ABSTRACT. Objective. To characterize cytokine and chemokine receptor profiles of T cells and monocytes in inflamed synovium and peripheral blood (PB) in patients with rheumatoid arthritis (RA) and other arthritides.

Methods. We studied PB and synovial fluid (SF) samples taken from 20 patients with RA and 9 patients with other arthritides. PB cells from 8 healthy adults were used as controls. CCR3, CCR5, and intracellular interferon-γ (IFN-γ) and interleukin 4 (IL-4) expression in CD8+ and CD8− T cell populations and in CD14+ cells were determined with flow cytometry.

Results. Expression of CCR5 and CCR3 by CD8−, CD8+ T cells and CD14+ monocytes was increased in SF compared to PB cells in patients with RA and other arthritides. The number of CD8+ T cells spontaneously expressing IL-4 and IFN-γ was higher in SF than in PB in RA patients. Spontaneous CCR5 expression was associated with intracellular IFN-γ expression in CD8+ T cells derived from SF in RA. In CD8− T cells the ratio of CCR5+/CCR3+ cells was increased in patients with RA compared to patients with other arthritides. The number of PB CD8− T cells expressing IFN-γ after mitogen stimulation was higher in controls than in patients. In PB monocytes the ratio of CCR5+/CCR3+ cells was increased in patients with RA compared to patients with other arthritides and controls.

Conclusion. T cells and monocytes infiltrating joints in RA and in other arthritides showed increased activation of both type 1 and type 2 immune markers. More pronounced type 1 immune response in joints, shown as an increased CCR5/CCR3 ratio, was found in the patients with RA versus those with other arthritides. Monocytes but not T cells in PB showed increased activation in RA. (J Rheumatol 2003;30:1928–34)

Key Indexing Terms: CHEMOKINE RECEPTORS RHEUMATOID ARTHRITIS CYTOKINES SYNOVIAL FLUID
Mononuclear cells were isolated from 1:2 diluted PB or SF. CCR3 and CCR5 antibodies were purchased from R&D, Abingdon, UK. IL-4 antibodies and isotype control and leukogate were purchased from Becton Dickinson. Cytokines are inducers of chemokine receptor ligands, chemokines. For example, CCL3, CCL4, and CCL5, which are ligands for CCR5, are induced by IFN-γ in association with tumor necrosis factor (TNF)10-13. CCL11 and CCL22, the ligands for CCR3 and CCR4, are induced by IL-4.14 Chemokines in turn activate the G-protein coupled signaling cascade as they bind to their receptors and in this way upregulate the expression of chemokine receptors on the cell surface.15

Chemokines and chemokine receptors are the key mediators of leukocyte recruitment to the sites of inflammation. The different migratory properties of type 1 and type 2 cells are related to the expression of different chemokine receptors that are regulated during the cell differentiation and activation process.9,17

Studies of the chemokine receptors in synovial inflammatory cells, and especially their relation to cytokines secreted by lymphocytes, might elucidate the role of type 1 and 2 immune response in the pathogenesis of RA. We studied the expression of CCR3 and CCR5 on CD8+ and CD8− T lymphocytes and on CD14 cells (monocytes) in SF and peripheral blood (PB) from patients with RA and from patients with other arthritides. We also analyzed the relation between CCR3 and CCR5 expression and intracellular cytokine expression (IL-4 and IFN-γ) in these cell populations.

MATERIALS AND METHODS

Patients. The study included 20 patients with RA (16 women, 4 men, mean age 54.5, range 29–69 yrs) who presented with acute swollen knee. Except for one, all the patients had chronic RA with mean disease duration of 13 years (range 0–43). They all fulfilled the 1987 criteria for RA. Among patients, 85% were seropositive and 90% had erosions. Five patients were not taking disease modifying antirheumatic drugs (DMARD) at the time of the study. The others were receiving methotrexate (MTX) either as a single drug (6 patients) or in combinations (5 patients, including one with infliximab), one was treated with leflunomide and infliximab, and 2 patients were receiving sulfasalazine (one as single DMARD, one in combination with auranofin). Low dose prednisone was used by 9 patients. As controls, we included 9 patients with other arthritides who also presented with acute synovitis in the knee (2 women, 7 men, mean age 44, range 31–64 yrs). Three patients had chronic reactive arthritis, 2 had spondyloarthritis, 2 had ankylosing spondylitis, and one had acute gout. Two patients (22%) were seropositive and 4 (44%) had erosions. Four patients were receiving sulfasalazine and 2 MTX. Low dose prednisone was used by 3 patients. From each patient, 20 ml PB and 5–20 ml SF was collected into EDTA tubes (Venoject, Tenumo, Leuven, Belgium).

The study was approved by the local ethics committee. All patients gave written informed consent.

Controls. PB samples from 8 age matched healthy adults were used as controls.

Materials. PerCP conjugated anti-human CD3, FITC conjugate anti-human CD8 and CD14, and phycoerythrin (PE) conjugated anti-human IFN-γ and IL-4 antibodies and isotype control and leukogate were purchased from Becton Dickinson, Erembodegem, Belgium. PE conjugated anti-human CCR3 and CCR5 antibodies were purchased from R&D, Abingdon, UK.

Cell extraction. Mononuclear cells were isolated from 1:2 diluted PB or SF (in phosphate buffered saline, PBS) by Ficoll-gradient centrifugation (2000 rpm, 25 min). The cells were then washed 3 times with pyrogen-free PBS and suspended in a concentration of 2 × 106 cells/ml into RPMI media containing 5% AB serum, 25 mM Heps, 2 mM glutamine, and 25 µg/ml gentamycin.

FACS for chemokine receptor analysis. Ex vivo SF and PB derived cells were washed with PBS containing 0.5% bovine serum albumin (PBS-BSA) and resuspended in 1 ml PBS-BSA. To detect CD3, CD8, CD14, CCR3, and CCR5, 2 × 105 cells were incubated 20 min in the dark with 5 µl of each labeled monoclonal antibody. The cells were then washed once with PBS-BSA and suspended into FACSTM Flow® solution for analysis.

FACS for intracellular cytokine expression. A quantity of 2 × 106 cells were stimulated with 25 ng/ml PMA and 1 µg/ml ionomycin for 16–18 h at 37°C; 10 µg/ml Brefeldin A (BFA) was also added. After incubation the cells were washed with PBS, fixed with 4% paraformaldehyde (5 min at 37°C), and washed with PBS-BSA. Unstimulated cells were also incubated 16–18 h without mitogen at 37°C and after incubation were treated as described above.

The surface markers (CD3 and CD8) were stained as described above, then the cells were permeabilized with FACS Permeabilizing solution®, washed once with PBS-BSA, incubated 30 min in the dark with 7.5 µl of anti-human IFN-γ (56 ng) or anti-human IL-4 (94 ng) antibody, and washed once before suspending cells into FACSTM Flow®.

A quantity of 20,000 stained cells were gated by side scatter and fluorochrome parameters for the T cell (CD3 perCP) and monocyte (CD14 FITC) analyses (FACS Calibur, Becton Dickinson).

The results were expressed as percentage of cells stained positive for different markers. The cutoff for positive staining for intracellular cytokines and chemokine receptors is above the level of control isotype antibody. The percentage of stained cells with control antibody varied between 0.00 and 0.1% in different samples. The statistics were calculated using paired t test for comparison of PB and SF derived cells from the same individual. We used the Kruskal-Wallis test for comparison of 3 groups (RA, other arthritides, and healthy subjects) and Mann-Whitney test for comparison of 2 groups. The p value < 0.05 was considered significant when the groups were compared. Spearman’s correlation test was used, and p value < 0.01 was considered significant due to the high number of comparisons performed.

RESULTS

CCR3 and CCR5 expression in SF and PB derived cells. As an example of FACS results, the expression of CCR5 on CD3 gated CD8− and CD8+ T cells is shown in Figure 1.

The expression of CCR5 and CCR3 was increased in SF derived CD8− and CD8+ T cells and CD14+ monocytes compared with the PB cell populations in patients with RA and in patients with other arthritides (Table 1). The CCR5/CCR3 ratio of SF derived CD8− T cells was increased in the patients with RA compared to patients with other arthritides (median 5.5 vs 2.4; p = 0.039) (Figure 2a). No such differences in the CCR5+/CCR3+ ratio of SF derived CD8+ T cells or CD14+ cells were found between patient groups (data not shown).

The CCR5+/CCR3+ ratio differed between the 3 groups in PB derived CD14+ monocytes and CD8+ T cells (p = 0.002 and p = 0.023, respectively, by Kruskal-Wallis test). In CD14+ cells the ratio of CCR5+/CCR3+ cells was higher in patients with RA than in patients with other arthritides or healthy controls (median 1.4 vs 0.6, p = 0.010, and median 1.4 vs 0.3, p = 0.001, respectively) (Figure 2b). The CCR5+/CCR3+ ratio of PB derived CD8+ T cells was
higher in patients with other arthritides than in healthy controls (median 0.9 vs 0.2; p = 0.011).

Intracellular cytokines in SF and PB derived cells. As an example of FACS results, the expression of IFN-γ in CD3 gated CD8– and CD8+ T cells is shown in Figure 3.

In patients with RA (Figure 4a), but not in patients with other arthritides (Figure 4b), the spontaneous IL-4 expression in CD8+ T cells was higher in SF than in PB (median 0.2 vs 0.1; p = 0.006). In patients with other arthritides (Figure 4d), but not in patients with RA (Figure 4c), the spontaneous IFN-γ expression in CD8+ T cells was higher in SF than in PB (median 0.2 vs 0.1; p = 0.030). Mitogen stimulated intracellular cytokine expression did not differ between SF and PB cells (data not shown).

We found no differences in the activation of intracellular cytokines in unstimulated or mitogen stimulated SF cells between the groups (data not shown).

The mitogen stimulated expression of IFN-γ between the groups differed in PB derived CD8– and CD8+ T cells (p = 0.004 and p = 0.006, respectively, by Kruskal-Wallis test) (Figure 5). Healthy controls had higher PMA + ionomycin stimulated IFN-γ expression in PB derived CD8– T cells than patients with RA or patients with other arthritides (median 16.9 vs 8.6, p = 0.001, and median 16.9 vs 6.7, p = 0.004, respectively) (Figure 5). Healthy controls and patients with RA showed higher IFN-γ expression in PB derived CD8+ T cells compared to patients with other arthritides after PMA + ionomycin stimulation (median 11.7 vs 3.1, p = 0.001, and median 7.8 vs 3.1, p = 0.025, respectively) (Figure 5).

Table 1. The percentage of CD3 gated CD8+ and CD8– T lymphocytes and CD14 gated monocytes expressing CCR5 and CCR3 in synovial fluid (SF) and peripheral blood (PB) from patients with RA and patients with other arthritides. Only PB was studied from healthy controls. PB and SF cells were compared by paired t test in different patient groups.

<table>
<thead>
<tr>
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<th>Patients with RA</th>
<th>Patients with Other Arthritis</th>
<th>Controls</th>
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<td></td>
<td>PB, median % (range)</td>
<td>SF, median % (range)</td>
<td>PB, median % (range)</td>
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<tr>
<td>CD8–/CCR5+</td>
<td>0.1 (0.0–0.3)*</td>
<td>6.2 (0.8–26.5)</td>
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<tr>
<td>CD8+/CCR5+</td>
<td>0.1 (0.0–0.6)*</td>
<td>5.3 (0.1–20.2)</td>
<td>0.1 (0.0–0.4)**</td>
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<tr>
<td>CD14+/CCR5+</td>
<td>0.4 (0.1–1.7)**</td>
<td>15.3 (2.2–45.3)</td>
<td>0.5 (0.2–2.2)**</td>
</tr>
<tr>
<td>CD8–/CCR3+</td>
<td>0.4 (0.0–1.6)**</td>
<td>1 (0.1–6.4)</td>
<td>0.3 (0.1–1.0)**</td>
</tr>
<tr>
<td>CD8+/CCR3+</td>
<td>0.2 (0.0–1.1)**</td>
<td>1 (0.3–9.7)</td>
<td>0.1 (0.0–0.4)**</td>
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<tr>
<td>CD14+/CCR3+</td>
<td>0.8 (0.2–2.9)**</td>
<td>2.3 (0.2–42.9)</td>
<td>0.6 (0.2–4.2)</td>
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* p < 0.0001; ** p < 0.005; *** p < 0.05.
Correlation between CCR5 and intracellular cytokines in SF cells. Spontaneous CCR5 expression correlated positively with spontaneous IFN-γ expression in CD8+ T cells in SF derived cells from patients with RA and patients with other arthritides, and (b) in peripheral blood (PB) CD14+ monocytes from patients with RA, patients with other arthritides, and controls (p = 0.002, Kruskal-Wallis test); p values from Mann-Whitney test are shown above.

**DISCUSSION**

We observed an association between the expression of CCR5 and intracellular IFN-γ activation in CD8 T cells from synovial fluid in patients with RA. This indicates that CCR5 expression is related to type 1 cytokine profile in vivo in human disease. Animal studies have revealed an association between CCR5 and type 1 cytokine profile. In *in vitro*
Figure 4. Percentage of cells with spontaneous cytokine (IL-4 and IFN-γ) production in synovial fluid (SF) and peripheral blood (PB) derived CD3 gated CD8− and CD8+ T cells in patients with RA (a, c) and with other arthritides (b, d). PB and SF derived cells were compared by paired t test.

Figure 5. Percentages of cells expressing IFN-γ in peripheral blood (PB) CD8− and CD8+ T cells in patients with RA, in patients with other arthritides, and in controls after 16–18 h stimulation with 25 ng/ml PMA, 1 µg/ml ionomycin, and 10 µg/ml Brefeldin A at 37°C. Percentages of stained cells were compared between 3 groups by Kruskal-Wallis test (p = 0.004 for CD8− and p = 0.006 for CD8+ T cells) and between 2 groups by Mann-Whitney test.
interestingly, CCR5 expression on SF monocytes was lower in RA patients treated with MTX, showing that MTX specifically modifies the cells in the target tissue. This kind of immunological effect of MTX treatment also suggests that CCR5 positive cells are associated with the development of the arthritic symptoms.

When stimulated with mitogen, the number of IFN-γ-expressing PB T cells was lower in the patient groups than in healthy controls. This may be a marker of exhaustion in vivo activated T cells in these diseases. Alternatively, poor mitogen response may be related to an immune activation defect in these diseases, as suggested by some studies. It has been shown that IL-2 production and proliferation response of PB T cells is defective in patients with RA. In 1997, Maurice, et al published data showing that the poor proliferation capacity, upon mitogenic and antigenic stimulation, of SF derived cells from patients with RA is due to impaired signal transduction via the TCR/CD3 complex. They showed that SF derived T cells exhibited a decreased overall tyrosine phosphorylation, and in particular the phosphorylation of tyrosine kinase p38 was virtually absent. The same kind of defects in the signaling cascade have been detected in PB mononuclear cells in type 1 diabetes. It was shown that the expression of tyrosine kinase p56lck is abnormally low in patients with type 1 diabetes and might be the cause of T cell hyporesponsiveness in patients with the disease. In our study poor mitogen response was restricted to PB cells, and no difference was seen between the patient groups or controls in the expression of IFN-γ in the mitogen stimulated T cells in the target tissue.

Although the type 1 immune response, indicated as CCR5 expression, was not enhanced in PB derived T cells in RA, the CCR5/CCR3 ratio of PB derived monocytes was significantly higher in patients with RA compared to patients with other arthritides and healthy controls. Monocytes in PB, instead of T cells in PB, may thus reflect the immune balance in the target tissue in RA. Katzschke, et al have also reported that activation of PB monocytes is found in RA, indicated as increased expression of CCR3, CCR4, and CCR5.

Treatment blocking TNF-α in RA has dramatic beneficial effects. The expression of many proinflammatory cytokines, such as IL-1 and IL-6, is enhanced by TNF-α, and blocking its function may thus inhibit the perturbation of the monocyte activation. On the other hand, treatment with agents blocking TNF-α causes activation of cytokine secretion, especially IFN-γ activation, in PB cells and has been reported to result in recovery of antigen-specific T cell response. Our findings in PB T cells and monocytes associated with RA — enhanced activation of monocytes combined with poor cytokine response to mitogen in the T cell population — are mechanisms that have been reported to be affected by TNF-α treatment. Thus, we suggest that these findings are fundamentally associated with the basic immune aberration in RA.
We conclude that T lymphocytes and monocytes that express CCR3 and CCR5 accumulate into joints in patients with RA and other arthritides. The type 1 cytokine response in target tissue is associated with the presence of CCR5-expressing lymphocytes. Peripheral blood monocytes, but not T cells, showed increased CCR5 expression and may thus reflect the immune balance in the target tissue in RA.

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REFERENCES