# Targeting of Gr-1+,CCR2+ Monocytes in Collagen-Induced Arthritis

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*Objective.* The chemokine receptor CCR2 is highly expressed on monocytes and considered a promising target for treatment of rheumatoid arthritis. However, blockade of CCR2 with a monoclonal antibody (mAb) during progression of collagen-induced arthritis results in a massive aggravation of the disease. In this study we investigated why CCR2 antibodies have proinflammatory effects, how these effects can be avoided, and whether CCR2+ monocytes are useful targets in the treatment of arthritis.

*Methods.* Arthritis was induced in DBA/1 mice by immunization with type II collagen. Mice were treated with mAb against CCR2 (MC-21), IgE, or isotype control antibodies at various time points. Activation of basophils and depletion of monocyte subsets were determined by fluorescence-activated cell sorter analysis and enzyme-linked immunosorbent assay.

*Results.* Crosslinkage of CCR2 activated basophils to release interleukin-6 (IL-6) and IL-4. In vivo, IL-6 release occurred only after exposure to high doses of MC-21, whereas application of low doses of the mAb circumvented the release of IL-6. Regardless of the dose level used, the antibody MC-21 efficiently depleted Gr-1+,CCR2+ monocytes from the synovial tissue, peripheral blood, and spleen of DBA/1 mice. Activation of basophils with high doses of MC-21 or with antibodies against IgE resulted in a marked aggravation of collagen-induced arthritis and an increased release of IL-6. In contrast, low-dose treatment with MC-21 in this therapeutic setting had no effect on IL-6 and led to marked improvement of arthritis.

*Conclusion.* These results show that depletion of CCR2+ monocytes may prove to be a therapeutic option in inflammatory arthritis, as long as the dose-dependent proinflammatory effects of CCR2 mAb are taken into account.

CCR2, a chemokine (CC motif) receptor, is considered a promising target for disorders such as multiple sclerosis, type II diabetes, and rheumatoid arthritis, and Phase I and II clinical trials are currently in progress (1). Data on CCR2 expression in humans and results from studies of CCR2-deficient mice (2–4) support the blockade of CCR2 as an effective strategy in the treatment of multiple sclerosis and diabetes. In patients with rheumatoid arthritis, we and other investigators have found an increased frequency of CCR2+ cells in the synovial fluid and synovial tissue (5–7). In addition, increased levels of the CCR2 ligand monocyte chemoattractant protein 1 (MCP-1; CCL2) were found in patients with rheumatoid arthritis (8). Monocytes are thought to play a major role in joint destruction, and their recruitment to sites of inflammation is crucially dependent on CCR2, as shown in several murine disease models (9–12).

However, thus far, data from murine models of collagen-induced arthritis do not support CCR2 as a target for treatment of rheumatoid arthritis. We have previously shown that treatment with a blocking mono-

clonal antibody (mAb) against CCR2, MC-21, administered at 500 μg every third day, improves arthritis, when the treatment is started before the first immunization with collagen (13). In contrast, when the treatment is started later, a marked aggravation of arthritis occurs. This negative effect is most pronounced when the MC-21 treatment is started at day 21 after the first immunization with collagen (13); however, even when the treatment is initiated at days 4 or 9 after the first immunization, worsening of arthritis is still observed (results not shown). A similar result was obtained in CCR2-deficient DBA/1 mice that displayed a more severe form of arthritis than that in wild-type mice (14).

To understand why the blockade of CCR2 during the progression phase of collagen-induced arthritis aggravates the disease, we analyzed the expression of CCR2 on murine leukocytes. We have previously reported that CCR2 is expressed on murine regulatory T cells, which may be compromised by treatment with MC-21 (13). In addition, we noted that DBA/1 mice treated with MC-21 during disease progression had massively elevated levels of interleukin-6 (IL-6) in their plasma (Brühl H, et al: unpublished observations).

IL-6 plays an important role in the pathogenesis of collagen-induced arthritis, as has been demonstrated in IL-6–deficient mice (15,16) and in studies using blocking antibodies against the IL-6 receptor (17). We therefore analyzed whether MC-21 is directly responsible for the increase in IL-6 release. We identified basophils as the cells that release IL-6 after exposure to MC-21, and found that direct activation of basophils with IgE antibodies led to high plasma levels of IL-6 and worsening of arthritis in DBA/1 mice. In addition, we demonstrated that the MC-21–induced IL-6 release could be avoided by injecting low doses of MC-21 (10 μg per day), which efficiently depleted Gr-1+,CCR2+ monocytes and significantly improved the severity of arthritis. These findings may help contribute to the development of a CCR2- and monocyte–based therapy for inflammatory joint diseases.

## MATERIALS AND METHODS

**Flow cytometry.** The following reagents were used for flow cytometry: leukocyte common antigen CD45–fluorescein isothiocyanate (FITC) (30-F11), CD11b-FITC (M1/70), CD11b–phycoerythrin (PE) (M1/70), CD19-FITC (1D3), CD19-PE (1D3), Gr-1–antigen-presenting cell (APC) (RB6-8C5), c-kit–PE (2B8), DX5-PE, DX5-biotin, anti–IgE-FITC (R35-72), mouse anti-rat IgG2b-biotin (R12-3), streptavidin-PE-Cy5 (all from BD Biosciences, San Jose, CA), CD19-PE–Cy5.5 (6D5) (Caltag, South San Francisco, CA), DX5-APC

(Miltenyi Biotec, Sunnyvale, CA), anti-mouse CCR2 mAb (clone MC-21) (9), and rat IgG2b isotype control antibody (clone MC-67) (9). To detect surface expression of CCR2, cells were incubated with MC-21 (5 μg/ml) or MC-67 (5 μg/ml) for 45 minutes on ice, followed by mouse anti-rat IgG2b–biotin (5 μg/ml). After blocking with 10% rat serum for 10 minutes, streptavidin-PE–Cy5 and a combination of directly labeled antibodies were added. Before basophils were stained with anti–IgE-FITC, blood, spleen, or bone marrow samples were washed 3 times in phosphate buffered saline (PBS) to remove unbound IgE. Finally, red blood cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). All FACS analyses were performed on freshly isolated cells, and samples were analyzed on a FACSCalibur (BD Biosciences) within 24 hours.

**Cell preparations and cell culture.** Blood was collected from the retroorbital plexus of anesthetized mice and anticoagulated with 1 mM EDTA. Plasma was obtained by centrifugation of blood for 10 minutes at 500g. Single cell suspensions of spleens were prepared in cold RPMI 1640 medium with 10% fetal calf serum (FCS) and filtered to remove cell debris. Bone marrow was obtained from the femur and tibia by flushing out the cells with cold RPMI 1640 medium with 10% FCS.

Basophils were depleted from total bone marrow cells with microbeads (Miltenyi Biotec). Cells were stained with anti–IgE-FITC, and then incubated with anti-FITC microbeads and applied to LS columns according to the manufacturer's recommendations (Miltenyi Biotec). More than 99% of IgE-expressing cells were depleted from the bone marrow. One million total bone marrow cells or basophil-depleted bone marrow cells were incubated in flat-bottom 96-well plates for 24 hours in RPMI 1640, 10% FCS, and 1% penicillin/streptomycin. Soluble antibodies were then added. For coating of culture plates with the antibodies, the plates were incubated overnight at 4°C with 100 μl antibody solution (10 μg/ml). Plates were washed 2 times with PBS before adding the cells.

**Isolation and stimulation of basophils.** Basophils were isolated from single cell suspensions of bone marrow and spleen from C57BL/6 wild-type mice and CCR2-deficient mice (18) that had been backcrossed for 10 generations to C57BL/6. Using microbeads against DX5 on LD columns (Miltenyi Biotec), basophils were first enriched and then stained with CD45-FITC and DX5-APC, and further separated from natural killer (NK) cells and other contaminating cells by FACS analysis (FACS Aria; BD Biosciences). Basophils were identified by their light-scatter properties, by moderate expression of CD45, and by high expression of DX5. The resulting cell population consisted of >95% basophils as determined by staining with anti–IgE-FITC. Basophils (10,000 per well) were cultured in round-bottom 96-well plates for 24 hours with the various antibodies. Coating of the plates with the antibodies was performed as described above.

**Collagen-induced arthritis.** Bovine type II collagen (Sigma C1188; St. Louis, MO) was dissolved overnight in 0.1M acetic acid at a concentration of 2 mg/ml and, for the first injection, was emulsified in an equal volume of Freund's complete adjuvant. Male DBA/1 mice were immunized with an intra-/subcutaneous injection of 100 μl emulsion at the base of the tail on day 0, and an intraperitoneal (IP) injection of 100 μg type II collagen without adjuvant at day 21. The

clinical score of arthritis was evaluated as follows: 0 = normal; 1 = swelling in 1 joint; 2 = swelling in >1 joint; 3 = swelling of the entire paw; 4 = deformity and/or ankylosis. Animal experiments were performed in accordance with the legal requirements of the government of Bavaria (Az. 55.2-1-54-2531-109-05).

**Histology.** Hind paws were fixed in 3.7% formalin for 24 hours, washed in PBS, and decalcified with RDO rapid decalcifier (Medite, Burgdorf, Germany). The tissue was then embedded in paraffin and cut in 5-$\mu$m–thick sections. After staining with hematoxylin and eosin, the following parameters were evaluated, in a blinded manner, on at least 10 sections of the tarsometatarsal joints, using a scale from 0 (normal) to 2 for all categories: synovial inflammation (1 = focal inflammatory infiltrates; 2 = inflammatory infiltrates dominating the histologic characteristics of the cell), synovial hyperplasia (1 = a single joint with continuous synovial lining of at least 3 layers in thickness; 2 = several joints with continuous, at least 3-layer–thick synovial lining), pannus formation and cartilage loss (1 = cartilage partially covered by pannus, no cartilage loss; 2 = cartilage loss), and bone destruction (1 = small areas of bone destruction; 2 = widespread bone destruction).

**Isolation and staining of leukocytes from synovial tissue.** Mice with an arthritis score of at least 3 in at least 1 paw were treated for 2 days with low-dose MC-21 (10 $\mu$g/day), high-dose MC-21 (100 $\mu$g/day), or MC-67 (10 $\mu$g/day) as control antibody, and tissue sections were analyzed 24 hours after the last antibody injection. The skin was removed from the inflamed paws and the remaining tissue was carefully recovered with a scalpel. All bones were left intact and were removed before digestion of the tissue with type I collagenase (Sigma) for 20 minutes at 37°C. Cells were stained with antibodies against CD11b, CD45, and Gr-1.

**Enzyme-linked immunosorbent assay (ELISA).** Levels of IL-4 and IL-6 in the cell culture supernatants and plasma of mice were measured with commercially available kits (OptEIA; BD PharMingen, San Diego, CA). Antibodies against type II collagen were quantified by ELISA. Type II collagen (20 $\mu$g/ml) was coated overnight on ELISA plates. Plasma samples were applied in a dilution of 1:1,000–1:20,000 in PBS/3% bovine serum albumin followed by a horseradish peroxidase (HRP)–labeled rabbit anti-mouse polyclonal antibody (P260; Dako, Carpinteria, CA) or HRP-labeled mAb specific for murine IgG1 (clone LO-MG1-2; Serotec, Oxford, UK) or for murine IgG2a (clone R19-15; BD PharMingen).

**Statistical analysis.** All statistical analyses were performed using Student's unpaired $t$-test. Results are expressed as the mean ± SEM.

## RESULTS

**Expression of CCR2 on murine leukocytes.** It has recently been shown that murine monocytes can be separated into 2 different populations by the presence or absence of surface marker Gr-1 (Ly6C/G) (19,20). The results of the present study showed that staining with the CCR2 mAb MC-21 selectively identified the subpopulation of Gr-1+ monocytes in the peripheral blood and spleen tissue from DBA/1 mice (Figure 1A). Monocytes
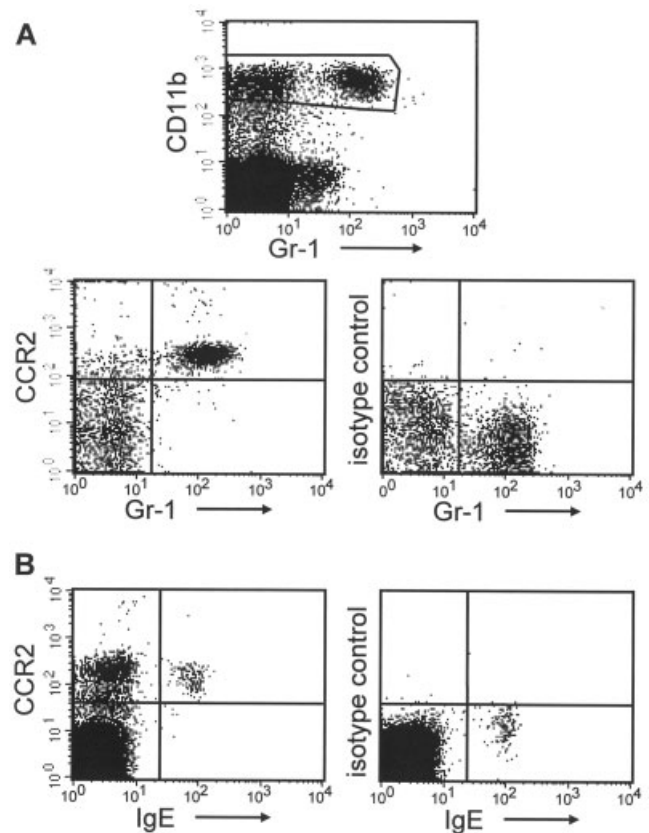
**Figure 1.** Expression of CCR2 on Gr-1+ monocytes and basophils in the peripheral blood of DBA/1 mice. **A,** Monocytes were distinguished from neutrophils by their light-scatter properties and from natural killer cells by their lack of DX5 expression, and were also identified by their expression of CD11b. The monocyte gate was set as detailed in Materials and Methods, and CCR2 expression on Gr-1+ and Gr-1− monocytes was detected using the CCR2 antibody MC-21 as compared with a rat IgG2b isotype control antibody. **B,** Basophils were identified by high expression of surface IgE. Expression of CCR2 is clearly detectable with the CCR2 antibody MC-21 as compared with the isotype control antibody. Results of staining for CCR2 on monocytes and basophils are representative of at least 3 experiments.

were identified by their specific light-scatter properties and expression of CD11b. Moreover, monocytes could be clearly distinguished from NK cells by the higher expression of CD11b and absence of DX5 in monocytes. Neutrophils and eosinophils were excluded on the basis of their light-scatter properties and the higher expression of Gr-1 on neutrophils compared with that on monocytes. Identical results (not shown) were obtained when monocytes were identified by their expression of CD115 instead of CD11b. Apart from monocytes, small populations of T cells, preferentially CD4+ T cells, express CCR2. We have previously shown that the

majority of CD4+,CCR2+ T cells also stain positive for the regulatory T cell markers CD25 and CD103 and fulfill functional criteria of regulatory T cells in vitro (13).

We also observed a homogeneous expression of CCR2 by basophils, which were identified by their high surface expression of IgE (Figure 1B) as well as their expression of CD123 and absence of c-kit (results not shown) (21). No CCR2 expression was found on B cells, NK cells, neutrophils, or eosinophils in the peripheral blood or spleen tissue from the mice.

**Depletion of Gr-1+ monocytes by the anti-CCR2 antibody MC-21.** In several cases, rat mAb with the isotype IgG2b have been described as capable of depleting their target cells in murine models. We analyzed whether the rat anti-mouse CCR2 mAb MC-21, which has the IgG2b isotype, is able to deplete CCR2+ cells from the peripheral blood and spleen of mice. By 8–24 hours after the first application of MC-21, a complete depletion of Gr-1+ monocytes was evident in the peripheral blood (Figure 2A), as well as the spleen, of DBA/1 mice and was also observed in other mouse strains (not shown). This complete depletion of Gr-1+ monocytes could be achieved with doses of MC-21 as low as 10 $\mu$g, administered IP. Following a single injection of 10 $\mu$g MC-21, monocytes reappeared after 48 hours and reached almost normal levels after 72 hours. After repeated daily injections of 10 $\mu$g MC-21, the depletion of monocytes could be sustained up to 5 days, whereas after 8 days of MC-21 treatment, no monocyte depletion was detectable (Figure 2B). Most likely, a humoral immune response against MC-21 is responsible for the failure of MC-21 to deplete monocytes after prolonged application, since mouse anti-rat antibodies became detectable after ~1 week of treatment with MC-21.

Although the Gr-1− monocytes did not express CCR2, their numbers were also reduced (~50%) by the MC-21 treatment after a few days (Figure 2B). It has been described that Gr-1+ monocytes can develop into Gr-1− monocytes (19,22–24). For this reason, depletion of Gr-1+ monocytes also might eventually result in a reduced frequency of Gr-1− monocytes.

Apart from the effects on monocytes, a small reduction (~30%) in the number of basophils occurred after treatment with MC-21, whereas the number of CD4+,CD25+ T cells remained constant. In lymph nodes, a partial depletion of Gr-1+ monocytes was observed. In contrast, in the bone marrow, no depletion of Gr-1+ monocytes occurred (results not shown).

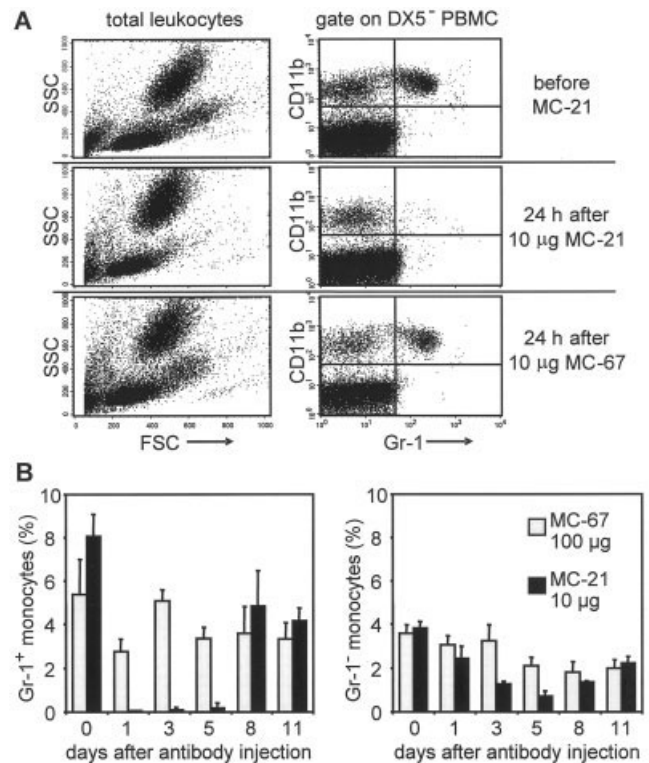Both the low dose (10 $\mu$g/day) and the high dose

**Figure 2.** Depletion of Gr-1+ monocytes with the CCR2 antibody MC-21, and kinetics of monocyte depletion with MC-21 in vivo. **A,** The peripheral blood of DBA/1 mice was analyzed before and 24 hours after injection of 10 $\mu$g MC-21 or the isotype control antibody MC-67. Total leukocytes and Cd11b+,DX5− peripheral blood mononuclear cells (PBMCs) were assessed for the presence of Gr-1+ monocytes. SSC = side scatter; FSC = forward scatter. **B,** DBA/1 mice were injected daily from day 0 to day 10 with 10 $\mu$g MC-21 or 100 $\mu$g isotype control antibody MC-67 (n = 5 per group), and the postinjection effects on Gr-1+ or Gr-1− monocytes in the peripheral blood were assessed. Results are the mean and SEM percentage of total leukocytes, representative of at least 10 experiments.

(100 $\mu$g per day) of MC-21 resulted in an identical depletion of cells. Depletion of Gr-1+ monocytes is mediated by antibody-dependent, cell-mediated cytotoxicity, as confirmed by studies showing that blockade of the low- and medium-affinity Fc receptor CD16/32 almost completely prevents the MC-21–induced depletion of monocytes (results not shown).

**Induction of release of IL-6 by MC-21 via CCR2-dependent activation of basophils.** In previous experiments we noted that the treatment of mice with high doses of MC-21 (500 $\mu$g every third day) during the progression phase of arthritis resulted in a high plasma level of IL-6. We therefore analyzed whether MC-21 per se induces a release of IL-6. Injection of high amounts of MC-21 (e.g., 100 $\mu$g IP) resulted in a rapid release of
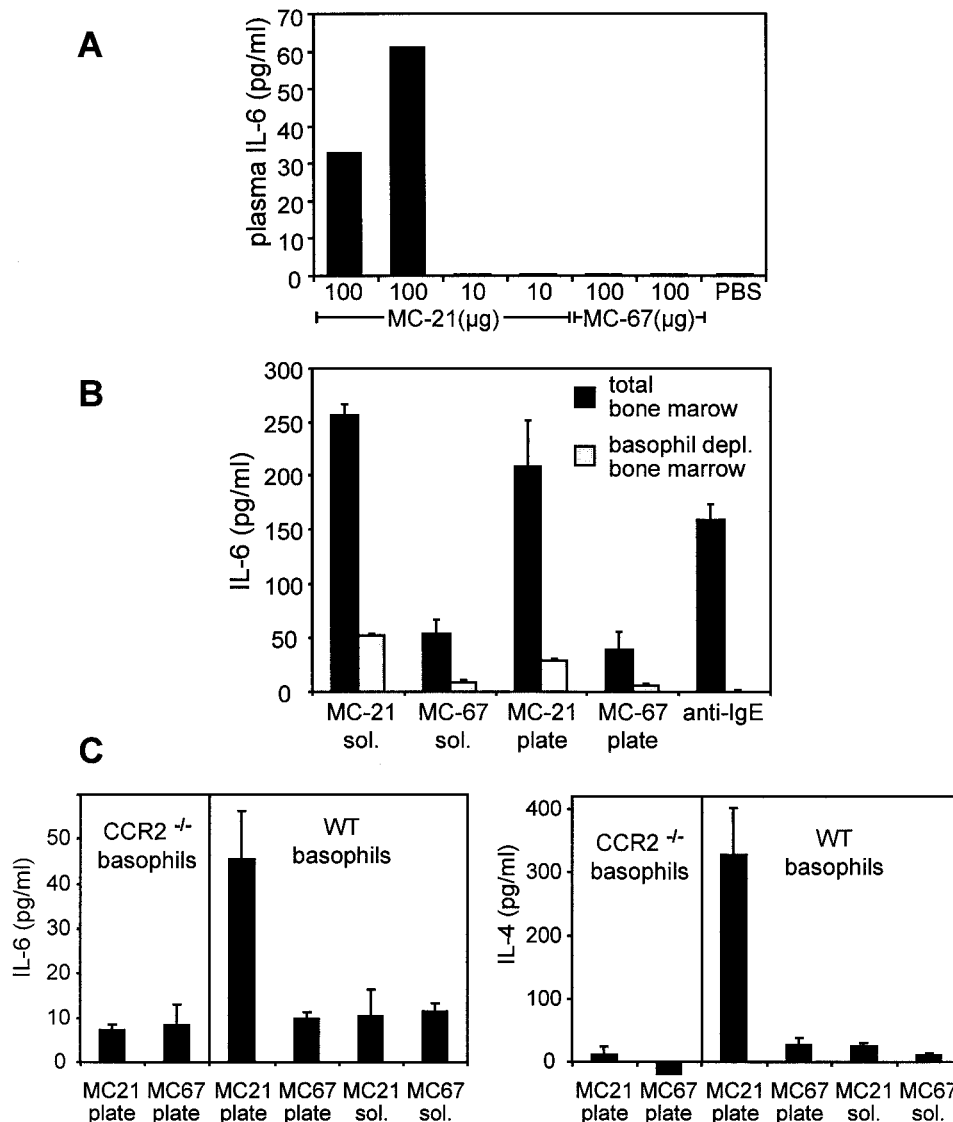
**Figure 3.** Activation of basophils by the CCR2 antibody MC-21. **A,** Plasma levels of interleukin-6 (IL-6) were assessed 2 hours after injection of high doses (100 $\mu$g) or low doses (10 $\mu$g) of MC-21, isotype control antibody MC-67 (100 $\mu$g), or phosphate buffered saline (PBS), administered intraperitoneally. Representative results from an individual mouse are shown. **B,** MC-21–induced release of IL-6 from basophils was assessed in the bone marrow of mice. Total bone marrow cells or basophil-depleted bone marrow cells were incubated for 24 hours with 10 $\mu$g/ml soluble MC-21 (MC-21 sol.), 10 $\mu$g/ml soluble MC-67 (MC-67 sol.), 0.5 $\mu$g/ml anti-IgE antibody (clone R35-72), plate-bound MC-21, or plate-bound MC-67. **C,** Basophils were isolated from the bone marrow of CCR2-deficient or wild-type (WT) C57BL/6 mice, using magnetic beads and fluorescence-activated cell sorting. Cells were incubated with plate-bound or soluble MC-21 or MC-67 (each at 10 $\mu$g/ml) for 24 hours, followed by measurement of the release of IL-6 or IL-4. Results in **B** and **C** are the mean and SEM, representative of at least 3 experiments.

IL-6, with plasma levels in the range of 50 pg/ml 2 hours after injection, while the injection of lower amounts (e.g., 10 $\mu$g MC-21 IP) did not lead to a measurable plasma level of IL-6 (Figure 3A). Injection of an isotype control antibody (100 $\mu$g MC-67 IP), which was produced under the same conditions as MC-21, did not result in a measurable release of IL-6. Both the MC-21 antibody and the isotype control antibody were virtually free of lipopolysaccharide (LPS) (LPS content <0.5 ng/mg antibody). The appearance of IL-6 within 2 hours after IP injection of MC-21 suggests that preformed IL-6 is released. IL-4 was undetectable in the plasma after stimulation with MC-21.

To identify the cells responsible for the IL-6

release, we incubated murine leukocytes in vitro with MC-21. Incubation of splenocytes with MC-21 resulted in only a marginal release of IL-6 (results not shown). However, incubation of bone marrow cells with MC-21 led to a pronounced increase in IL-6 (Figure 3B). In view of the CCR2 expression on basophils and the preferential localization of basophils in the bone marrow, we analyzed basophils as a potential source of the MC-21–induced IL-6 release. Depletion of basophils from the bone marrow almost completely prevented the MC-21–induced, as well as the IgE antibody–induced, IL-6 release (Figure 3B). However, when the cells were stimulated with LPS (100 ng/ml), depletion of basophils from the bone marrow did not result in reduced IL-6 release (1,080 pg/ml and 1,189 pg/ml IL-6 in total and basophil-depleted bone marrow cells, respectively), indicating that LPS-induced IL-6 release is not dependent on basophils.

To further demonstrate that basophils are responsible for the IL-6 release, we isolated basophils from the bone marrow with magnetic beads and FACS analysis, as described in Materials and Methods. Incubation of basophils with plate-bound MC-21, but not with plate-bound MC-67 control antibody, induced a release of both IL-6 and IL-4 (Figure 3C). Soluble MC-21 was unable to induce cytokine release from isolated basophils, suggesting that the activation of basophils occurs due to crosslinkage of CCR2.

Using basophils from CCR2-deficient mice, we investigated whether activation of basophils by the antibody MC-21 is mediated via CCR2 or is the result of a cross-reactivity of MC-21 with other surface molecules on basophils. No release of IL-6 or IL-4 was detectable when CCR2-deficient basophils were stimulated with MC-21, thus indicating the crucial involvement of CCR2 (Figure 3C). Whereas both IL-6 and IL-4 were detectable after the stimulation of isolated basophils with MC-21, no release of IL-4 was detectable when total bone marrow cells were activated with MC-21. This correlates with the finding that after injection of MC-21 in vivo, only IL-6, but not IL-4, was detectable in the plasma.

**Influence of basophil activation and monocyte depletion on the course of collagen-induced arthritis.** For in vivo blockade of CCR2, high amounts (500 $\mu$g every third day) of the CCR2 antibody MC-21 had to be injected to achieve plasma levels of the antibody that are above the 50% inhibitory concentration of MC-21 (9,13). However, depletion of Gr-1+ monocytes is possible with much lower doses (e.g., 10 $\mu$g), administered once per day. Injection of low doses of MC-21 circum-

vents the potentially unfavorable release of IL-6, and thus provides an opportunity to analyze the depletion of CCR2+ monocytes as a treatment strategy in arthritis.

Depletion of monocytes with low doses of MC-21 requires daily injection of the antibody, as indicated by our results showing that Gr-1+ monocytes reappeared after 48 hours. Therefore, we analyzed the effects of a daily injection of high amounts (50 $\mu$g/day) or low amounts (10 $\mu$g/day) of MC-21 during the progression phase of collagen-induced arthritis. In our previous experiments, injection of 500 $\mu$g MC-21 every third day beginning at day 21 after the first immunization significantly increased the severity of arthritis as compared with the effects of injection of an isotype control antibody (MC-67) (13) or PBS (results not shown). Similarly, daily injection of 50 $\mu$g MC-21 beginning at day 21
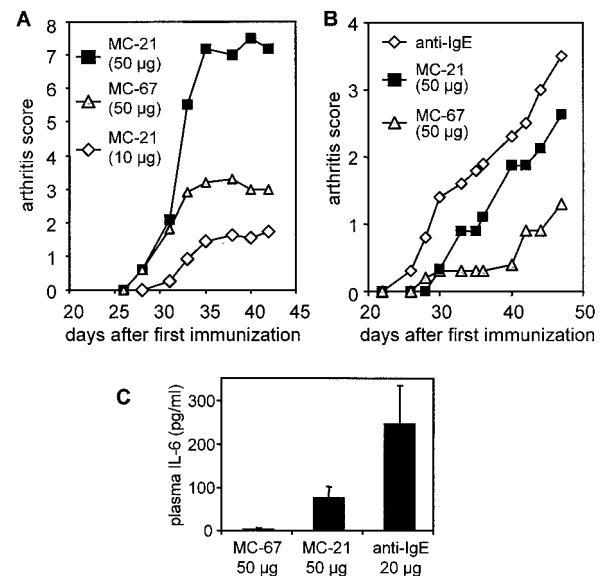


**Figure 4.** Influence of basophil activation and monocyte depletion on collagen-induced arthritis. **A,** The dose-dependent effects of MC-21 on collagen-induced arthritis were assessed in DBA/1 mice (n = 10 per group). Mice were immunized with type II collagen on day 0 and day 21 and treated over days 21–32 with daily intraperitoneal (IP) injections of low doses of MC-21 (10 $\mu$g), high doses of MC-21 (50 $\mu$g), or isotype control antibody MC-67 (50 $\mu$g). The arthritis score was evaluated every 2–3 days. **B,** Effects of basophil activation with IgE antibodies or high doses of MC-21 on arthritis were evaualied in DBA/1 mice (n = 10 per group). Mice were treated over days 21–32 with daily IP injections of IgE antibodies (clone R35-72) (20 $\mu$g), high doses of MC-21 (50 $\mu$g), or isotype control antibody MC-67 (50 $\mu$g). Results in **A** and **B** are the mean score per group, and differences between groups were statistically significant ($P < 0.05$). **C,** Plasma levels of interleukin-6 (IL-6) were measured on day 30 in mice treated as described above (n = 10 mice per group). Results are the mean and SEM, representative of at least 2–5 experiments.

after the first immunization resulted in a marked increase in arthritis as compared with the effects of MC-67 (Figure 4A). In contrast, when a lower dose of MC-21 (10 $\mu$g/day) was injected beginning from day 21, a clear reduction of arthritis was achieved (Figure 4A).

To determine whether the activation of basophils with reagents other than MC-21 also results in increased arthritis, we treated mice from day 21 to day 32 with daily injections of an anti-IgE antibody (20 $\mu$g/day), a high dose of MC-21 (50 $\mu$g/day), or the MC-67 isotype control antibody (50 $\mu$g/day) (Figure 4B). Activation of basophils with the anti-IgE antibody resulted in an even more pronounced aggravation of arthritis than that resulting from injection of 50 $\mu$g MC-21 (Figure 4B).

We also measured the plasma levels of IL-6 in mice treated with MC-21 (50 $\mu$g/day), IgE antibody (20 $\mu$g/day), or MC-67 isotype control antibody. On day 30 after the first immunization with type II collagen, mice treated with high doses of MC-21 had several-fold higher plasma IL-6 levels than did mice treated with the isotype control antibody (Figure 4C). As expected, mice treated with IgE antibodies also had very high plasma IL-6 levels.

In a separate set of experiments, we analyzed whether the depletion of monocytes with low doses of MC-21 is still beneficial when treatment is started after the clinical onset of arthritis (beginning, on average, 30.5 days after the first immunization with type II collagen). Mice with established arthritis (clinical arthritis score of ~3.0) were randomly assigned to receive treatment with the antibody MC-21 or the isotype control antibody MC-67 (Figure 5). The time point of the first application of MC-21 or isotype control antibody was designated day 0, and daily treatment was thereafter continued for 6 days. Treatment with the control antibody resulted in a highly significant progression of arthritis, with a rise in the clinical arthritis score from 2.9 on day 0 to 5.1 on day 6. In contrast, no significant progression of arthritis occurred in the MC-21–treated group (clinical arthritis score 3.1 at day 0 and 3.4 at day 6) (Figure 5A). As soon as day 2 of treatment, the clinical arthritis score in the MC-21–treated mice was significantly lower than that in the control group, and remained lower until the end of the experiment at day 6.

After treating mice for 3–5 days with low-dose MC-21 or control antibodies, we determined the percentage of Gr-1+ monocytes in the peripheral blood and the plasma levels of IL-4, IL-6, anti–type II collagen IgG1, and anti–type II collagen IgG2a. The percentage of blood CD11b+ monocytes was significantly lower
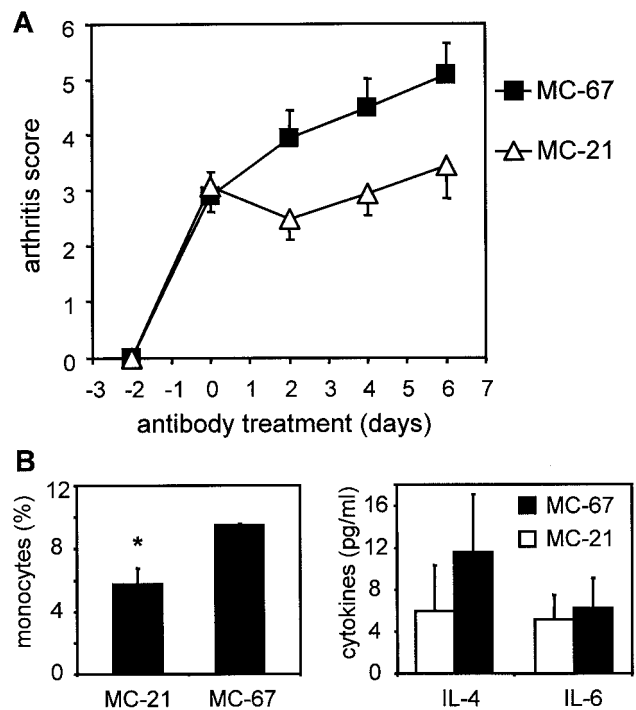


**Figure 5.** Improvement of established arthritis in mice following treatment with low-dose MC-21. **A,** DBA/1 mice were immunized with type II collagen on day 0 and day 21, and then observed for the development of arthritis. Starting on days 28–33 (1–2 days after arthritis onset), mice were randomly assigned to receive daily intraperitoneal treatment with low-dose MC-21 (10 $\mu$g) or isotype control antibody MC-67 (10 $\mu$g) (n = 17 per group). At each time point, equal numbers of mice were evaluated in the 2 groups. The day of the first application of MC-21 or isotype control antibody was designated day 0, and the treatment was continued for 6 days. Mice were killed on day 6 for histologic assessment. From day 0 to day 6, the arthritis score significantly increased in the control group only ($P < 0.01$). Results are representative of at least 2 experiments. Differences between the groups were significant ($P < 0.05$) on days 2–6. **B,** Blood was withdrawn from the mice on days 3–5 after treatment with the antibodies. The percentage of CD11b+ monocytes in relation to total leukocytes, and plasma levels of interleukin-4 (IL-4) and IL-6 were compared between the groups. ∗ = $P < 0.05$ versus MC-67–treated mice. Results are the mean and SEM.

($P < 0.05$) in mice treated with low-dose MC-21 (5.7% of total leukocytes) compared with mice treated with the control antibody (9.5% of total leukocytes) (Figure 5B). The majority of monocytes expressed Gr-1 in both groups. The plasma levels of IL-4 and IL-6 were equally low in both groups (Figure 5B). Moreover, the anti–type II collagen IgG1 and IgG2a levels were not different between groups (results not shown).

We also examined the histologic changes that occurred in the hind paws in mice treated for 6 days after
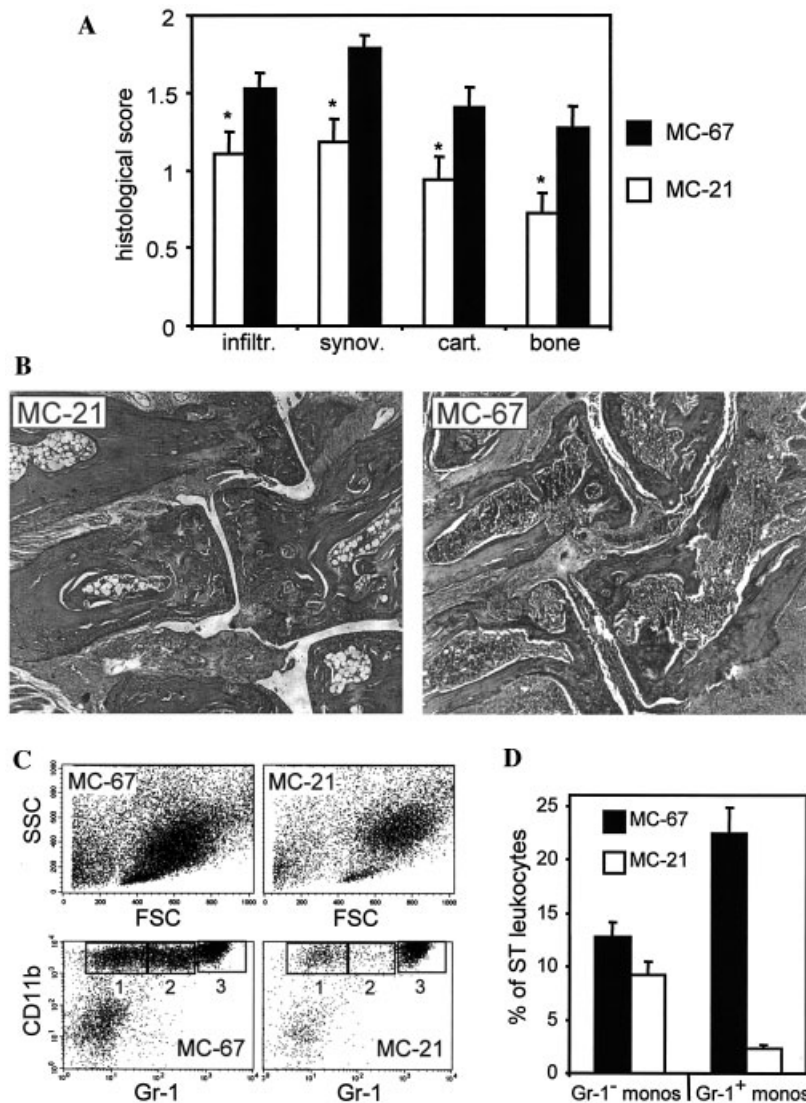
**Figure 6.** Histologic changes in mice treated with low-dose MC-21 after the onset of arthritis. Mice (n = 17 per group) were treated as described in Figure 5, and the histologic changes in the lower tarsometatarsal joints were determined on day 6 after the initiation of treatment with low-dose MC-21 or isotype control antibody MC-67. **A,** Leukocyte infiltration (infiltr.), synovial hyperplasia (synov.), cartilage erosion (cart.), and bone destruction (bone) were evaluated by histologic scoring on a scale of 0–2. ∗ = $P < 0.01$ versus MC-67–treated mice. **B,** Hematoxylin and eosin–stained tissue sections of the tarsometatarsal joints from an MC-21–treated mouse show synovial hyperplasia without cartilage or bone destruction, whereas control MC-67–treated joint tissue shows heavy bone and cartilage destruction, infiltration, and synovial hyperplasia. **C** and **D,** Mice with established arthritis were treated for 2 days with a daily injection of 10 $\mu$g MC-21 or MC-67 (n = 3 per group). After 24 hours, the composition of the synovial tissue infiltrates was analyzed by flow cytometry. **C,** Representative plots from fluorescence-activated cell sorter analysis show the light-scatter properties of the infiltrating cells (top) and the identification of Gr-1− monocytes, Gr-1+ monocytes, and neutrophils (inset populations designated 1, 2, and 3, respectively). Only results in CD45+ cells are shown. SSC= side scatter; FSC = forward scatter. **D,** The percentage of Gr-1− and Gr-1+ monocytes (monos) in relation to total CD45+ synovial tissue (ST) leukocytes was compared between MC-67–treated and MC-21–treated mice. Results in **A** and **D** are the mean and SEM.

the onset of arthritis with low doses of MC-21 or MC-67 (each at 10 $\mu$g/day) (Figures 6A and B). Mice were killed on day 6 of antibody treatment, and the tarsometatarsal joints were evaluated after decalcification and staining with hematoxylin and eosin. In correlation with the changes in the clinical arthritis score, the joints of MC-21–treated mice displayed significantly less leukocyte infiltration and synovial hyperplasia as well as

reduced bone and cartilage erosion as compared with the control group.

We also analyzed the cellular composition of the infiltrating cells. Mice with an arthritis score of at least 3 in at least 1 paw were treated for 2 days by daily injection of 10 $\mu$g MC-67 or 10 $\mu$g MC-21, and 24 hours later the synovial tissue leukocytes were analyzed by FACS (Figures 6C and D). In mice treated with MC-21, the subpopulation of Gr-1+ monocytes was markedly reduced in the synovial tissue, whereas there was little influence on Gr-1− monocytes or neutrophils. Gr-1+ monocytes were also depleted in the peripheral blood and, to a lesser degree, in the spleens after treatment with MC-21 (results not shown).

Taken together, these data indicate that treatment with low doses of MC-21 starting at day 21 after the first immunization with type II collagen or even after the clinical onset of arthritis significantly improves arthritis. Treatment of mice with high doses of MC-21 or with antibodies against IgE results in release of IL-6, which is attributable to the activation of basophils, and leads to a marked increase in the severity of arthritis.

## DISCUSSION

In the present study we analyzed why the blockade of CCR2 with the antibody MC-21 during the progression phase of collagen-induced arthritis has proinflammatory effects, how these proinflammatory effects can be avoided, and what are the mechanisms of action of CCR2+ monocytes in the progression of arthritis. Our findings showed that the CCR2 antibody MC-21 activates basophils to release IL-6 and IL-4. In vivo, IL-6 release was observed only at high doses of the CCR2 antibody (e.g., 50–100 $\mu$g) and could be avoided by injecting lower doses of MC-21 (e.g., 10 $\mu$g). Activation of basophils by daily application of high doses of MC-21 or by injecting antibodies against IgE resulted in a markedly aggravated arthritis and an increased IL-6 release. In contrast, the application of low doses of the MC-21 CCR2 antibody (10 $\mu$g/day) circumvented the release of IL-6, allowed the depletion of Gr-1+ monocytes from the peripheral blood, spleen, and synovial tissue, and markedly improved established arthritis.

An aggravation of arthritis due to activation of basophils has not been previously described in the literature. However, it is known that mast cells and IL-6 play an important role in the development of arthritis. Mast cell–deficient or IL-6–deficient mice are, to a large extent, protected from antibody- or collagen-induced arthritis, respectively (15,16,25). Some reports describe

the expression of CCR2, as determined by reverse transcriptase–polymerase chain reaction, on cultured bone marrow–derived murine mast cells (26), and an activation of pulmonary mast cells by the CCR2 ligand MCP-1 (27). We found only a marginal expression of CCR2 on c-kit+ cells in the bone marrow, using the antibody MC-21 (mean channel fluorescence 18.5 on c-kit+ cells versus 490 on monocytes).

We also showed that depletion of basophils from total bone marrow, with magnetic beads directed against IgE, almost completely prevented the MC-21–induced IL-6 release. The only cells in freshly isolated bone marrow that expressed surface IgE were basophils, but not mast cells. This observation helps rule out the possibility of a substantial contribution of mast cells to the MC-21–induced release of IL-6.

Thus far, it is not known how many basophils are present in the synovial tissue, either in mice or in patients with arthritis. However, in the bone marrow, large numbers of basophils are present. In histologic sections of joint tissue from mice with severe collagen-induced arthritis, we clearly observed expansion of synovial tissue and inflammatory infiltrates extending into the bone marrow. In addition, a recent study in humans with rheumatoid arthritis has shown that inflamed tissue is present in the bone marrow, and that the inflammatory process extends to the bone marrow cavity (28). It is therefore conceivable that cytokines released from bone marrow cells (e.g., IL-6 produced by basophils) might contribute to the severity of arthritis.

It is not widely recognized that basophils also release IL-6 (21). In our experiments with isolated basophils, we observed a release of IL-6 and IL-4 after stimulation with anti-IgE or MC-21 antibodies. When total bone marrow cells were stimulated with anti-IgE or MC-21, and when plasma cytokines were measured after injection of MC-21, only IL-6, but not IL-4, was detectable. This might be due to the detection limit of our ELISA or due to a consumption or degradation of IL-4.

The stimulation of basophils with the CCR2 antibody MC-21 is dependent on the crosslinkage of CCR2. When isolated basophils were assessed, only plate-bound MC-21, but not soluble MC-21, induced a release of IL-6 and IL-4. When total bone marrow cells were assessed, both plate-bound and soluble MC-21 induced basophil activation. We assume that the large number of Fc receptor–positive cells present in the total bone marrow immobilizes the MC-21 antibody, and thereby allows crosslinkage of CCR2. It is well accepted that the oligomerization of chemokine receptors affects their activation state. With antibodies against CCR5, we

have previously shown that crosslinkage results in partial activation of CCR5 (29). In several assays (calcium flux, cell migration, receptor down-modulation), we were unable to detect agonistic activity of the CCR2 antibody MC-21. However, it is important to note that partial activation might become detectable only if multiple signal transduction pathways are analyzed in several cell types. Eotaxin/CCL11, a natural antagonist of CCR2, was recently shown to induce signal transduction via the ERK-1/2 pathway (30). Human basophils also express CCR2 (31) and can be activated via CCR2, resulting in shape changes (31) and the release of cytokines and leukotriene $C_4$ (32).

In vivo, the release of IL-6 by MC-21 was dose dependent and was only detectable after administration of high doses of MC-21, whereas the depletion of Gr-1+ monocytes from the peripheral blood, spleen, and synovial tissue was almost identical after high doses (100 $\mu$g) or low doses (10 $\mu$g) of MC-21. This enabled us to study the effects of monocyte depletion without activating basophils. The application of low doses of MC-21 resulted in a significant improvement of arthritis, using 2 different treatment regimens. Starting the treatment at day 21 after the first immunization, and even starting the treatment after the onset of arthritis, resulted in an improved clinical arthritis score in the MC-21–treated group. Following treatment with MC-21, the number of Gr-1+ monocytes was reduced in the peripheral blood, in the spleen, and in the synovial tissue of mice with arthritis. We were somewhat surprised that the depletion of peripheral blood monocytes in DBA/1 mice with arthritis was not as complete as in naive DBA/1 mice (>95% depletion of Gr-1+ monocytes). This difference might result from a higher turnover of monocytes in DBA/1 mice with arthritis. This finding and the fact that the depletion of monocytes can only be sustained for a few days may explain why we missed the MC-21–induced depletion of monocytes in our previous experiments, in which the depletion of monocytes was analyzed only after 10 days of MC-21 application in DBA/1 mice with arthritis (13).

Several lines of evidence suggest that the depletion of CCR2+ monocytes, but not the blockade of CCR2, is responsible for the improvement of arthritis after low-dose MC-21 treatment. First, the application of 10 $\mu$g MC-21 is unlikely to fully block CCR2. Second, CCR2-deficient DBA/1 mice show increased progression of arthritis as compared with wild-type mice (14). The underlying mechanisms of the disease in CCR2-deficient mice are unclear. Apart from findings of an impaired activation-induced cell death (14), the in-

creased number of Gr-1+ monocytes in the bone marrow of CCR2-deficient mice (33) might contribute to the aggravation of arthritis, provided that the leukocytes in the bone marrow have access to the inflamed joints, which appears possible in view of the extensive destruction of the bone architecture in this model. In addition, a recent clinical trial showed that blockade of the MCP-1–CCR2 interaction by a humanized antibody against MCP-1 was associated with a worsening of rheumatoid arthritis (34). Indeed, CCR2 might not be the ideal target for an antibody-mediated depletion of monocytes as a therapeutic approach in arthritis, since crosslinkage of CCR2 on basophils activates basophils to release IL-6, and activation of basophils markedly aggravates arthritis. Other surface structures on monocytes may be more suitable for the recognition and depletion of monocytes.

By low-dose treatment with an anti-CCR2 antibody, we were able to significantly improve collagen-induced arthritis in mice by depleting CCR2+ monocytes. Potential proinflammatory effects resulting from the blockade of CCR2 and the activation of basophils were prevented. The beneficial effect of depleting CCR2+ monocytes may thus help in the design of new strategies for the treatment of arthritis.

## AUTHOR CONTRIBUTIONS

Dr. Mack had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
**Study design.** Brühl, Mack.
**Acquisition of data.** Brühl, Cihak, Plachý, Kunz-Schughart, Niedermeier, Denzel, Gomez, Talke, Luckow, Stangassinger, Mack.
**Analysis and interpretation of data.** Brühl, Kunz-Schughart, Niedermeier, Denzel, Gomez, Talke, Luckow, Stangassinger, Mack.
**Manuscript preparation.** Brühl, Mack.
**Statistical analysis.** Brühl, Mack.

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