Selective accumulation of CCR5\(^+\) T lymphocytes into inflamed joints of rheumatoid arthritis

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Abstract
Chemokines and their receptors play critical roles in the selective recruitment of various subsets of leukocytes. Recent studies have indicated that some chemokine receptors are differentially expressed on T\(_{h1}\) and T\(_{h2}\) cells. However, available data concerning the presence of T cells with a T\(_{h1}\) or a T\(_{h2}\) character and the expression of chemokine receptors on infiltrating T cells in the rheumatic joint are still limited. In this study, we investigated the expression of CC chemokine receptor 4 (CCR4) and CCR5, which have been shown to be preferentially expressed on T\(_{h2}\) and T\(_{h1}\) respectively in T cells from rheumatoid arthritis (RA) patients. Although both CCR5\(^+\) and CCR4\(^+\) CD4\(^+\) T cell populations were observed in peripheral blood mononuclear cells from healthy controls and osteoarthritis patients, these cell populations were decreased in patients with active RA. In contrast, the vast majority of synovial fluid (SF) T cells from active RA patients expressed CCR5 but not CCR4. CCR5 ligands, MIP-1\(\alpha\) and RANTES, were found in RA SF at high levels. CCR5\(^+\) CD4\(^+\) T cells from SF mononuclear cells of RA patients produced IFN-\(\gamma\) but not IL-4 in response to anti-CD3 stimulation in vitro. These results indicated that differential expression of chemokine receptors plays a critical role for selective recruitment of pro-inflammatory T cells into the joints of RA.

Introduction
Rheumatoid arthritis (RA) is an autoimmune disease characterized by the infiltration of various leukocyte subpopulations into both the developing pannus and synovial space. The chronic nature of this disease results in multiple joint inflammation with subsequent destruction of cartilage and joint. The etiologic cause of RA has not been clearly understood, but T cell-mediated autoimmune responses are considered to play a crucial role in the pathogenesis of RA (1). In fact, the importance of infiltrating T cells in synovitis has recently been underscored by the demonstration that synovial T cells from RA patients could cause inflammatory arthritis in severe combined immunodeficiency mice (2).

Differential cytokine production allows the subdivision of mouse and human CD4\(^+\) (and CD8\(^+\)) T lymphocytes into two major subsets: T\(_{h1}\) (T\(_{c1}\)) cells that secret IL-2, lymphotoxin and IFN-\(\gamma\) but not IL-4, IL-5, IL-10 or IL-13; and T\(_{h2}\) (T\(_{c2}\)) cells that secret the opposite set of cytokines (3,4). Development of the appropriate T\(_h\) subset during immune response is physiologically important because certain pathogens are most effectively controlled by either a cellular (T\(_{h1}\)) or a humoral (T\(_{h2}\)) immune response. In some autoimmune diseases, dominance of either T\(_h\) subsets can exacerbate the disease (5,6). Accumulating evidence supports the concept that a T\(_{h1}\)-type response is associated with the pathogenesis of RA (7). In animal models of RA, T\(_{h1}\) cells appear to play pro-inflammatory roles, while T\(_{h2}\) cells may play an anti-inflammatory role (8,9). However, it is still unclear how these T lymphocytes with a T\(_{h1}\) or a T\(_{h2}\) character are recruited into the rheumatic joint. Recent observations indicated that activated T lymphocytes acquire different migrating capacities...
have indicated that Th1 and Th2 cells differentially responded.

The control of T cell migration depends on the combined actions of various adhesion molecules and a vast array of chemotactic factors (chemokines). The role of adhesion molecules in T cell migration is well characterized (11,12), but that of chemokines and their receptors is still unclear. The chemokine receptors comprise two major groups; the CC chemokine receptors 1–8 (CCR1–8) that bind CC chemokines and the CXC chemokine receptors 1–4 (CXCR1–4) that bind CXC chemokines (13–15). In general, the CC chemokines and their receptors are involved in the migration of monocytes, eosinophils, basophils and T cells, while CXCR1 and CXCR2 are involved in the migration of neutrophils. The chemokines produced at the sites of inflammation are likely to play a major role in the recruitment of particular cell types that infiltrate and participate in the pathological lesions. Presumably chemokines and their receptors are also important for the selective migration of particular T cell subsets. Recent reports have indicated that T11 and T12 cells differentially responded to different chemokines (16) and expressed different chemokine receptors. CXCR3 and CCR5 were preferentially expressed in human T1, cells (17–19), and CCR3 and CCR4 were preferentially expressed in T2 cells (20–22). These observations suggest that the differential expression of chemokine receptors may be useful for discriminating pathogenic T cells. In fact, it has recently been reported that CCR1 T cells were found in the synovial tissue of RA patients (17,19). However, these studies contained only limited numbers of patients. Furthermore, available data concerning the presence of T cells with a T1 or T2 character and the expression of chemokine receptors on T cells in the rheumatoid joint are still limited.

In this study, we examined the expression of CCR4 and CCR5 on T cells from either peripheral blood (PB) or synovial fluid (SF) of larger numbers of RA patients to generalize the finding. Our results show that CCR5 T cells, but not CCR4 T cells, were selectively accumulated in the inflamed joints of RA, while both of these cell populations were decreased within PB. The ligands of CCR5, macrophage inflammatory protein (MIP)-1α and regulated on activation, normal T cell expressed and secreted (RANTES), were found at high levels in SF of RA, suggesting that CCR5 T cells were selectively accumulated in response to these chemokines. CCR5 T cells from inflamed joints of RA had a typical T1 character that produced IFN-γ but not IL-4. These results support the concept that pro-inflammatory T1 cells are selectively recruited into inflamed tissues by differential expression of chemokine receptors.

**Methods**

**Patients**

Patients with RA were diagnosed according to the revised criteria of the American College of Rheumatology (23). Most patients were receiving disease modifying anti-therapeutic drugs, but none were receiving prednisolone at ≥5 mg/day. RA was in a chronic phase (disease duration ≥2 years) and active (number of swollen joints ≥4, CRP ≥2.0 mg/dl, erythrocyte sedimentation rate >40 mm/hr) in all of the RA patients studied. Flow cytometric analysis for PB mononuclear cells (PBMC) was performed in 21 RA patients, 15 osteoarthritis (OA) patients and 12 healthy donors as controls. Paired PBMC and SF mononuclear cells (SMC) were isolated from 10 RA patients and six OA patients. The age of RA patients ranged from 30 to 76 years, with a mean (± SD) of 56 ± 11 years old, and that of controls or OA patients was 50 ± 10 or 58 ± 10 years old respectively. At the time of blood drawing, an aliquot was subjected to whole blood leukocyte count and hemogram on a Micro Diff 18 cell counter (Coulter, Hialeah, FL) in our central blood test laboratory. PBMC and SFMC were isolated by Ficoll-Hypaque gradient centrifugation within 3 h after sample drawing.

**mAb**

FITC- or phycoerythrin (PE)-conjugated mAb against the following antigens were obtained from PharMingen (San Diego, CA); CD4 (RPA-T4, mouse IgG1), CD8 (RPA-T8, mouse IgG1), CD14 (M5E2, mouse IgG2a), CD20 (2H7, mouse IgG2b), CCR5 (2D7, mouse IgG2a), IL-4 (MP4-25D2, mouse IgG1), and IFN-γ (B27, mouse IgG1). An anti-human CCR4 mAb (KM2160, mouse IgG1) has been established and characterized as described previously (22), and was conjugated with FITC by a standard technique. Isotype-matched control mAb were also obtained from PharMingen (mouse IgG1, 107.3; IgG2a, G155-178; IgG2b, 49.2).

**Immunofluorescence and flow cytometry**

Immunofluorescent staining for surface markers, flow cytometry and data analysis were performed as described previously (24). Cytoplasmic staining for IFN-γ and IL-4 was performed as described by Openshaw et al. (25). Briefly, cells were incubated with Brefeldin A (10 µg/ml; Epicentre Technologies, Madison, WI) for the last 6 h of culture. The harvested cells were fixed with 2.5% paraformaldehyde in PBS for 20 min, and then washed with PBS containing 1% BSA and 0.5% saponin (S-7900, Sigma). After washing twice, the cells were incubated with either PE-conjugated anti-IL-4 or anti-IFN-γ mAb for 30 min and then washed twice with 1% BSA/PBS without saponin. Samples were analyzed on a FACSscan (Becton Dickinson, San Jose, CA).

**Cell isolation and stimulation**

CCR5 CD4 T cells were isolated by using the MACS anti-FITC MultiSort Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). At first, CCR5 T cells were isolated from SFMC using FITC-conjugated anti-CCR5 mAb for the positive selection. After release of the magnetic label, second positive selection was performed with CD4 MicroBeads according to the manufacturer’s instruction. The isolated cells were >90% CCR5 CD4 as analyzed by flow cytometry. Isolated CCR5 CD4 T cells were stimulated with or without immobilized anti-CD3 mAb (OKT3, 5 µg/ml; ATCC, Rockville, MD) in 24-well flat-bottomed microplates in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and antibiotics. After 24 h, the cells were harvested and analyzed for intracellular cytokines.
Accumulation of CCR5$^+$ T cell in RA joints

Fig. 1. Expression of CCR4 and CCR5 on peripheral blood CD4$^+$ or CD8$^+$ T lymphocytes from normal controls, OA patients and RA patients. PBMC from healthy adults, OA patients and RA patients were stained with FITC-conjugated anti-CCR4 (a) or anti-CCR5 (b) mAb and PE-conjugated anti-CD4 or anti-CD8 mAb. Samples were analyzed by two-color flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as dot-plots (four-decade scale) and quadrant markers were positioned to include >98% of control Ig-stained cells in the lower left. Representative FACS profiles are shown.

Quantitation of MIP-1α, RANTES and IFN-γ

Cytokine contents in the SF were measured by using specific ELISA kits for MIP-1α and RANTES (R & D Systems, Minneapolis, MN) or those for IL-4 and IFN-γ (BioSource Europe, Fleurus, Belgium) according to the manufacturer’s instructions. Each recombinant cytokine was used as a standard.

Statistical analysis

Mann-Whitney rank-sum test was performed to determine the statistical significance. $P < 0.05$ was considered significant.

Results

Selective decrease of CCR5$^+$ and CCR4$^+$ T cells in PB lymphocytes from RA patients

We first examined the surface expression of CCR5 and CCR4, which have been reported to be preferentially expressed on T$_{h}1$ and T$_{h}2$ cells respectively on PB T cells from RA patients. Two-color immuno-fluorescence analysis of PB lymphocytes from 21 RA patients, 15 OA patients and 12 healthy controls was performed to examine the CCR5 and CCR4 expression on CD4$^+$ and CD8$^+$ T cell subsets. As represented in Fig. 1(a), CCR4 was expressed on a substantial part of CD4$^+$ T cells in both normal controls and OA patients, but this population was decreased in RA patients. As represented in Fig. 1(b), CCR5 was expressed on substantial parts of both CD4$^+$ and CD8$^+$ T cells in normal controls and OA patients, but these populations were largely lost in RA patients. Mean percentages of CCR4$^+$ or CCR5$^+$ cells within CD4$^+$ or CD8$^+$ T cells are summarized in Fig. 2. This clearly indicated that both CCR4$^+$ and CCR5$^+$ T cells were generally decreased in PB of RA patients.

Accumulation of CCR5$^+$ T cells in SF of RA patients

The presence of many infiltrating leukocytes in inflamed joints is a characteristic of RA. We then examined the expression of CCR4 and CCR5 on T cells from SF of RA patients. As represented in Fig. 3, major populations of either CD4$^+$ or CD8$^+$ T cells from SF expressed CCR5. We evaluated paired PBMC and SFMC from 10 RA patients and the results are summarized in Fig. 4. Approximately 68% of CD4$^+$ T cells and 87% of CD8$^+$ T cells in SF expressed CCR5, suggesting a preferential accumulation of CCR5$^+$ T cells in SFMC as compared to PBMC. In contrast, no significant accumulation of CCR4$^+$ T cells was observed in SFMC as compared to PBMC. We also tried to examine SFMC from OA patients but...
Accumulation of CCR5⁺ T cell in RA joints

Fig. 4. Accumulation of CCR5⁺ T cells in SFMC from RA patients. Mean percentages of CCR4⁺ or CCR5⁺ cells within CD4⁺ or CD8⁺ T cells in PBMC or SFMC from 10 RA patients are shown as described in Fig. 2. Bars show the mean ± SEM. **P < 0.01 compared with PBMC.

Fig. 5. Increase of MIP-1α and RANTES in SF from RA patients. Concentrations of MIP-1α and RANTES in SF from patients with RA (n = 15) or OA (n = 10) were determined by ELISA. Bars show the mean ± SEM. Horizontal dashed line represents the limit of detection. ***P < 0.001 compared with SF from OA patients.

The majority was CD14⁺ monocytes and we could not evaluate the T cells in OA samples conclusively.

Presence of CCR5 ligands in SF of RA patients

Previously, increased levels of several chemokines were found in inflamed joints of RA patients (26). We next verified whether the ligands for CCR5 were indeed increased in inflamed joints of RA. Since MIP-1α, MIP-1β and RANTES have been shown to be the ligands of CCR5, we evaluated the contents of MIP-1α and RANTES in SF from either RA or OA patients. As shown in Fig. 5, concentrations of MIP-1α and RANTES were much higher in RA SF than those in OA SF. These results indicated that CCR5⁺ T cells might be accumulated in response to MIP-1α and RANTES produced in the RA joint.

Production of IFN-γ by accumulated CCR5⁺ T cells in SF of RA patients

We finally verified whether the accumulated CCR5⁺ T cells in SF from RA patients did have a Th1-like character. In this context, we first examined concentrations of IFN-γ and IL-4 in SF from RA or OA patients by ELISA. As shown in Fig. 6(a), significant levels of IFN-γ were detected in RA SF but not in OA SF. IL-4 was not detectable in either RA or OA SF (data not shown). This suggests that a Th1-type immune response occurred in rheumatoid inflamed joints. We next examined the production of IFN-γ by CCR5⁺ T cells by intracellular staining. CCR5⁺ CD4⁺ T cells were isolated from SFMC and stimulated with immobilized anti-CD3 mAb for 24 h, fixed and permeabilized cells were stained with PE-conjugated anti-IFN-γ or anti-IL-4 mAb (closed histogram). PE-conjugated isotype control was included as a control (open histogram). The data are representative of three experiments using three individual RA samples with similar results.

Discussion

Preferential expression of CCR5 on human Th1 cells and the increased CCR5⁺ T cells in RA joints have recently been reported (17–20). However, these studies contained only limited numbers of patients (17,19). In the present report, we studied a larger number of patients to generalize the finding. In addition, previous studies have not compared PB samples from RA to those from OA and normal individuals. Furthermore, the expression of CCR4 on T cells from RA patients has not been investigated in previous studies. We demonstrated in this report that CCR5⁺ T cells but not CCR4⁺ T cells are selectively accumulated into inflamed joints of RA patients where the CCR5 ligands such as MIP-1α and RANTES are abundantly produced. In contrast, we found that both CCR5⁺ T cells and CCR4⁺ T cells were decreased in PB from RA patients as compared with PB from OA patients and normal individuals. Furthermore, our results also showed that CCR5⁺
CD4+ T cells in RA SFMC were indeed T1,1 cells producing IFN-γ but not IL-4.

The contribution of T cells to the pathogenesis of RA was recently brought into question by the disappointing results of a clinical trial using anti-CD4 mAb for the treatment of RA (27). However, others have demonstrated that such depleting anti-CD4 mAb might selectively eliminate naive and T2,2-type CD4+ T cells (28,29). T1,1-type immune responses have been suggested to be important for the pathogenesis of organ-specific autoimmune diseases such as multiple sclerosis, insulin-dependent diabetes mellitus and RA (6). Infiltration of pathogenic T cells of T1,1 phenotype into target organs is a characteristic of these diseases. Dolhain et al. demonstrated that the mean percentage of T1,1 cells was significantly increased in SFMC compared with PBMC (30), which supports the notion that T1,1 cells specifically migrated into the inflamed joint of RA patients. The importance of T cells in synovitis has recently been underscored by Mima et al. who demonstrated that T cells isolated from either SF or synovial tissues of RA patients could cause inflammatory arthritis in severe combined immunodeficiency mice (2). Therefore, while the contribution of infiltrating T cells to the pathogenesis of RA is unquestionable, it is not fully understood how these pathogenic T cells, particularly of the T1,1 type, are recruited to the inflamed joint of RA. It has been shown that T1,1 cells, but not T2,2 cells, express a functional ligand for P- and E-selectin, and therefore are selectively recruited to the inflammatory sites where P- and E-selectins are expressed on the vascular endothelium (31,32). Although the selectins are efficient mediators initiating leukocyte–endothelial cell interaction, chemokines play a critical role in subsequent adhesion and transendothelial migration of leukocytes by activating integrins (11). Recently, differential responsiveness of T1,1 and T2,2 cells to chemokines was reported by Siveke et al. (16). They demonstrated that CC chemokines MIP-1α, MIP-1β and RANTES were efficient chemoattractants for T1,1 cells but not for T2,2 cells. Our present results demonstrated that substantial levels of MIP-1α and RANTES were produced in inflamed joints of RA but not OA patients, suggesting that T1,1 cells could be recruited in response to these chemokines. Consistent with this notion, T1,1-type cells expressing CCR5, but not T2,2-type cells expressing CCR4, were accumulated in the inflamed joints of RA.

A characteristic feature of chemokines is a complexity in ligand and receptor binding. In some instance, RANTES has been reported to be a ligand for not only CCR5 but also for CCR3 (12,13), which has been recently demonstrated to be selectively expressed on human T2,2 cells (21). However, the specific CCR3 ligand is eotaxin rather than RANTES. It has been reported that only half of IL-4-producing T2,2 cells expressed CCR3 among PB T cells and some IFN-γ-producing cells also expressed CCR3. It should be also noted that <1% of PB T cells from healthy individuals expressed CCR3 (21). Since our present study demonstrated that relatively higher percentages of PB T cells express CCR4 and CCR5, it is plausible that CCR5+ T cells predominate over CCR3+ T cells in response to RANTES. Another possible explanation is that CCR3+ T cells that co-express CCR5 may respond to RANTES. Recent papers demonstrated that production of RANTES was regulated by T1,1 cytokines (33) and neutralization of RANTES could ameliorate adjuvant arthritis which has been known to be mediated by the T1,1 response (34). Therefore, RANTES appears to contribute to T1,1 immune responses rather than T2,2. However, some IL-4-producing T1,2 cells in Fig. 6 might express CCR3 that might accumulate in response to RANTES. Further studies will be needed to address these possibilities.

Recent observations indicated that CCR5 and CXCR3 were preferentially expressed on T1,1 cells, while CCR4 and CCR3 were preferentially expressed on T2,2 cells (17-19,21,22). We demonstrated here that CCR5+ T cells were selectively accumulated in the inflamed joints of RA while CCR4+ T cells did not. In contrast, both CCR5+ and CCR4+ T cells were decreased in PBMC from RA patients as compared to PBMC from normal controls and OA patients. The decrease of CCR5+ T cells in PBMC from RA patients may be simply explained by the preferential accumulation of CCR5+ T cells in the inflamed joint of RA patients. In contrast, the decrease of CCR4+ T cells in PBMC from RA patients cannot be simply explained since no significant accumulation of CCR4+ T cells was observed in SFMC of RA patients. The precise mechanism for the decrease in CCR4+ T cells in PB from RA patients remains unclear. However, recent observations suggested that organ-specific autoimmune disease might be provoked by systemic immune deviation (35). It is well known that vigorous T1,1-type immune responses lead to inhibition of T2,2 cells. Therefore, the observed decrease of CCR4+ T cells in PBMC from RA patients may represent the systemic T1,1 deviation of RA patients. Alternatively, it is also possible that CCR4+ T cells might selectively accumulate in synovial tissue but not in SF. To address this possibility, we are now examining the expression of CCR4 and its ligands (TARC and MDC) in RA synovial tissue.

Although CCR5 has been shown to be a T1,1 marker (17,18), Sallusto et al. demonstrated that CCR5 was expressed not only on T1,1 cells but also on T2,2 cells (20). This discrepancy is not surprising since Loetscher et al. reported that some T2,2 clones expressed CCR5 despite at low frequency (17). Therefore, in a certain condition, T2,2 cells may have the ability to express CCR5. However, our present results indicated that the vast majority of CCR5+ CD4+ T cells in SFMC were IFN-γ-producing cells. We could detect only a low frequency of IL-4-producing cells in this cell population, indicating that CCR5 is a useful marker for T1,1 cells in clinical samples.

At present, accumulating evidence supports the concept that a T cell response with either a T1,1 or T2,2 character is associated with different manifestations of disease and therapies that induce a shift of the immune response from T1,1 to T2,2 may result in the amelioration of diseases (36-38). From this point of view, chemokines and chemokine receptors that influence the recruitment of T1,1 cells will be a potential target of new strategies for the treatment of autoimmune diseases. In fact, recent reports demonstrated that the development of T1,1-mediated experimental autoimmune diseases was efficiently inhibited by anti-RANTES or anti-MIP-1α mAb (34,39). However, when one chemokine is inactivated, other chemokines may compensate for its function since different chemokines bind the same receptor.
Therefore, our results imply that CCR5 will be a good target for the treatment of RA.

In conclusion, we demonstrated here the preferential accumulation of CCR5+ T cells producing IFN-γ in inflamed joints of RA patients. This supports the concept that Th1 cells are involved in the inflammation and destruction of RA joints. In addition, our results indicated that differential expression of chemokine receptors plays a critical role in selective recruitment of pro-inflammatory Th1 cells into the joints of active RA.

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Abbreviation

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
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<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<td>MC</td>
<td>mononuclear cell</td>
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<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
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<td>OA</td>
<td>osteoarthritis</td>
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<td>PB</td>
<td>peripheral blood</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RANTES</td>
<td>regulated on activation, normal T cell expressed and secreted</td>
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<td>SF</td>
<td>synovial fluid</td>
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References

Accumulation of CCR5$^+$ T cell in RA joints


