CCR3, CCR5, Interleukin 4, and Interferon-γ Expression on Synovial and Peripheral T Cells and Monocytes in Patients with Rheumatoid Arthritis

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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
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The inflamed synovium in rheumatoid arthritis (RA) is infiltrated by mononuclear effector cells, among which T cells play a prominent role. The cells are recruited to the inflammation site by adhesion molecules and chemoattractants. The mechanisms of the pathogenesis of RA have been studied by characterization of the cytokine, chemokine, and chemokine receptor (CXCR or CCR) profile of infiltrating T cells and monocytes. It has been shown that chemokine concentrations for chemokine receptors CXCR3 and CCR5 were elevated in synovial fluid (SF) of patients with RA compared to healthy controls[1]. In addition, the majority of T cells homing to SF expressed CXCR3 and CCR5, and monocytes expressed CCR5 and CCR3[1-4]. It has also been shown that stimulation of SF-derived CD4+ CCR5+ T cells from RA patients with anti-CD3 resulted in production of interferon-γ (IFN-γ) but not interleukin 4 (IL-4)[2].

T cells are divided into type 1 and type 2 lymphocytes that have different cytokine and chemokine receptor profiles. Type 1 T cells produce high concentrations of IL-2 and IFN-γ cytokines and express high concentrations of CCR5 and CXCR3 chemokine receptors on their cell surface[5,6]. The type 2 response is characterized by the IL-4, IL-5, IL-6, IL-10, and IL-13 production and high expression of CCR3, CCR4, and CCR8[7-9]. The balance between type 1

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and 2 responses regulates the choice between cellular and antibody mediated immune responses.

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[12,14,15]. 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[16].

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[18]. 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 spondyloarthropathy, 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, Terumo, 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 2×10^6 cells/ml into RPMI media containing 5% AB serum, 25 mM Hepes, 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, 2×10^5 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 FACS Flow® solution for analysis.

FACS for intracellular cytokine expression. A quantity of 2×10^6 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 FACS 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+ relation on 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

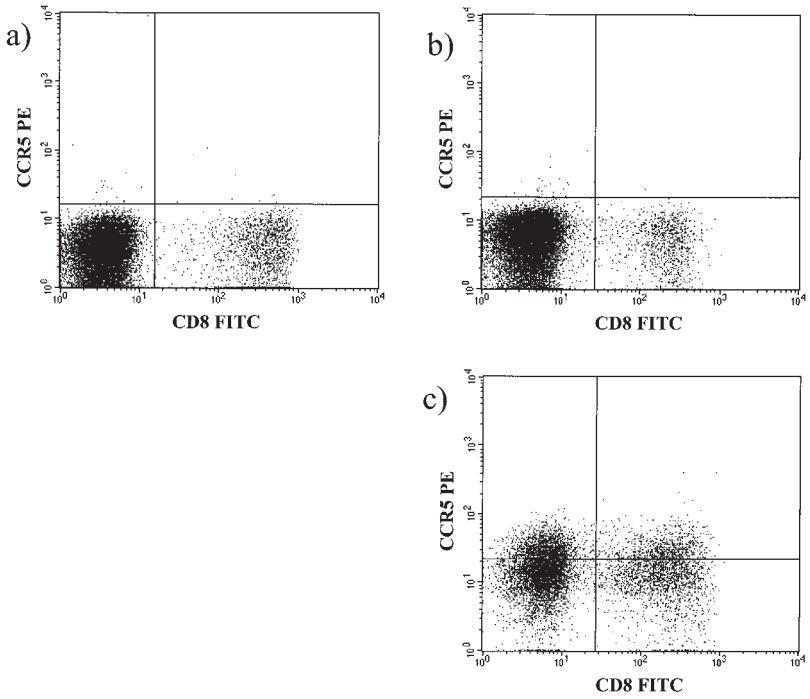

Figure 1. FACS dot plots show spontaneous expression of CCR5 in CD3 gated CD8+ and CD8– T cells in (a) peripheral blood cells from a healthy control, and (b) peripheral blood and (c) synovial fluid cells from one patient with RA.

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.

	Patients with RA		Patients with Other Arthritides		Controls
	PB, median % (range)	SF, median % (range)	PB, median % (range)	SF, median % (range)	PB, median % (range)
CD8–/CCR5+	0.1 (0.0–0.3)*	6.2 (0.8–26.5)	0.2 (0.1–0.3)	1.4 (0.2–23.7)	0.1 (0.1–0.3)
CD8+/CCR5+	0.1 (0.0–0.6)*	5.3 (0.1–20.2)	0.1 (0.0–0.4)***	2.1 (0.05–9.6)	0.1 (0.0–0.1)
CD14+/CCR5+	0.4 (0.1–3.7)*	15.3 (2.2–45.3)	0.5 (0.0–2.2)***	16.3 (0.0–30.2)	0.7 (0.4–2.3)
CD8–/CCR3+	0.4 (0.0–1.6)**	1 (0.1–6.4)	0.3 (0.1–1.0)*	0.6 (0.1–2.6)	0.8 (0.2–1.6)
CD8+/CCR3+	0.2 (0.0–1.1)***	1 (0.3–9.7)	0.1 (0.0–0.4)***	0.6 (0.0–1.8)	0.3 (0.1–0.6)
CD14+/CCR3+	0.8 (0.2–2.9)***	2.3 (0.2–42.9)	0.6 (0–2.4)	1.2 (0–11.6)	2.4 (1.0–7.1)

* $p < 0.0001$; ** $p < 0.005$; *** $p < 0.05$.

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).

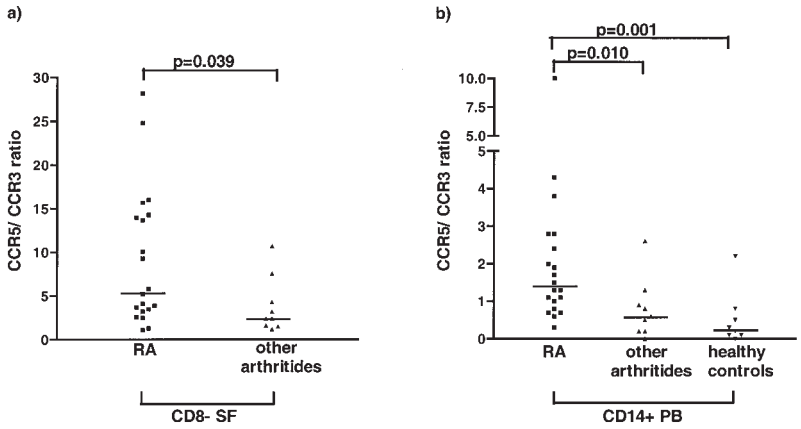

Figure 2. The ratio of CCR5-expressing and CCR3-expressing CD3 gated CD8– T cells (a) in synovial fluid (SF) from patients with RA and patients with other arthritides, and (b) in peripheral blood (PB) CD14+ monocytes from patients with RA, patients with other arthritides, and controls (p = 0.002, Kruskal-Wallis test); p values from Mann-Whitney test are shown above.

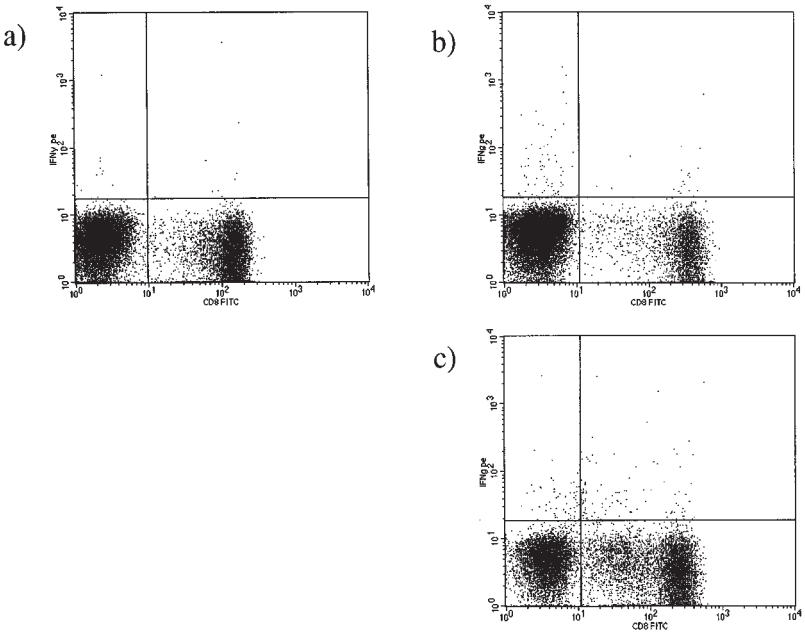

Figure 3. FACS dot plots show spontaneous expression of IFN-γ in CD3 gated CD8+ and CD8– T cells in (a) peripheral blood cells from a healthy control, and (b) peripheral blood and (c) synovial fluid cells from one patient with RA.

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 (r = 0.604, p = 0.005) (Figure 6). No such correlations were seen in the patients with other arthritides.

Effect of MTX treatment on CCR and cytokine expression in patients with RA. We analyzed the effect of MTX treatment on CCR expression and cytokine activation. The expression of CCR5 on SF derived monocytes was lower in RA patients treated with MTX than in RA patients not receiving MTX (median 10.8 vs 21.3; p = 0.031).

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[9]. In *in vitro*

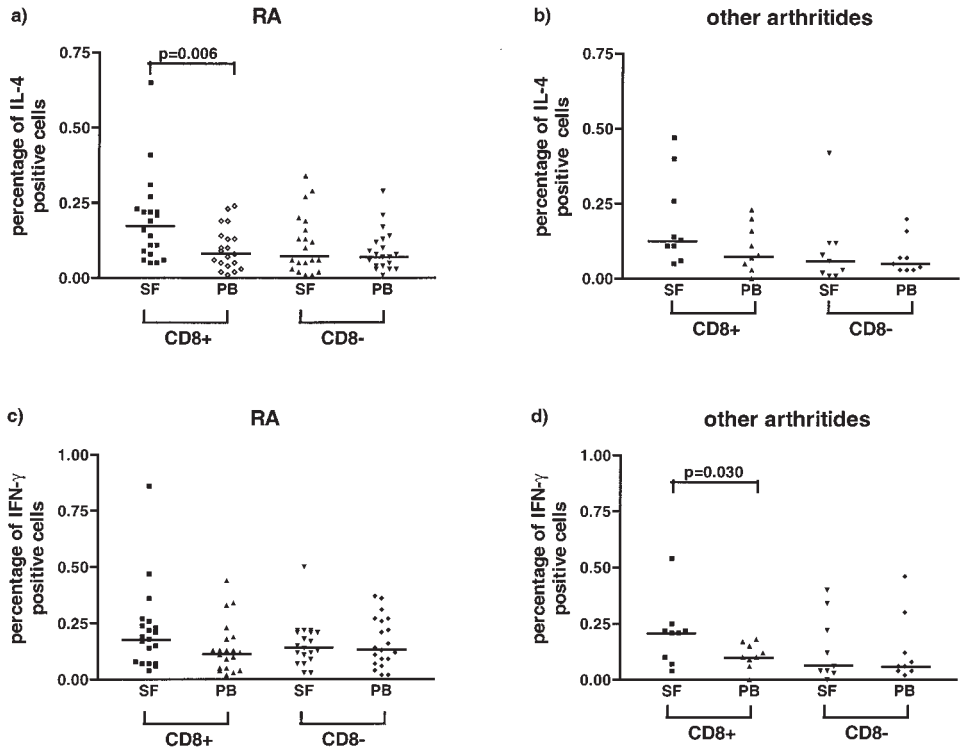

Figure 4. Percentage of cells with spontaneous cytokine (IL-4 and IFN-γ) production in synovial fluid (SF) and peripheral blood (PB) derived CD3 gated CD8– and CD8+ T cells in patients with RA (a, c) and with other arthritides (b, d). PB and SF derived cells were compared by paired t test.

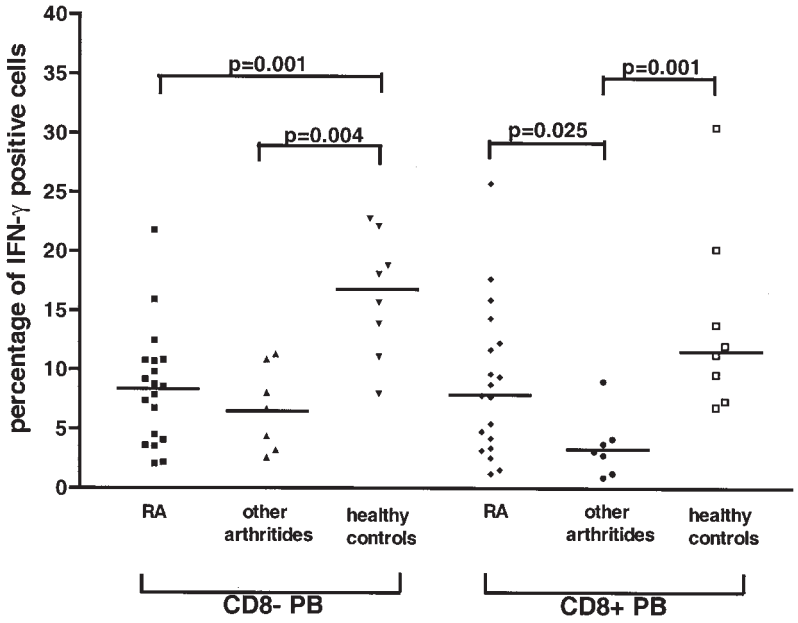

Figure 5. Percentages of cells expressing IFN-γ in peripheral blood (PB) CD8– and CD8+ T cells in patients with RA, in patients with other arthritides, and in controls after 16–18 h stimulation with 25 ng/ml PMA, 1 µg/ml ionomycin, and 10 µg/ml Brefeldin A at 37°C. Percentages of stained cells were compared 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.

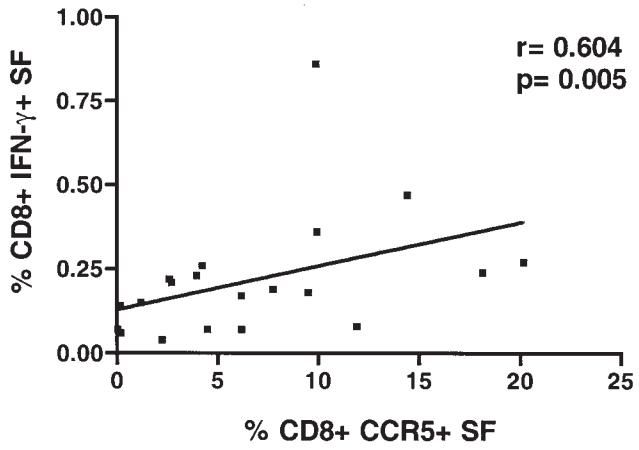

Figure 6. Correlation between spontaneous CCR5 and IFN-γ expression in synovial fluid (SF) derived CD8+ cells in patients with RA. Result of Spearman correlation test is shown.

culture experiments, CCR5 expression has been linked to Th1 phenotype in humans[2,6-9], but studies of *ex vivo* derived cells are few.

Although synovial tissue-infiltrating T cells are the effector cells in the inflamed joint, SF T cells from RA patients have been shown to be pathogenic in transfer experiments and thus represent the cell population present in the target tissue[19]. As reported by other groups, we found CCR3- and CCR5-expressing T cells and monocytes infiltrating the joints of patients with RA[2,3]. In our study, this was also true for patients with other arthritides, as recently suggested by Bruhl, *et al*[20]. The accumulation of both CCR5 and CCR3 positive cells in SF indicates the presence of type 1 and type 2 cells in the target tissue. Supporting this, we found that the intracellular levels of both IL-4 and IFN-γ were higher in target tissue derived CD8 T cells than in PB derived CD8 T cells in both patient groups. That the CCR5/CCR3 ratio of SF derived CD8– T cells was higher in patients with RA than in patients with other arthritides suggests a more vigorous type 1 immune response in the inflamed tissue of patients with RA compared to patients with other arthritides.

It has been reported that a 32 base pair deletion at the coding region of the CCR5 gene leads to reduced expression of CCR5 protein in heterozygous individuals[21]. These heterozygous CCR5-delta 32 carriers have been reported to be at a higher frequency in patients with the non-severe form of RA compared to those with the severe form: 17% vs 4%[22]. Studies on the chemoattraction of lymphocytes, however, suggest that accumulation of CCR5 positive cells in SF is not dependent on a functional CCR5 receptor, but CCR5 is a marker of a lymphocyte subset infiltrating into SF[23]. The importance of the phenotype of CCR5-expressing T cells in SF is further supported by Hisakawa, *et al,* who showed that the majority of CCR5-expressing T cells in SF are CD45RO positive memory cells[24]. Our results suggest that the CCR5-expressing T cells in SF represent the Th1 phenotype.

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[25,26]. 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 *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[27,28]. In 1997, Maurice, *et al*[29] 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 also in PB mononuclear cells in type 1 diabetes. It was shown that the expression of tyrosine kinase $p56^{lck}$ is abnormally low in patients with type 1 diabetes and might be the cause of T cell hyporesponsiveness in patients with the disease[30]. 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. Katschke, *et al*[3] 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[31,32]. 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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