Chemokine and chemokine receptor expression in paired peripheral blood mononuclear cells and synovial tissue of rheumatoid arthritis, osteoarthritis and reactive arthritis patients.


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ABSTRACT

Objective: As chemokine receptors and chemokines play a crucial role in leukocyte recruitment into inflamed tissue, we examined the expression of an extensive number of chemokines and receptors in a unique bank of paired samples of synovial tissue (ST) and peripheral blood (PB) from patients with different forms of arthritis. These studies will assist in identifying suitable targets for therapeutic intervention.

Methods: Synovial biopsies were obtained from patients with RA (n=23), OA (n=16), and ReA (n=8). ST chemokine (CCL2/MCP-1, CCL5/RANTES, CCL7/MCP-3, CCL8/MCP-2, CCL14/HCC-1, CCL15/HCC-2, CCL16/HCC-4), chemokine receptor (CCR1, CCR2b, CCR5, CXCR4) and CD13 expression was analyzed by immunohistochemistry and 2-color immunofluorescence. Chemokine receptor expression (CCR1, CCR3, CCR5, CCR6, CCR7) on PB cells was studied by flow cytometry. For statistical analysis non-parametric tests were used.

Results: There was abundant expression of CCR1, CXCR4 and CCR5 in all forms of arthritis, with a specific increase of CCL5 and CCL15 in RA. CCL7, CCL8, CCL14, CCL15 and CCL16 were detected for the first time in ST. The results for PB analysis were comparable among different arthritides. Interestingly, compared to healthy controls we observed significantly lower expression of CCR1 (P<0.005) and CCR5 (P<0.05) by PB monocytes in the patient groups.

Discussion: These results indicate that a variety of chemokines and receptors could play an important role in several inflammatory joint disorders. Although other receptors are involved as well, migration of CCR1+ and CCR5+ cells toward the synovial compartment might be involved in the effector phase of various forms of arthritis.

INTRODUCTION

Chemokines are small chemotactic proteins that play a central role in the recruitment of leukocytes into inflamed tissue (1-3). To date about 50 chemokines have been identified signaling through some 20 distinct receptors (4). Besides the ability to directly recruit leukocytes by providing a chemotactic gradient, chemokines can also activate integrins, stimulate mediator release, and modulate vascularisation thereby playing a central role in the inflammatory process (5).

Chemokines and chemokine receptors have been shown to be involved in a broad number of inflammatory and infectious diseases (6). Since the discovery of their existence, targeting chemotactic proteins has been suggested as potential therapy in many disorders. Especially due to the development of low molecular weight antagonists directed against chemokine receptors, which could be used as an oral treatment, the chemokine family may be an attractive therapeutic target (7). The first clinical study using a specific CCR1 antagonist in patients with rheumatoid arthritis (RA) confirmed the potential of this approach (8).

The analysis of synovial tissue (ST) from affected joints could assist in identifying the most important ligands and receptors in RA and other joint diseases. Especially the analysis of their expression in both ST and PB of the same patients will provide additional evidence on their possible suitability as future therapeutic targets. Therefore, the objective of this study was to determine the expression of an extensive number of chemokines and chemokine receptors in a unique bank of paired samples of ST and peripheral blood (PB) in patients with RA, inflammatory osteoarthritis (OA) and reactive arthritis (ReA). In addition we studied the expression of CD13/Aminopeptidase N, which has been described to be involved in the mechanism of lymphocyte recruitment in inflamed joints of RA patients (9). In light of the recent interest in the development of CCR1 and CCR5...
blockade for treatment of RA, we had a special interest in the detection of the ligands for these receptors in the synovium. In addition, we used immunofluorescence double staining techniques to further elaborate the expression of several novel chemokines on different cell types in ST.

PATIENTS and METHODS

Patients
Forty-seven patients with different forms of arthritis were included in the study. The patients fulfilled established criteria for RA (n=23) (10), inflammatory OA (n=16) (11), and ReA (n=8) (12). The patients were followed for at least one year to allow confirmation of the diagnosis. All patients had active knee arthritis defined by pain and swelling at the time of evaluation. Most patients were treated with nonsteroidal anti-inflammatory drugs. None were treated with corticosteroids or immunosuppressive drugs at the time of the synovial biopsy. Laboratory assessments included ESR and CRP measurements, as well as serum levels of rheumatoid factor. Clinical assessments on the day of the biopsy procedure included the Ritchie articular index (13) and number of swollen joints. All patients gave written informed consent and the study protocol was approved by the Medical Ethics Committee.

Synovial tissue samples
An average of 20 biopsy specimens was taken from the supra-patellar pouch with a Parker Pearson needle (14). All samples were snap frozen together in Tissue Tek OCT (Miles Diagnostics, Elkhart, IN, USA) by immersion in methylbutane (-70°C). The frozen blocks were stored in liquid nitrogen until sectioned for staining. Of each tissue sample, consisting of at least 6 different biopsy samples, five micrometer serial sections were cut with a cryostat and mounted on glass slides (Start Frost, Knittgelaser, Braunschweig, Germany). The glass slides were sealed and stored at -80°C until immunohistochemical analysis could be performed.

Antibodies
For immunohistochemical analysis the following monoclonal antibodies (mAb) were used: anti-CD68 (EBM11, Dako, Glostrup, Denmark), anti-CD3 (SK7, Becton-Dickinson, San Jose, CA), anti-CD13 (NCL-CD13, Novocastra), anti-CCR1 (MAB145, R&D systems Europe Ltd., Avingdon, UK), anti-CCR2b (sc-6228, Santa Cruz Biotechnology), anti-CXCR4 (MAB172, R&D), anti-CCR5 (MAB145, R&D systems), anti-CCL2/MCP-1 (sc-1304, Santa Cruz Biotechnology), anti-CCL5/RANTES (MAB278, R&D systems), anti-CCL7/MCP-3 (sc-1308, Santa Cruz Biotechnology), anti-CCL8/MCP-2 (sc-1307, Santa Cruz Biotechnology), anti-CCL14/HCC-1 (BAF324, R&D), anti-CCL15/HCC-2 (sc-8582, Santa Cruz Biotechnology), and anti-CCL16/HCC-4 (AF802, R&D). Goat-anti-mouse-HRP (P0447, Dako) and swine-anti-goat-HRP (ACI3404, Biosource (TAGO)) were used to detect bound mAb.

For tissue immunofluorescence staining the following mAb were used: anti-CD3 fluorescein isothiocyanate (FITC)-conjugated (345763, BD), anti-CD55-FITC (M2192, CLB, The Netherlands), anti-CD68-IgG3 (M0876, Dako), anti-CCL7/MCP-3 (MAB282, R&D), anti-CCL8/MCP-2 (MAB281, R&D), and CCL15/hCC2 (MAB363, R&D). Streptavidin-TRITC (43-4314, Zymed laboratories), rabbit-anti-FITC (058, Dako), goat-anti-rabbit Alexa 488 (99D1-1, Molecular probes), and goat-anti-mouse Alexa 488 (73D1-1, Molecular probes) were used to detect bound mAb.
For flow cytometry anti-chemokine receptor mAb were generated in mice against their respective human proteins by Millennium Pharmaceuticals (Cambridge, MA) and generously provided. Anti-CCR1 (clone designation 2D4) and anti-CCR6 (clone designation 9H7) were mouse IgG1, anti-CCR3 (clone designation 7B11) and anti-CCR5 (clone designation 2D7) were mouse IgG2a, and anti-CCR7 (clone designation 7H12) was IgG2b. All other antibodies and controls were obtained commercially. These included anti-CD14 allophycocyanin (APC) (catalog number 555399, Becton Dickinson [BD] Pharmingen, San Diego, CA), IgG1- R-phycocerythrin (PE) isotype control (349043, BD), IgG1-FITC isotype control (554679, BD), IgG2a-PE isotype control (349053, BD), IgG2a-APC isotype control (555576, BD), IgG2b-PE isotype control (555058, BD), and goat anti-mouse IgG R-PE (115-116-146, Jackson ImmunoResearch, West Grove, PA).

Immunohistochemical analysis
Serial sections were stained and sections with non-assessable tissue, defined by the absence of an intimal lining layer, were omitted before analysis. For control sections, the primary antibodies were omitted or irrelevant isotype-matched antibodies were applied. Staining was performed according to a 3-step immunoperoxidase method as previously described in detail (15). After immunohistochemical staining, all coded sections were randomly analyzed by computer-assisted image analysis. For all markers, 18 high-power fields were analyzed as described earlier (16). All sections were coded and analyzed in a random order by an independent observer who was blinded for the clinical data.

Immunofluorescence tissue staining
For further quantification of chemokine expression on CD3+ T-cells, CD55+ fibroblast-like-synoviocytes (FLS) cells and CD68+ macrophages, we randomly selected five ST samples from the RA group. Tissue sections were cut, stored, and fixed as described above. Sections were washed with phosphate buffered saline (PBS) and the primary antibodies or isotype-matched controls were added and incubated overnight at 4 °C. After incubation sections were washed and goat-anti-mouse-HRP antibodies were applied for 30 minutes in the dark at room temperature (RT). After washing and incubation with biotin-tyramine for 15 minutes streptavidin-TRITC was added to the tissue sections for 30 minutes in the dark at RT. After blocking with normal mouse serum 10% in PBS the anti-CD3, anti-CD55, and anti-CD68 antibodies were applied for 60 minutes in the dark at RT. After washing rabbit-anti-FITC antibodies were added to the sections, which were previously incubated with the anti-CD3-FITC and anti-CD55-FITC antibodies. Finally, after washing steps with PBS, the sections were incubated with goat-anti-rabbit-Alexa 488 for the sections previously incubated with anti-CD3 and anti-CD55, and with goat-anti-mouse-Alexa 488 for the sections previously incubated with anti-CD68. After a 30 minute incubation at RT the slides were washed and coverslipped with Vectashield (Vector). The sections were examined under a fluorescent photomicroscope. Two observers independently quantified the number of double staining positive cells by manual counting. On average between 100 and 200 cells which were positive for CD3, CD55, or CD68 were counted by each observer. The mean percentage of double staining cells with the anti-chemokine-antibodies was calculated from the results of the 2 observers.

Flow cytometry
PB mononuclear cells (PBMCs) were obtained at the same day as the ST samples from the same patient groups, as well as from 5 healthy controls and were isolated from PB samples by Ficoll-Hypaque gradients and directly stored in liquid nitrogen. Previously it was shown that isolated PBMC can be frozen and stored in liquid nitrogen without affecting
chemokine receptor expression on CD14+ monocytes. Chemokine receptor expression on these cells, measured by flow cytometry, was comparable to the chemokine receptor expression in paired fresh whole blood samples [Haringman et al. unpublished data]. The vials were removed from the liquid nitrogen storage and thawed at room temperature until only a small clot was still present. The contents of each vial were transferred immediately into 10 ml tubes and then slowly filled with DMEM + 20% Fetal Calf Serum (FCS) until 10 ml. Tubes were centrifuged for 10 minutes at 500 g (1700 rpm) at 4 °C. The supernatant was discarded and the cells resuspended. Next 10 ml of DMEM + 10% FCS was added into each tube and mixed to make a homogeneous PBMC suspension. Cells were counted and resuspended at a concentration of 2 x 10⁶/ml with DMEM + 10% FCS.

After thawing the cells were incubated for 30 minutes at 4 °C in the dark with primary antibodies directed against CCR1, CCR3, CCR5, CCR6, and CCR7, or appropriate IgG isotype controls. After several washing steps the cells were incubated with goat-anti-mouse IgG R-phycoerythrin for 30 minutes. After washing steps APC-conjugated CD14 monoclonal antibodies were added for 30 minutes at 4 °C in the dark. Cells were analyzed using a FACSCaliber flow cytometer and CellQuest software (BD Biosciences) and the percentages of positive monocytes were determined. Monocytes were differentiated by characteristic side and forward light-scatter properties and confirmed by CD14 staining. The threshold level was based on the maximum staining of a matched isotypic antibody with irrelevant specificity used in the same concentration. Isotype-control antibody bound to <1% of cells, and results are reported as the proportion of cells with fluorescence signals above the cut-off, as defined by the isotype controls.

**Statistical Analysis**

Patient data were analyzed using non-parametric tests (Kruskal-Wallis H test (KWH) and the Mann-Whitney U test (MWU)) for the comparison of chemokine/receptor expression between the 3 diagnostic groups. P values less than 0.05 were considered significant.

**RESULTS**

**Clinical and demographic features**

The clinical and demographic characteristics (obtained at the time of sample collection) of the patients are presented in Table 1. On average patients in the inflammatory OA group were older than the patients in the other 2 groups. Thirteen of the 23 RA patients were rheumatoid factor positive, while none of the patients from the OA and ReA group were rheumatoid factor positive.

| Table 1. Clinical and demographic data of 23 patients with rheumatoid arthritis (RA), 16 patients with inflammatory osteoarthritis (OA) and 8 patients with reactive arthritis (ReA). The data represent the mean ± standard error of the mean (range). |
|-----------------|-----------------|-----------------|
|                 | RA N=23 | OA N=16 | ReA N=8 |
| **Sex (m/f)**   | 9/14    | 2/14   | 6/2 |
| **Age (years)** | 58 ± 3 (24-76) | 72 ± 2 (54-81) | 33 ± 4 (16-47) |
| **Disease duration (months)** | 7 ± 1 (2-12) | - | 5 ± 2 (1-18) |
| **Total Ritchie score** | 10 ± 1 (3-26) | 4 ± 1 (0-15) | 1 ± 1 (0-5) |
| **Swollen joint count** | 8 ± 1 (3-18) | 2 ± 1 (0-9) | 2 ± 0 (1-4) |
| **ESR (mm per hr)** | 63 ± 7 (13-125) | 33 ± 6 (10-89) | 15 ± 4 (3-28) |
| **CRP (mg per L)** | 51 ± 9 (11-214) | 21 ± 7 (3-112) | 14 ± 7 (3-50) |
| **RF (+/-)**    | 13/10   | 0/16   | 0/8 |
ESR: Erythrocyte Sedimentation Rate, CRP: C-Reactive Protein, RF: Rheumatoid Factor IgM.

**Immunohistochemical analysis**

**Chemokine receptors**

Slides were negative when the primary antibody was omitted or irrelevant antibodies were applied. All chemokine receptors could be detected in the ST in all groups. The mean scores (± standard error of the mean [sem]) and representative examples of chemokine receptor expression in ST are demonstrated in Table 2 and Figure 1A, respectively. On average there was abundant ST expression of CCR1, CCR5 and CXCR4 in RA, OA and ReA patients. CD13 and CCR2b were also present in ST of all patients, although at a lower expression level.

Immunohistologic analysis revealed no statistically significant differences in chemokine receptor expression between RA, inflammatory OA and ReA indicating that these CKRs are upregulated in all forms of arthritis.

**Table 2. Immunohistologic features of synovial tissue from patients with rheumatoid arthritis (RA), inflammatory osteoarthritis (OA) and reactive arthritis (ReA) for CD3+ T lymphocytes, CD68+ macrophages, CD13+ cells, CCR1+ cells, CCR2b+ cells, CCR5+ cells, CXCR4+ cells. The data represent the mean number of positive cells per 2.1 square mm ± standard error of the mean.**

<table>
<thead>
<tr>
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<th>RA (N=23)</th>
<th>OA (N=16)</th>
<th>ReA (N=8)</th>
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<tbody>
<tr>
<td>CD3+ T lymphocytes *</td>
<td>331 ± 83</td>
<td>46 ± 13</td>
<td>75 ± 26</td>
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<tr>
<td>CD68+ macrophages</td>
<td>1015 ± 178</td>
<td>383 ± 111</td>
<td>601 ± 256</td>
</tr>
<tr>
<td>CD13+ cells</td>
<td>640 ± 172</td>
<td>381 ± 122</td>
<td>547 ± 236</td>
</tr>
<tr>
<td>CCR1+ cells</td>
<td>1232 ± 231</td>
<td>1274 ± 182</td>
<td>955 ± 317</td>
</tr>
<tr>
<td>CCR2b+ cells</td>
<td>262 ± 71</td>
<td>114 ± 37</td>
<td>266 ± 152</td>
</tr>
<tr>
<td>CCR5+ cells</td>
<td>1335 ± 315</td>
<td>982 ± 248</td>
<td>730 ± 201</td>
</tr>
<tr>
<td>CXCR4+ cells</td>
<td>815 ± 202</td>
<td>784 ± 282</td>
<td>706 ± 353</td>
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* Kruskal Wallis test p < 0.05, Mann-Whitney test, comparison between RA and OA: p < 0.05, comparison between RA and ReA: not significant, comparison between OA and ReA: not significant.

**Chemokines**

All chemokines could be detected in inflamed synovium. The mean scores (±sem) for the chemokine markers investigated in this study are depicted in Table 3 and representative examples of chemokine expression in ST are demonstrated in Figure 1B. There was abundant expression of especially CCL2/MCP-1, CCL5/RANTES, CCL8/MCP-2 and CCL15/HCC-2 in the ST of RA patients. The differences between RA and the other two groups did not reach statistical significance for CCL2/MCP-1, CCL7/MCP-2, CCL8/MCP-2, CCL14/HCC-1 and CCL16/HCC-4. The expression of CCL5/RANTES was higher in RA than in the OA and ReA groups. Similarly, CCL15/HCC-2 expression was significantly increased in RA (Table 3).
Table 3. Immunohistologic features of synovial tissue from patients with rheumatoid arthritis (RA), inflammatory osteoarthritis (OA) and reactive arthritis (ReA) for CCL2/MCP-1, CCL5/RANTES, CCL7/MCP-3, CCL8/MCP-2, CCL14/HCC-1, CCL15/HCC-2 and CCL16/HCC-4. The data represent the mean integrated optical density (IOD) per 2.1 square mm ± standard error of the mean.

<table>
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<th>RA</th>
<th>OA</th>
<th>ReA</th>
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<tbody>
<tr>
<td>CCL2</td>
<td>6867 ± 2823</td>
<td>1020 ± 343</td>
<td>687 ± 379</td>
</tr>
<tr>
<td>CCL5 *</td>
<td>11202 ± 2360</td>
<td>8759 ± 2930</td>
<td>2081 ± 1181</td>
</tr>
<tr>
<td>CCL7</td>
<td>1471 ± 650</td>
<td>1555 ± 758</td>
<td>1208 ± 663</td>
</tr>
<tr>
<td>CCL8</td>
<td>10653 ± 3877</td>
<td>3819 ± 1384</td>
<td>1664 ± 716</td>
</tr>
<tr>
<td>CCL14</td>
<td>1072 ± 313</td>
<td>435 ± 314</td>
<td>1071 ± 999</td>
</tr>
<tr>
<td>CCL15 **</td>
<td>4993 ± 1601</td>
<td>1738 ± 573</td>
<td>1253 ± 919</td>
</tr>
<tr>
<td>CCL16</td>
<td>2213 ± 1137</td>
<td>1674 ± 801</td>
<td>1323 ± 650</td>
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</tbody>
</table>

* Kruskal Wallis test p < 0.05, Mann-Whitney test, comparison between RA and OA: not significant, comparison between RA and ReA: p < 0.05, comparison between OA and ReA: p < 0.05.
** Kruskal Wallis test p < 0.05, Mann-Whitney test, comparison between RA and OA: not significant, comparison between RA and ReA: p < 0.05, comparison between OA and ReA: not significant.

**Immunofluorescence double staining**

As some of the investigated chemokines are described here for the first time in ST of RA patients, we performed double immunofluorescence to phenotype positive cells. The expression of CCL7/MCP-3, CCL8/MCP-2 and CCL15/HCC-2 by T-cells, FLS and macrophages, respectively, was determined. None of the CD3+ T-cells showed co-expression with CCL7/MCP-3 or CCL8/MCP-2 while on average 57% (±4) of the CD3+ lymphocytes showed co-expression with CCL15/HCC-2 (Figure 2). The immunohistochemical analysis indicated that CCL7/MCP-3 and CCL8/MCP-2 were mainly expressed in the intimal lining layer. Double immunofluorescence showed that 45% (±15) and 50% (±12) of the CD55+ FLS co-expressed CCL7/MCP-3 and CCL8/MCP-2, respectively. Seventy-two % (±11) of the CD55+ FLS were positive for CCL15/HCC-2. Of the CD68+ macrophages on average 27% (±12) co-expressed CCL7/MCP-3, 25% (±4) CCL8/MCP-2, and 55% (±12) of these cells co-expressed CCL15/HCC-2 (Figure 2).

**Flow cytometry analysis**

For all patients as well as for 5 healthy controls we evaluated the expression of CCR1, CCR3, CCR5, CCR6, and CCR7 on CD14+ PB monocytes with antibodies available for the detection of chemokine receptors in peripheral blood (Figure 3). In all samples chemokine receptor expression could be detected. The results of chemokine receptor expression by CD14+ monocytes were comparable among the different disease groups. Of interest, when the results were compared to the healthy controls we observed significantly lower expression of CCR1 in the arthritis groups (P<0.005). Patients in the RA group showed on average also lower expression of CCR5 compared to healthy controls (P<0.05) (Figure 3).

**DISCUSSION**

The present study investigated the expression of a broad variety of chemokines and chemokine receptors in paired samples of ST and PB from patients with RA, inflammatory OA, and ReA. Of interest, the percentages of CCR1 and CCR5 positive monocytes were decreased in PB of RA patients compared to normal individuals. There was abundant
expression of CCR1 and CCR5 in the ST of these patients indicating upregulation of these receptors and/or accumulation of CCR1 and CCR5 positive cells in the inflamed synovium. Chemokines and chemokine receptors are important mediators of leukocyte trafficking in inflammatory disorders and many family members may be potential targets for biological intervention in a variety of diseases (17). As many of the chemokines and receptors play a role in cell migration and inflammation, it is difficult to predict which ligands or receptors are the best candidates to target. Studying their expression in paired ST and PB of the same patients will assist in the process of selecting the best candidates for therapeutic intervention.

Both CCR1 and CCR5 have been shown to play a specialized role in the recruitment of monocytes and Th1-type T cells under inflammatory conditions (18). In RA CCR1 and CCR5 have been implicated as potential therapeutic targets as they seem strongly involved in monocyte and T-lymphocyte recruitment towards the joints (19). Although both cell types are intimately involved in the pathogenesis of RA, it was recently shown that especially ST-macrophages are related to clinical signs and symptoms (20-23). Both CCR1 and CCR5 have been described to be expressed on a large number of ST macrophages. Interference with the migration of these cells by CKR blockade is therefore suggested as a novel therapeutic approach to reduce synovial inflammation. The only published study on chemokine blockade in patients so far indicated that short term treatment with a specific CCR1 antagonist resulted in an evident reduction in the number of ST macrophages (8).

The results of the present study confirm the expression of both CCR1 and CCR5 in the ST and PB of patients with RA and other arthritides, but also show that other chemokine receptors and chemokines are involved as well. Although these data do not prove a functional role of these CKRs in arthritis, it can be anticipated that they are involved in the inflammatory process based on reported functional studies (18).

Except for CCL5/RANTES and CCL15/HCC-2, which were present at higher levels in RA ST, there were on average no significant differences in the expression of the analyzed chemokine receptors and chemokines in RA compared to the disease controls, indicating that chemokines and chemokine receptors are not uniquely restricted to inflammation in RA. Thus based on these expression data, chemokine blockade might not only be a potential treatment for RA patients, but also for other inflammatory joint disorders, since this approach is directed at common final pathways.

In addition to CKRs, we investigated the expression of many of their ligands in the synovium. Our results confirm earlier reports that there is abundant expression of CCL2/MCP-1, mainly in the intimal lining layer, and CCL5/RANTES, diffusely expressed in ST, especially in RA patients (24;25). CCL7/MCP-3 and CCL8/MCP-2, which are ligands of both CCR1 and CCR2, were expressed abundantly in ST of both RA patients and disease controls. These ligands are structurally similar to CCL2/MCP-1 and influence migration of especially lymphocytes and monocytes (26). This is the first description of CCL7/MCP-3 and CCL8/MCP-2 in ST. The expression of CCL7/MCP-3 and CCL8/MCP-2 resembles that of CCL2/MCP-1, with marked expression by FLS and macrophages in the intimal lining layer.

In addition, the present study shows for the first time the expression of CCL14/HCC-1, CCL15/HCC2 and CCL16/HCC-4, ligands of CCR1, in the inflamed synovium. CCL14/HCC-1 is an exception to most chemokines as it is present in high concentrations in human plasma (27). CCL14/HCC-1 is a low-affinity agonist of CCR1 which is converted into a high-affinity agonist of CCR1 (and CCR5) by serine proteases (28). CCL15/HCC-2 which binds to CCR1 and CCR3, has a chemoattractant role for neutrophils, lymphocytes and monocytes (29). Besides having chemotactic and pro-
inflammatory effects, CCL15/HCC-2 is also known to promote homeostasis and was reported to be expressed only in the gut and the liver (30;31). CCL16/HCC-4 is another liver-expressed chemokine, which is also known to be upregulated in colonic biopsy samples from patients with ulcerative colitis (32). This chemokine has been suggested to be an effective inducer of cell adhesion and has been shown to activate angiogenic programs in vascular endothelial cells (33;34). The results of our study show that these inflammatory mediators are present in the ST of all patient groups. CCL15/HCC-2 was expressed at higher levels in the ST of RA patients. Immunofluorescence double staining showed that more than half of the CD3+ T-cells and CD68+ macrophages and more than 70% of the CD55+ FLS co-expressed CCL15/HCC-2. This indicates that CCL15/HCC-2 may be an important contributor to cell migration in synovitis.

Taken together, the data indicate that activation of the chemokine network represents a pivotal common final pathway in synovial inflammation. The strong expression of CKRs in the ST may be explained by upregulation of the receptors or increased migration of cells expressing these receptors towards the inflammation, or a combination of both. The abundant expression of CCR1 and CCR5 in rheumatoid ST in combination with their decreased expression on monocytes from paired PB suggests a possible role in the migration of mononuclear cells from the PB toward the joints. Both monocytes and lymphocytes may be involved in this process.

In addition, based on our data, CCR1 also appears to play a role in other joint diseases. Interference with this mechanism may result in decreased infiltration of leukocytes into the joints and a subsequent improvement in signs and symptoms, as recently was described for the effects of a specific CCR1 antagonist in RA patients (8). Although other receptors and ligands are involved as well, blockade of CCR1 and CCR5 may be a potentially effective treatment for a variety of arthritides. This study provides the rationale for current and future functional studies and subsequently for clinical trials investigating the true potential of their application as therapeutic targets in patients.

**Competing Interests**
The authors have no competing interests to disclose.

**Author contributions**
JJH was contributed to the experiments and was responsible for data analysis and interpretation and wrote the manuscript.

TJMS and PRB were responsible for both the set-up and performance of the experiments. PPT was responsible for the planning of the work and contributed to data analysis, interpretation and write-up.

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**Statement**
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Legends to the Figures

**Figure 1 A.** Expression of the chemokine receptors CCR1, CCR2b, CCR5 and the CD13/Aminopeptidase N in synovial tissue of patients with RA, OA and ReA. Original magnification 400x, 400x, 400x, 200x respectively.

**Figure 1B.** Representative expression of the chemokines CCL2/MCP-1, CCL5/RANTES, CCL7/MCP-3 and CCL15/HCC-2 in synovial tissue of patients with RA, OA and ReA. Original magnification 400x, 200x, 400x, 400x respectively.

**Figure 2A.** Mean (±sem) percentage of double staining CD3+ T-cells, CD55+ FLS and CD68+ macrophages with the chemokines CCL7/MCP-3, CCL8/MCP2 and CCL15/hCC-2 in the synovium of patients with RA (n=5).

**Figure 2B.** Representative example of immunofluorescence double staining of CD55+FLS (green) and CCL7/MCP-3 (red) in synovial tissue of a RA patient (original magnification 1000x).

**Figure 3A.** Percentages (±sem) of CD14+ peripheral blood monocytes expressing the chemokine receptors CCR1, CCR3, CCR5, CCR6 and CCR7 in patients with RA (n=20), OA (n=16), ReA (n=8) and healthy controls (n=5).

**Figure 3B.** Representative flow cytometry dot plots showing chemokine receptor 1 (CCR1) expression on CD14+ cells in Healthy control and Rheumatoid Arthritis peripheral blood (PB) together with the isotype matched control (IgG2b). CD14+ monocytes were defined by allophycocyanin (APC) and side scatter properties. A gate was drawn around the CD14+ population and CCR1 positively staining cells were chosen by the phycoerythrin (PE) fluorescence over isotype-matched control (IgG2b).

Reference List


Figure 1A

- CCR1
- CCR2b
- CCR5
- CD13

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<thead>
<tr>
<th>RA</th>
<th>OA</th>
<th>ReA</th>
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Figure 1B

- CCL2
- CCL5
- CCL7
- CCL15

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