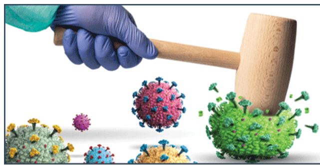

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Expression and Characterization of the Chemokine Receptors CCR2 and CCR5 in Mice[1]

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The chemokine receptors CCR2 and CCR5 play important roles in the recruitment of monocytes/macrophages and T cells. To better understand the role of both receptors in murine models of inflammatory diseases and to recognize potential problems when correlating these data to humans, we have generated mAbs against murine CCR2 and CCR5. In mice CCR2 is homogeneously expressed on monocytes and on 2–15% of T cells, closely resembling the expression pattern in humans. In contrast to humans, murine NK cells are highly CCR5 positive. In addition, CCR5 is expressed on 3–10% of CD4 and 10–40% of CD8-positive T cells and is weakly detectable on monocytes. Using a model of immune complex nephritis, we examined the effects of inflammation on chemokine receptor expression and found a 10-fold enrichment of $CCR5^+$ and $CCR2^+$ T cells in the inflamed kidneys. The activity of various chemokines and the antagonistic properties of the mAbs were measured by ligand-induced internalization of CCR2 and CCR5 on primary leukocytes. The Ab MC-21 (anti-CCR2) reduced the activity of murine monocyte chemotactic protein 1 by 95%, whereas the Ab MC-68 (anti-CCR5) blocked over 99% of the macrophage-inflammatory protein 1α and RANTES activity. MC-21 and MC-68 efficiently blocked the ligand binding to CCR2 and CCR5 with an IC_{50} of 0.09 and 0.6–1.0 μg/ml, respectively. In good correlation to these in vitro data, MC-21 almost completely prevented the influx of monocytes in thioglycollate-induced peritonitis. Therefore, both Abs appear as useful reagents to further study the role of CCR2 and CCR5 in murine disease models. ***The Journal of Immunology,* 2001, 166: 4697–9704.**

Chemokine receptors belong to the family of G protein-coupled hepta-helical receptors and are primarily expressed on hemopoietic cells. In humans, the expression of chemokine receptors has been extensively studied on RNA and protein level using in situ hybridization, RNase protection assays, FACS analysis, and immunohistochemistry. Particularly the use of mAbs provided valuable insights into the role of chemokine receptors in diseases, such as rheumatoid arthritis (1–3), multiple sclerosis (4, 5), and inflammatory kidney diseases, including transplant rejection (6) and inflammatory bowel diseases (7). However, in mice, little is known about the expression of chemokine receptors on the protein level due to the lack of mAbs. Receptor-deficient mice and receptor antagonists are widely used to study the functional role of certain chemokine receptors in disease models. The correlation of these results to humans depends on a comparable expression pattern for chemokine receptors in both species. Moreover, interpretation of data obtained in mice would be facilitated by a detailed knowledge of receptor expression. Therefore, we aimed to develop mAbs against the murine chemokine receptors CCR2 and CCR5. We have chosen these receptors because they play an important role in various inflammatory diseases, infections, and arteriosclerosis. CCR5-deficient mice were significantly more susceptible to infections with the parasites *Listeria monocytogenes* (8), *Cryptococcus neoformans* (9), and *Toxoplasma gondii* (10). They also displayed an increased mortality in influenza A virus infection (11). In contrast, the lack of CCR5 reduced the number of ulcerations in dextran-sulfate-mediated colitis (12) and increased the tolerance to LPS (8). CCR2 knockout mice show a reduced delayed-type hypersensitivity reaction and granulomata formation (13, 14) and were more susceptible to infections with *L. monocytogenes* (15), *Leishmania major* (16), and *C. neoformans* (17). The deficiency of CCR2 had a protective effect against arteriosclerosis in $apoE^{-/-}$ mice (18), allergen-induced bronchial hyperreactivity (19), dextran sulfate-mediated colitis (12), and thioglycollate-induced peritonitis (13–15). Both, the CCR5 and CCR2 knockout mice appear to have a partial defect in macrophage function and show a decreased Th1 immune response, as measured by a reduced production of Th1 cytokines, such as IFN-γ (12, 13) and IL-12 (10). An association of chemokine receptors with Th1 and Th2 cells has also been found in humans, where CCR5 and CXCR3 are primarily expressed on Th1 cells (20), whereas CCR3, CCR4, and CCR8 predominate on Th2 cells (21–23).

Apart from knockout mice, chemokine receptor antagonists were used to study the role of chemokines in various models of inflammation. The chemokine analog methionine (Met)[3]-RANTES together with an antiserum against monocyte chemotactic protein (MCP)-1 was used in crescentic nephritis and resulted in a significant reduction of leukocyte infiltration, proteinuria, and tissue damage (24). Met-RANTES (3) showed a beneficial effect in

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[3] Abbreviations used in this paper: Met, methionine; MCP, monocyte chemotactic protein; MIP, macrophage-inflammatory protein; AOP, aminooxypentane; CHO, Chinese hamster ovary.

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collagen-induced arthritis (25) and the MCP-1 analog (MCP-1 9-76) prevented arthritis in the MRL-lpr mouse (26). Experimental nephritis in rats was improved by the virally encoded broad-spectrum chemokine antagonist macrophage-inflammatory protein (MIP)-II (4, 27) and the RANTES analog aminooxypentane (AOP)-RANTES (5, 28). Interpretation of those experiments is often difficult because chemokine analogs might bind to more than one chemokine receptor and the activity of most chemokine analogs has not been fully characterized on murine receptors. To better understand the effects of various chemokines and chemokine analogs on murine receptors, we analyzed ligand-induced internalization of CCR5 and CCR2 and determined the agonistic profile of various ligands.

The problem arising with the ill-defined receptor specificity of chemokine analogs could be overcome with receptor-specific inhibitory mAbs. Therefore, we investigated the inhibitory potential of our Abs and found a robust blockade of CCR2 and CCR5 with two of the clones.

Materials and Methods

Cell lines and chemokines

Chinese hamster ovary (CHO) (6) cells were stably transfected with murine CCR2 (29) or CCR5 (30, 31) using the eucaryotic expression vector PEF-DHFR (32). The coding region of the receptors was amplified from genomic DNA and subcloned in the vector PEF-DHFR with *Xba*I and *Sal*I. Transfected CHO cells were selected in nucleoside-free medium (α-MEM; Life Technologies, Rockville, MD) and expression of the receptors was further amplified by addition of increasing concentrations of methotrexate. The cells were cloned several times and expression of CCR2 and CCR5 was quantified with a serum generated in rabbits against N-terminal peptides of the receptors.

The murine chemokines MIP-1α and MCP-1 were obtained from R&D Systems (Minneapolis, MN) and ^{125}I-labeled chemokines from Amersham Pharmacia (Uppsala, Sweden). Met-RANTES and AOP-RANTES were produced as described (33, 34).

Generation of mAbs in rats

Wistar rats were immunized with at least six intraperitoneal injections of 10^7 transfected CHO cells in 3- to 4-wk intervals. Four days after the last injection, the spleens were removed and splenocytes fused to X63-Ag8 plasmocytoma cells. The resulting hybrids were plated on six 96-well plates at an approximate frequency of 10 clones/well. Positive clones were identified by FACS analysis on transfected and nontransfected CHO cells and cloned twice by limited dilution.

Preparation of cells and FACS analysis

Blood was obtained from anesthetized mice by puncture of the retro-orbital plexus or by cardiac puncture. Leukocytes were isolated from the kidneys using a slightly modified protocol as described by Cook et al. (35). Trypsin was omitted in the isolation procedure to avoid degradation of cell surface Ags, especially CD4 and CD8.

For FACS analysis, anti-coagulated full blood was incubated with 5 μg/ml of the mAbs MC-21 or MC-68 for 60 min on ice. Isotype controls (rat IgG2b; BD PharMingen, San Diego, CA) were always included in the assays. After three washing steps, the cells were incubated for 1 h on ice with a biotin-labeled anti-rat polyclonal Ab (Dako, Hamburg, Germany, and BD PharMingen) followed by PE-labeled streptavidin (Dako) and a combination of directly conjugated Abs. These were CD11b fluorescein-isothiocyanate (clone M1/70), CD8 Cy-Chrome and CD4 allophycocyanin, or Pan-NK FITC (clone DX-5), CD8 Cy-Chrome, and CD4 allophycocyanin (BD PharMingen). After lysis of erythrocytes with FACS lysing solution (Becton Dickinson, Franklin Lakes, NJ), the cells were analyzed on a flow cytometer (FACSCalibur; Becton Dickinson). Monocytes were identified by their light-scatter properties and expression of CD11b. NK cells were identified by gating the lymphocyte population and expression of DX-5 and CD11b as well as absence of the T cell Ags CD4 and CD8. The cutoff to define chemokine receptor-positive cells was set according to the staining with the isotype control Ab.

Down-modulation of chemokine receptors

Anti-coagulated full blood was incubated for 30 min at 37°C with various concentrations of chemokines as described previously (36). Two samples were incubated with medium as control. The cells were then placed on ice and stained for chemokine receptor expression as described above. One of the medium controls was stained with an isotype control Ab. Relative surface expression of chemokine receptors was determined by [(mean channel chemokine] − (mean channel isotype control)]/[(mean channel medium) − (mean channel isotype control)]. To demonstrate that reduced surface expression of chemokine receptors is due to internalization of the receptor, we also incubated the cells in parallel with chemokines on ice. Under these conditions, the binding of the mAbs MC-21 and MC-68 was not significantly reduced, excluding a chemokine-mediated inhibition of Ab binding (data not shown).

To investigate whether MC-21 or MC-68 induce receptor internalization by themselves, we incubated murine leukocytes with the Abs for 30 min on ice or at 37°C and then measured surface expression of CCR2 and CCR5 as described above.

Chemokine binding assays

CHO transfectants expressing CCR2 and CCR5 were harvested at 80% confluence and detached using PBS containing 1 mM EDTA. Equilibrium competition binding assays were conducted in 96-well plates by preincubating 1×10^5 cells in 50 mM HEPES (pH 7.2), containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA (binding buffer), and appropriate dilutions of the Abs in binding buffer, for 1 h on ice. The radiolabeled chemokines used were ^{125}I-labeled MCP-1 for CCR2 and ^{125}I-labeled MIP-1β, ^{125}I-labeled MIP-1α, and ^{125}I-labeled RANTES for CCR5, which were added to a final concentration of 0.1 nM. The cells were incubated for an additional 4 h on ice, and the unbound iodinated chemokine removed by washing three times with 200 μl of ice-cold binding buffer containing 0.5 M NaCl under aspiration. Scintillant (50 μl) was added to each well and the plates counted in a β scintillation counter for 1 min per well. Data were analyzed using Grafit 3.01 software using the following equation: $B = B_0/1 + [L]/IC_{50}$.

Thioglycollate-induced peritonitis and apoferritin-induced glomerulonephritis

For the induction of peritonitis in BALB/c mice, Brewer-Thioglycollate (1.5 ml, 4% solution in distilled water; Difco, Detroit, MI) was injected i.p. Before this injection, the treatment group (three mice) received 500 μg of MC-21 and the control group (three mice) received the same volume of PBS as i.p. injection. After 3 days, a peritoneal lavage was performed with ice-cold PBS containing 1 mM EDTA. The recovered cells were counted with a cell counter (Coulter Counter; Coulter Pharmaceutical, Palo Alto, CA) and monocytes/macrophages were identified by flow cytometry using light-scatter properties.

Immune complex-mediated glomerulonephritis was induced in BALB/c mice by daily i.p. injections of 4 mg of horse spleen apoferritin (Aldrich Chemical, Steinheim, Germany) in 80 μl of 0.1 M of sodium chloride as described (37). After 14 days, the kidneys were removed and leukocytes isolated and stained as described above. All animal experiments were approved by the governmental boards.

Results

Generation of mAbs against murine CCR2 and CCR5

Rats were immunized with CHO cells stably transfected with murine CCR2 or CCR5. By several rounds of amplification and cloning, we obtained CHO cell clones with a high expression of chemokine receptors. A high receptor density is considered important for successful immunization. Chemokine receptor expression was quantified by FACS analysis using an antiserum generated in rabbits against N-terminal peptides of CCR2 or CCR5.

From 23 fusion reactions we identified 3 mAbs against CCR2 and 15 mAbs against CCR5. The Ab MC-21 directed against CCR2 and the Ab MC-68 directed against CCR5 (both IgG-2b) showed the best binding properties and were used for further analysis.

The specificity of the Abs was determined on CHO cells overexpressing closely related human and murine chemokine receptors (Fig. 1). The clone MC-21 binds to murine CCR2 but does not cross-react with murine CCR5 or human CCR2 and CCR5. Similarly, the clone MC-68 only binds to murine CCR5 and not to murine CCR2 or the human homologues. In addition, both Abs showed no detectable binding to human PBMC. Further evidence

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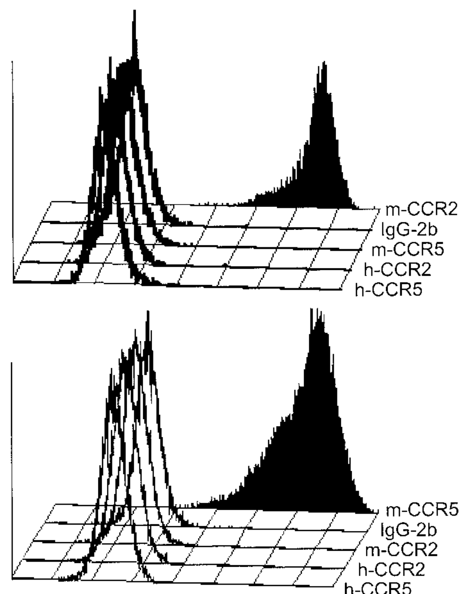

FIGURE 1. Specificity of the mAbs MC-21 and MC-68 on CHO cells overexpressing various chemokine receptors. The clone MC-21 (*top*) specifically recognizes murine CCR2 and shows no cross-reactivity to other receptors. The clone MC-68 only binds to murine CCR5 (*bottom*).

for the specificity of the mAbs is obtained with down-modulation experiments described below.

Expression of CCR2 and CCR5 on mouse leukocytes

A central aim of our study was to compare the chemokine receptor expression in mice and humans. The mAbs MC-21 and MC-68 showed excellent binding to CCR2 and CCR5, respectively, on primary mouse leukocytes (Fig. 2). To further verify that both Abs indeed recognize the appropriate chemokine receptors, we induced internalization of the receptors with murine MCP-1 and murine MIP-1α in parallel assays. The Abs MC-21 and MC-68 did not induce receptor internalization by themselves (data not shown).

The expression pattern of the chemokine receptor CCR2 was almost identical in mice and humans (1). Approximately 5–15% of the $CD4^+$ T cells express CCR2 in BALB/c mice, whereas only 2–10% of the $CD8^+$ T cells are CCR2 positive (Fig. 2*A, left*). Moreover, the expression level for CCR2 was higher on Th cells than on CTLs. Monocytes stained homogeneously positive for CCR2. Preincubation with murine MCP-1 caused a marked down-modulation of CCR2 (Fig. 2*A, right*).

In contrast, the expression pattern of CCR5 significantly differed between the two species. Murine NK cells ($DX\text{-}5^+$, $CD11b^+$, $CD4^-$, $CD8^-$) homogeneously express high levels of CCR5 (Fig. 2*B*), whereas only a small subpopulation of human NK cells weakly express CCR5 (1). The CCR5 expression on T cells and monocytes was comparable in both species (38, 39). In mice, CCR5 is also expressed on a higher percentage of $CD8^+$ T cells (10–40%) than on $CD4^+$ T cells (3–10%) and freshly isolated monocytes very weakly express CCR5 (Fig. 2*B, left*). Down-modulation of CCR5 with MIP-1α almost completely prevented binding of the Ab (Fig. 2*B, right*).

In addition, we compared the CCR5 and CCR2 expression on leukocytes isolated from the peripheral blood, spleen, and lymph nodes of two different mouse strains, namely BALB/c and C57BL/6. The fraction of CCR5-positive T cells was somewhat higher in the spleen than in the two other compartments. Interestingly, the two strains differed in their chemokine receptor expression, because the percentage of CCR5- and CCR2-positive T cells was almost two times higher in the Th1-prone C57BL/6 mice than in the Th2-prone BALB/c mice (data not shown).

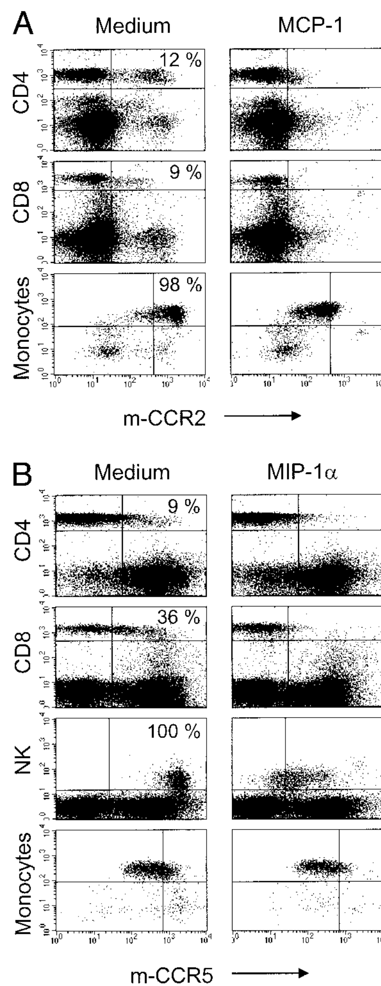

FIGURE 2. Detection of CCR2 and CCR5 on murine peripheral blood leukocytes with the Abs MC-21 and MC-68. *A*, Monocytes homogeneously stain positive for CCR2, whereas only a small subpopulation of $CD4^+$ and $CD8^+$ T cells express CCR2 (*left*). Murine MCP-1 (1 μg/ml) almost completely down-modulates CCR2, further demonstrating the specificity of the Ab MC-21 (*right*). *B*, CCR5 is highly expressed on NK cells and only weakly detectable on monocytes. Among T cells, the $CD8^+$ cells have a higher percentage of CCR5-positive cells than $CD4^+$ cells (*left*). Down-modulation with murine MIP-1α (1 μg/ml) significantly reduced surface expression of CCR5 (*right*).

Blockade of CCR2 with the Ab MC-21 inhibition of thioglycollate-induced peritonitis

To analyze a potential blockade of CCR2 by the mAb MC-21, we studied the inhibition of MCP-1-induced receptor internalization. As shown in Figs. 2*A* and 3, murine MCP-1 efficiently down-modulates CCR2 from the surface of T cells and monocytes. At a concentration of 100 ng/ml, ~50% of CCR2 is internalized within 30 min at 37°C. Parallel incubation of cells on ice served as control to exclude a sterical blockade of MC-21 binding by MCP-1 (Fig. 3*A*). A representative histogram showing the internalization of CCR2 on monocytes with 1 μg/ml of MCP-1 is depicted in Fig. 3*B*.

We then investigated whether preincubation of monocytes with MC-21 can reduce MCP-1-induced receptor internalization. In general, internalization of chemokine receptors depends on receptor activation and a reduced internalization would indicate blockade of the receptor. Preincubation of murine blood cells with

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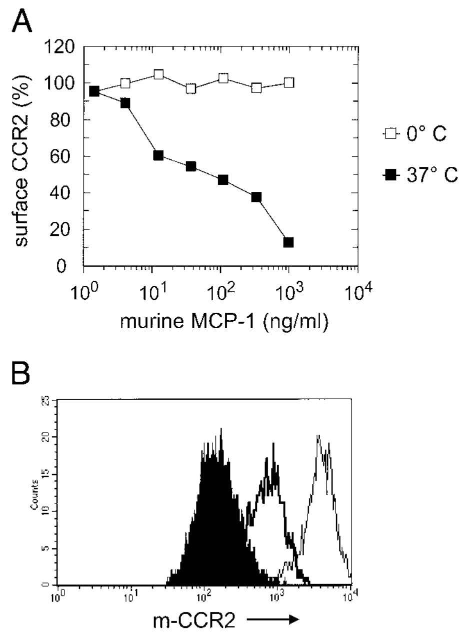

FIGURE 3. FACS analysis showing down-modulation of CCR2 from the surface of monocytes with various concentrations of murine MCP-1. *A*, The incubation at 37°C allows internalization of CCR2, whereas the incubation on ice was performed to exclude a steric blockade of MC-21 binding by MCP-1. *B*, A representative histogram shows the reduced surface expression of CCR2 on monocytes after incubation with 1 μg/ml MCP-1 (thick line) in comparison with the positive control (light gray area) and the negative control (dark gray area).

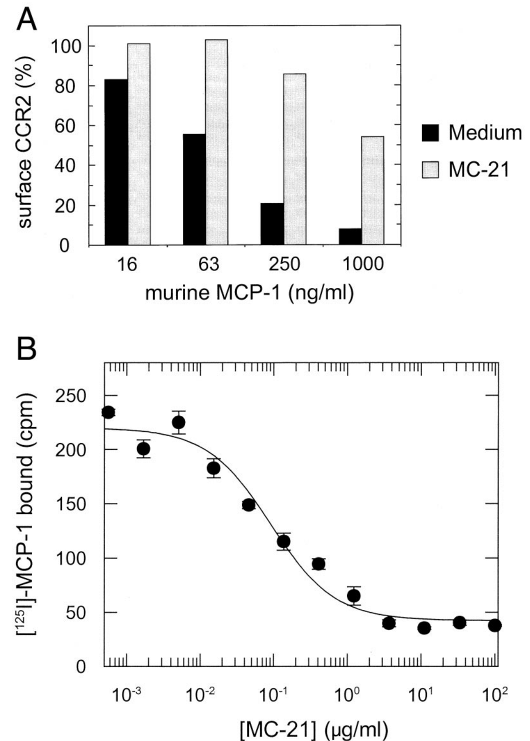

FIGURE 4. Blockade of CCR2 by the Ab MC-21. *A*, MC-21 efficiently prevents the down-modulation of CCR2 from the surface of monocytes. The cells were preincubated with MC-21 (10 μg/ml) or medium and the down-modulation of CCR2 induced with various concentrations of murine MCP-1 was measured by flow cytometry. *B*, MC-21 blocks the binding of radiolabeled MCP-1 to CCR2 on stably transfected CHO cells with an IC_{50} of 0.09 μg/ml. Cells were preincubated with various concentrations of MC-21 and binding of MCP-1 was measured as described.

MC-21 considerably diminished CCR2 internalization by MCP-1 (Fig. 4*A*). When monocytes were preincubated with 10 μg/ml of MC-21, only 14% of the surface CCR2 could be internalized by 250 ng/ml of MCP-1, whereas 80% of the receptors were internalized in the absence of MC-21.

In addition, we examined whether the Ab MC-21 blocks the binding of MCP-1 to murine CCR2. Using CCR2-transfected CHO cells and radiolabeled MCP-1, we could demonstrate that MC-21 efficiently blocks the binding of MCP-1 with an IC_{50} of 0.09 μg/ml (Fig. 4*B*).

To demonstrate that the Ab MC-21 is able to block CCR2 in vivo, we studied the effect of MC-21 on thioglycollate-induced peritonitis. Experiments with CCR2 knockout mice have demonstrated that CCR2 is essential for the influx of monocytes/macrophages into the peritoneal cavity in this model (13–15). Shortly before injection of thioglycollate, we injected either 500 μg of MC-21 or PBS as a control. After 3 days a peritoneal lavage was performed and the total number of cells counted. As shown in Fig. 5*A*, treatment of mice with MC-21 significantly reduced the number of peritoneal leukocytes. In an additional experiment, we determined the relative number of lymphocytes, granulocytes, and monocytes/macrophages in the peritoneal lavage by flow cytometry (Fig. 5*B*). Treatment with MC-21 decreased the total number of monocytes/macrophages in the peritoneal fluid by >90% (MC-21 group 7.0×10^6 vs control 0.55×10^6; $p < 0.001$). In contrast, treatment with MC-21 had little influence on the number of infiltrating granulocytes and lymphocytes indicating that MC-21 efficiently blocks CCR2 in vivo and that the influx of monocytes/macrophages is dependent on CCR2.

Blockade of CCR5 with the Ab MC-68

Down-modulation of CCR5 by various chemokines and the inhibitory effect of MC-68 were measured on murine peripheral blood leukocytes. Quantitative down-modulation of CCR5 was determined on NK cells because these cells homogeneously express high levels of CCR5. Comparable results were also obtained for T cells.

From all chemokines tested, murine MIP-1α most efficiently internalized CCR5 (Fig. 6*A*). It is approximately one order of magnitude more active than the two human chemokines RANTES and MIP-1β. More than sixty percent of surface CCR5 was internalized with 12 ng/ml of murine MIP-1α, whereas >200 ng/ml of human RANTES were necessary for a comparable degree of internalization. A representative histogram shows the internalization of CCR5 on NK cells with 1 μg/ml MIP-1α (Fig. 6*B*). The ability of MIP-1α, MIP-1β, and RANTES to induce receptor internalization correlates well with their activity in Ca-flux experiments (30). The two N-terminal modifications of RANTES, Met-RANTES, and AOP-RANTES were also included in the assay because they are commonly used as antagonists in rodent studies. Met-RANTES and AOP-RANTES were approximately an order of magnitude less efficient than unmodified RANTES or human MIP-1β, indicating that the modified versions of RANTES have only a weak agonistic activity on murine CCR5. Met-RANTES and AOP-RANTES bind to murine CCR5 with about the same affinity as unmodified RANTES (A.E.I.P., unpublished observations).

We then investigated whether MC-68 is able to block internalization of murine CCR5 induced by RANTES and MIP-1α (Fig. 7*A*). For that purpose, murine blood cells were preincubated with MC-68 at a concentration of 10 μg/ml followed by incubation with various concentrations of the chemokines. Internalization of CCR5

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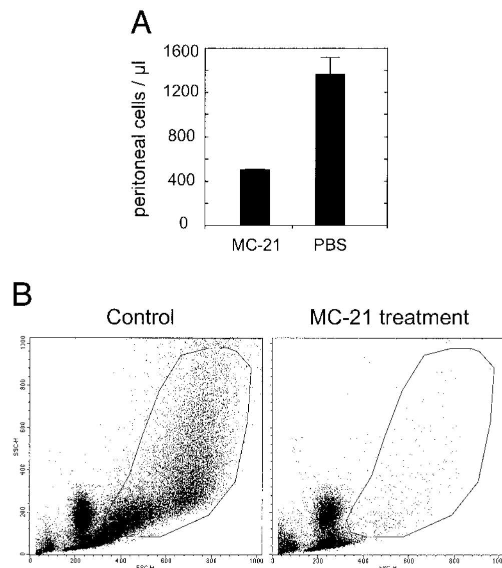

FIGURE 5. Effect of MC-21 on the influx of monocytes in the model of thioglycollate-induced peritonitis. *A*, A single i.p. injection of 500 μg of MC-21 significantly reduces the number of cells obtained in the peritoneal lavage. *B*, Flow cytometry of the peritoneal lavage shows that MC-21 almost completely blocks the influx of monocytes/macrophages (indicated by a circle), while it has little influence on the number of infiltrating granulocytes and lymphocytes.

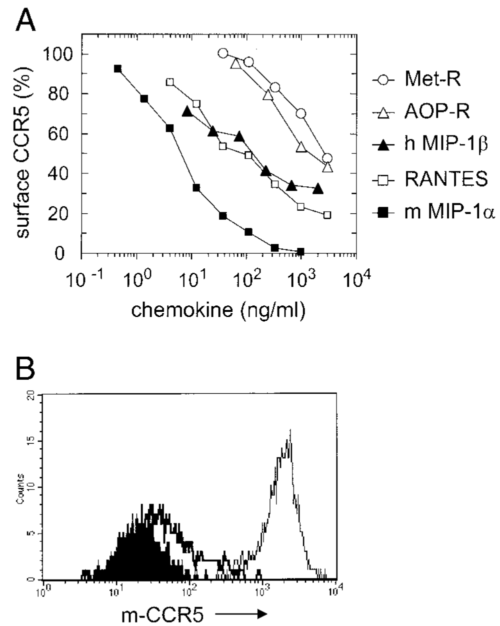

FIGURE 6. FACS analysis showing down-modulation of CCR5 from the surface of murine NK cells with various chemokines. *A*, Murine MIP-1α is 10 times more active than human RANTES or MIP-1β and 100 times more active than Met-RANTES (Met-R) or AOP-RANTES (AOP-R). *B*, A representative histogram shows the down-modulation of CCR5 with 1 μg/ml of MIP-1α (thick line) compared with the positive control (light gray area) and the negative control (dark gray area).

with both chemokines was significantly blocked by MC-68. Internalization of CCR5 with 1 μg/ml of RANTES was reduced from >80% to <20% by MC-68. Comparable results were obtained for murine MIP-1α (100 ng/ml), where MC-68 reduced the internalization of CCR5 from 96% to 20%.

Competitive binding assays using radiolabeled ligands confirmed that the Ab MC-68 efficiently blocks murine CCR5. The binding of MIP-1β to CCR5 was blocked with an IC_{50} of 0.6 μg/ml (Fig. 7*B*). MC-68 also blocked the binding of MIP-1α and RANTES with an IC_{50} of ~1 μg/ml (data not shown). These results indicate that MC-68 is a suitable reagent to specifically block CCR5.

Expression of CCR2/CCR5 on T cells in apoferritin-induced glomerulonephritis

A detailed knowledge of chemokine receptor expression in murine models of inflammation can provide important insights into the pathogenesis of certain diseases and help to fully understand the results obtained with knockout mice and receptor blockade by chemokine-analogs or small molecular antagonists. Moreover, we were interested whether in mice an accumulation of CCR5- and CCR2-positive cells is a hallmark for certain types of inflammation.

Therefore, we investigated the expression of CCR2 and CCR5 in a model of immune complex-induced kidney inflammation, the apoferritin-induced glomerulonephritis. Previously we have shown by RT-PCR and in situ hybridization that both receptors are expressed in the kidneys (37). Using FACS analysis, we now were able to quantify the expression of both receptors on leukocytes isolated from the peripheral blood and from the inflamed kidneys. As shown in Fig. 8, CCR5-positive and to a lesser extent CCR2-positive T cells accumulate in the inflamed kidney. Although in the peripheral blood only 3% of the $CD4^+$ and 20% of the $CD8^+$ T cells express CCR5, the corresponding percentages in the kidney were 69% and 92%. A similar distribution was found for CCR2, where only 4% of the $CD4^+$ and 3% of the $CD8^+$ T cells in the peripheral blood expressed CCR2 as opposed to 63% and 30% in the kidney with glomerulonephritis.

Discussion

We describe the generation and characterization of mAbs directed against the murine chemokine receptors CCR2 and CCR5. The Abs specifically recognize the receptors on primary mouse cells and allow the analysis of receptor expression in healthy and diseased animals. Murine models are widely used to study the role of chemokines and their receptors in a variety of diseases. The neutralizing Abs will be of value for the comparison of data generated with receptor-deficient mice, where compensation can always be hypothesized to have occurred. In addition, the appropriate transfer of these data to humans depends on a detailed knowledge of receptor expression in both species. Therefore, we analyzed the expression of CCR2 and CCR5 on murine leukocytes isolated from the peripheral blood, spleen, lymph nodes, and inflamed kidneys. The chemokine receptor CCR2 is expressed on monocytes and a small subpopulation of T cells and exhibits an almost identical expression pattern in mice and humans. In contrast, CCR5 is highly expressed on murine NK cells, whereas in humans only a small subpopulation of NK cells weakly express CCR5 (1). Reagents to block CCR5 or the targeted disruption of CCR5 could, therefore, considerably alter NK cell function and innate immunity in mice. Based on the differential expression, one would expect less effect of CCR5 on NK cells in humans. Apart from NK cells, the expression of CCR5 on T cells and monocytes is comparable in mice and humans. CCR5 is expressed on a subpopulation of T cells with a higher fraction of CCR5-positive $CD8^+$ than $CD4^+$ T cells. Monocytes show only a very weak expression of CCR5, as is observed in human PBMC (1). As far as T cells and monocytes/macrophages are involved in the pathogenesis of a disease, the

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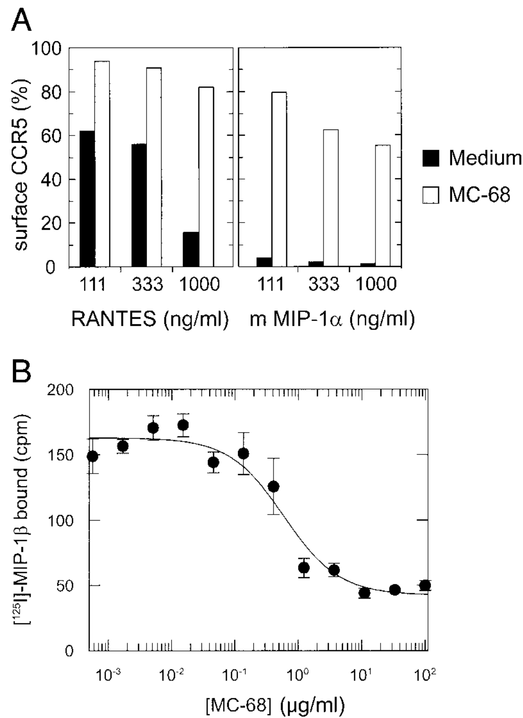

FIGURE 7. Blockage of murine CCR5 by the Ab MC-68. *A*, Preincubation of murine NK cells with MC-68 significantly reduces the down-modulation of CCR5 induced with RANTES or murine MIP-1α. *B*, MC-68 blocks the binding of radiolabeled MIP-1β on CCR5-transfected CHO cells with an IC_{50} of 0.6 μg/ml.

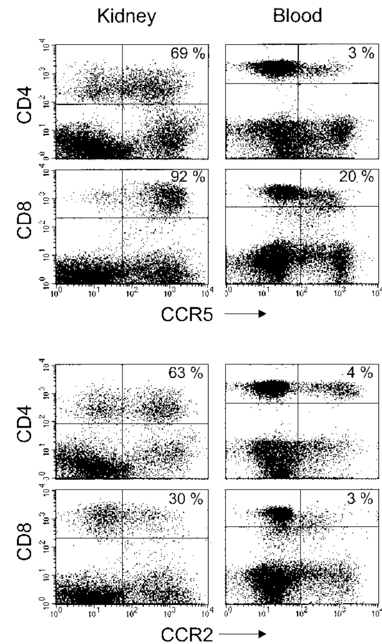

FIGURE 8. Accumulation of CCR5- and CCR2-positive T cells in the inflamed kidneys during immune complex-induced nephritis. Expression of CCR5 and CCR2 was determined by FACS analysis on leukocytes obtained from the peripheral blood (*right*) and the kidneys (*left*) of mice with apoferritin-induced glomerulonephritis.

blockade of CCR5, therefore, might have similar effects in mice and humans.

In humans the chemokine receptors CCR5 and CCR2 are highly expressed in chronic inflammation. In various types of arthritis, we found an accumulation of CCR5- and CCR2-positive T cells in the inflamed joints compared with their frequency in the peripheral blood (1). Both receptors also predominate on T cells in inflammatory kidney diseases including transplant rejection (6). In addition, multiple sclerosis (4, 5) and inflammatory bowel disease (7) are characterized by an accumulation of CCR5-positive cells. To demonstrate that a comparable enrichment of CCR2- and CCR5-positive T cells can be found in the inflamed kidneys of mice, we used the model of apoferritin-induced glomerulonephritis. FACS analysis of T cells isolated from the peripheral blood and the inflamed kidneys allowed a precise quantification of the percentage of CCR2- and CCR5-positive cells in both compartments. Although only 4% or 8% of the T cells in the peripheral blood expressed CCR2 or CCR5, respectively, the respective percentages in the inflamed kidney were 49% and 76%, corresponding to a 10-fold enrichment of CCR2- and CCR5-positive T cells. These data indicate that in mice a pronounced accumulation of CCR5- and CCR2-positive T cells also occurs after an inflammatory stimulus and that the roles of both chemokine receptors for T cells appear to be conserved in both species. Moreover, we found that the percentage of CCR5- and CCR2-positive T cells is almost two times higher in the Th1-prone mouse strain C57BL/6 than in the Th2-prone strain BALB/c. The strain-dependent expression correlates to a reduced Th1 response described in CCR5- and CCR2-deficient mice.

Using internalization assays with primary cells, we analyzed the activation of CCR2 and CCR5 by various chemokines and chemokine analogs. In most cases, internalization of chemokine receptors shows a good correlation to other activation assays such as Ca-influx or migration (36). The RANTES analog, Met-RANTES, has been used extensively to block chemokine receptors in mice, whereas the potent anti-HIV analog, AOP-RANTES, is less well documented for its anti-inflammatory properties, although several studies are currently ongoing. The results of these experiments are often difficult to interpret because little is known about the effects of these reagents on murine CCR5, and some of the reagents may influence receptors other than CCR5. To get insight into the first issue, we performed down-modulation assays measuring the agonistic activity of various chemokines. On murine CCR5, the murine chemokine MIP-1α shows a $>$10-fold higher activity than the two human chemokines RANTES and MIP-1β. The chemokine analogs Met-RANTES and AOP-RANTES were 100-fold less active than murine MIP-1α. Interestingly, the effect of AOP-RANTES on murine CCR5 differs completely from its effect on human CCR5, because AOP-RANTES efficiently internalizes the human receptor (36) but is only weakly active on the murine receptor. AOP-RANTES and Met-RANTES were shown to suppress disease activity in models of experimental nephritis (28) and collagen-induced arthritis (25). Consistent with these findings, we show that both ligands are 100-fold less active on CCR5 than MIP-1α as measured by receptor internalization. Because AOP-RANTES and Met-RANTES bind with about the same affinity to CCR5 (A.E.I.P., unpublished observations), the two RANTES analogs may be considered CCR5 antagonists.

The mAbs MC-21 and MC-68 show a profound blockade of CCR2 and CCR5, respectively. As described above, chemokine analogs often have the disadvantage of acting on several receptors and knockout mice might have partially adapted to the lifelong deficiency of the receptor. mAbs would allow to specifically block one receptor at any given time point. Therefore, we investigated

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whether the Abs MC-21 and MC-68 are able to block ligand binding and activation of CCR2 and CCR5 measured by chemokine-induced receptor internalization. The Ab MC-21 blocked the binding of MCP-1 to murine CCR2 with an IC_{50} of 0.09 μg/ml. When murine monocytes were preincubated with MC-21, ~20 times more MCP-1 was necessary to obtain the same degree of receptor internalization as in the absence of MC-21 indicating that MC-21 blocks nearly 95% of the MCP-1 activity. In good correlation to these in vitro data, MC-21 almost completely prevented the influx of monocytes in the model of thioglycollate-induced peritonitis, whereas the influx of granulocytes and lymphocytes was not significantly altered. This indicates that MC-21 efficiently blocks CCR2 in vivo and that the influx of monocytes in contrast to other leukocytes is dependent on the expression of CCR2.

We could also show that the Ab MC-68 efficiently blocks murine CCR5. The binding of MIP-1α, MIP-1β, and RANTES was blocked with an IC_{50} between 0.6 and 1.0 μg/ml. In addition MC-68 neutralized >99% of the MIP-1α and RANTES activity as measured by receptor internalization assays. Therefore, MC-68 appears as useful reagent for inhibition of CCR5.

The mAbs against murine CCR2 and CCR5 are valuable tools to further study the role of these receptors in mice and to recognize potential limitations when transferring results from murine models to human diseases.

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