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J Immunol 2000; 165:6590-6598; doi: 10.4049/jimmunol.165.11.6590

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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

Toshihiro Nanki, Kenji Hayashida, Hani S. El-Gabalawy, Sharon Suson, Kenrin Shi, Hermann J. Girschick, Sule Yavuz and Peter E. Lipsky

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 (CXCR4) 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.

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™ 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 MgCl₂; 1 mM DTT; 10 μM dNTP (Sigma); 5 U/ml Prime RNease Inhibitor (5 Prime → 3 Prime, Boulder, CO); 300 U/ml RNase Guard (Pharmacia Biotech); 200 ng/ml oligo(dT)₁₂₋₁₈ (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

Received for publication December 30, 1999. Accepted for publication September 7, 2000.

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‡ This work was supported by National Institutes of Health Grant AR-36169.

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Abbreviations used in this paper: RA, rheumatoid arthritis; OA, osteoarthritis; SDF, stromal cell-derived factor; CCR, CC chemokine receptor; TCR, CD3; TCRβ, TCR Cβ; CD45RO, T cells are memory T cells that express CD45RO.

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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 CoCl₂; 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 MgCl₂; 0.01% Triton X-100; 200 μM dNTP, and 2 μM X(dT)₁₅ primer (ATG TCG TAC CGG CTC TGG ACA AAA TAT GAA TTC-dT₂₄) (Integrated DNA Technologies). Twenty-five cycles of amplification were performed with 1 min at 94°C, 2 min at 45°C, and 7 min at 72°C plus 10 s extension per cycle. Afterward, 5 μl 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 MgCl₂; 0.01% Triton X-100; 200 μM dNTP, and 0.625 μl 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 MgCl₂; 0.01% Triton X-100; 200 μM dNTP; and 1.25 μl 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 β (TCRB) 5'-CTA AGT GCA TTA CGG CCT GCT C, 3'-TCA TAG AGG ATG GTG GCA GCA A, 5'- (nested)-CTC TGA GAG AAT GAC GAG TAC CC; CCR1 5'-CTG GTC TCA AGT CAG TGT ACA GGT AA) or SDF-1 (5'-GTC AGT ACA AGG CAT ATG GGT ACG AG; 3'-GTC CTG AGA GTC CTT TTG CG). For nested PCR, 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 MgCl₂; 0.01% Triton X-100; 200 μM dNTP, and 1.25 μl Taq DNA polymerase). The cloning program was 94°C for 1 min, 60°C for 1 min, and 72°C for 2 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 MgCl₂; 0.01% Triton X-100; 200 μM dNTP, and 1.25 μl Taq DNA polymerase). The cloning 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 β (TCRB) 5'-CTA AGT GCA TTA CGG CCT GCT C, 3'-TCA TAG AGG ATG GTG GCA GCA A, 5'- (nested)-CTC TGA GAG AAT GAC GAG TAC CC; CCR1 5'-CTG GTC TCA AGT CAG TGT ACA GGT AA) or SDF-1 (5'-GTC AGT ACA AGG CAT ATG GGT ACG AG; 3'-GTC CTG AGA GTC CTT TTG CG).

To confirm the sensitivity of the single cell RT-PCR method, purified CD4⁺ T cells were prepared using a commercially available purification method (StemSep: StemCell Technologies). Purity of the separated cells was >95%. The purified peripheral CD4⁺ cells were cultured in RPMI 1640 with 10% BSA supplemented where indicated with 1000 ng/ml of M-1 (R&D Systems) was added to the lower wells, and 5 × 10⁵ mononuclear cells obtained from synovial tissue and subjected to single cell RT-PCR described above, except that the annealing temperature was decreased. 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 65°C.

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)₁₅ 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 then β-actin PCR products were analyzed by using diolylated dDNA. The cDNA was amplified with primers 5'-GTC GCT TCA AGT TCG TAC TTT GGT ACG ACT GC; 3'-GTC ACT GAG ACT AGG GCT CCA GGT GTG GTG CAC TCC (4890111) (R&D Systems, Minneapolis, MN), and then run on agarose gel and visualized by ethidium bromide staining. The PCR products were then separated by agarose gel and visualized by ethidium bromide staining. The PCR products were run on agarose gel and visualized by ethidium bromide staining.

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; Dako), anti-CXCR4-PE (35L12; Sigma), and anti-CCR3-FITC (4890111) (R&D Systems, Minneapolis, MN), and anti-CXCR4-PE (12G5; PharMin- gen). Synovial tissue or peripheral blood mononuclear cells were adjusted to 1 × 10⁵ cells and incubated with two or three fluorochrome-labeled mAbs for 30 min, rinsed with PBS-3% FCS, and analyzed with a FACSScan (Becton Dickinson).
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 biotin-labeled 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 χ² 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 TCRβ 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 × 10⁻¹¹, p < 0.05, χ² 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 × 10⁻¹⁰, χ² 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, χ² 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 ± 10.7% of CD4⁺ synovial T cells expressed CXCR4, mean ± 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 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 CXCR5 mRNA expression was analyzed by the RT-PCR method. Cell number expected from the dilution is indicated.

*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 TCRβ 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.
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. 5C). The OA samples demonstrated occasional isolated CD4+ T cells or small perivascular lymphocytic aggregates (Fig. 5H). CXCR4 expression was widespread in all RA samples, with intense staining of the synovial lining cell layer and the sublining macrophages (Fig. 5A). 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. 5B). 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 strata (Fig. 5I). 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. 5E). 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 patients with RA and in peripheral blood from a normal donor

<table>
<thead>
<tr>
<th>Synovial tissue from RA</th>
<th>CCR1</th>
<th>CCR2</th>
<th>CCR3</th>
<th>CCR4</th>
<th>CCR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1 (n = 50)</td>
<td>35%</td>
<td>6%</td>
<td>5%</td>
<td>10%</td>
<td>20%</td>
</tr>
<tr>
<td>RA2 (n = 39)</td>
<td>5%</td>
<td>1%</td>
<td>3%</td>
<td>16%</td>
<td>32%</td>
</tr>
<tr>
<td>RA3 (n = 63)</td>
<td>7%</td>
<td>2%</td>
<td>1%</td>
<td>25%</td>
<td>33%</td>
</tr>
<tr>
<td>Total (n = 152)</td>
<td>17%</td>
<td>8%</td>
<td>5%</td>
<td>21%</td>
<td>39%</td>
</tr>
<tr>
<td>Peripheral blood from RA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA3 (n = 24)</td>
<td>3%</td>
<td>2%</td>
<td>0%</td>
<td>17%</td>
<td>11%</td>
</tr>
<tr>
<td>RA4 (n = 24)</td>
<td>2%</td>
<td>1%</td>
<td>0%</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>Total (n = 48)</td>
<td>5%</td>
<td>3%</td>
<td>0%</td>
<td>4%</td>
<td>12%</td>
</tr>
<tr>
<td>Peripheral blood from normal donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor (n = 33)</td>
<td>1%</td>
<td>1%</td>
<td>2%</td>
<td>9%</td>
<td>12%</td>
</tr>
</tbody>
</table>

The frequencies of CCR5-positive synovial tissue CD4+CD45RO+ T cells were significantly greater than that of RA peripheral CD4+CD45RO+ T cells ($p < 0.05$, χ2 test).

* Number of positive cells.

# Frequency of chemokine receptor-positive cells in TCRBC-positive cells.

* The frequencies of synovial tissue CD4+CD45RO+ T cells expressing CCR2 were significantly higher than that of RA peripheral CD4+CD45RO+ T cells ($p < 0.05$).

* The frequency of synovial tissue CD4+CD45RO+ T cells expressing CXCR4 was significantly higher than that found in RA peripheral CD4+CD45RO+ T cells ($p < 5 \times 10^{-11}$, $p < 0.05$, χ2 test, Student’s t test, respectively).
induces adhesion of naive and memory T cells to ICAM-1 (CD54) and is tant for both naive and memory T cells (43). In addition, SDF-1 was originally identified as a growth factor for murine pre-B cells (41, 27), and its unique ligand was subsequently found to be SDF-1, CXCR4 (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 naïve 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 naïve 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.

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 stimulates 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 chemotactic signal 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 naïve 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 naïve 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.

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

<table>
<thead>
<tr>
<th></th>
<th>CCR5</th>
<th>CXCR2</th>
<th>CXCR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 152)</td>
<td>7+</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Peripheral Blood (n = 48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (17%)</td>
<td>4 (3%)</td>
<td>7 (21%)</td>
</tr>
<tr>
<td></td>
<td>5 (4%)</td>
<td>3 (21%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<td></td>
<td>83 (69%)</td>
<td>10 (71%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>121*</td>
<td>80%</td>
<td>29%</td>
</tr>
</tbody>
</table>

* Number of positive cells.

† Frequency of cells expressing either CCR5, CXCR2, or CXCR4 in the total number of cells.

‡ Frequency of CCR5 only expressing CD4+CD45RO+ T cells in peripheral blood was significantly higher than in synovial tissue (p < 0.05).

§ Number of cells expressing either CCR5, CXCR2, or CXCR4.

¶ 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 × 10−3).

![FIGURE 3](http://www.jimmunol.org/) 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](http://www.jimmunol.org/) 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 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.
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 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).
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 naïve 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.

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 synovocytes 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
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 G2M/G0/G1 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-1a (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.

Acknowledgments

We thank Angie Mobley for assistance with cell sorting, and Rehana Hussain and Michelle McGuire for their technical support.

References


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.


