGT AA. The PCR products were then separated by electrophoresis through 2.0% agarose.

To confirm that the PCR products were amplified from the corresponding genes, the nucleotide sequences of the PCR products were analyzed. More than four PCR products of each chemokine receptor from a total of two donors were sequenced. All the sequences of the PCR products were identical with the previously published sequences (data not shown).

To confirm the sensitivity of the single cell RT-PCR method, purified peripheral $CD4^+CD45RO^+$ T cells were prepared using a commercially available purification method (StemSep; StemCell Technologies, Vancouver, Canada). The cells were adjusted to 1000 cells in lysis buffer and serially diluted. CXCR4 and CCR5 mRNA expression was analyzed by the method described above. CXCR4 mRNA was routinely detected from one cell, whereas CCR5 expression was detected in 2.5 cells (see Fig. 1). Because the frequency of cell surface expression of CXCR4 by the $CD4^+CD45RO^+$ T cells was 10–37% and that of CCR5 was 17–20% (see below), the sensitivity of this method appeared to be sufficient to detect mRNA from individual cells.

Cell surface expression of chemokine receptors

For analysis of cell surface chemokine receptor expression, the following mAbs were used: anti-CD4-FITC, Quantum-Red (Q4120), anti-CD45RA-FITC (F8-11-13; Sigma), anti-CD45RO-PE (UCHL-1), anti-CCR5-FITC (2D7; PharMingen, San Diego, CA), and anti-CXCR3-FITC (49801.111; R&D Systems, Minneapolis, MN), and anti-CXCR4-PE (12G5; PharMingen). Synovial tissue or peripheral blood mononuclear cells were adjusted to 1×10^5 cells and incubated with two or three fluorochrome-labeled mAbs for 30 min, rinsed with PBS-3% FCS, and analyzed with a FACScan (Becton Dickinson).

Immunohistochemistry

Synovial tissue immunohistochemistry. Synovial tissue samples were obtained from seven additional RA patients fulfilling the American College of Rheumatology criteria, and from three patients with OA at the time of arthroplasty ($n = 5$), or using closed percutaneous needle biopsy ($n = 5$). All samples were of adequate size for histological analysis and had a visible synovial lining layer. Immunohistochemistry was conducted on OCT-embedded sections of frozen synovial samples. Briefly, 8-μm-thick cryostat sections were fixed in acetone for 15 min and then air dried for 10 min. The samples were rehydrated in 1× TBS, pH 7.4, for 10 min. Nonspecific binding was blocked with Peroxidase Blocking Reagent (Dako, Carpinteria, CA) for 10 min, then with Protein Block Serum-Free (Dako) for 20 min. Serial sections were then incubated overnight at 4°C with the primary Abs: anti-CXCR4 mAb (44716.111; R&D Systems) at 20 μg/ml, anti-stromal cell-derived factor (SDF)-1 mAb (79014.111; R&D Systems) at 50 μg/ml, or anti-CD4 mAb (MT310; Dako) at 2 μg/ml. The incubations included 10% normal human serum, 10% normal goat serum, and 80% 1× TBS, pH 7.4. The samples were then washed twice for 5 min in 1× TBS, pH 7.4, and then incubated with peroxidase-conjugated goat anti-mouse IgG (Dako) with 10% normal human serum for 1 h at room temperature. After washing in 1× TBS, pH 7.4, for 5 min, diaminobenzidine chromogen and a buffered substrate (Dako) were used for visualization. The sections were then counterstained in Mayer's hematoxylin for 5 s and washed in tap water for 10 min.

Migration assay

Cell migration was assessed in 24-well chemotaxis chambers (6.5-mm diameter, 5-μm pore polycarbonate transwell culture insert; Costar, Cambridge, MA). RPMI 1640 with 0.5% BSA supplemented where indicated with 1000 ng/ml of SDF-1α (R&D Systems) was added to the lower wells, and 5×10^5 mononuclear cells obtained from synovial tissue and suspended in RPMI 1640 with 0.5% BSA were added to the upper wells. After incubation, the membrane was removed, and migrated cells were stained with FITC-conjugated anti-CD4 mAb (Q4120). CD4-positive cells were then counted by FACScan.

Apoptosis

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation from healthy donors. $CD4^+$ T cells were isolated by negative selection (StemSep; StemCell Technologies). Purity of the separated cells was >95%. The purified peripheral $CD4^+$ cells were cultured in RPMI 1640 with 10% FCS for 6 h because this induced spontaneous CXCR4 expression on peripheral T cells; then, they were incubated in medium supplemented where indicated with 1000 ng/ml of SDF-1α (R&D Systems) for 2 h. Subsequently, the $CD4^+$ cells were transferred to anti-CD3-coated 96-well microtiter plates (400 ng OKT3 per microwell) and stimulated for 8 h. The stimulated cells were then resuspended with 2.5% propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. The frequency of hypodiploid nuclei was measured by flow cytometry using the FACScan.

SDF-1 mRNA expression by synovial tissue

Total RNA was prepared from whole synovial tissue of five RA and five OA patients using TRIzol (Life Technologies). The RNA was treated with DNase I (Life Technologies), and first strand cDNA was synthesized using oligo$(dT)_{12–18}$ primers (Pharmacia Biotech) and SuperScript II reverse transcriptase (Life Technologies). The amount of cDNA for amplification was adjusted by the amount of RNA measured by OD meter and also β-actin PCR products using serially diluted cDNA. The cDNA was amplified with primers for β-actin (5′-GTC CTC TCC CAA GTC CAC ACA; 3′-CTG GTC TCA AGT CAG TGT ACA GGT AA) or SDF-1 (5′-CTG GGC AAA GCC TAG TGA AG; 3′-GTC CTG AGA GTC CTT TTG CG). The PCR conditions were the same as were used for gene-specific PCR by single cell RT-PCR described above, except that the annealing temperature was 56°C.

SDF-1 production by RA synovial fibroblasts and anti-CD40 stimulation

Synovial fibroblasts established from RA synovium were cultured in 96-well plates (3.5×10^4 cells/well) with DMEM (Life Technologies) supplemented with 10% FCS for 6 days. The cells were incubated with 20 μg/ml anti-CD40 mAb (G28.5; American Type Culture Collection, Manassas, VA) or control mAb (MOPC-31C) for 30 min. To cross-link the

FIGURE 1. Sensitivity of the single cell RT-PCR method. Peripheral $CD4^+CD45RO^+$ T cells were adjusted to 1000 cells in lysis buffer and were serially diluted. CXCR4 and CCR5 mRNA expression was analyzed by the RT-PCR method. Cell number expected from the dilution is indicated.

mAb, 10 μg/ml biotin-conjugated rabbit anti-mouse IgG Ab (Sigma) was added and incubated for 30 min. Subsequently, 10 μg/ml streptavidin (Sigma) was added to cross-link the rabbit anti-mouse Ab. After a 3-day culture, SDF-1α concentration in the culture supernatant was measured by sandwich ELISA. Anti-SDF-1 mAb (79018.111; R&D Systems) and biotinylated anti-SDF-1α Ab (R&D Systems) were used for the ELISA.

Stimulation of cell surface CXCR4 expression by IL-15

Peripheral $CD4^+$ T cells from healthy donors were cultured in RPMI 1640 supplemented with 10% FCS and, where indicated, 100 ng/ml recombinant IL-15 (R&D Systems). Cultured $CD4^+$ T cells were stained with PE-conjugated anti-CXCR4 mAb (12G5), and CXCR4 expression was analyzed by flow cytometry with a FACScan.

Statistical analysis

The χ^2 test and Student's t test were used to compare the frequencies of chemokine receptor expression between synovial tissue and peripheral $CD4^+CD45RO^+$ T cells at the single cell level and by patient samples, respectively. Student's t test was used to compare frequencies of apoptosis and SDF-1α production by RA synovial fibroblasts.

Results

Chemokine receptor mRNA expression by $CD4^+CD45RO^+$ T cells from synovial tissue and peripheral blood of RA patients, and from peripheral blood of a healthy donor

We analyzed chemokine receptor mRNA expression by $CD4^+CD45RO^+$ T cells from synovial tissue and peripheral blood of RA patients and from peripheral blood of a healthy donor using a single cell RT-PCR technique. The presence of chemokine receptor mRNA was analyzed in 152 individual synovial tissue $CD4^+CD45RO^+$ T cells sorted from three RA patients, 48 individual peripheral $CD4^+CD45RO^+$ T cells from two RA patients, and 33 individual peripheral $CD4^+CD45RO^+$ T cells from a normal donor in which TCRBC mRNA was detected. Chemokine receptor mRNA expression by 50 synovial tissue $CD4^+$ memory T cells (RA1) is shown in Fig. 2.

Seventy-six percent of $CD4^+CD45RO^+$ T cells from synovial tissue expressed CXCR4 mRNA (Table I), a significantly higher frequency than that in peripheral blood $CD4^+CD45RO^+$ T cells ($p < 5 \times 10^{-11}$, $p < 0.05$, χ^2 test, Student's t test, respectively). The frequencies of synovial tissue $CD4^+CD45RO^+$ T cells expressing CCR5 and CXCR2 mRNA were also higher than that expressed by peripheral $CD4^+CD45RO^+$ T cells (Tables I and II). Frequencies of chemokine receptor mRNA expression were not significantly different between peripheral blood of RA patients and that of a normal donor.

Eighty percent of synovial tissue $CD4^+CD45RO^+$ T cells expressed either CCR5, CXCR2, or CXCR4 mRNAs (Table III), which is significantly higher than that expressed by peripheral $CD4^+CD45RO^+$ T cells ($p < 1 \times 10^{-10}$, χ^2 test). Of the cells that expressed at least one of these, 6% of synovial tissue $CD4^+CD45RO^+$ T cells expressed all three chemokine receptor mRNAs, 17% expressed both CCR5 and CXCR4 mRNAs, 4% expressed both CXCR2 and CXCR4 mRNAs, and 69% expressed only CXCR4 mRNA. The frequency of synovial tissue $CD4^+CD45RO^+$ T cells expressing CCR5 only was less than expressed by peripheral $CD4^+CD45RO^+$ T cells ($p < 0.05$, χ^2 test).

Cell surface chemokine receptor expression by synovial tissue and peripheral blood $CD4^+$ T cells of RA patients

Synovial tissue $CD4^+$ T cells were analyzed for chemokine receptor expression by flow cytometry. Results of one patient are shown in Fig. 3. Most of RA synovial tissue $CD4^+$ T cells expressed CD45RO (88% of the synovial tissue $CD4^+$ T cells expressed CD45RO) by flow cytometry, as was previously reported (1–3). Moreover, 77% of the synovial tissue $CD4^+$ T cells also expressed CXCR4 (Fig. 3). Similar results were noted in two other patient samples (75.3 $\pm$ 10.7% of $CD4^+$ synovial T cells expressed CXCR4, mean $\pm$ SEM, $n = 3$). Of note, 10–28% of RA peripheral $CD4^+$ memory ($CD45RA^-$) T cells and 43–61% of RA peripheral $CD4^+$ naive ($CD45RA^+$) T cells expressed CXCR4 ($n = 2$). By comparison, 40–65% of normal peripheral $CD4^+$ naive T cells and 28–37% of memory T cells expressed CXCR4 ($n = 3$). These frequencies were not significantly different between RA and healthy donors.

The frequency of CCR5 expressing synovial tissue $CD4^+CD45RO^+$ T cells was significantly higher than noted for

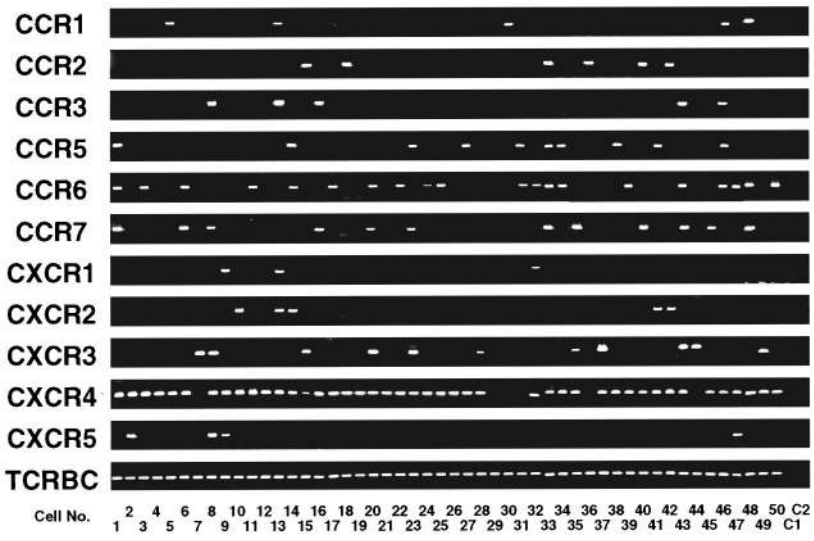

FIGURE 2. Chemokine receptor expression by $CD4^+CD45RO^+$ T cells from RA synovial tissue (RA1). RA synovial tissue $CD4^+CD45RO^+$ T cells were sorted, and chemokine receptor expression was determined in the TCRBC mRNA-positive wells using single cell RT-PCR. Amplified PCR products were separated by electrophoresis through 2.0% agarose. As negative controls, one well that did not contain a cell (C1) and one well that was not prepared with reverse transcriptase (C2) were analyzed.

Table I. *Frequency of CXCR-expressing CD4$^+$ CD45RO$^+$ T cells in synovial tissue and peripheral blood of patients with RA and in peripheral blood from a normal donor*

	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5
Synovial tissue from RA					
RA1 (n = 50)	3[a] (6%)[b]	5 (10%)	11 (22%)	44 (88%)	4 (8%)
RA2 (n = 39)	1 (3%)	0 (0%)	5 (13%)	21 (54%)	1 (3%)
RA3 (n = 63)	6 (10%)	8 (13%)	9 (14%)	51 (81%)	11 (17%)
Total (n = 152)	10 (7%)	13 (9%)[c]	25 (16%)	116 (76%)[d]	16 (11%)
Peripheral blood from RA					
RA3 (n = 24)	3 (13%)	0 (0%)	2 (8%)	8 (33%)	1 (4%)
RA4 (n = 24)	0 (0%)	0 (0%)	4 (17%)	3 (13%)	0 (0%)
Total (n = 48)	3 (6%)	0 (0%)	6 (13%)	11 (23%)	1 (2%)
Peripheral blood from normal donor					
Donor (n = 33)	1 (3%)	2 (6%)	2 (6%)	6 (18%)	1 (3%)

[a] Number of positive cells.
[b] Frequency of chemokine receptor-positive cells in TCRBC-positive cells.
[c] The frequencies of synovial tissue CD4$^+$CD45RO$^+$ T cells expressing CXCR2 were significantly higher than that of RA peripheral CD4$^+$CD45RO$^+$ T cells ($p < 0.005$, χ^2 test).
[d] The frequency of synovial tissue CD4$^+$CD45RO$^+$ T cells expressing CXCR4 was significantly higher than that found in RA peripheral CD4$^+$CD45RO$^+$ T cells ($p < 5 \times 10^{-11}$, $p < 0.05$, χ^2 test, Student's t test, respectively).

peripheral CD4$^+$CD45RO$^+$ T cells (synovial tissue, 65%; peripheral blood, 17–20%), whereas the frequencies of CXCR3 expressing synovial tissue and peripheral CD4$^+$CD45RO$^+$ T cells were similar (synovial tissue, 24%; peripheral blood, 20–41%) (Fig. 4).

All RA samples demonstrated the presence of lymphocytic aggregates of various sizes, primarily in a perivascular location. These aggregates were composed primarily of CD4$^+$ T cells (Fig. 5*C*). The OA samples demonstrated occasional isolated CD4$^+$ T cells or small perivascular lymphocytic aggregates (Fig. 5*H*). CXCR4 expression was widespread in all RA samples, with intense staining of the synovial lining cell layer and the sublining macrophages (Fig. 5*A*). The aggregates of CD4$^+$ T cells also expressed CXCR4, intensely in all cases with some aggregates including a majority of CXCR4-positive T cells (Fig. 5*B*). Notably, there was incomplete concordance between expression of CD4 and CXCR4. In contrast, OA samples demonstrated minimal expression of CXCR4 by scattered cells in the lining layer and occasional cells in the sublining stroma (Fig. 5*I*). A similar pattern of dense expression of CXCR4 was seen in all seven RA samples, whereas scant expression of CXCR4 was noted in all three OA samples. In the RA samples, SDF-1 expression was found in the regions of the perivascular aggregates of CD4$^+$ T cells and was largely confined to cells with a fibroblastic appearance (Fig. 5, *D* and *E*). In addition, there was staining of extracellular material in some regions (Fig. 5*E*). In contrast, SDF-1 expression was virtually absent in the OA samples. It should be noted that similar patterns of SDF-1 expression were observed in all seven RA samples, whereas minimal expression was found in the OA samples.

Migration of RA synovial tissue CD4$^+$ T cells stimulated by SDF-1α

The capacity of SDF-1α, the ligand of CXCR4, to stimulate migration of CD4$^+$ memory cells from rheumatoid synovium was measured. The number of synovial tissue CD4$^+$ T cells stimulated to migrate by SDF-1α was found to be ~10 times greater than that induced by medium alone (Fig. 6). These results show that the expressed CXCR4 on RA synovial tissue CD4$^+$ memory T cells is functional.

SDF-1α inhibits activation-induced apoptosis induced by anti-CD3 stimulation

In addition to the impact on migration, we analyzed the impact of SDF-1 on activation-induced apoptosis of CD4$^+$ T cells induced by anti-CD3 stimulation. As shown in Fig. 7, SDF-1α significantly reduced anti-CD3-induced apoptosis.

SDF-1 expression by RA and OA synovial tissue

SDF-1 mRNA expression by whole synovial tissue from RA and OA patients was analyzed by RT-PCR. SDF-1 mRNA was expressed by four of five RA patients and two of five OA patients, although one OA synovium expressed SDF-1 mRNA very weakly (Fig. 8).

Table II. *Frequency of CCR-expressing CD4$^+$ CD45RO$^+$ T cells in synovial tissue and peripheral blood of RA patients and in peripheral blood from a normal donor*

	CCR1	CCR2	CCR3	CCR5	CCR6	CCR7
Synovial tissue from RA						
RA1 (n = 50)	5[a] (10%)	6 (12%)	5 (10%)	10 (20%)	20 (40%)	12 (24%)
RA2 (n = 39)	5 (13%)	1 (3%)	1 (3%)	6 (15%)	6 (15%)	9 (23%)
RA3 (n = 63)	7 (11%)	1 (2%)	1 (2%)	16 (25%)	33 (52%)	8 (13%)
Total (n = 152)	17 (11%)	8 (5%)	7 (5%)	32 (21%)[a]	59 (39%)	29 (19%)
Peripheral blood from RA						
RA3 (n = 24)	3 (13%)	2 (8%)	0 (0%)	4 (17%)	11 (46%)	4 (17%)
RA4 (n = 24)	2 (8%)	1 (4%)	0 (0%)	0 (0%)	2 (8%)	5 (21%)
Total (n = 48)	5 (10%)	3 (6%)	0 (0%)	4 (8%)	13 (27%)	9 (19%)
Peripheral blood from normal donor						
Donor (n = 33)	1 (3%)	1 (3%)	2 (6%)	3 (9%)	4 (12%)	2 (6%)

[a] The frequencies of CCR5-positive synovial tissue CD4$^+$CD45RO$^+$ T cells were significantly greater than that of RA peripheral CD4$^+$CD45RO$^+$ T cells ($p < 0.05$, χ^2 test).

Table III. *Expression of the combination of CCR5, CXCR2, and CXCR4 by synovial tissue and peripheral blood CD4$^+$CD45RO$^+$ T cells of patients with RA*

CCR5	CXCR2	CXCR4	Synovial Tissue (n = 152)	Peripheral Blood (n = 48)
+	+	+	7[a] (6%)[b]	0 (0%)
+	+	−	0 (0%)	0 (0%)
+	−	+	21 (17%)	1 (7%)
−	+	+	5 (4%)	0 (0%)
+	−	−	4 (3%)	3 (21%)[c]
−	+	−	1 (1%)	0 (0%)
−	−	+	83 (69%)	10 (71%)
Total			121[d] (80%)[e]	14 (29%)

[a] Number of positive cells.
[b] Frequency of cells expressing either CCR5, CXCR2, or CXCR4 in the total number of cells.
[c] Frequency of CCR5 only expressing CD4$^+$CD45RO$^+$ T cells in peripheral blood was significantly higher than that in synovial tissue ($p < 0.05$).
[d] Number of cells expressing either CCR5, CXCR2, or CXCR4.
[e] Frequency of cells expressing either CCR5, CXCR2, or CXCR4 by synovial tissue CD4$^+$CD45RO$^+$ T cells was significantly higher than that by peripheral blood CD4$^+$CD45RO$^+$ T cells ($p < 1 \times 10^{-10}$).

Anti-CD40 stimulation enhances SDF-1 production by RA synovial fibroblasts

Synovial fibroblasts established from RA synovium expressed surface CD40 (data not shown) as previously reported (36, 37). RA synovial fibroblasts produced SDF-1α protein, and production was markedly enhanced by CD40 engagement using an anti-CD40 mAb (Fig. 9).

Enhancement of CXCR4 expression by IL-15

It has been reported that IL-15 is produced by synoviocytes and synovial endothelial cells from RA patients, and that IL-15 simulates T cells to proliferate (38, 39). Therefore, we analyzed the effect of IL-15 on CXCR4 expression by CD4$^+$ T cells. Purified peripheral CD4$^+$ T cells expressed a low level of CXCR4. After 1 day of in vitro incubation, CXCR4 expression was up-regulated even when the cells were cultured with medium alone, as previously reported (40). CXCR4 expression gradually decreased as the incubation in medium was prolonged beyond 1 day. In contrast, after the first day, CXCR4 expression was further amplified when cells were stimulated with IL-15 (Fig. 10).

Discussion

CXCR4 was markedly up-regulated on synovial tissue CD4$^+$ memory T cells compared with that expressed by peripheral CD4$^+$ memory T cells. CXCR4 was cloned as an orphan receptor (24–27), and its unique ligand was subsequently found to be SDF-1, originally identified as a growth factor for murine pre-B cells (41, 42). SDF-1 occurs in two alternative splicing variants, SDF-1α and SDF-1β (41). SDF-1 is a highly efficient and potent chemoattractant for both naive and memory T cells (43). In addition, SDF-1 induces adhesion of naive and memory T cells to ICAM-1 (CD54) (44) by up-regulating the binding activity of LFA-1 (CD11a/CD18). Thus, SDF-1 and CXCR4 interactions might allow specific subsets of T cells to be recruited to specific sites.

Previously, it was reported that CXCR4 was mainly expressed by naive T cells in the periphery (45–47), suggesting that antigenic stimulation or differentiation to a memory phenotype might lead to down-regulation of CXCR4 expression. Consistent with this, our data show that, in RA patients, CXCR4 was mainly expressed by naive CD4$^+$ T cells in the peripheral blood, as it was in normal patients. Notably, however, most of RA synovial tissue CD4$^+$CD45RO$^+$ T cells expressed CXCR4, although only ~30% of peripheral CD4$^+$CD45RO$^+$ T cells expressed this chemokine receptor. Thus, peripheral CD4$^+$CD45RO$^+$ T cells expressing CXCR4 might be selected for migration to the inflamed synovium. Alternatively, CXCR4 expression might be up-regulated during and/or after migration into the synovium. In this regard, IL-15, which is produced in the rheumatoid synovium by endothelial cells and synovial fibroblasts (38, 39), was found to up-regulate CXCR4 expression. Previously, IL-15, along with interaction with endothelial cells, has been shown to up-regulate expression of the activation Ag, CD69, by migrating memory T cells (39). In a similar manner, IL-15 may up-regulate expression of CXCR4 by memory T cells at inflammatory sites, permitting them to migrate into the tissue in response to SDF-1.

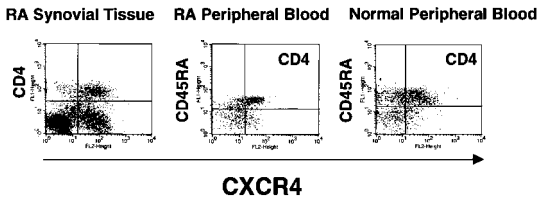

FIGURE 3. Cell surface CXCR4 expression by RA synovial tissue, RA peripheral blood, and normal peripheral T cells. Synovial tissue (RA3) mononuclear cells were stained with anti-CD4 mAb and anti-CXCR4 mAb, and peripheral blood (RA3 and normal donor) mononuclear cells were stained with anti-CD4 mAb, anti-*CD45RA* mAb, and anti-CXCR4 mAb, and the expression was analyzed by FACS.

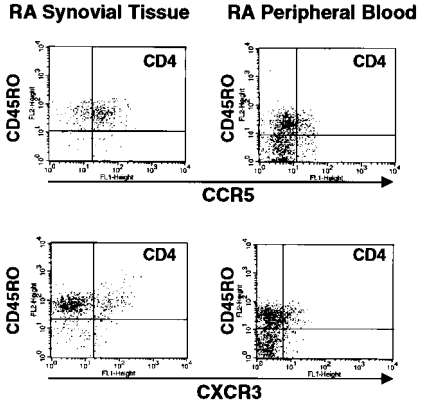

FIGURE 4. Cell surface CCR5 and CXCR3 expression by RA synovial tissue and peripheral blood. Synovial tissue (RA3) and peripheral blood (RA3) mononuclear cells were stained with anti-CD4 mAb, anti-CD45RO mAb, and anti-CCR5 mAb or anti-CXCR3 mAb, and the expression was analyzed by FACS.

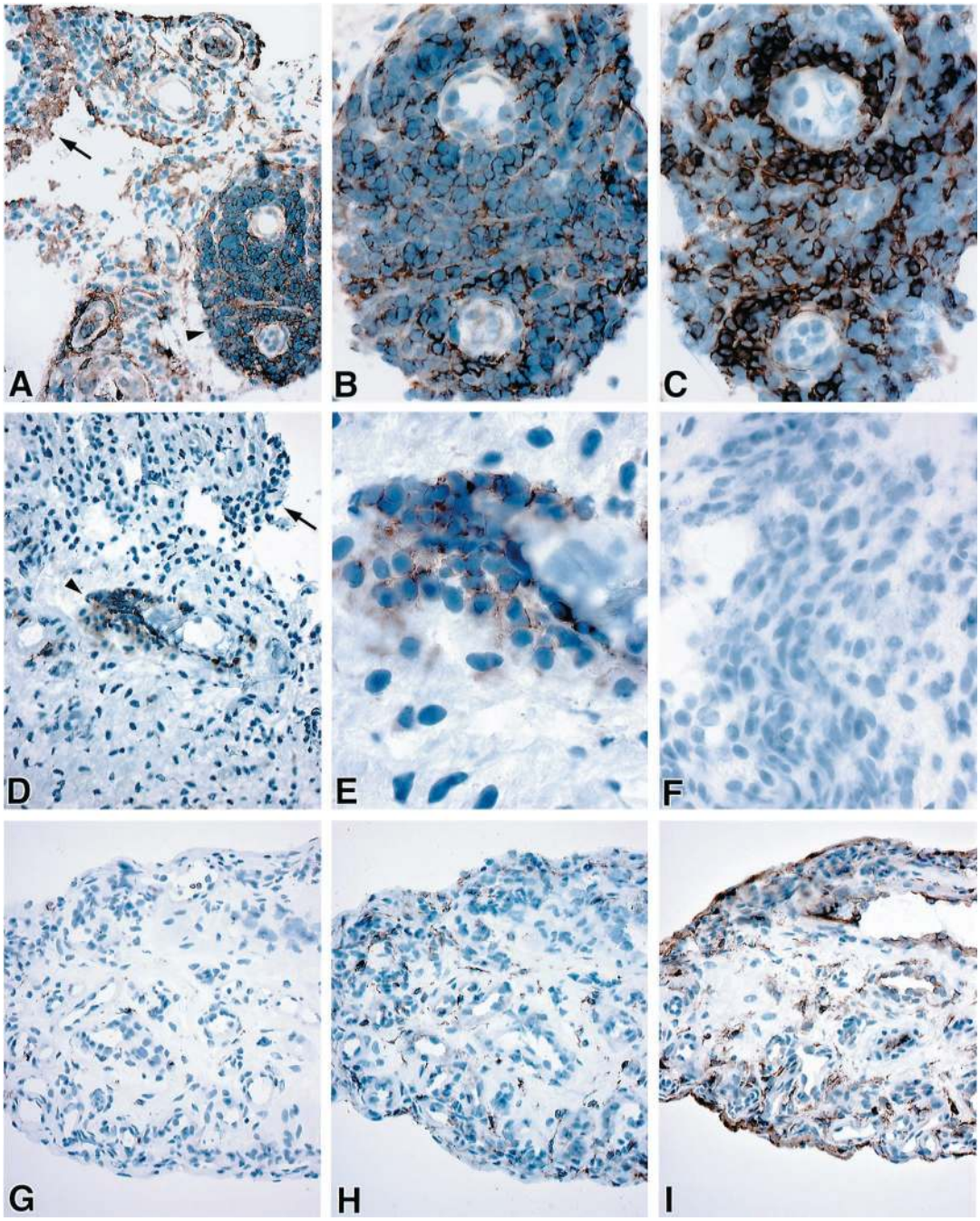

FIGURE 5. Synovial tissue samples from seven patients with RA (*A–F*) or three with OA (*G–I*) were stained for CXCR4 (*A, B,* and *I*) SDF-1 (*D, E,* and *G*), CD4 (*C* and *H*), or an isotype-matched control Ab (*F*). CXCR4 was widely expressed in the RA tissue sections (*A*), both in the synovial lining layer (arrow) and in the lymphocytic aggregates (arrowhead). Higher power view of a lymphocytic aggregate (*B*) shows that most of the perivascular lymphocytes are CXCR4 positive. A sequential section shows that there is marked but incomplete concordance with CD4 staining (*C*). SDF-1 was only expressed in the region of perivascular aggregates of lymphocytes (*D*, arrowhead) and was not expressed by cells in the lining layer (*D*, arrow). Higher power view (*E*) shows that the SDF-1 staining was intense in cells with abundant cytoplasm and a fibroblastic appearance, with some extracellular staining noted. SDF-1 staining was virtually absent in all of the OA tissues (*G*). All sections were counterstained with hematoxylin. Original magnifications for the photomicrographs were ×100 (*A, D, G, H,* and *I*) or ×250 (*B, C, E, F*).

SDF-1 was expressed by most of RA synovial tissues, and less frequently by OA synovium. In addition, SDF-1 mRNA was expressed by cultured RA synoviocytes. Of note, previous studies indicated that SDF-1 mRNA expression was unique to RA and not OA synoviocytes (48). The current data show that cultured RA fibroblasts produce SDF-1 protein. Moreover, anti-CD40 stimulation markedly enhanced SDF-1 production. It has previously been reported that CD40 engagement up-regulated TNF-α, macrophage inflammatory protein (MIP)-1α, and GM-CSF production by cultured synovial fibroblasts from RA synovium (36, 37). These data clearly add SDF-1 to the list of RA synoviocyte-derived effector molecules whose production is up-regulated by CD40 engagement. CD40 stimulation appears to play an important role in the activation of synovial fibroblasts in the rheumatoid synovium. In this regard, CD40 ligand (CD154) is expressed by stimulated $CD4^+$ T cells, and ~10% of $CD4^+$ T cells in RA synovial tissue express

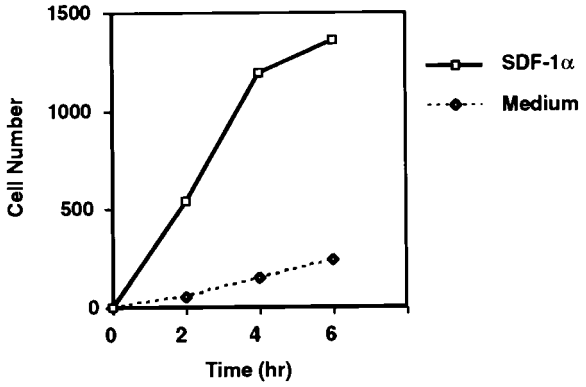

FIGURE 6. Migration of synovial tissue $CD4^+$ T cells in response to SDF-1α. Mononuclear cells from RA synovial tissue were cultured with or without SDF-1α (1000 ng/ml) for various lengths of time. Migrated cells were stained with anti-CD4-FITC, and the number of migrated $CD4^+$ T cells was assessed.

this molecule (49, 50). These results suggest that CD40 ligand expressed by $CD4^+$ memory T cells in the rheumatoid synovium may stimulate synovial fibroblasts to produce SDF-1 and that $CD4^+CD45RO^+$ T cells, which express CXCR4 possibly after stimulation by IL-15, may respond by migration into the inflamed tissue. These results provide an interesting example of the bidirectional modulation of function between endogenous synovial cells and migrating memory T cells that may contribute to the evolving inflammatory response characteristic of RA.

It is noteworthy that these findings suggest a new role for SDF-1/CXCR4 interactions. Previously, these interactions have been thought to be primarily involved in normal homeostasis by playing a role in the homing of naive T cells to secondary lymphoid organs (51). These results suggest an additional proinflammatory role for this interaction. Mice lacking SDF-1 or CXCR4 exhibited cardiovascular, vascular, and neurologic defects as well as defective B cell lymphopoiesis and a severe impairment of bone marrow myelopoiesis (52–54). SDF-1 attracts progenitor B cells into the microenvironment of stromal cells where growth and differentiation factors are released (52, 55–57). However, $CXCR4^{-/-}$ mice exhibit normal T cell development and distribution into peripheral lymphoid organs although their capacity to enter inflammation sites has not been examined.

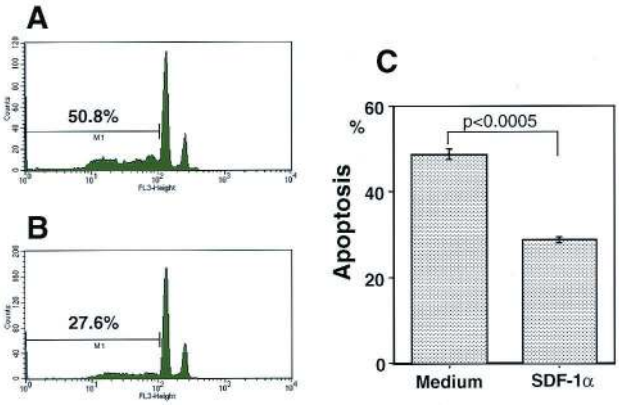

FIGURE 7. Inhibition of apoptosis of $CD4^+$ T cells induced by anti-CD3 stimulation by SDF-1α. Peripheral $CD4^+$ T cells were cultured with medium for 6 h, and then the T cells were stimulated in medium supplemented with or without SDF-1α (1000 ng/ml) for 2 h. Subsequently, the T cells were transferred to anti-CD3 coated plates and cultured for 8 h. Then the cells were stained with propidium iodide, and apoptosis was measured by flow cytometry. The results of anti-CD3-induced apoptosis of cells cultured without (*A*) or with SDF-1α (*B*) are depicted. Representative mean data from one of three independent experiments analyzed in triplicate are shown (*C*).

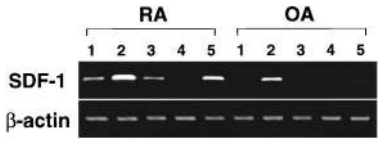

FIGURE 8. SDF-1 mRNA expression in RA and OA synovium. SDF-1 mRNA expression was analyzed in five RA and five OA synovial tissues using RT-PCR. PCR products were separated by electrophoresis through 2.0% agarose.

SDF-1 is expressed constitutively by various tissues (41, 58). Moreover, SDF-1 mRNA expression in spinal cord was not changed by experimental allergic encephalomyelitis, although expression of inflammatory chemokines, such as regulated on activation, normal T cell-expressed and -secreted (RANTES), MIP-1α, MIP-1β, IFN-γ-inducible protein 10 (IP-10), and monocyte chemoattractant proteins 1 and 2, were up-regulated (59). Thus, interaction of SDF-1 and CXCR4 has been thought to exert an essential developmental function rather than a role in inflammation. However, the current results strongly imply that interaction of SDF-1 and CXCR4 plays an important role in T cell accumulation in the inflamed RA synovium. Moreover, the data also show that production of SDF-1 may be regulated in inflammatory sites.

It has been reported that stimulation with IL-2 or IL-4 enhances surface CXCR4 expression by T cells (40, 46, 60). In this study, we found that IL-15 also enhances surface CXCR4 expression on $CD4^+$ T cells. It has been reported that IL-15 is expressed by RA synoviocytes and synovial endothelial cells, and that expression is up-regulated by stimulation with TNF-α, IL-1β, or IFN-γ (38, 39, 61, 62). These results suggest that the complex cytokine network present in the rheumatoid synovium might facilitate expression of CXCR4 by memory T cells.

Recent reports showed that CCR5 expression was up-regulated on RA synovial fluid T cells (29–31). However, the expression by synovial tissue T cells was not analyzed. Our data showed that the frequency of CCR5-expressing synovial tissue $CD4^+$ memory T cells was also increased compared with that on peripheral $CD4^+$ memory T cells. Therefore, RANTES, MIP-1α, and MIP-1β, the ligands of CCR5, might also play a role in attracting $CD4^+$ memory T cells from the periphery and, after migration, the T cells may express CXCR4 as a result of IL-15 stimulation. However, because some RA patients do not have functional CCR5 because of a homozygous Δ32 deletion (63, 64), CCR5 may not be necessary for the development of RA. Alternatively, IL-15 may up-regulate CXCR4 directly and facilitate migration of memory T cells in

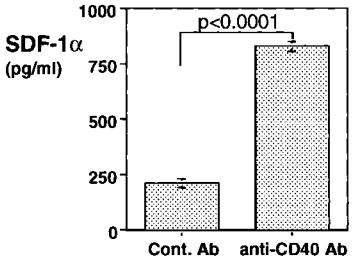

FIGURE 9. SDF-1 production by cultured RA synovial fibroblasts and enhancement by anti-CD40 stimulation. Supernatants of the synovial fibroblasts cultured for 3 days with anti-CD40 or control mAb were assessed for SDF-1α by ELISA. Representative mean data from one of four experiments analyzed in triplicate are shown.

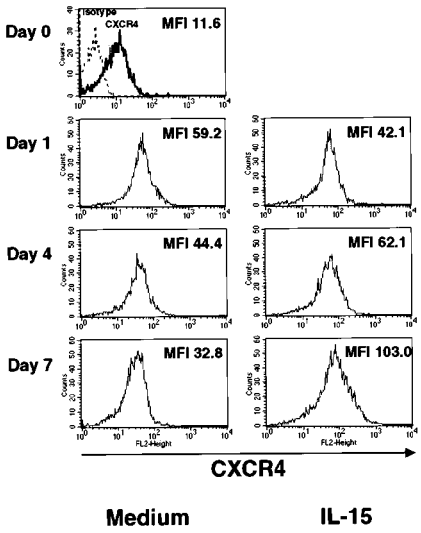

FIGURE 10. IL-15-mediated enhancement of surface CXCR4 expression by $CD4^+$ T cells. Purified peripheral $CD4^+$ T cells were cultured with medium alone or with IL-15 (100 ng/ml) for various lengths of time and stained with isotype-matched control mAb or anti-CXCR4 mAb. The surface expression of CXCR4 was then analyzed by flow cytometry. Representative data from one of three independent experiments are shown. Mean fluorescence intensity (MFI) values are indicated.

response to SDF-1. It was reported that synovial fluid $CD3^+$ T cells highly express CXCR3 by flow cytometry (29), and synovial tissue T cells also express CXCR3 by immunohistochemistry (32). However, the frequency of CXCR3-positive cells in synovial tissue has not been analyzed. These data show that the frequency of CXCR3 expression by $CD4^+$ memory T cells was not significantly different between synovial tissue and peripheral blood. Therefore, these data provide no evidence that expression of CXCR3 is uniquely involved in the accumulation of memory T cells in the rheumatoid synovium.

Of note, SDF-1 inhibited activation-induced apoptosis of $CD4^+$ T cells. It has been reported that few T cells in the rheumatoid synovium are apoptotic (65). Although a variety of influences in the inflamed synovium may limit apoptosis, interaction of SDF-1 and CXCR4 might additionally contribute to the rescue of $CD4^+$ T cells from apoptosis after TCR stimulation. The mechanism of this phenomenon is unclear. It has been shown that SDF-1 stimulation inhibited anti-CD3-stimulated phosphorylation of the TCR signaling molecules, ZAP-70, SLP-76, and pp36 in Jurkat cells, suggesting that SDF-1 could regulate the threshold for T cell activation (66). The altered nature of the anti-CD3 stimulation might serve to limit apoptosis. However, after anti-CD3 stimulation, the ratio of cells in the $G_2{\cdot}M/G_0{\cdot}G_1$ stage of the cell cycle was not altered by SDF-1 (data not shown) as might be expected if signaling was altered. Thus, stimulation with SDF-1 might exert other effects to protect $CD4^+$ T cells from apoptosis. Recently, we found that SDF-1α (50–1000 ng/ml) enhanced IL-2 production by anti-CD3-stimulated peripheral $CD4^+$ T cells (67). It is possible that the IL-2 produced may inhibit apoptosis.

CXCR2 expression by synovial tissue $CD4^+$ memory T cells was also up-regulated compared with that expressed by peripheral $CD4^+$ memory T cells. The ligand of CXCR2 is IL-8, which is also produced by synovial fibroblasts (68, 69). This suggests that the interaction of IL-8 and CXCR2 also could play a role in the migration of $CD4^+$ memory T cells into the inflamed synovium.

The single cell RT-PCR method is a powerful tool to analyze the expression of many genes from one cell and to assess correlations between expression of various genes (34, 35). Differences in amplification frequencies of different cDNAs are minimized by limiting the length of the cDNA and tailing the cDNA. However, it should be noted that cell surface expression of CCR5 and CXCR3 was higher than detected by this mRNA analysis. This may be related to differential regulation of mRNA and protein expression. Alternatively, the sensitivity of the detection of CCR5 or CXCR3 mRNAs may be less than that of the surface protein by flow cytometric analysis. However, this is unlikely because mRNA expression by individual cells could be routinely detected by this method (Fig. 1). Moreover, the frequency of CXCR4 mRNA expression was comparable to that of cell surface protein expression, indicating that the single cell RT-PCR method appears to provide a reasonably accurate assessment of chemokine receptor mRNA expression by individual memory T cells.

In conclusion, functional CXCR4 expression was up-regulated by RA synovial tissue $CD4^+$ memory T cells. The expression was up-regulated by IL-15, and SDF-1, the ligand of CXCR4, induced migration of synovial tissue $CD4^+$ T cells and inhibited apoptosis of $CD4^+$ T cells induced by anti-CD3 stimulation. Moreover, RA synovial tissue and synoviocytes produced SDF-1, and the latter was enhanced by CD40 engagement. Therefore, CXCR4-SDF-1 interactions might play a central role in memory T cell migration into the inflamed RA synovium and for persisting inflammation at this site mediated by $CD4^+$ T cells. These findings emphasize the complex interactions between endogenous tissue cells and migrating T cells in the evolving synovial inflammation of RA.

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