

# Aberrant Responsiveness to RANTES in Synovial Fluid T Cells from Patients with Rheumatoid Arthritis

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**ABSTRACT. Objective.** To study expression and function of the chemokine receptor CCR5 in synovial fluid (SF) T cells from patients with rheumatoid arthritis (RA).

**Methods.** Expression of CCR5 was studied by flow cytometry and immunoblotting. The chemotactic response of T cells to chemokines was studied in cell migration assay. Tyrosine phosphorylation of Crk-associated substrate lymphocyte-type (CasL) was evaluated in immunoprecipitation and immunoblotting.

**Results.** SF T cells showed an increase in the population of CCR5, CXCR4, and CD45RO positive cells and exhibited an increase in chemotactic activity, which was not augmented with RANTES but stromal cell-derived factor-1 $\alpha$ . Tyrosine phosphorylation per CasL molecule was markedly enhanced in SF T cells. In H9 cells, tyrosine phosphorylation of not only focal adhesion kinase but also CasL was induced after treatment with RANTES. Downmodulation of CCR5 by RANTES was decreased and recycling of CCR5 was accelerated in SF T cells when compared with peripheral blood (PB) T cells. When CD45RO positive PB T cells were cultured with interleukin 2, blunted responsiveness to RANTES-induced chemotaxis was reproduced as well as spontaneous chemotaxis, increased expression of CCR5, and aberrant receptor dynamics, after RANTES stimulation as observed in SF T cells.

**Conclusion.** Synovial fluid T cells highly positive for CCR5 show aberrant characteristics; resistant to RANTES in terms of migration, but responsive in terms of dynamics of CCR5. (J Rheumatol 2002;29:1124–34)

## Key Indexing Terms:

RHEUMATOID ARTHRITIS  
CCR5 RANTES

SYNOVIAL FLUID

T CELL LYMPHOCYTES  
CRK-ASSOCIATED SUBSTRATE LYMPHOCYTE

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints characterized by leukocyte invasion and synovial cell activation, leading to cartilage and bone

destruction. Various mediators, including inflammatory cytokines and adhesion molecules, have been implicated in the pathogenesis of RA<sup>1-3</sup>, and the importance of T cell-mediated autoimmune response has been documented in the initiation and maintenance of the disease<sup>4,5</sup>.

The chemokines are a group of signal peptides produced in a variety of cells that induce, for example, chemotaxis of leukocyte subsets<sup>6</sup>. These chemokines seem to play key roles in inflammatory and immune responses; for instance, release of chemokines from inflammatory cells is a crucial step in the recruitment of the cells required to establish local inflammatory responses<sup>6</sup>. All chemokine receptors belong to the same class of 7 transmembrane receptors and recognize more than one chemokine; and several chemokines bind to more than one receptor, indicating that redundancy and versatility are characteristic for the chemokine–chemokine receptor system. The binding of chemokines to their receptors is followed by involvement of a number of intracellular signaling molecules including heterotrimeric G proteins, adenylyl cyclase, phospholipases, protein tyrosine and serine/threonine kinases, lipid kinases, the Rho family of small GTPases<sup>7,8</sup>, Stat1 and Stat3<sup>9</sup>. Multiple and distinct signaling pathways, therefore, exist for individual chemokine receptors, which are differentially regulated depending on the ligands and cell types involved. In the case of the CC chemokine receptor CCR5, after binding its

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cognate ligands, e.g., RANTES, macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) and MIP-1 $\beta$ , 2 distinct signaling cascades are activated: one associated with recruitment of G proteins and the other with protein tyrosine kinase activation, resulting in variable biological responses including receptor internalization and recycling, chemotaxis, T cell proliferation, and interleukin 2 (IL-2) receptor expression<sup>10–12</sup>. Development of modified CCR5 ligand such as amino terminal modifications of RANTES, aminooxypentane (AOP)-RANTES, has facilitated the understanding of coupling between such distinct signaling pathways and subsequent biological actions<sup>13</sup>. In contrast to RANTES, for example, AOP-RANTES is able to induce internalization of CCR5 but unable to trigger the association of focal adhesion kinase (FAK) and migration<sup>10,13,14</sup>. These results strongly argue that downstream signals of CCR5 could be conditionally variable, the biological or pathological significance of which remains unclear.

Growing evidence suggests that the coordinated production of chemokines is likely to be important in the orchestration of the inflammatory responses observed in patients with RA<sup>15</sup>. It has recently been shown that T cells in the synovial membrane and fluid express high levels of the inflammatory chemokine receptors CCR5 and CXCR3 compared to peripheral blood (PB) T cells<sup>16–18</sup>. RANTES and MIP-1 $\alpha$  and  $\beta$  are expressed in the joints of patients with RA and detected in synovial fluid (SF) at high levels<sup>19,20</sup>. Collectively, these results may suggest that T cells expressing CCR5 migrate in response to its ligands, and take part in inflammatory reactions, particularly of Th1 type, since the production of interferon- $\gamma$  and IL-2 in CCR5-positive SF T cells has been reported<sup>20,21</sup>. Clinical importance of CCR5 has also been suggested; the frequency of such genetic alteration that decreases affinity of RANTES is low in patients with RA<sup>22</sup>, and a blockade of RANTES-CCR5 interaction, e.g., by a CCR5 antagonist met-RANTES, gives beneficial effects on joint inflammation<sup>23</sup>. On the other hand, SF T cells from patients with RA are chronically activated, bearing the “memory” phenotype (CD45RO+, CD29<sup>bright</sup>) and exhibiting an increase in migratory capacity without chemoattractants *in vitro*<sup>24,25</sup>. Crk-associated substrate lymphocyte-type (CasL) is shown to be preferentially expressed in T cells<sup>26</sup> and tyrosine phosphorylated by the ligation of  $\beta$ 1 integrins and plays a role in chemotaxis of T cells<sup>26–28</sup>. It was reported that RANTES induces tyrosine phosphorylation of FAK, tyrosine kinase zeta-associated protein 70, and paxillin<sup>29</sup>, and FAK has been shown to be directly associated with CasL<sup>30</sup>. However, the relationship between chemokine system and chemotaxis activity is not clearly understood and whether stimulation of CCR5 influences CasL remains unknown.

We describe that CCR5 expression is increased in SF T cells with corresponding increase in expression of CXCR4 and CD45RO, and that spontaneous chemotactic activity of

SF T cells is augmented along with enhanced tyrosine phosphorylation of CasL. In our investigation of the underlying mechanism, SF T cells highly positive for CCR5 showed aberrant characteristics; were resistant to RANTES in terms of migration, but were at least partially responsive in terms of internalization and recycling of CCR5.

## MATERIALS AND METHODS

**Patients.** Seventeen patients who fulfilled the American College of Rheumatology criteria<sup>31</sup> for diagnosis of RA and 17 healthy subjects were studied. All patients registered at the Institute of Medical Science, the University of Tokyo and Tokyo Women’s Medical University. All samples were obtained after informed consent was given.

**Monoclonal antibodies (Mab).** The following Mab were purchased: anti-CCR5 (2D7, PharMingen, San Diego, CA; C20, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-CXCR4 (12G5, PharMingen); anti-CCR2 (48607.211, R & D Systems, Inc., Minneapolis, MN, USA); anti-CD45RO (UCLH-1); anti-CD45RA (HI100); anti-CD19 (1F5); anti-CD11b (ICRF44); anti-CD56 (B159, PharMingen); anti-CD26 (4EL-1C7, Beckman Coulter, Inc., Fullerton, CA, USA); PerCP-conjugated anti-CD3 (Leu-4); PerCP-conjugated anti-CD4 (Leu-3a); PerCP-conjugated anti-CD8 (Leu-2a, Becton Dickinson, Thousand Oaks, CA, USA); anti-mouse IgG1 (MOPC-21); anti-mouse IgG2a (R19-15, PharMingen); Biotin-conjugated donkey anti-goat IgG, HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA); anti-Cas, anti-FAK (Transduction Laboratories, Lexington, KY, USA); anti-phosphotyrosine (p-Tyr) 4G10 (Upstate Biotechnology, Lake Placid, NY, USA).

**Cell preparation.** PB mononuclear cells (PBMC) were prepared from heparinized venous blood samples by density gradient centrifugation with Ficoll-Paque (Pharmacia Biotech Inc., Piscataway, NJ, USA) and washed 3 times with phosphate buffered saline (PBS). SF was aseptically withdrawn from the knees of 9 patients who had active synovitis with marked effusion. SF mononuclear cells (SFMC) were prepared in the same manner as described for PBMC. For the isolation of T cells, PBMC were cultured in plastic dishes for 1 h at 37°C with 5% CO<sub>2</sub> and non-adherent cells were further purified by negative selection with anti-CD19, anti-CD11b, and anti-CD56 Mab using magnet beads (BioMag Goat Anti-Mouse IgG, PerSeptive Biosystems, Framingham, MA, USA). The purity of CD3 positive cells were about 95% as determined by flow cytometry. For isolation of CD45RO positive cells, anti-CD45RA Mab was added to this process for deprivation of CD45RA positive cells. The obtained cells were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM Hepes buffer, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin (Life Technologies Inc., Grand Island, NY, USA), unless otherwise specified. When pretreatment of CD45RO positive cells was needed, cells were cultured in medium with 10 ng/ml IL-2 (IL-2, Peprotech, London, UK), 1 ng/ml or 5 ng/ml tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , Dainippon Pharmaceutical Co., Osaka, Japan).

H9 cells (human leukemic T cell line) and ECV304 cells (human endothelium cell line) were obtained from American Type Culture Collection. H9 cells and ECV304 cells were cultured in RPMI 1640 and Medium 199 (Life Technologies Inc.), respectively, supplemented with 10% FBS at 37°C with 5% CO<sub>2</sub>.

**Flow cytometry.** Flow cytometry was performed on FACS Caliber (Becton Dickinson) after direct immunofluorescent staining of the cells using the indicated Mab. Briefly,  $1 \times 10^5$  to  $5 \times 10^5$  lymphocytes from PB and SF were washed twice with 2.5% human serum (Sigma Chemical Co.) in PBS, 0.1% sodium azide, and 10% Blockace (Snow Brand Co., Sapporo, Japan). After washing, cell suspensions were stained with saturating concentrations of the appropriate Mab at 4°C for 30 min. Cells were washed twice and analyzed for immunofluorescence using FACS Caliber. To determine the individual percentage in T cells, lymphocytes were first gated according to

their light scatter properties. A second gate was set on CD3+ lymphocyte subset, and the number of positive cells was calculated after defining a cutoff value according to isotype control.

**Immunoprecipitation and immunoblot analysis.** Cells were lysed in a detergent buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenyl methylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µM sodium orthovanadate, 1 mM EGTA, 10 mM glycerophosphate, and 10 mM sodium fluoride) for 30 min at 4°C with continuous rocking, then centrifuged (5,000 rpm, 5 min, 15,000 rpm, 15 min). The protein concentration of the lysates was determined using a protein detection kit (Pierce, Rockford, IL, USA). Protein extracts (500 µg protein) precleared by incubation with 10 µg of anti-goat IgG-protein G sepharose (Pharmacia Biotech Inc.) were briefly centrifuged, immunoprecipitated with protein G sepharose-conjugated anti-CCR5 antibody C20 (10 µg/sample, 120 min, 4°C). Immunoprecipitates were separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk (Wako Pure Chemical Co., Osaka, Japan) in Tris buffered saline containing 0.1% Tween 20 (TBS-T), followed by incubation with anti-CCR5 antibody C20 at 1 µg/ml. After further washing in TBS-T, membranes were incubated with a 1/10,000 dilution of biotin-conjugated donkey anti-goat IgG in 2% bovine serum albumin (BSA, Sigma Chemical Co.)/TBS-T, followed by washing and incubated with a 1/20,000 dilution of streptavidin horseradish peroxidase (HRP, Sigma Chemical Co.) in 5% skim milk /TBS-T.

For detection of phosphorylated tyrosine, immunoprecipitates or lysates were separated in SDS-PAGE and electrophoretically transferred to Immobilon-P membranes and reacted with anti-CasL, anti-FAK, or anti-phosphotyrosine Mab 4G10 at 0.1 µg/ml. A HRP-conjugated goat anti-mouse IgG was used as the second antibody and the blot was developed with enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL, USA).

**Cell migration assay.** Cell migration was assayed using 24 well plates with 3 µm pore size cell culture inserts (Transwell, Corning Costar Corp., Cambridge, MA, USA) as described<sup>28</sup>. In brief, confluent monolayers were established on the inserts by adding  $1 \times 10^5$  ECV304 cells/well and incubated overnight at 37°C with 5% CO<sub>2</sub>. After gently removing culture media from the inserts, 100 µl of  $1 \times 10^7$  cells/ml of PB or SF T cells, or H9 cells in RPMI 1640 with 0.6% BSA were added to each insert. Recombinant human chemokines, RANTES, SDF-1α (Peprotech) were diluted in 0.6 ml of RPMI 1640 with 0.6% BSA to 100 ng/ml and added to the lower wells. Plates were incubated for 180 min at 37°C with 5% CO<sub>2</sub> and the cells that migrated to the lower well were harvested and counted using FACS Caliber. Cell migration was expressed as the mean cell number counted for 30 s on high flow.

**Downmodulation and recycling of CCR5 on the surface of synovial fluid T cells by RANTES.** Downmodulation and recycling of CCR5 on the cell surface were assessed by a described method<sup>14</sup>. Cells were first incubated for 60 min at 37°C with 1 µg/ml RANTES diluted in RPMI 1640 with 0.6% BSA. Cells were washed 3 times in medium at room temperature and incubated further in RPMI 1640 with 0.6% BSA at 37°C. Aliquots were taken at the indicated time points, stained with anti-CCR5 Mab 2D7, and analyzed using FACS Caliber. Relative fluorescence intensity was calculated as (mean channel fluorescence [chemokine] – mean channel fluorescence [negative control]) / (mean channel fluorescence [medium] – mean channel fluorescence [negative control]).

**Statistical analysis.** Differences among 3 groups were analyzed for statistical significance using analysis of variance. Student's t test was used for comparison of 2 groups. P values < 0.05 were considered statistically significant.

## RESULTS

**CCR5 in SF T cells from patients with RA.** We examined expression and function of CCR5, in SF T cells from

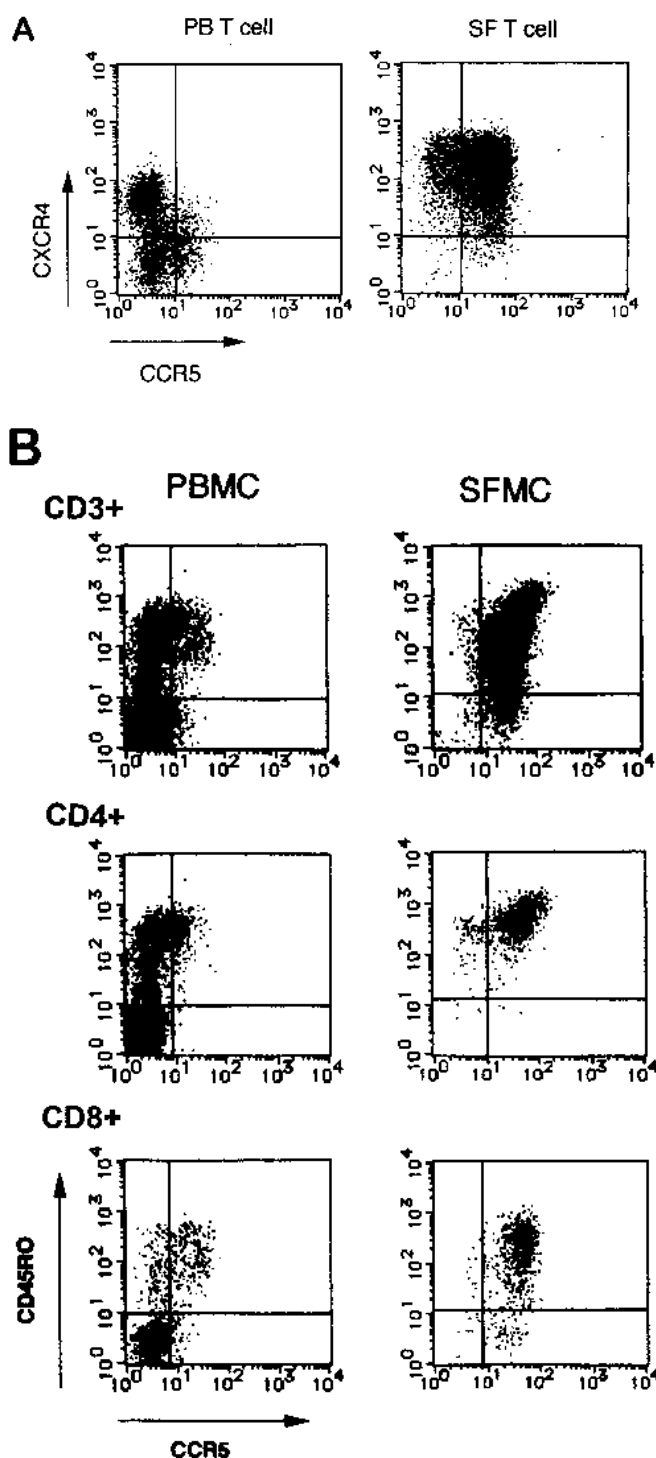
patients with active RA. Three color flow cytometric analysis revealed that surface expression of various molecules so far examined are not distinct in PB T cells between healthy subjects and patients with RA except for mild decrease in CCR5 in the latter (Table 1). SF T cells, however, showed a significant increase in the population of CCR2, CXCR4, and CD45RO positive cells with a reciprocal increase in CCR5 positive population when compared with PB T cells (Table 1). Moreover, in clear contrast to PB T cells in which reciprocal expression pattern of CCR5 and CXCR4 has been reported<sup>32</sup>, a majority of SF T cells revealed dual positivity for CCR5 and CXCR4 (Figure 1A). Most CCR5 positive T cells, irrespective of bearing either CD4 or CD8, were CD45RO positive, thus characterized as a memory subset (Figure 1B). Modestly increased CD26 expression may support this (Table 1). Of note, the mean fluorescence intensity of CCR5 in SF T cells was increased by about 5 fold when compared with that in PB T cells from either healthy subjects or patients with RA (Table 1). Increment of CCR5 expression was also confirmed at protein levels by immunoprecipitation and consecutive immunoblot assay using anti-CCR5 antibody. As shown in Figure 2, in not only PB T cells from patients with RA but also SF T cells, anti-CCR5 antibody reacted with the protein bands of 43 kDa, which corresponds to the expected molecular weight of CCR5. Obviously, SF T cells (lanes 3 and 4) contained a greater amount of CCR5 protein when compared with PB T cells in both patients (lanes 1 and 2).

**Effect of RANTES on migration of synovial fluid T cells.** We next asked whether increment in CCR5 expression influenced RANTES-induced chemotactic activity in SF T cells with flow cytometry as described in Materials and Methods. As reported<sup>24,25</sup>, we found that SF T cells from patients with RA showed increase in spontaneous migratory activity (Figure 3), while SF T cells from patients with osteoarthritis did not show increase in spontaneous migratory activity (data not shown). After stimulation with RANTES, PB T

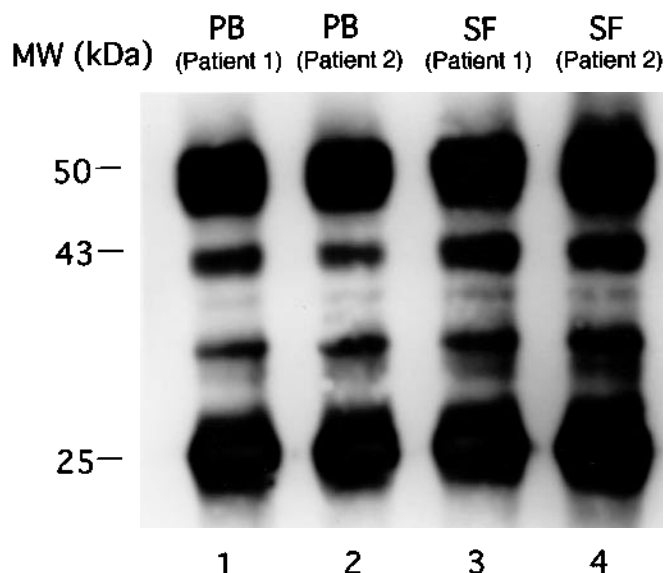
**Table 1.** Flow cytometric analysis of T cells from peripheral blood and synovial fluid from patients with RA. Values are % positive expressed as the mean ± SEM.

	Peripheral Blood T Cells Normal, n = 14 RA, n = 9		Synovial Fluid T Cells, n = 7
Surface Antigen			
CCR5	18.5 ± 1.4*	9.3 ± 1.4*	79.2 ± 7.4**
CCR5 MFI	6.7 ± 0.4	5.8 ± 0.8	27.4 ± 5.2**
CXCR4	60.6 ± 2.8	66.9 ± 2.9	92.6 ± 3.3**
CXCR4 MFI	32.4 ± 3.7	37.8 ± 3.4	145.5 ± 18.4**
CCR2	9.5 ± 0.9	4.3 ± 0.4	19.7 ± 5.0**
CCR2 MFI	7.7 ± 0.5	6.4 ± 0.6	10.7 ± 2.1
CD45RO	32.8 ± 3.0	40.6 ± 6.7	95.3 ± 1.2**
CD26 high	11.1 ± 1.0	8.2 ± 3.0	15.3 ± 2.4

MFI: mean fluorescence intensity. \* p < 0.05; \*\* p < 0.01 compared to peripheral blood from healthy subjects.



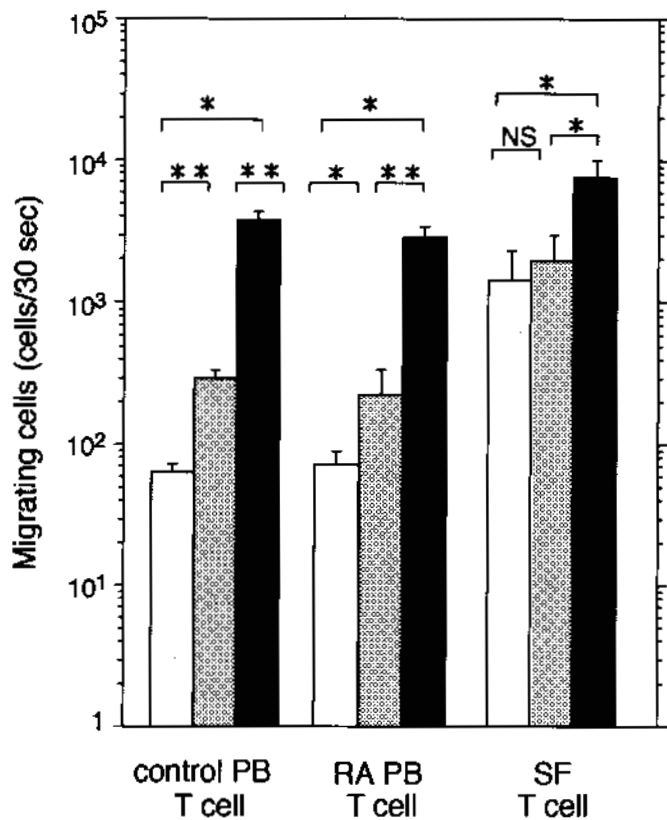
**Figure 1.** Dot plot flow cytometry analysis showing CCR5 expression on peripheral blood (PB) T cells and synovial fluid (SF) T cells from a patient with rheumatoid arthritis (RA). A. Three color staining protocol was used to assess for expression of CCR5 (horizontal axis) and CXCR4 (vertical axis) on CD3-gated T cells. B. Expression of CCR5 (horizontal axis) and CD45RO (vertical axis) on the CD3+, CD4+, and CD8+ cells, respectively, was analyzed by 3 color immunofluorescence using mononuclear cells (MC) isolated from patients with RA. Quadrant markers were positioned to include > 98% of control Ig-stained cells in the lower left. Representative profiles are shown.



**Figure 2.** Identification of CCR5 in peripheral blood (PB) and synovial fluid (SF) T cells. Whole cell lysates from PB T cells (lanes 1 and 2) and SF T cells (lanes 3 and 4) of 2 representative patients with rheumatoid arthritis (RA) were immunoprecipitated, then analyzed in immunoblot using anti-CCR5 antibody C20 as described in Materials and Methods. Molecular weights (MW) were determined according to the marker run in parallel. One representative experiment of 5 is shown.

cells from either healthy subjects or patients with RA showed increased migration (Figure 3). In clear contrast, however, stimulation with RANTES did not further enhance the migratory activity of SF T cells (Figure 3). This unresponsiveness to RANTES was paradoxical since a majority of SF T cells expressed its cognate receptor CCR5 as described above. Loss of bioavailability of RANTES is not the likely reason, since PB T cells showed significant response to the same reagent (Figure 3). SF T cells, as well as PB T cells, were responsive to SDF-1 $\alpha$  in migration assay (Figure 3), indicating that viability of SF T cells was preserved during experiments. Together with the fact that stimulation with either MIP-1 $\alpha$  or MIP-1 $\beta$  did not enhance chemotaxis in SF T cells (data not shown), we may conclude that chemotaxis of SF T cells is spontaneously increased but unresponsive to further stimulation with RANTES.

**CasL in SF T cells from patients with RA.** We then addressed the underlying mechanism for enhancement of spontaneous migratory activity of SF T cells. As we reported, CasL with its tyrosine phosphorylation is one of the essential components for T cell migration, for example, after ligation of  $\beta$ 1 integrins<sup>28</sup>. Thus, we performed immunoblot assay for CasL using SF T cells. Figure 4 shows that Mab against Cas reacted 105 kDa proteins in the cell extracts from either PB T cells or SF T cells (Figure 4A, I), indicating that CasL is expressed not only in PB T cells but also in SF T cells. The protein amounts of CasL appeared to be slightly decreased in SF T cells (Figure 4A, lane 3). However, additional protein bands with the molecular mass of about 76 (Figure

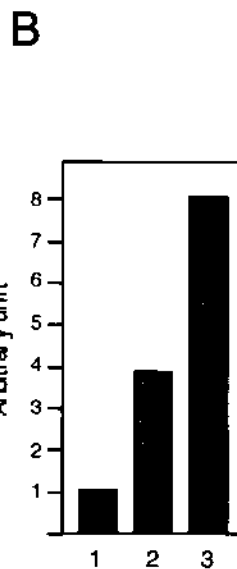
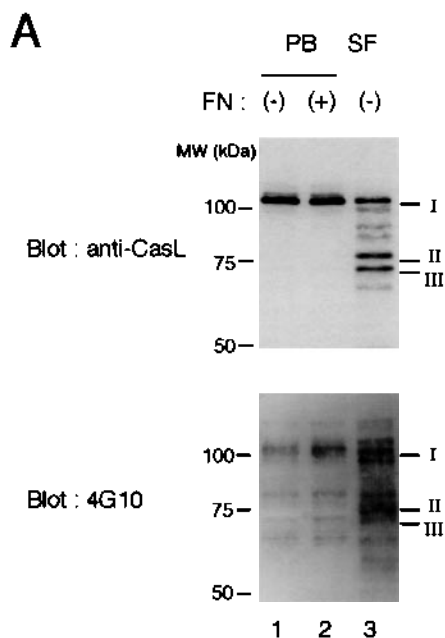


**Figure 3.** Effect of RANTES and stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) on migration of peripheral blood (PB) and synovial fluid (SF) T cells. Migrating cells of PB T cells from healthy subjects and patients with rheumatoid arthritis (RA), and SF T cells in control medium (open columns), media containing RANTES (100 ng/ml, shaded columns) and SDF-1 $\alpha$  (100 ng/ml, filled columns) were counted using flow cytometry. Values are the mean and SEM from 17 healthy subjects and 11 RA patients. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

4A, II) and 68 kDa (Figure 4A, III) were observed only in SF T cells. Blot with anti-p-Tyr Mab revealed that this assay could detect tyrosine phosphorylation of CasL, since stimulation of PB T cells with fibronectin increased intensity of CasL immunoreactivity against anti-p-Tyr Mab without significant alteration in protein levels of CasL (Figure 4A, lanes 1 and 2). Interestingly, not only 105 kDa but also 76 and 68 kDa bands reacted with anti-p-Tyr Mab in SF T cells, indicating these smaller protein bands are also CasL-related. Densitometric analysis demonstrated that, despite the apparent decrease in 105 kDa-protein amount in SF T cells, tyrosine phosphorylation per CasL molecule was markedly augmented in SF T cells when compared with that in fibronectin-stimulated PB T cells (Figure 4B). Similar results were obtained in the separate studies of 5 additional patients and we produced the same results in repeated experiments, suggesting that these phenomena may be characteristic in SF T cells.

**Induction of tyrosine phosphorylation of CasL by RANTES in H9 cells.** Although several signal transduction pathways

**Figure 4.** Tyrosine phosphorylation of Crk-associated substrate lymphocyte-type (CasL) in peripheral blood (PB) and synovial fluid (SF) T cells. A. PB T cells from healthy subjects were incubated in fibronectin-coated or non-coated dishes for 30 min at 37°C. Cellular lysates of non-stimulated PB T cells (lane 1), PB T cells stimulated with fibronectin (lane 2), non-stimulated SF T cells (lane 3) were run on 8% SDS-polyacrylamide gel and subjected to immunoblotting with anti-Cas monoclonal antibody (Mab) (upper panel), followed by anti-phosphotyrosine Mab 4G10 (lower panel). Molecular weights (MW) were determined according to the marker run in parallel. B. Densitometric analysis. Fold increase represents calculated values of 105 kDa protein after densitometric scanning of radiographic film in the lower panel compared with upper panel and the value of lane 1 arbitrarily serves as control. One representative experiment of 6 is shown.

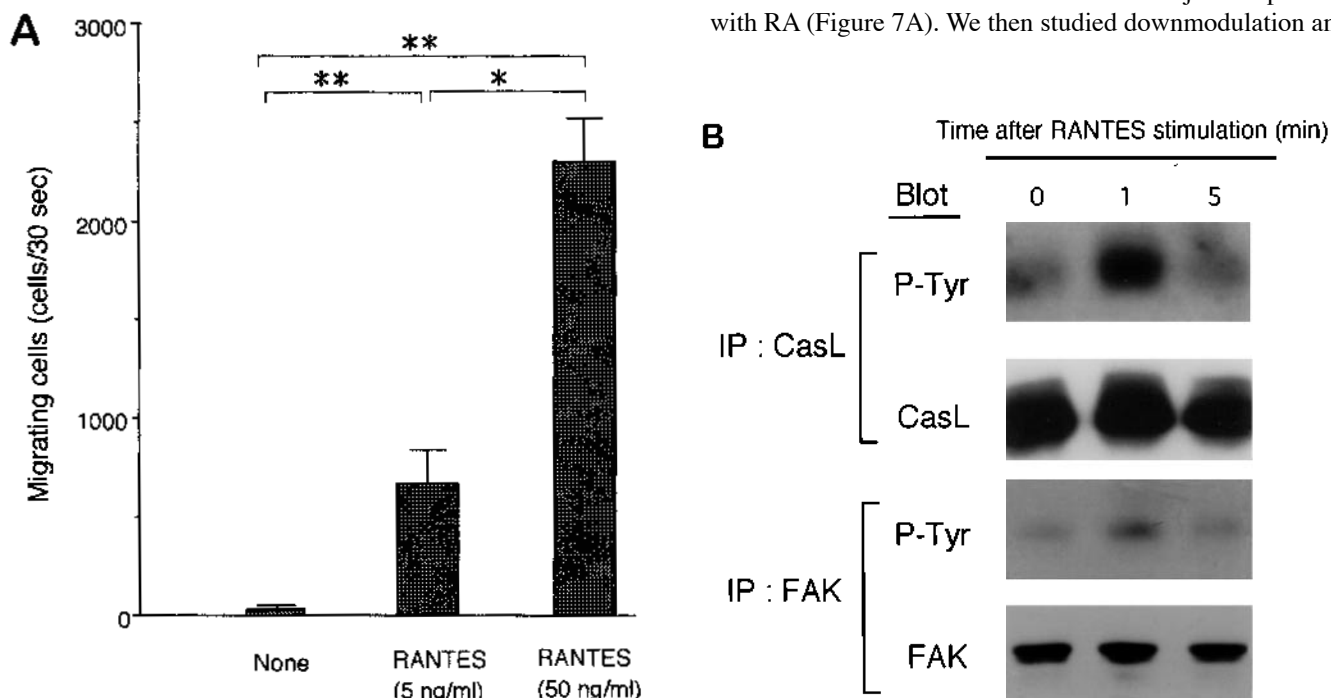


have been reported to converge onto tyrosine-phosphorylation of CasL, effect of RANTES on tyrosine-phosphorylation of CasL has not been evaluated. To test this, we assayed migratory activity and tyrosine phosphorylation of CasL and FAK after stimulation with RANTES in human T cell leukemia-derived H9 cells. We found that stimulation with RANTES significantly increased migration of H9 cells (Figure 5A). To examine protein levels and tyrosine phosphorylation of CasL and FAK, we immunoprecipitated whole cell lysates of H9 cells after stimulation with RANTES, and analyzed in immunoblot experiments. Figure 5B shows that protein levels of either CasL or FAK were unaffected before and after RANTES stimulation. In contrast, tyrosine phosphorylation of CasL and FAK was dramatically increased in 1 minute, but rapidly disappeared in about 5 minutes after stimulation with RANTES (Figure 5B). We, therefore, may suggest that T cells respond to RANTES through its interaction with CCR5 and show increased migratory activity, at least in part, via tyrosine phosphorylation of CasL and FAK. It should be noted that, as in the case of PB T cells, CasL-related 76 or 68 kDa bands were not observed in our experiments using H9 cells before or after stimulation with RANTES.

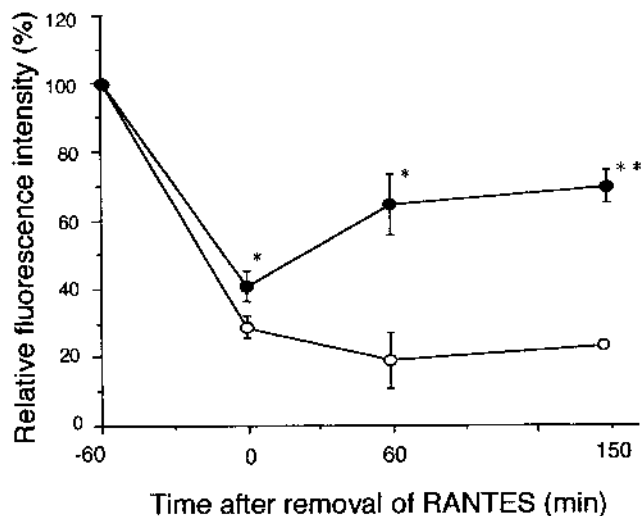
*Downmodulation and recycling of CCR5 in SF T cells from patients with RA.* Next, we addressed the discrepancy between increased expression of CCR5 and diminished

migratory response to the cognate ligand RANTES in SF T cells. For this purpose, we studied downmodulation and recycling of CCR5, both of which are also considered to be cellular responses to RANTES (see introduction). After PB and SF T cells were stimulated with RANTES, media were changed for deprivation of RANTES, and expression of CCR5 on T cell surface was analyzed at the indicated time points. As shown in Figure 6, stimulation with RANTES induced down modulation of CCR5 not only in PB T cells but also in SF T cells. However, the extent of downmodulation was significantly decreased in SF T cells. There was no difference in downmodulation of CCR5 between PB T cells from healthy subjects and those from patients with RA (data not shown). Moreover, receptor recycling appeared to be accelerated in SF T cells when compared with PB T cells (Figure 6).

*Pretreatment of PB T cells with IL-2 showed aberrant responsiveness to RANTES.* Finally, to clarify the mechanism for this aberrant responsiveness to RANTES in SF T cells, we focused on the effect of cytokines, since there is generally thought to be an abnormality in cytokine profile of Th1 type in SF from patients with RA<sup>20,21</sup>. Indeed, treatment of T cells with certain cytokines and chemokines has been shown to variably modulate signal transduction cascades in T cells<sup>33</sup>. We found that pretreatment with IL-2 increased expression of CCR5 in a CD45RO positive subpopulation of T cells isolated from PB of either control subjects or patients with RA (Figure 7A). We then studied downmodulation and



**Figure 5.** Effect of RANTES in H9 cells. **A.** Migration of H9 cells by RANTES. Migrating H9 cells to control medium and RANTES (5 ng/ml and 50 ng/ml) containing medium were counted by flow cytometry. Values are the mean and SEM of 5 experiments. \* $p < 0.05$ ; \*\*  $p < 0.01$ . **B.** Tyrosine phosphorylation of Crk-associated substrate lymphocyte-type (CasL) and focal adhesion kinase (FAK) in H9 cells by RANTES. H9 cells were stimulated for the indicated periods by RANTES (50 ng/ml). Cellular lysates of H9 cells were immunoprecipitated with anti-Cas monoclonal antibody (Mab) or anti-FAK Mab. The immune complexes were run on 8% SDS-polyacrylamide gel and subjected to serial immunoblotting with anti-Cas and anti-FAK Mab, followed by anti-phosphotyrosine Mab 4G10. One representative experiment of 3 is shown.



**Figure 6.** Downmodulation and recycling of CCR5 on the surface of peripheral blood (PB) T cells and synovial fluid (SF) T cells by RANTES. PB T cells from healthy subjects (open circles) and SF T cells (closed circles) were incubated for 1 h in RPMI 1640 with 0.6% BSA containing 1  $\mu$ g/ml RANTES at 37°C. After incubation, RANTES were removed by 3 washing steps with medium, and cells were further cultured in medium at 37°C for the indicated periods of time. Surface expression of CCR5 was detected by flow cytometry. Results are expressed as the percentage of relative fluorescence intensity before stimulation by RANTES, with SEM indicated. \* $p < 0.05$ ; \*\* $p < 0.01$ , compared to PB T cells at the corresponding time points. Values are the mean of 3 experiments.

recycling of CCR5 using these T cells. As shown in Figure 7B, we found that these IL-2-pretreated CD45RO positive T cells again showed reduced downmodulation and accelerated recycling of CCR5 after stimulation with RANTES when compared with non-treated CD45RO positive T cells. To determine the effect of IL-2 on chemotactic activity, a migration assay was performed using these IL-2-pretreated or TNF- $\alpha$ -pretreated CD45RO positive T cells. While TNF- $\alpha$ -pretreated T cells did not show increase in spontaneous migratory activity and preserved migratory response by RANTES, IL-2 pretreated T cells showed increase in spontaneous migratory activity, which was not enhanced after additional stimulation with RANTES (Figure 7C). Taken together, it is suggested that IL-2-pretreated T cells, not only phenotypically but also functionally, could mimic SF T cells.

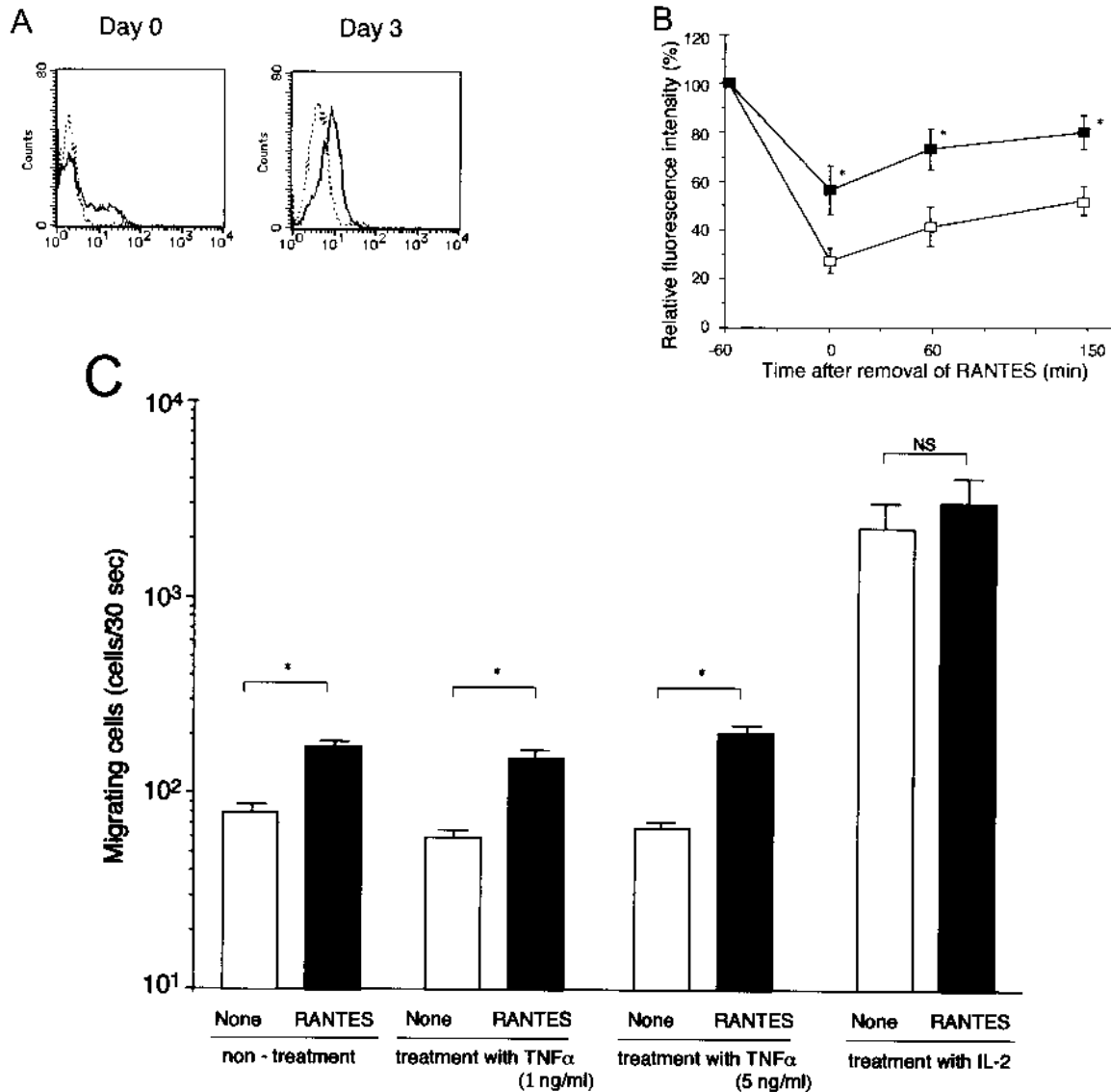
## DISCUSSION

We described that CCR5 expression is increased in SF T cells with its corresponding decrease in PB T cells. SF T cells also bear CD45RO, indicating that those T cells are considered as a memory subset. Since the concentrations of ligands for CCR5 are reported to be high in SF in patients with RA<sup>20</sup>, our results, together with previous reports<sup>16-20</sup>, may indicate that PB T cells are recruited to the inflammatory sites in the joints toward the gradient of chemokines, constituting a pool of memory T cells. Expression of CCR5

has been shown to be associated with polarization to Th1 phenotype<sup>17,34</sup>, suggesting that these SF T cells play an important role in pathogenesis of RA. We also showed increased expression of other chemokine receptors, for example, CXCR4, in SF T cells. Although the redundancy of chemokine signal transduction has been reported (see introduction), the pathological role of simultaneous expression of several chemokine receptors on SF T cells remains unclear.

It is believed that T cells trafficking to the inflammatory sites are regulated by a number of pathways including the interaction between adhesion molecules (e.g., VCAM1) and their receptors on T cells surface (e.g., VLA-4). Although it has already been shown that SF T cells show an increase in spontaneous chemotactic activity<sup>24,25</sup>, the role of chemokine-chemokine receptor system in SF T cells is poorly understood. We have reported that tyrosine phosphorylation of CasL is one of the essential components for T cells to achieve  $\beta$ 1-integrin-dependent migration<sup>28</sup>. In addition, we studied whether expression and tyrosine phosphorylation of CasL are associated with increase of spontaneous migratory activity in SF T cells from patients with RA. Although protein levels of CasL were not increased, tyrosine phosphorylation per molecule of CasL appeared to be augmented in SF T cells. Moreover, we found that additional CasL-related 78 kDa and 65 kDa proteins were also tyrosine phosphorylated. Those proteins were not detected in PB T cells from healthy subjects, patients with RA, or H9 cells. Although further studies are needed, it is interesting to speculate that alternative splicing and/or proteolysis may occur in SF T cells to produce these low molecular weight fragments of CasL. It has recently been reported that alternative splicing could conditionally occur, e.g., by cell stretch<sup>35</sup> and play an important role in controlling certain cellular processes. Alternatively, limited proteolysis might be also conditionally switched on for fine tuning of cellular function. In any case, we suggest that increased and/or aberrant tyrosine phosphorylation of CasL is involved in spontaneous augmentation of chemotaxis of SF T cells. It remains to be clarified, however, whether stimulation of CCR5 with its ligand could transduce signals down to CasL. Our studies with H9 cells clearly show that stimulation with RANTES induces tyrosine phosphorylation of not only FAK but also CasL. Together with the report showing that CCR5 colocalizes with FAK<sup>36</sup>, it is strongly suggested that CasL in SF T cells is tyrosine phosphorylated, at least in part, via continuous RANTES-CCR5 signaling, resulting in increase of chemotactic activity.

Unexpectedly, response to stimulation with those ligands for CCR5 (RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ ) was blunted in migration assay. These results are extremely paradoxical since SF T cells have increased expression of CCR5 and may migrate through chemokine-chemokine receptor interaction<sup>16-20</sup>. In addition to chemotaxis, RANTES evokes



**Figure 7.** IL-2 modulates CCR5 expression and migratory activity in CD45RO positive T cells. **A.** IL-2 up regulates CCR5 expression on CD45RO positive T cells. CD45RO positive cells were maintained in the media containing 10 ng/ml of IL-2 for 3 days. In each plot, the solid profile represents staining with the anti-CCR5 monoclonal antibody (Mab), and the dotted profile staining with an isotype matched control Mab. **B.** Downmodulation and recycling of CCR5 on freshly isolated CD45RO positive cells (open squares), and CD45RO positive cells cultured with IL-2 (closed squares) after treatment with RANTES. Freshly isolated CD45RO positive cells and CD45RO positive cells cultured with IL-2 for 3 days were incubated for 1 h in RPMI 1640 with 0.6% BSA containing 1  $\mu$ g/ml RANTES at 37°C. After incubation, RANTES were removed, and cells were further cultured at 37°C for various periods of time and surface expression of CCR5 was detected by flow cytometry. Results are expressed as the percentage of relative fluorescence intensity before stimulation by RANTES, with SEM indicated. \* $p < 0.05$  compared to freshly isolated CD45RO positive cells at the corresponding time periods. Values are the mean of 3 experiments. **C.** Effect of pretreatment with IL-2 or TNF- $\alpha$  on migration of CD45RO positive cells. Migrating CD45RO positive cells cultured in the medium containing 10 ng/ml of IL-2, 1 ng/ml or 5 ng/ml of TNF- $\alpha$  for 3 days to control medium (open columns) and RANTES (100 ng/ml, shaded columns) were counted using flow cytometry. Values are the mean and SEM of 3 experiments. \* $p < 0.05$ .

various cellular responses including internalization and recycling of CCR5 in T cells. We found that, despite deficit in RANTES-triggered migratory response, SF T cells partially demonstrated the other responses similar to PB T

cells. We thus considered that not only expression of but also downstream signals from CCR5 were differentially modulated in T cells after docking to the inflammatory sites, i.e., joints. Decreased rate of internalization and accelerated



recycling of CCR5 may support this notion. Moreover, sustained elevation of CCR5 expression in SF T cells might, at least in part, be due to this aberrant receptor recycling. It has already been shown that differential regulation of downstream signals from CCR5 is accomplished between internalization and migration<sup>13</sup>. We can now consider a number of possibilities to account for the observed aberrant responsiveness to RANTES. It should be noted that T cell migration is controlled by mechanisms other than chemokine-chemokine receptor signaling. If downstream signals from CCR5 converge with other CCR5-unrelated signaling pathways onto migration machinery, so called heterologous desensitization could occur. Heterologous desensitization is characterized by loss of receptor function after phosphorylation induced by second messenger-activated kinases, as a result of activation of different receptors or signaling processes<sup>37</sup>. This phenomenon is best described for G protein coupled receptors and functional loss of CCR5 by IL-16 without modulating CCR5 expression has already been reported<sup>33</sup>. On the other hand, since chemokine-induced cell migration is a complex process, mediated by multiple signaling mechanisms, signal transduction could be modulated in a disease-related manner. In fact, dysregulation of G-protein-related kinases (GRK) have also been documented in PBMC from patients with RA<sup>38</sup> and adjuvant arthritis mice<sup>39</sup>, and it is suggested that inflammation induces a tissue-specific down regulation of GRK<sup>39</sup>. Given that GRK2 as well as GRK3 are generally thought to determine CCR5 internalization and its sensitivity to ligand<sup>40,41</sup>, evaluation of GRK might be useful to determine an underlying mechanism for aberrant responsiveness to RANTES in SF T cells. Further studies, however, are clearly needed to identify molecular mechanism of aberrant responsiveness to RANTES in SF T cells from patients with RA.

Various humoral factors have been shown to modulate the expression of chemokine receptors on both monocytes and lymphocytes<sup>16,32,42-44</sup>; expression of CCR5 is induced by IL-2 in cultured lymphocytes<sup>16,32,44</sup>, and by interferon- $\gamma$  in monocytes and macrophages<sup>43</sup>. Moreover, it is also known that cytokines variably influence cellular signal transduction pathways as well<sup>33</sup>. We, therefore, searched such factors that affect responsiveness to RANTES in SF T cells and found that treatment of PB T cells with IL-2 resulted in aberration in RANTES-CCR5 signaling as well as elevated expression of CCR5, while treatment of PB T cells with TNF- $\alpha$  only did not result in characteristics of SF T cells. Although a pathological role of IL-2 in RA remains controversial<sup>45-48</sup> and involvement of other factors cannot be excluded, we may postulate that, during migration from peripheral circulation to synovial membrane, PB T cells are conditioned by IL-2 so that RANTES-CCR5 signaling pathway appears to be modulated as observed in SF T cells. In any case, our results strongly argue that expression of CCR5 and its downstream signal transduction could be modulated *in situ*

by at least certain mediator(s) of immunological and/or inflammatory reactions.

Finally, what is the pathological significance of increased expression and this aberrant responsiveness to RANTES of CCR5 in SF T cells? Despite an activated phenotype, SF T cells have reduced proliferative responses and cytokine production after mitogenic stimulation<sup>49</sup>. On the other hand, we and others showed that expression of CXCR4 is increased<sup>50</sup> and stimulation with its cognate ligand SDF-1 significantly induces migration even in SF T cells. Tilton, *et al* have reported that signal downstream of SDF-1 $\alpha$ -CXCR4 involves prolonged protein kinase B and extracellular signal-regulated kinase 2 activation, both of which are independent from RANTES-CCR5 signaling<sup>51</sup>. It is therefore possible that not RANTES-CCR5 system but SDF-1 $\alpha$ -CXCR4 system operates in terms of chemokine-dependent migration of T cells in SF, together with the report that SDF-1 $\alpha$  is expressed in the synovial membrane in patients with RA<sup>52</sup>. Collectively, it can be suggested that PB T cells enter into SF along with the gradient of ligands for CCR5; they do not respond to additional exposure by these ligands but constitute a reservoir of Th1-deviated memory T cells, thereafter recruited to the ongoing inflammatory sites in an SDF-1 $\alpha$ -dependent manner. Such spatially-defined usage of distinct chemokine signalings may contribute to ordered responsiveness of pathogenic T cells in certain disorders including RA. On the other hand, we should also keep in mind the possibility that CCR5 may have as yet an unidentified function. For example, it has recently been shown that in astrocytes RANTES-CCR5 signaling serves as an important regulator of cell survival and growth<sup>53</sup>. Further identification of the function of CCR5 in patients with RA, therefore, would contribute to understanding the pathogenesis and development of novel therapeutic approach of RA.

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