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Increased expression of CCL18, CCL19 and CCL17 by dendritic cells from patients with rheumatoid arthritis (RA) and regulation by Fc gamma receptors.

Extended report

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Keywords
Rheumatoid arthritis, CCL18 (DC-CK1), CCL19 (ELC), CCL17 (TARC), synovial tissue.
Abstract

Objective: Dendritic cells (DC) play an essential role in the regulation of both immunity and tolerance. DC orchestrate the attraction of inflammatory cells by the production of various chemokines (CK). Recently, it was suggested that Fc gamma receptors (FcγR) are involved in the regulation of DC function. The aim of the study was to assess the expression of a panel of CK by immature (iDC) and mature DC (mDC) and its regulation by FcγR both in RA patients and healthy donors (HC).

Methods: The expression CK by DC from RA patients and HC was determined using Real Time quantitative PCR and ELISA techniques. DC were derived from monocytes following standardized protocols. To study the potential regulation by FcγR, iDC were stimulated with immune complexes (IC) during LPS-induced maturation. The presence of CK was studied in synovial tissue from patients with RA, OA and healthy individuals by using RT-PCR techniques and immunohistochemistry.

Results: iDC from RA patients show markedly increased mRNA levels of the CK CCL18 and CXCL8. Upon maturation with LPS, the expression of CCL18, CCL19, CXCL8, CCL3 and CCL17 increased dramatically and reached significantly higher levels in RA patients. Monocytes failed to express these CK. IC mediated triggering of the FcγR on DC from RA patients with high disease activity resulted in a downregulation of all CK, whereas the opposite effect was seen upon stimulation of DC from RA patients with low disease activity and healthy donors. Moreover, CCL18, CCL19, CCL17 and CXCL8 are significantly increased in the RA synovial tissue.

Conclusion: In this study we provide evidence for elevated CK expression levels by DC in RA patients. The expression of these CK is, at least partly regulated by FcγR triggering. These data suggest a potential role for CK producing DC in RA pathogenesis.
Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease mainly characterized by inflammation of the synovial joints leading to progressive cartilage breakdown and destruction of underlying bone. Within both the synovial tissue and fluid there is a massive infiltration of activated leukocytes. Furthermore, in RA the synovium is characterised by features such as widespread hyperplasia and angiogenesis. Many factors have been implicated in this process including growth factors, adhesion molecules and chemokines. Chemokines (CK) are members of a complex superfamily of at least 50, low molecular weight (6-14 kDa), proteins of which the importance in autoimmune diseases, neoplasia and cardiovascular diseases have been demonstrated recently (1). CK play an essential part in the interaction between leukocytes and endothelial cells. CK induce the directional migration of cells in homeostatic and inflammatory conditions. Lymphocyte homing to secondary lymphoid tissue, frequently present in RA synovium, involves a sequela of events which is believed to be tailored by CK (2).

Dendritic cells (DC) are producers of a large set of different CK (1;3;4). DC are professional antigen presenting cells and play a major role in the orchestration of both innate as well as adaptive immune responses. In the immature state, DC (further designated as iDC) are highly specialized in antigen uptake and processing whereas upon maturation DC (further designated as mDC) become fully equipped for antigen presentation (5-7). Intriguingly, these different functions are also performed at different anatomical sites in the body. IDC are scattered throughout the body and are therefore often referred as “sentinels of the immune system”. Upon maturation however, DC migrate to secondary lymphoid organs to attract and interact with T cells. The trafficking of DC, T cells and other lymphocytes during inflammatory processes is a key event and is critically dependent on the production of CK and expression of their corresponding receptors. IDC, produce low levels of inflammatory chemokines (e.g. DC-CK1 (CCL18), ELC (CCL19), MDC (CCL22), TARC (CCL17)) and express the inflammatory chemokine receptors CCR1, CCR2, CCR5 CXCR2 and CCR8 (8). Upon triggering by inflammatory mediators such as TNF-α and IL-1, DC mature and down-regulate the inflammatory receptors while upregulating CCR7. In addition, mature DC produce large amounts of CK to attract T cells, B cells, monocytes and macrophages.

DC have also been implicated to play a role in RA (9-13). Furthermore, inflamed rheumatoid synovium has several characteristics resembling secondary lymphoid tissue suggesting a role for CK (14;15). However the involvement and role of DC and DC produced CK in these processes during RA remains to be clarified (16-19). Until now, the role of chemokines in RA has mainly focused on the CK fractalkine (CXC3CL1)(18), IL-8 (CXCL8) (20-22), RANTES (CCL5) (23), MCP-1 (CCL2) (24) and MIP-1α (CCL3) (25) and their related receptors.

For antigen uptake DC use a wide variety of molecules including Fc gamma receptors (FcγR). FcγR bind IgG molecules or IgG containing immune complexes (IC) which are abundantly present in serum and synovial fluid from RA patients. All FcγR except for FcγRIIb mediate cellular activation signals upon triggering (26;27). Triggering of these receptors plays a central role in immune mediated tissue injury and the immunological outcome and is partly dependent on the balance between activating and inhibitory FcγR (28). The balance between activating or inhibiting FcγR has been shown to effect the Th1/Th2 balance (29). We have demonstrated recently that DC from RA patients express elevated levels of FcγRIIb compared with those of healthy controls (30). This altered balance led to aberrant production of pro-
inflammatory and anti-inflammatory cytokines such as TNF-α, IL-6 and IL-10 upon incubation with IC (30;31).

In the present study we explored whether CK production by DC is altered in RA. Furthermore, we investigated the potential role of FcγR in the regulation of chemokine expression by DC, including several DC specific and non-specific CK. The results demonstrated an increased expression of chemokines by DC obtained from RA patients in comparison to those of healthy individuals. Furthermore, we show that DC from patients with active disease, decreased their CK production upon triggering of FcγR by IC.

**Materials and methods**

**Patients and samples**

Peripheral blood mononuclear cells (PBMC) were obtained from 26 patients with RA attending the outpatient clinics of the University Medical Center (UMC) Nijmegen. A total of 22 healthy individuals were included as control in the present study. All patients fulfilled the American College of Rheumatology criteria for RA and gave informed consent for the study (32). The activity of the disease was assessed using the 28 joint disease activity score (DAS) (33). For the present study, RA patients having a DAS of > 3.2 were considered as active RA patients. Remission in this study was characterized as having a disease activity lower than 2.6. The number of DMARDs used by RA patients were taken into account and patients which used prednisolone or biological agents such as TNF-α and IL-1 blocking agents were excluded. For immunohistochemical analysis, synovial biopsy specimens were obtained from 3 RA and osteo-arthritis patients using and 3 healthy individuals. Synovial tissue from RA patients was obtained by using percutaneous biopsies of the knee joint by using a Parker Pearson needle under local anesthesia. An average of 30 biopsy samples were obtained at each occasion. The synovial tissue from healthy individuals and patients with osteo-arthritis was isolated during arthroscopic procedures performed by the orthopaedic surgeons.

For determination of synovial chemokine mRNA expression, synovial tissue of 5 RA patients, 5 patients with osteo-arthritis and 5 healthy controls were compared. These synovial samples were obtained by using small needle arthroscopic procedures and were kindly gifted by Dr. B. Ostendorf (Dusseldorf, Germany). Synovial fluid of RA patients (n=7), OA patients (n=5) and patients with gout (n=5) were collected during arthroscopic procedures or joint pucture at our centre. The study was approved by the Ethics Committee of the University Medical Center in Nijmegen.

**Generation and culture of monocyte-derived DC**

Monocyte-derived dendritic cells were cultured using standardized protocols as previously described (30). Briefly, PBMC were isolated from heparinised venous blood by using density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Roosendaal, The Netherlands). The interphase was collected and washed with citrated PBS whereafter the cells were allowed to adhere for 1 hour at 37°C in RPMI-1640 (Life Technologies, Breda, The Netherlands) supplemented with 2% human serum in 6-well culture plates (Costar, Badhoevedorp, The Netherlands). Adherent monocytes were cultured in RPMI-1640 Dutch modification supplemented with 10% fetal calf serum (FCS) and antibiotic-antimycotic (Life Technologies) in the
presence of IL-4 (500 U/ml, Schering-Plough, Amstelveen, The Netherlands) and GM-CSF (800 U/ml, Schering-Plough) for 6 days. Fresh culture medium with the same supplements was added at day 3 whereafter iDC were harvested at day 6. To generate mDC, iDC were resuspended in fresh cytokine-containing culture medium and transferred to new 6 well culture plates. For the maturation process, 2µg/ml LPS (\textit{E. coli}, Sigma, Chemical Co., St. Louis, MO) was added. mDC were harvested after two more days of culture.

**Phenotypic characterization of DC**

Expression of cell surface markers on both iDC and mDC was measured by indirect immunofluorescence staining as described in detail previously \(30\). Briefly, the first layer was attached after incubation of \(1*10^5\) monocyte-derived DC for 30 min at \(4°C\) with the monoclonal anti-human antibodies against, CD14, CD16, CD32, CD64 (all DAKO), DC-SIGN (8), CD80 (Becton Dickinson), CD83 (Coulter), CD86 (Pharminen), MHC-I (clone W6/32), MHC-II DQ (clone SPV-L3) and MHC-II DP/DR (clone Q1513). After a washing step, the cells were incubated with fluorescein isothiocyanate conjugated goat anti-mouse IgG (GAM-FITC) for 30 min at \(4°C\) in complete darkness. Cells were gated according to their forward- and side-scattering patterns. For each marker \(10^4\) cells were counted in the gate.

**Purification of monocytes**

The monocytes used for detection of chemokine mRNA expression were purified by density gradient centrifugation and immunomagnetic separation techniques (MACS). At first, the pellet originating from the Ficoll gradient was resuspended and incubated with CD14 positive labeled MACS microbeads (Miltenyi Biotec, Bergische Gladbach, Germany). Positively labeled cells were then isolated using separation columns that were placed in a strong magnetic field. After removal of the magnetic field, the cells were flushed out using RPMI-1640 supplemented with 10% FCS, 1% glutamate and 1% antibiotic-antimycotic (all life Technologies). Thereafter, the cells were centrifuged and the pellet was solved in the Trizol reagent and stored at -70°C.

**Chemokine mRNA expression by DC after stimulation with heat aggregated gamma globulins (HAGG).**

In certain experiments DC were cultured in the presence of heat aggregated gamma immunoglobulins (HAGGS) which bind and crosslink FcγR. HAGGS were obtained by heating human IgG (Sigma Chemicals) at 63°C for 30 min. Thereafter the solution was centrifuged at 12000 G for 10 min and the concentration of HAGGS was determined by reading the absorbance at 280 nm. HAGGS (final concentration 100µg/ml) were then added to the culture medium for 48 hours.

**Primers and probes**

The sequence of the primers (Life technologies) and Taqman™ probes (PE Biosystems, Branchburg, New Jersey) used in this study are given in table 1. The chemokine-specific probes were labeled at the 5’ end with a FAM fluorescent group and at the 3’ end with a TAMRA quencher group. The probes specific for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinoogen deaminase (PBGD) were labeled with a VIC fluorescent group at the 5’ end.
Table 1. Primers and Taqman™ probes used for real-time quantitative PCR analysis (4)

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>5' primer</th>
<th>3'</th>
<th>5' probe</th>
<th>3'</th>
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<tr>
<td>DC-CK1 (CCL18)</td>
<td>CCTGGAGGCGCACCTCTTCTAA</td>
<td>AGTCCCATCTGCTATGCCCAGCCAC</td>
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<tr>
<td>ELC (CCL19)</td>
<td>CAGAGGACCTCAGCCAAGATG</td>
<td>CCTATGACCGGAGGAGGAGCC</td>
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<tr>
<td>IL-8 (CXCL8)</td>
<td>AGAAGTTTCTGAGGGCTGAGA</td>
<td>TCCAAGATCTGAGATGAGGAGCCAG</td>
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<td></td>
</tr>
<tr>
<td>Lymphotactin (XCL1)</td>
<td>GGACAGGAAATCACCACAGAGACTACT</td>
<td>CAACAGGAGGAGGAGGAGCC</td>
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<td></td>
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<tr>
<td>MDC (CCL22)</td>
<td>GTCTCGTCCCATCAGCGTGAT</td>
<td>CCATGACTCCCACACTGGAAGCT</td>
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<tr>
<td>MIP-1α (CCL3)</td>
<td>TGTGTTTGTGATTTGTCATCTGA</td>
<td>CTTCCCTCACACCCCGCTCTGA</td>
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<tr>
<td>MIP-3α (CCL20)</td>
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<td>TCCATGACTTGCAAGTGAAGAGCCTCA</td>
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<tr>
<td>SDF-1α (CXCL12)</td>
<td>ACTAAACCTTGGAGAGATGA</td>
<td>CTTAAACCAGGAGGACAGGT</td>
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<td>SDF-1β (CXCL12)</td>
<td>ACAAGAGGTTCAGAATGAGTGA</td>
<td>TAGGAGCAGCTCTGAAACCAG</td>
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<tr>
<td>SLC (CCL21)</td>
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<td>ACTGTCCTTCTGAGAGGAGACCA</td>
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<td></td>
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<tr>
<td>TARC (CCL17)</td>
<td>GGGAGCCCTATCAGGTCGA</td>
<td>GGA</td>
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| GAPDH | GGAAGGTGGATGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
Measurement of chemokines in culture supernatant, synovial fluid and serum
Chemokine protein level of CCL18 was measured in the supernatant of the cell cultures, synovial fluid and serum of patients with RA and healthy individuals. The secretion of CCL18 was analyzed using a specific sandwich ELISA as previously described (4). In the CCL18 ELISA, mouse anti-human CCL18 mAb (AZN-CK18B) and goat anti-human PARC Ab (R&D systems, Abingdon, UK) were used as primary and secondary antibody respectively. The detection antibodies were stained with horse radish peroxidase (HRP)-conjugated donkey anti-goat IgG (Jackson Immunoresearch Lab Inc, West Grove, PA, USA) or HRP-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc, San Fransisco,CA). HRP was visualized by incubation with TMB-substrate. Thereafter, the absorption was measured at 450 nm using a Titertek multiscan ELISA reader (Bio-Rad Laboratories, Veenendaal, The Netherlands). The detection limit of the ELISA was 40pg/ml.

Immunohistochemical analysis of CCL18 expression in synovial tissue
Percutaneous synovial tissue biopsies of RA knee joints and synovial tissue from controls obtained during arthroscopic procedures were embedded in Tissue Tek OCT (Miles Inc) and snap frozen in liquid nitrogen. Cryostat sections (7 µm) were mounted on superfrost slides and stored at -70°C until processing. Immunohistochemical localization of CCL18 (TIL) and CD32 (FcγRII, DAKO, Glostrup, Denmark) was performed using serial sections. Briefly, cryostat sections were dried, fixed in 100% cold acetone (10 min) and dried again. Thereafter, endogenous peroxidase was blocked using H2O2 in PBS and incubated with the first antibody (5 µg/ml, 60 min at RT). Subsequently, sections were incubated with normal horse serum and with biotinylated horse anti-murine IgG (Vectorlabs, Burlingame, CA, USA) and ABC complexes ( Vectastain, Vectorlabs). Slides were developed with diaminobenzidine and counterstained with hematoxylin for 3 minutes. Controls consisted of irrelevant primary isotype-specific IgG1 and IgG2a (DAKO) and omission of the secondary antibodies.

Statistical analysis
Differences between the DC from RA or healthy controls were calculated by using Mann-Whitney U test and Students T-test as appropriate. P values were two-sided and the level of significance was set at P < 0.05. The Wilcoxon matched pairs test was used to calculate the effects of HAGG stimulation on paired samples. For statistical analysis, Graphpad Prism® version 4 was used.

Results
Chemokine expression by immature and mature dendritic cells
We evaluated the expression of a set of 11 CK known to be abundantly expressed by DC (table 1). Expression levels were related to GAPDH and presented relative to another house keeping gene PBGD which was set to one. We first analyzed chemokine expression by iDC which were characterised by high expression of FcγRI, II and III and DC-SIGN, low levels of MHC-I and II molecules and CD80 and CD86, and absence of CD83 (data not shown)(30). iDC of RA patients (n=16, mean DAS 5.71 ± 1.2) produce low levels of the chemokines ELC (CCL19) and MIP-1α (CCL3)
and high levels of MDC (CCL22) and TARC (CCL17) which were similar when compared with DC from healthy controls (n=12) (figure 1). However, expression of CCL18 (35-fold, $P < 0.0001$) and IL-8 (CXCL8) (6-fold, $P = 0.02$) was significantly higher in iDC from RA patients compared to their normal counterparts. The chemokines SDF-1α (CXCL12) SLC (CCL21), MIP-3α (CCL20) and lymphotactin (CXCL1) were expressed at lower levels then PBGD (not shown).

As expected, stimulation of iDC with LPS for 48 hours resulted in fully matured DC (mDC) which express high levels of co-stimulatory, MHC molecules and CD83 (data not shown). After full maturation the mRNA CK expression by DC from RA patients and healthy controls increased dramatically except for CXCL8 and CCL3 (figure 1). The expression of CCL18 (46-fold, $P < 0.0001$), CCL19 (19-fold, $P < 0.0001$) CXCL8 (62-fold, $P < 0.01$), CCL3 (13-fold, $P = 0.0002$) and CCL17 (5-fold, $P < 0.0001$) was significantly higher in mDC from RA patients compared to those from healthy donors. CCL22 expression also increased upon maturation but the increase was similar in RA and controls. As with iDC, the expression of CCL21, CXCL12, CCL20 and XCL1 was low or not detectable (data not shown).

**Chemokine expression and relation to disease activity**

To investigate whether the increased expression of CK in RA patients was related to the disease activity we studied the CK expression in another group of patients having active RA (n=10, mean DAS 6.1 ± 1.6) and compared that with patients with RA in remission (n=6, mean DAS 2.3 ± 0.2) and healthy individuals (n=10). As shown in figure 2a, CCL18 ($P = 0.0002$) and CXCL8 ($P = 0.003$) expression is significantly increased by iDC from active RA patients compared with DC from RA patients in remission and controls. The same phenomenon was observed when CK expression profiles of CCL18 ($P = 0.0002$), CCL19 ($P = 0.0002$), CXCL8 ($P = 0.0002$) and CCL3 ($P = 0.005$) of mDC was related to disease activity (figure 2b). CCL17 was the only chemokine which was significantly higher expressed in patients both with active (6-fold, $P = 0.02$) and inactive (2-fold, $P = 0.03$) RA in comparison with their normal counterparts. Of note, no differences between the number of DMARDs or NSAIDs used between patients with active or inactive disease could be detected.

**Chemokine expression by monocytes**

Since we used monocyte-derived DC in our study, we investigated whether the increased expression of several chemokines was specific for DC or already present on their precursor cells. Therefore we purified monocytes and subsequently analyzed their CK expression profile. In line with the literature CXCL8 (6-fold, $P = 0.03$) and CCL3 (6-fold, $P = 0.01$) were expressed at significantly higher levels in RA (figure 3). The chemokines CCL18, CCL19, CCL20 and CCL17 were produced at very low levels whereas CCL21, CXCL12 and XCL1 were not detectable.

**Diminished chemokine production in RA after immune complex stimulation.**

In a previous study we demonstrated that DC from RA patients express FcγRII at increased levels resulting in an aberrant production of TNF-α upon FcγR mediated triggering of these DC (30). In accordance with this finding, in this study, DC from active RA patients (n=9) expressed significantly higher FcγRII levels (MFI ± SD; 410 ± 61) when compared with those having inactive disease (n=5, 233 ± 89, $P = 0.008$) and healthy donors (n=6, 151 ± 36, $P = 0.002$) (data not shown). No other DC phentotypic differences were observed between the groups. To determine whether chemokine production is affected by triggering of FcγR receptors, we stimulated both
iDC and DC during maturation (LPS stimulation) via FcγR dependent pathways by using heat aggregated IgG immunoglobulins (HAGGS). The stimulation of FcγR resulted in a significantly decreased expression of CCL18 (3-fold, \( P = 0.03 \)), CCL22 (8-fold, \( P = 0.04 \)), CCL17 (5-fold, \( P < 0.01 \)), CCL19 (3-fold, \( P < 0.01 \)) and CXCL8 (2-fold, \( P = 0.02 \)) by DC from RA patients (figure 4). Although not significant, the same trend was observed for CCL3 (2-fold, \( P = 0.1 \)). In contrast, the opposite effect was seen for CCL18 (4-fold, \( P = 0.04 \)), CCL17 (2-fold, \( P < 0.01 \)), CCL19 (2-fold, \( P = 0.04 \)), CXCL8 (17-fold, \( P = 0.02 \)) and CCL3 (3-fold, \( P = 0.02 \)) in healthy donors (figure 4). CCL22 was the only chemokine in which a decreased expression (4-fold, \( P = 0.01 \)) was observed after FcγR triggering in both groups suggesting different mechanisms responsible for the expression of various chemokines. Noteworthy is the fact that despite a clear decreased expression upon stimulation with IC in RA and the opposite effect in healthy donors the absolute level of CK expression by DC was still higher in RA.

Chemokine mRNA expression in RA synovial tissue
Since DC of patients with active RA express extremely high levels of CCL18, CCL19, CCL17 we examined the expression of these three chemokines as well as of CXCL8 in RA synovial tissue (n=5) and compared this with synovial tissue of patients with osteoarthritis (n=5) and healthy individuals (n=5). As illustrated in figure 5, CCL18 (234-fold, \( P < 0.001 \)) and CCL19 (406-fold, \( P < 0.001 \)) are expressed at significantly higher levels in RA synovium when compared with synovial tissue of healthy individuals. Furthermore, synovial tissue from patients with osteoarthritis showed a significantly higher expression of CCL19 (110-fold, \( P = 0.03 \)) than in healthy synovial tissue but still less than in active RA patients. Although the same trend was observed for CCL17 (3-fold, \( P = 0.06 \)) it failed to reach the level of significance. CXCL8 was highly expressed in RA synovial tissue (36-fold, \( P = 0.03 \)), which is in concordance with the literature.

Protein levels of CCL18 in supernatant and synovial fluid of RA patients and healthy controls.
Since CCL18 was highly expressed by cultured DC and in the synovium of patients with active RA, the secretion of CCL18 protein was tested in the supernatant, synovial fluid and serum of RA patients and healthy controls. In the DC supernatant, the CCL18 protein production paralleled the expression of CCL18 mRNA as measured by real-time PCR (figure 6a). CCL18 production was significantly higher in both iDC (12-fold, \( P = 0.02 \) and 17-fold, \( P = 0.01 \)) and mDC (25-fold, \( P < 0.01 \) and 4-fold, \( P = 0.02 \)) from RA patients when compared with that of patients with inactive disease and healthy individuals, respectively. Triggering of FcγR using IC led to a decreased production of CCL18 protein (median 63%, \( P = 0.02 \)) in 8 of the 9 RA patients tested with active RA whereas an increase (median 137%, \( P = 0.04 \)) in CCL18 protein production was observed in 6 healthy individuals which is in line with the real-time PCR data (figure 6b). Furthermore, high CCL18 protein levels were present in synovial fluid of patients with active RA (n=7) (Med [range]; 1100 pg/ml [800-1340]) when compared with synovial fluid of patients with gout (n=5) (300 pg/ml [150-473]) and osteo-arthritis (n=5) (410 pg/ml [254-684]) (data not shown).

CCL18 in synovial tissue, an immunohistochemical analysis
So far, we have shown that CCL18 is secreted in much higher quantities by mature DC from patients with RA, that the mRNA expression of this chemokine is highly
expressed in RA synovial tissue, and the levels in synovial fluid of RA patients was increased when compared with that of controls. To analyse whether the expression of CCL18 in RA synovial tissue was increased when compared with synovium of OA patients and healthy controls, consecutive sections of synovium from 3 RA and osteo-arthritis patients and healthy controls were stained for CCL-18 and DC-LAMP, a marker for mature DC. As shown in figure 7, CCL-18 was highly expressed in the perivascular regions and showed an overlapping pattern with DC-LAMP both in rheumatoid and osteoarthritis synovium which is in line with the high production of DC-CK1 by fully matured DC. In contrast, healthy individuals showed hardly any presence of CCL-18 and DC-LAMP positive cells were essentially absent.

Discussion

In the present study we investigated the involvement of DC specific and non-specific chemokines in the disease process of RA. We demonstrate for the first time that both immature monocyte derived DC and mature DC from active RA patients express significantly higher levels of CCL18 (DC-CK1), CCL19 (ELC), CXCL8 (IL-8), CCL3 (MIP-1α) and CCL17 (TARC) when compared with those of healthy individuals. Intriguingly, this increased expression was present exclusively during the active phase of the disease. To confirm these findings at the site of inflammation we also investigated the level of mRNA expression of CCL18, CCL19, CXCL8 and CCL17 in synovial tissue and showed that patients with RA have faremost the highest levels in comparison with osteoarthritis patients or healthy individuals. Next to this, we confirmed this result by showing a high expression of the protein CCL18 at the protein level. Analysis of supernatants of DC cultures and synovial fluid of patients with RA and controls confirmed the elevated production of CCL18 at the protein level. These data clearly showed an increased production of CCL18 by iDC and mDC exclusively from patients with active RA. The increased production of CCL18 in RA was further substantiated by the finding that a significantly higher level of this pivotal chemokine is present in the synovial tissue of these patients which show an overlapping pattern with mature DC.

Dendritic cells-specific chemokine 1 (DC-CK1, also known as PARC or CCL18), is a chemokine preferentially produced by DC and involved in the selective chemoatraction of CD45RA T lymphocytes and CD38-B cells (3;36). CCL19 (Epstein-Barr virus induced molecule 1 ligand, or MIP-3β) is primarily involved in the CCR7 mediated triggering of lymphocyte arrest and plays a pivotal role in the recruitment and localization of lymphocytes within secondary lymphoid tissues (37;38). Thymus and activation-regulated cytokine (TARC, CCL17) is able to attract CCR4 positive cells which are mainly Th2 cells and therefore, appears to be important in the development of an acquired Th2 response. The attraction of different effector cells to the site of inflammation might be important in RA pathology and possibly contribute to the formation of secondary lymphoid follicles that are often observed in RA patients. Indeed, there is accumulation of T cells, B cells, monocytes as well as follicular dendritic cells in RA synovium and they often form lymphocyte aggregates which reflects certain features of germinal centers (GC) (2;16;39;40). Recently, it was demonstrated that a complex interplay between T cells, DC and probably B cells results in the formation of secondary lymphoid follicles in rheumatoid synovium (41;42). Interestingly, analysis of CCR7 deficient mice revealed severe morphological alterations in all secondary lymphoid organs (43;44). Therefore, it is
tempting to speculate that the abundant expression of CCL18 and CCL19 in RA synovium might be part of the explanation why lymphoid structures arise in RA patients. Especially, the expression of IL-4 and IL-13 in the synovial compartment is intriguing as they are known to strongly induce/upregulate the expression of the expression of CCL18 (3;45). The observation that the elevated production of chemokines is exclusively present during the active phase of disease is interesting and provides support to recent data suggesting the presence of specific environmental factors during the active phase (11;46;47). Unfortunately, these studies did not address differences between active or inactive disease states. Another interesting observation in the present study was the decreased expression of chemokines observed after 48 hours of FcγR engagement DC from active RA patients. In contrast, FcγR dependent stimulation of DC from healthy individuals resulted in the opposite effect, namely a clear increased expression of chemokines. The latter is consistent with the observation that stimulation of monocytes with IC from healthy donors induced an increased expression of MIP-1α, MIP-1β and IL-8 mRNA levels (48). We hypothesize that the opposite effects between DC from RA patients with active RA and healthy donors upon engagement of FcγR is the result of an altered FcγR balance in patients with active disease. This is in agreement with our previous report in which we demonstrate a clearly increased expression of the inhibitory FcγRII, exclusively during the active phase of disease (30). However, despite the clear decrease in CK expression in RA upon FcγR triggering and opposite effect in healthy donors, the level of chemokine expression was still much higher in RA. Our results are in line with recent findings which suggest an dominant role for both the activation and inhibitory FcγR and their concerted actions in determining the balance between Th1/Th2 responses and the discrimination between immunity and tolerance (29; 49-51). In fact, systemic injection of DC genetically modified to express IL-4 or IL-13, cytokines known to regulate FcγR balances towards the inhibitory subtype, completely abrogated the detrimental effects of arthritis even after the development of a full blown collagen induced arthritis (52;53).

In conclusion, our study strongly supports the proposed mechanisms by which DC and their expression of chemokines are involved in the pathogenesis of RA. Our data further strengthen the potential important role of these chemokines in the inflammatory process, and indicate that the potential blockade of these chemokines is a highly interesting novel therapeutic approach. Detailed knowledge concerning the role and mode of action of CK in RA might herald new opportunities for the manipulation of (auto) inflammatory responses in auto-immune diseases such as RA.
Legends to the figures

Figure 1. Chemokine mRNA expression by immature and mature monocyte-derived dendritic cells from RA patients and healthy controls. Chemokine expression of immature DC of RA patients (black bars) healthy individuals (light grey bars) and after full maturation (RA patients (dark gray bars) and healthy controls (white bars)). The mRNA expression is related to PBGD expression set to level 1. Note the log-scale of the Y-axis. The mean level of mRNA expression is indicated with the number above the bar.

Figure 2. Chemokines expressed by iDC and mDC in relation with RA disease activity. a) chemokine mRNA expression by immature DC from patients with active RA (black bars, n=10), with inactive disease (grey bars, n=6) and healthy controls (white bars, n=12). b) Production of chemokines by mature DC from the same group of active (black bars), inactive (grey bars) and healthy donors (white bars). * P-values lower than 0.05. The mRNA expression is related to PBGD expression set to level 1. Note the log-scale of the Y-axis. The mean level of mRNA expression is indicated with the number above the bar.

Figure 3. Chemokine expression by monocytes from RA patients and healthy controls. Relative expression of chemokines CXCL8 and CCL3 by monocytes of patients with active RA (black bars, n=5) and healthy donors (white bars, n=5), respectively. * P-values lower than 0.05. The mRNA expression is related to PBGD expression set to level 1. Note the log-scale of the Y-axis. The mean level of mRNA expression is indicated with the number above the bar.

Figure 4. Chemokine mRNA expression after triggering of FcγR on mature DC. Level of CCL18, CCL22, CCL17, CCL19, CXCL8 and CCL3 mRNA expression in the absence (black bars) or presence (white bars) of immune complexes by mature DC from RA patients (n=8) and healthy controls (n=6), respectively. * P-values lower than 0.05. The mRNA expression is related to PBGD expression set to level 1. Note the log-scale of the Y-axis. The mean level of mRNA expression is indicated with the number above the bar.

Figure 5. Expression of chemokines in synovial tissue from patients with RA compared with patients with osteoarthritis and healthy controls. mRNA expression level of the chemokines CCL18, CCL19, CCL17 and CXCL8 in synovial tissue from RA patients (black bars, n=5), patients with osteoarthritis (grey bars, n=5) and healthy donors (white bars, n=5). * P-values lower than 0.05. The mRNA expression is related to PBGD expression set to level 1. Note the log-scale of the Y-axis. The mean level of mRNA expression is indicated with the number above the bar.

Figure 6. CCL18 (DC-CK1) production by immature and mature DC of RA patients and healthy individuals before and after stimulation with anti-IgG complexes (HAGGS). a) CCL18 protein production in supernatants of immature and mature DC from patients with active RA (black bars, n=9), inactive RA (grey bars, n=5) and healthy donors (white bars, n=6). b) CCL18 secretion by iDC after stimulation with LPS alone or LPS + HAGGS in patients with active RA (left panel, n=9) and healthy donors (right panel (n=6).
Figure 7. Expression of CCL18 (DC-CK1) in synovial tissue of patients with RA, OA and healthy donors. The top row represents immunostaining for CCL18 of synovial tissue from a RA patient, a patient with osteoarthritis and healthy donor, respectively. The bottom row corresponds with immunostaining for DC-LAMP from the same individuals.

Reference list


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Figure 1. Chemokine expression of immature and mature dendritic cells obtained from RA patients compared with those of healthy controls.
Figure 2. Expression of chemokines in relationship with RA disease activity by immature DC (panel a) and mature DC (panel b) compared with that of healthy donors.

<table>
<thead>
<tr>
<th></th>
<th>Active RA</th>
<th>Inactive RA</th>
<th>Healthy control</th>
</tr>
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<tbody>
<tr>
<td><strong>CCL18</strong></td>
<td>673</td>
<td>39</td>
<td>19</td>
</tr>
<tr>
<td><strong>CXCL8</strong></td>
<td>1000</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

* P-values < 0.05

*P-values*
**Figure 3.** Chemokine expression by monocytes from RA patients and healthy controls.

- **CXCL8**
  - RA patients: 80
  - Healthy controls: 14

- **CCL3**
  - RA patients: 143
  - Healthy controls: 26

* *P*-values < 0.05
Figure 4. Expression of chemokines by DC upon LPS-induced maturation without or with the presence of immune complexes (IC).

- **CCL18**
  - DC maturation without IC: RA 1985, C 599
  - DC maturation with IC: RA 20, C 81

- **CCL19**
  - DC maturation without IC: RA 55, C 20
  - DC maturation with IC: RA 5, C 11

- **CCL22**
  - DC maturation without IC: RA 3396, C 416
  - DC maturation with IC: RA 1475, C 408

- **CCL17**
  - DC maturation without IC: RA 4377, C 805
  - DC maturation with IC: RA 932, C 1513

- **CCL3**
  - DC maturation without IC: RA 30, C 17
  - DC maturation with IC: RA 3, C 8

- **CXCL8**
  - DC maturation without IC: RA 39, C 5
  - DC maturation with IC: RA 4, C 0.6
Figure 5. Expression of chemokines in synovial tissue from RA patients, OA patients and healthy donors.

* P-values < 0.05
Figure 6. CCL18 protein secretion by immature and mature DC from RA patients (panel a). Secretion of CCL18 upon LPS-induced maturation on the absence or presence of immune-complexes.
Increased expression of CCL18, CCL19, and CCL17 by dendritic cells from patients with rheumatoid arthritis and regulation by Fc gamma receptors

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