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Chemokine receptor blockade in rheumatoid arthritis

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Why did CCR2 and CCR5 blockade fail in rheumatoid arthritis?

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ABSTRACT

Objective: The aim of this study was to investigate why CCR2 as well as CCR5 blockade failed in the treatment of rheumatoid arthritis (RA) by using an *in vitro* monocyte migration system model.

Methods: Monocytes from healthy donors (HD; n = 8) or from RA patients (for each chemokine receptor antibody n = 8) were isolated from peripheral blood and pre-incubated with different concentrations of either anti-CCR2 or anti-CCR5 blocking antibodies (or medium or isotype controls) prior to chemotaxis. Chemotaxis was induced by CCL2/MCP-1 (CCR2 ligand), or by CCL5/RANTES (CCR5 ligand), or by a mix of 5 RA synovial fluids (SFs) and compared to chemotaxis medium alone.

Results: Anti-CCR2 antibody treatment blocked CCL2/MCP-1-induced chemotaxis of both HD and RA monocytes compared to isotype control. Similarly, anti-CCR5 antibody treatment blocked CCL5/RANTES-induced chemotaxis of RA monocytes. However, none of the blocking antibodies was able to block SF-induced monocyte chemotaxis.

Conclusion: The RA synovial compartment contains several ligands for both CCR2 and CCR5 as well as other chemokine receptors involved in monocyte recruitment to the site of inflammation. The results indicate that redundancy might indeed account for the observed chemokine receptor blockade failure in clinical trials.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by massive infiltration of synovial tissue and synovial fluid with immune cells that is mediated by chemokines and adhesion molecules(1;2). It is well accepted that monocyte/macrophage numbers are increased in clinically affected joints and these numbers correlate with the clinical signs and symptoms(3). Accordingly, clinical improvement after effective antirheumatic therapy is consistently associated with reduced macrophage numbers in the synovium(4). Taken together, synovial macrophages are considered key effector cells in the pathogenesis of RA(5;6).

Chemokines play an important role in the accumulation of these cells at the site of inflammation. They belong to a superfamily of small (6-14 kDa) structurally related proteins that regulate the traffic of various leukocytes (7). Inflammatory chemokines are expressed in inflamed tissues by resident and infiltrated cells upon stimulation by pro-inflammatory mediators present in situ. RA synovial tissue and fluid contain high concentrations of a variety of inflammatory chemokines (1;8)(9-13) that can interact with chemokine receptors on the surface of monocytes/macrophages contributing to their accumulation at these sites. Specifically, CCR2 (major ligand CCL2/MCP-1) and CCR5 (ligands CCL3/MIP-1 α , CCL5/RANTES and CCL7/MCP-3) are abundantly expressed by RA monocytes/macrophages (1;10) suggesting that interference with the migration of these cells by cytokine receptor blockade might be a successful therapeutic approach to reduce synovial inflammation. Although CCR2(14) and CCR5(15) receptor blockade has shown positive results in animal models of RA, targeted CCR2(16) and CCR5(17;18) blockade was not effective in RA patients. Therefore, we investigated the effect of specific CCR2 or CCR5 blocking antibodies on monocyte migration in an in vitro model, to provide insight into the question as to why CCR2 and CCR5 blockade may have failed in RA patients.

MATERIAL AND METHODS

Patients

Peripheral blood was obtained from RA patients (19) with active disease, defined by the presence of at least one clinically inflamed joint (for each CCR antibody n = 8) and healthy subjects (n = 8). None of the patients was being treated with biologicals. The study protocol was approved by the Medical Ethics Committees of the AMC/University of Amsterdam and all patients gave their written informed consent. Demographic and clinical features are shown in Table 1.

Table 1. Demographic and clinical data of patients

	Anti-CCR2	Anti-CCR5
Sex, female/male (n)	8/8	7/8
Age in years, mean (range)	56.1 (44-72)	57.1 (41-78)
Disease duration, mean (range)	35.5 (2-108)	46 (4-120)
Rheumatoid factor positive, n (%)	4 (50%)	7 (87.5%)
ACPA positive, n (%)	4 (50%)	6 (75%)
SJC, mean (range)	6.7 (0-13)	1.6 (0-3)
TJC, mean (range)	8.5 (0-15)	5.8 (0-15)
ESR mm/h , mean (range)	21.6 (7-62)	32.7 (5-110)
CRP mg/liter, mean (range)	6.7 (1-21.7)	3.6 (2-4.8)

ACPA, anti-citrullinated protein/peptide antigens; SJC, swollen joint count; TJC, tender joint count; ESR, erythrocyte sedimentation rate; CRP, C reactive protein.

Monocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient as previously described (20). Monocytes were purified by negative selection using Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. The purified cells were > 95% pure as determined by FACS analysis. Isolated cells were phenotyped using: CD3-FITC (BD Biosciences, Oxford, United Kingdom), CD14-APC (BD Biosciences) and one of the following chemokine receptors CCR2-PE (R&D systems, Abingdon, United Kingdom) or CCR5-PE (BD Biosciences).

Neutralizing antibodies and isotype controls

The following neutralizing antibodies were a gift from Millennium Pharmaceuticals Inc.: mouse anti-human CCR2 (mouse IgG2a; clone m1D9) and mouse anti-human CCR5 (mouse IgG1; clone 2D7). Functional grade mouse IgG1 and mouse IgG2a antibodies were used as isotype controls (both from eBioscience, San Diego, CA) for CCR5, and for anti-CCR2, respectively.

In vitro chemotaxis

Monocytes were first washed in chemotaxis medium (PBS with 1% albumin low toxin), incubated for 30 minutes in the absence or in the presence of various concentrations of anti-CCR antibodies (anti-CCR5: 1 or 5 μ g/ml; anti-CCR2: 1, 5 or 25 μ g/ml) or respective isotype controls (25 or 5 μ g/ml). After incubation, 1×10^5 monocytes were transferred into the upper chamber of 5 μ M pore size transwell plates (96 well ChemoTX[®], NeuroProbe, Gaithersburg, MA). Chemotaxis medium was added to the lower chamber together with recombinant chemokines CCL2/MCP-1 (100 ng/ml; R&D systems) or CCL5/RANTES (500 ng/ml; Peprotech, Rocky Hill, NJ) or pooled RA synovial fluid (SF; 50% diluted in chemotaxis medium, n = 5 RA patients). After 2 hours at 37°C, migration was quantified by staining the cells that were attached to the

membrane. Briefly, after aspiration and removal of the cells from the top wells the membrane was fixed in pre-chilled methanol (bottom side down) followed by addition of DAPI solution to the membrane. After the membrane was dried, it was mounted on an OptiPlate (bottom side up) and the number of DAPI positive cells (cells that were trapped in the membrane = migrated cells) was quantified using a multilabel reader Victor3_{TM} (PerkinElmer, Inc., Waltham, MA). The DAPI counts of empty wells (no addition of cells; background) were subtracted from the wells containing cells. Data are expressed as mean \pm SD of migrated cells.

Statistical analysis

Differences between groups were determined by unpaired t-test using the program GraphPad Prism (version 4) and ($P < 0.05$ considered statistically significant).

RESULTS

Anti-CCR2 antibody treatment blocks CCL2/MCP-1-induced HD or RA monocyte migration

As expected, CCL2/MCP-1 induced significant migration of both HD (Fig 1A) and RA (Fig. 1B) monocytes pre-incubated with medium (HD $P = 0.0358$; RA $P = 0.0205$) or isotype control (HD $P = 0.0483$; RA $P = 0.0005$). Monocyte pre-incubation with anti-CCR2 antibodies resulted in blockade of CCL2/MCP-1-induced migration of cells derived from either HD (Fig. 1A; 5 or 25 $\mu\text{g/ml}$, $P = 0.0147$ and $P = 0.0035$, respectively) or RA patients (Fig. 1B; 5 and 25 $\mu\text{g/ml}$, $P = 0.0226$ and $P = 0.0009$, respectively). There was no effect of CCR2 blockade on spontaneous migration, except for HD monocytes at the antibody concentration of 25 $\mu\text{g/ml}$ (Fig. 1A; $P = 0.0294$).

Anti-CCR2 antibody treatment does not affect SF-induced HD or RA monocyte migration

Since in RA circulating monocytes are recruited to the synovial compartment under the influence of chemotactic agents, we mimicked this situation by using SF as chemoattractant in our in vitro model. SF induced significant migration of both HD (Fig 1C) and RA (Fig. 1D) monocytes pre-incubated with medium (HD $P = 0.0153$; RA $P < 0.0001$ compared to migration in medium control groups). Contrary to results for CCL2/MCP-1-induced migration, SF-induced migration could not be blocked by anti-CCR2 antibody treatment at the different concentrations used.

Anti-CCR5 antibody treatment blocks CCL5/RANTES-induced RA monocyte migration

CCL5/RANTES induced significant migration of RA monocytes (Fig. 2A) pre-incubated with medium (RA $P = 0.0199$). Anti-CCR5 antibody treatment blocked CCL5/RANTES-induced migration of RA monocytes (5 $\mu\text{g/ml}$, $P = 0.0198$ compared to isotype control).

Anti-CCR5 antibody treatment does not affect SF-induced RA monocyte migration

While SF induced significant migration of RA monocytes (Fig. 2B) pre-incubated with medium (RA $P < 0.0002$), this migration could not be blocked by anti-CCR5 antibody treatment.

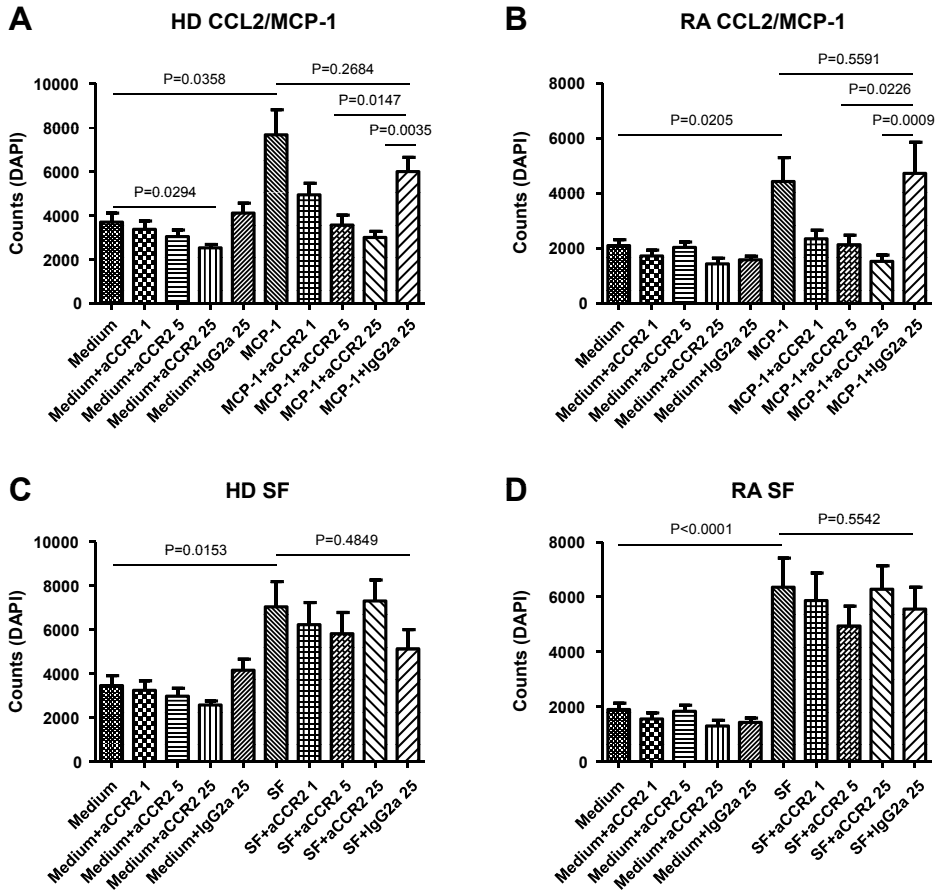


Figure 1. Anti-CCR2 blocks CCL2/MCP-1- but not SF-induced HD or RA monocyte migration. (A) HD monocyte migration induced by CCL2/MCP-1. (B) HD monocyte migration induced by SF. (C) RA monocyte migration induced by CCL2/MCP-1. (D) RA monocyte migration induced by SF. Data are expressed as mean \pm SEM (HD $n = 8$; RA $n = 8$).

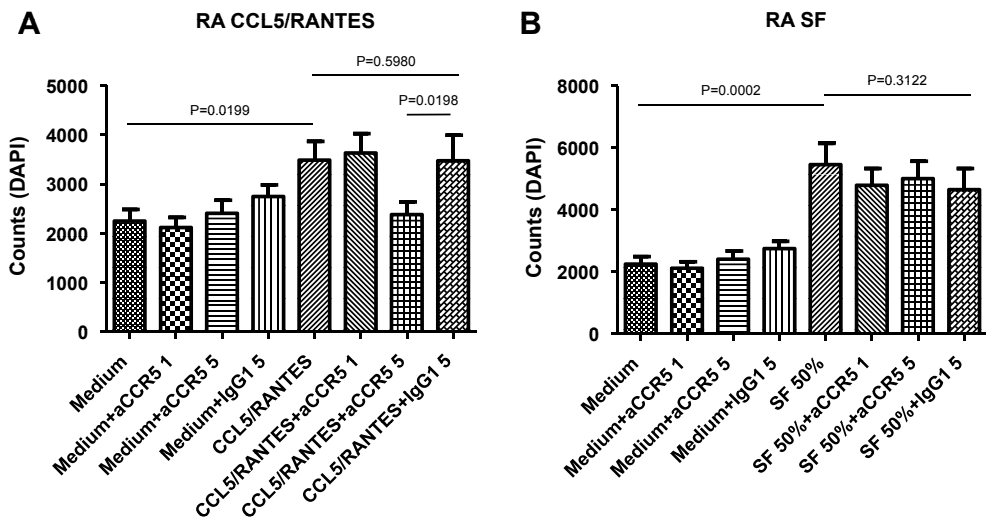


Figure 2. Anti-CCR5 blocks CCL5/RANTES- but not SF-induced RA monocyte migration. (A) RA monocyte migration induced by CCL5/RANTES. (B) RA monocyte migration induced by SF. Data are expressed as mean \pm SEM (n = 8).

DISCUSSION

In the present study we showed that ligand-induced monocyte migration could be blocked by the respective receptor blocking antibody (CCL2: anti-CCR2 or CCL5: anti-CCR5) but not when RA SF (that contains several chemokines) was used as chemoattractant.

Chemokines and their receptors have been shown to participate in a number of various biological processes and due to their diverse role in autoimmune diseases have been considered good therapeutic targets, in particular CCR2 and CCR5 for immune-mediated inflammatory diseases of which RA is a prototype disease (21-23). In view of these observations, a number of chemokine receptor antagonists (small molecule receptor antagonists and neutralizing antibodies to the receptor) have been designed and tested in animal models and several clinical trials (21;24). While CCR2 and CCR5 receptor antagonists have shown initial promise in pre-clinical studies (14;15;21), blockade of CCR2 (16), its ligand CCL2 (25), and CCR5 (17;18) have failed in clinical trials in RA patients(16-18).

There may be different explanations for the negative results in the clinical trials. First, we cannot completely exclude the possibility that the levels receptor occupancy needed to effectively block the CCR2 or CCR5 were not achieved in the clinical trials. Second, CCR5 is expressed by T regulatory cells in humans (26). Therefore, the lack of efficacy of treatment with a CCR5 antagonist could perhaps be explained by inhibition of T regulatory cells. This may also be

relevant for the observation with the CCR2 antagonists, as CCR2 and CCR5 are very close in homology, and inhibitors often target both (27).

Apart from these and other mechanisms, the results presented here clearly show that there is redundancy in this system and that *in vivo* another chemokine receptor may substitute for CCR2 or CCR5. We found that SF-induced monocyte chemotaxis was not affected when one chemokine receptor was blocked, opposed to ligand (CCL2 or CCL5)-induced monocyte chemotaxis. As in RA patients the synovial joint (tissue and SF) contains several ligands for both CCR2 and CCR5 (1;8-13) that are responsible for monocyte recruitment to these compartments (via many receptors such as CCR1(28)), this redundancy might account for the observed chemokine receptor blockade failure in both our *in vitro* model and in the clinical trials. However, we might consider other possible explanations for the lack of efficacy of blockade of both chemokine receptors individually. In line with these observations, it is tempting to speculate that in certain inflammatory pathologies it might be beneficial to block several chemokine receptors simultaneously.

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Competing interests

The study was financed by Millennium Pharmaceuticals Inc.

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Ethical approval

This study was conducted with the approval of the Medical Ethical Committee of the Academic Medical Center/University of Amsterdam.

Patient consent

Obtained.

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