

Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2

(gene targeting/monocyte chemoattractant protein 1/trafficking/peritonitis/granuloma)

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Contributed by Oliver Smithies, September 3, 1997

ABSTRACT CC chemokine receptor 2 (CCR2) is a prominent receptor for the monocyte chemoattractant protein (MCP) group of CC chemokines. Mice generated by gene targeting to lack CCR2 exhibit normal leukocyte rolling but have a pronounced defect in MCP-1-induced leukocyte firm adhesion to microvascular endothelium and reduced leukocyte extravasation. Constitutive macrophage trafficking into the peritoneal cavity was not significantly different between CCR2-deficient and wild-type mice. However, after intraperitoneal thioglycollate injection, the number of peritoneal macrophages in CCR2-deficient mice did not rise above basal levels, whereas in wild-type mice the number of macrophages at 36 h was ≈ 3.5 times the basal level. The CCR2-deficient mice showed enhanced early accumulation and delayed clearance of neutrophils and eosinophils. However, by 5 days neutrophils and eosinophils in both CCR2-deficient and wild-type mice had returned to near basal levels, indicating that resolution of this inflammatory response can occur in the absence of macrophage influx and CCR2-mediated activation of the resident peritoneal macrophages. After intravenous injection with yeast β -glucan, wild-type mice formed numerous large, well-defined granulomas throughout the liver parenchyma, whereas CCR2-deficient mice had much fewer and smaller granulomas. These results demonstrate that CCR2 is a major regulator of induced macrophage trafficking *in vivo*.

Accumulation of leukocytes at inflammatory sites is regulated by a family of small, discrete chemotactic proteins called chemokines (1, 2). Two major chemokine subfamilies are distinguished by whether the first two conserved cysteine residues occur together (CC) or are separated by another amino acid (CXC). The monocyte chemoattractant proteins (MCPs) share about 65% amino acid identity and comprise a subgroup within the CC chemokine subfamily (3–5). MCP-1 is the most prominent and best studied of these molecules. MCP-1 is produced by a wide variety of cell types in response to pro-inflammatory stimuli (1, 6, 7), and its chemotactic and activating functions are directed at monocytes, T lymphocytes, natural killer (NK) cells and basophils (8–14).

The CC chemokine receptors (CCRs) are structurally related, seven-transmembrane-spanning proteins that signal through heterotrimeric G-protein complexes (15). In the mouse, MCP-1 appears to bind solely to CCR2 (16), although CCR2 also serves as a receptor for the other MCP subfamily members (17–20). CCR2 gene expression is highly regulated. Differentiation of monocytes to macrophages or treatment of monocytes with interferon γ , tumor necrosis factor α plus

interleukin 1, or with lipopolysaccharide decreases CCR2 gene transcription whereas interleukin 2 treatment of T lymphocytes and monocytes augments CCR2 expression (21–24).

MCP-1, the major ligand for CCR2, is abundantly expressed in a number of pathological conditions characterized by monocyte infiltration, including atherosclerosis (25, 26), rheumatoid arthritis (27, 28), experimental autoimmune encephalomyelitis (29, 30), idiopathic pulmonary fibrosis (31), psoriasis (32), glomerulonephritis (33), and allograft rejection (34). With respect to HIV-1 infection and AIDS, CCR2 can serve as a coreceptor for some HIV-1 strains (35); however, a point mutation in the CCR2 gene that leads to a single, conservative amino acid change in the first transmembrane domain of CCR2 was found to correlate with significantly delayed progression to AIDS (36).

To determine the *in vivo* biological functions of CCR2 and the potential consequences of chronic CCR2 deficiency, we used homologous recombination in embryonic stem (ES) cells to disrupt expression of the mouse CCR2 gene. Our results demonstrate that CCR2 deficiency severely diminishes leukocyte adhesion to the microvasculature and monocyte extravasation in response to pro-inflammatory stimuli.

MATERIALS AND METHODS

Cloning and Targeted Disruption of the Mouse CCR2 Gene.

A 748-bp PCR fragment was made from human genomic DNA using primers corresponding to unique regions of the human CCR2 gene: 5'-GCTTTCACAGTTACTCAGGCCGAA-3' and 5'-CATAAATTTGACGTGAAGCAA-3' (37). This fragment was radiolabeled with [α -³²P]dCTP and used to screen mouse strain 129/Ola genomic libraries. A single clone was identified that contained a 2.6-kb EcoRI fragment which included 60% of the N-terminal coding sequence for mouse CCR2 (16, 18) and upstream sequences. Alignment of the mouse CCR2 genomic sequence with the cDNA sequence revealed a splice site 11 bp in front of the putative translation start codon and a noncoding exon located ≈ 2 kb upstream of the coding exon. The 2.6-kb EcoRI fragment cloned in pBlue-script served as the basis for the construction of a targeting plasmid (see Fig. 1).

Control Animals. Southern blot analysis of bacterial artificial chromosome (BAC) clones containing large (50–240 kb) fragments of strain 129 genomic DNA indicated that the CCR1, CCR2, and CCR5 genes are closely linked (ref. 16 and

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Abbreviations: CCR, CC chemokine receptor; MCP, monocyte chemoattractant protein; ES cell, embryonic stem cell; NK, natural killer.

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our unpublished results), consistent with the clustering of human *CCR* genes (39). To eliminate the potential influence of strain-specific effects linked to the *CCR2* gene (40), a control strain in which the *CCR* gene cluster is derived from the 129/Ola strain was developed by crossing wild-type F₁ progeny of 129/Ola × C57BL/6J matings (40). A *Bgl*III restriction fragment length polymorphism was found that distinguishes the *CCR5* allele in 129/Ola and C57BL/6J; a probe made from a 1.5-kb *Xba*I fragment located about 1.8 kb upstream of the *CCR5* coding exon detects a 3.3-kb *Bgl*III fragment in 129/Ola and a 3.6-kb *Bgl*III fragment in C57BL/6J genomic DNA. All work with animals was done in accordance with National Institutes of Health guidelines and protocols approved by Animal Care and Use Committees at the University of North Carolina at Chapel Hill, the University of Texas at Austin, and the University of Virginia.

Competitive Binding Assays. Radioiodinated mouse JE (an MCP family member and a ligand for *CCR2*) was purchased from New England Nuclear. To obtain macrophages for binding assays, mice 8–12 weeks old were injected i.p. with 1 ml of 4% Brewer's thioglycollate broth (Becton Dickinson). Three days later mice were killed by exposure to CO₂ and the peritoneal cells were harvested by lavage with 7 ml of PBS. Cells were counted with a hemocytometer. Binding assays were done in duplicate in round-bottom 96-well plates and a final volume of 200 μ l. Binding buffer was RPMI 1640 medium, 40 mM Hepes (pH 7.5), and 1% BSA. Two-fold serial dilutions of cold JE (R & D Systems) were made beginning at a final concentration of 10 nM and radiolabeled chemokine was added at a final concentration of 0.2 nM. After addition of cells, the plates were incubated for 3 h at 4°C, at which time bound ligand was separated from free by briefly microcentrifuging the cells through 100 μ l of a mixture of 80% dibutylphthalate/20% olive oil. The tubes were frozen and the cell pellets were clipped into counting tubes. Both cell-associated and free radioactivity were measured in a gamma counter. PRISM software (Graphpad, San Diego) was used for calculations, statistical analysis, and graphics.

Intravital Microscopy. Intravital microscopy of the mouse cremaster muscle was performed as described (41). Briefly, mice were anesthetized by i.p. injection with 30 mg/kg sodium pentobarbital (Nembutal; Abbott) and 0.1 mg/kg atropine (Elkins-Sinn, Cherry Hill, NJ), followed by 100 mg/kg ketamine hydrochloride (Ketalar; Parke-Davis). All mice were pretreated for 2 h before surgery with an intrascrotal injection of 300 ng recombinant murine MCP-1 (PharMingen) in 0.3 ml isotonic saline, the dose sufficient to induce significant leukocyte adhesion in wild-type mice. The trachea, carotid artery, and jugular vein were cannulated for blood sampling, blood pressure monitoring, and injection of supplemental anesthetic. The cremaster was dissected as described (41) and placed on an intravital microscope (Axioskop; Carl Zeiss) with a saline immersion objective (SW 40, 0.75 numerical aperture). Venules with diameters between 20 and 60 μ m were recorded through a charged-coupled device (CCD) camera system (model VE-1000CD; Dage-MTI, Michigan City, IN) on an S-VHS video recorder. Microvessel diameters were measured from video recordings using a customized digital image processing system (42). The centerline erythrocyte velocity in recorded microvessels was measured using a dual photodiode and a digital on-line cross-correlation program (42) running on an IBM-compatible computer system. Centerline velocities, V_c , were converted to mean blood flow velocities, V_b , by dividing the centerline velocity by an empirical factor of 1.6 (43). Wall shear rates were estimated as $2.12 (8 V_b/d)$, where V_b is the mean blood flow velocity, d is the diameter of the vessel, and 2.12 is a median empirical correction factor obtained from actual velocity profiles measured in microvessels *in vivo* (44). The venules were stratified for a similar distribution of hemodynamic parameters (wall shear rate range be-

tween 400 and 1,500 s⁻¹). In these venules, the number of adherent leukocytes was determined per 200 μ m segment of venule from the intravital video recordings. For differential counts, whole mount preparations were prepared as described (45) and emigrated leukocytes were counted in a semicircular field of view. The velocity of rolling leukocytes was determined by measuring the distance traveled during a constant 2-s time interval. A constant time interval was chosen because previous research had indicated that varying time intervals of velocity measurements can introduce experimental artifacts (46). The mean rolling velocities and numbers of adherent or emigrated leukocytes were compared between genotypes using the two-tailed Student's *t* test with correction for unequal variances. Statistical significance was set at $P < 0.01$.

Cytospin Preparations. Peritoneal exudate cells were harvested at various times after thioglycollate injection, counted, and cytospun onto slides using a cytocentrifuge (StatSpin; Shandon Scientific, Runcorn, U.K.). Cells were stained with Diff-Quik (Baxter Scientific Products, McGaw Park, IL) and leukocyte differentials were determined by scoring at least 300 cells. Peripheral blood was obtained from the retro-orbital sinus using heparinized capillary tubes. Red blood cells were lysed by using an erythrocyte lysis kit (R & D Systems). White blood cells were counted with a hemocytometer, cytospun onto slides and stained with Diff-Quick. Statistics were calculated as described above.

Liver Granulomas and Histology. Groups of 6- to 12-week-old mice were given a single tail vein injection of 200 μ l of PBS containing 2 mg of sonicated Zymocel, a purified yeast β -glucan preparation composed of nonantigenic micron-sized particles (Alpha-Beta Technology, Pullman, WA). After 10 days, the peak time for hepatic granuloma formation, the mice were sacrificed by exposure to CO₂ and the livers were excised intact and fixed in Bouin's solution. Sections were cut and stained with hematoxylin/eosin. Slides were examined under a light microscope at various magnifications.

RESULTS

Targeted Disruption of the Mouse *CCR2* Gene. The targeting strategy used to disrupt *CCR2* gene expression is shown in Fig. 1A. The neomycin-resistance cassette was inserted in front of the coding sequence for the first transmembrane domain of *CCR2* in opposite transcriptional orientation (16, 18). Male chimeras generated from correctly targeted ES cells transmitted the mutation through the germ line when mated with C57BL/6J females (Fig. 1B). Heterozygote matings then produced homozygous *CCR2*^{-/-} mice at the expected Mendelian frequency. The *CCR2*^{-/-} mice were viable, exhibited normal growth and development and were fertile. In competitive binding assays using ¹²⁵I-labeled JE, thioglycollate-elicited peritoneal exudate cells from *CCR2*^{-/-} mice showed a dramatic reduction in specific binding relative to the elicited cells from control animals (Fig. 1C). The *CCR2*^{-/-} mice have been observed for up to 15 months in both pathogen-free conditions and in viral-free conventional caging without observable differences with wild-type mice, suggesting that they are not severely immunocompromised.

Defective Leukocyte Adhesion to the Microvasculature. Current models of induced leukocyte extravasation propose a multistep process that includes rolling of leukocytes along the vessel wall, firm adhesion, and arrest at sites of high chemoattractant concentration, transendothelial cell migration, and chemotaxis (47). We therefore compared leukocyte rolling in venules of MCP-1-treated cremaster muscle of *CCR2*-deficient and wild-type mice. The numbers of rolling cells per unit of time and the distribution of rolling velocities were not different between the two groups (Fig. 2) and are similar to those seen without MCP-1 treatment (45). This finding shows that the rolling of the majority of leukocytes is unaffected by a defi-

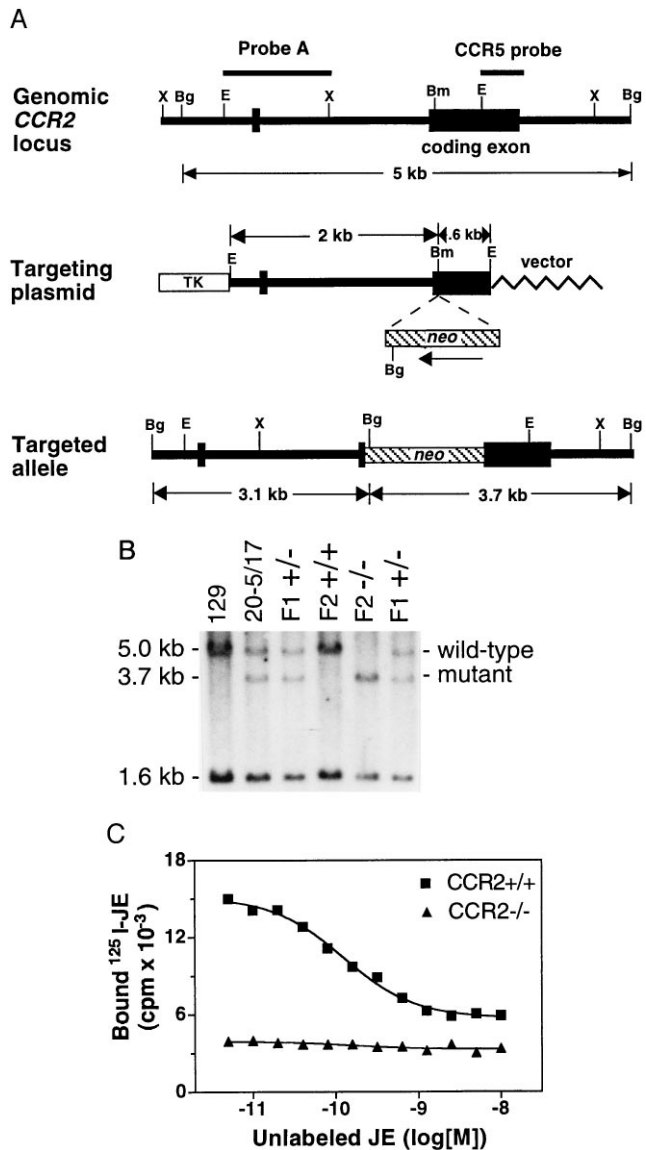


FIG. 1. Strategy for targeted disruption of the mouse *CCR2* gene. (A) (Top) *CCR2* gene locus showing the single coding exon on a 5.0-kb *Bgl*III (*Bg*) fragment and a noncoding exon about 2 kb upstream of the coding exon. Other restriction enzyme sites are *Xba*I (*X*), *Bam*HI (*Bm*), and *Eco*RI (*E*). A 420-bp PCR fragment of the 3' coding region of the cross-hybridizing *CCR5* gene was used as a probe to screen for correctly targeted ES clones and to detect germ-line transmission of the mutation. Probe A was used to confirm correct recombination at the 5' end. (Middle) The targeting plasmid. A 1.8-kb pgk-neo cassette was then inserted into the unique *Bam*HI site in the *CCR2* coding region. This insertion created homology arms of 2.0 kb and 0.6 kb. A pgk-thymidine kinase (TK) cassette on a *Kpn*I fragment was inserted into the *Kpn*I site of the pBluescript polylinker. The targeting plasmid was linearized by digestion with *Not*I, transfected by electroporation into E14TG2a ES cells (38), and the transfected cells were placed under double selection in 200 μ g/ml G418 and 2 μ M ganciclovir. (Bottom) Structure of the correctly targeted allele. There is a unique *Bgl*III site near the 3' end of the neo gene which results in a reduction of the germ-line 5.0-kb *Bgl*III fragment to 3.7 kb when the *CCR5* probe is used and 3.1 kb when probe A is used. (B) Southern blot showing correct targeting and germ-line transmission of the mutation. Genomic DNA was prepared from mouse strain 129/Ola liver, the correctly targeted ES clone 20-5/17 used for blastocyst injection, and from the tails of F1 +/– heterozygote, F2 homozygote +/+, and F2 homozygote –/– mice. The DNA was digested with *Bgl*III and probed with the *CCR5* coding region PCR fragment, detecting bands of 5.0 kb (wild-type *CCR2* fragment), 3.7 kb (mutant *CCR2* fragment), and 1.6 kb (wild-type *CCR5* fragment). (C) Competitive binding assay using ¹²⁵I-labeled murine JE on peritoneal exudate cells harvested 3 days

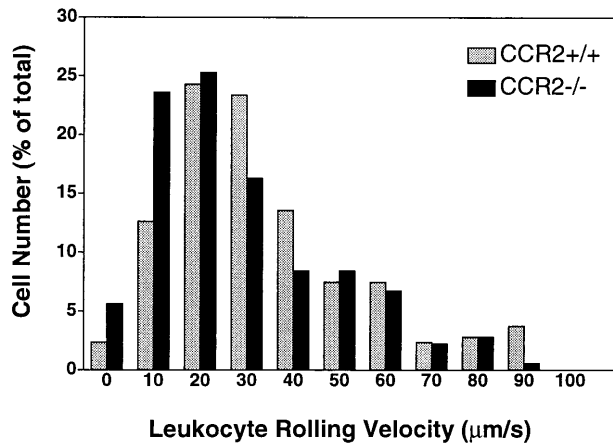


FIG. 2. Rolling leukocyte velocities measured in venules of the exposed cremaster muscle treated with MCP-1 for 3 h. The rolling velocities (in μ m/s) of 214 leukocytes in *CCR2*^{+/+} mice (gray bars) and 178 leukocytes in *CCR2*^{-/-} mice (black bars) were measured and showed a similar distribution.

ciency in *CCR2*, although our data do not exclude the possibility that the rolling velocity of a small subpopulation of circulating leukocytes—e.g., monocytes or NK cells—might be altered in *CCR2*-deficient mice.

In contrast to these results with rolling, the number of firmly adherent leukocytes inside cremaster muscle venules was significantly reduced ($P < 0.01$) in *CCR2*-deficient mice after MCP-1 treatment (Fig. 3A). This reduction in leukocyte adhesion was accompanied by a diminished accumulation of leukocytes in the tissue space surrounding the venules (Fig. 3B). Whereas in wild-type mice an average of 32 cells was found next to a venule in a high power microscopic field, an average of only 20 cells was observed in *CCR2*-deficient animals ($P < 0.01$). These differences could not be attributed to differences in hemodynamic parameters between the venules in wild-type and *CCR2*-deficient mice as judged by our finding no significant differences in diameter, centerline velocity, and shear rate in the two types of mice (data not shown).

Induced But Not Constitutive Monocyte Accumulation Is Severely Reduced in *CCR2*-Deficient Mice. An i.p. injection of thioglycollate is a well-established mouse model of acute peritonitis, which is characterized by successive waves of granulocyte and macrophage accumulation. To determine whether *CCR2* deficiency affects this dynamic inflammatory process, control and *CCR2*-deficient mice were injected with thioglycollate and the number and type of leukocytes present in the peritoneal cavity at 4, 24, 36, 72, and 120 h after injection were determined. As shown in Fig. 4, the absence of *CCR2* led to multiple changes in leukocyte accumulation. In both wild-type and *CCR2*-deficient mice, the vast majority of resident peritoneal leukocytes were macrophages, and 4 h after thioglycollate injection, the number of macrophages in both strains was decreased by >40% (Fig. 4A). After this time point, however, the two strains differed dramatically in number of peritoneal macrophages. By 24 h, the macrophages in wild-type mice were \approx 2 times basal levels, peaked at 36 h at 3.5 times basal levels, and then declined at 72 h and 120 h, at which time

after thioglycollate injection of *CCR2*^{+/+} (■) and *CCR2*^{-/-} (▲) animals. Cells (1.7×10^6 cells per well) were incubated with 0.2 nM labeled JE, and the indicated concentration of unlabeled JE for 3 h at 4°C. Each data point represents the average of duplicate determinations of bound labeled JE. In the experiment shown, the *CCR2*^{-/-} cells exhibited a 93% reduction in specific binding (72% after normalization for the number of macrophages in the cell populations; see text). In three similar experiments, the mean \pm SEM reduction in binding was $89.1 \pm 3.5\%$.

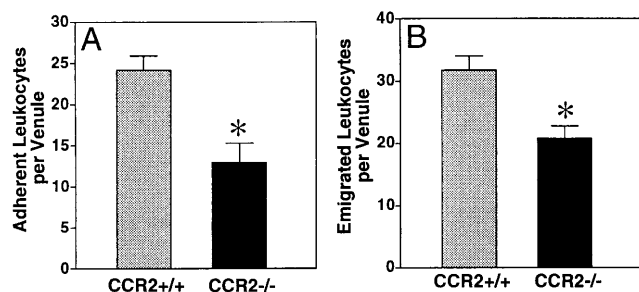


FIG. 3. (A) Leukocyte adhesion, measured as firmly adherent cells per 200- μ m segment of venule. A total of 368 cells in 16 venules adhered in CCR2^{+/+} mice (gray bar) and 246 cells in 19 venules adhered in CCR2^{-/-} mice (black bar). In mice receiving saline only, fewer than two adherent leukocytes were observed per venule. (B) Leukocyte emigration, expressed as number of cells emigrated per venule. A total of 493 cells were observed next to 31 venules of CCR2^{+/+} mice (gray bar) and 323 cells were observed next to 31 venules of CCR2^{-/-} mice (black bar). No emigrated leukocytes were seen in mice receiving saline only. Data are presented as mean \pm SEM. Significant difference: *, $P < 0.01$.

macrophage numbers were about 1.5 times the basal level. In contrast, the peritoneal macrophages in the CCR2-deficient mice never rose above the constitutive level at any time point, and at 120 h the number of macrophages was only about 17% of the constitutive number. The differences between control and CCR2-deficient mice were significant ($P < 0.01$ at 24, 36, and 120 h and $P < 0.001$ at 72 h) and clearly demonstrate that CCR2 is essential for macrophage migration in this model of acute inflammation.

Neutrophils and eosinophils comprise only about 2% of the resident peritoneal leukocytes in both mouse strains, but after thioglycollate injection, the two strains again showed different patterns of accumulation and clearance (Fig. 4B). In both strains there was a large influx of neutrophils and eosinophils at 4 h but the CCR2-deficient mice showed a mean of 40% more cells than control animals. Peak accumulation, ≈ 75 times basal levels in both strains, was reached at 24 h. Neutrophils and eosinophils declined sharply in control mice at 36 and 72 h, but they remained elevated in the CCR2-deficient mice until the 120-h time point. At both the 36- and 72-h time points the differences in cell number were significant ($P < 0.001$). By

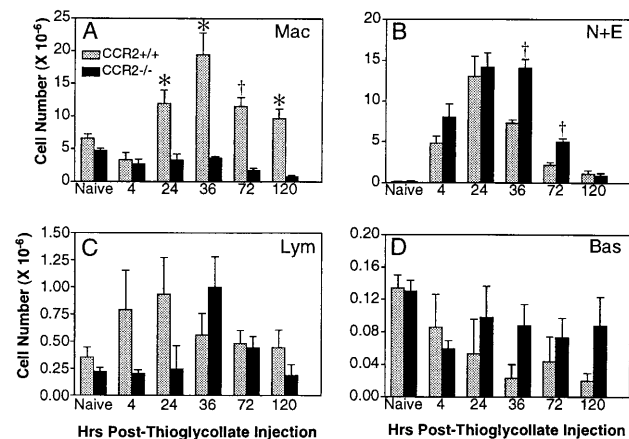


FIG. 4. Peritoneal leukocyte accumulation in CCR2^{+/+} mice (gray bars) and CCR2^{-/-} mice (black bars) following thioglycollate injection. The number of each leukocyte type was determined on cytospin slides at the times indicated after thioglycollate injection: (A) Mac, macrophages; (B) N+E, neutrophils and eosinophils; (C) Lym, lymphocytes; (D) Bas, granulated basophils ($n \geq 5$ CCR2^{+/+} and CCR2^{-/-} animals at each time point). Data are presented as mean \pm SEM; significant difference: *, $P < 0.01$; †, $P < 0.001$. Differences in C and D did not reach statistical significance.

120 h both strains had returned to ≈ 5 times basal level. Because MCP-1 does not have a direct effect on neutrophil and eosinophil chemotaxis (1), these differences could be related to the reduced macrophage numbers or to effects on another cell type.

Lymphocytes and granulated basophils together account for $< 5\%$ of peritoneal leukocytes (Fig. 4C and D). After thioglycollate injection, changes in these cell populations followed clearly distinct trends in wild-type and CCR2^{-/-} mice. Control mice showed a substantial early increase in lymphocytes at 4 h, reaching a peak at 24 h of 2.6 times the basal level (Fig. 4C). CCR2-deficient animals did not have this early increase but showed a 4.5-fold increase at 36 h. In both cases lymphocytes returned to approximately basal levels by 120 h. Both strains had similar constitutive numbers of basophils (Fig. 4D). After thioglycollate injection, control animals showed a gradual reduction in basophilic cells at each time point and by 120 h only about 15% of the constitutive number remained. CCR2-deficient mice showed a reduction in basophils of 55% at 4 h, but no further decline through 120 h. The results summarized in Fig. 4 demonstrate that CCR2 deficiency, either directly or indirectly, alters the accumulation of multiple leukocyte subtypes in this acute inflammatory process.

Reduced Liver Granuloma Formation in CCR2^{-/-} Mice.

Previous studies by others have shown that 10 days after i.v. injection of yeast glucan, about 10% of the total liver parenchymal volume becomes granulomatous, with the typical lesion consisting predominantly of macrophage-derived cells (48, 49). Because MCP-1 has been implicated in hepatic and pulmonary granuloma formation (50, 51), we assessed granuloma formation in the livers of control and CCR2-deficient mice after intravenous injection of the β -glucan extract Zymocel. The livers of uninjected mice showed no evidence of granuloma formation. Ten days after glucan injection of control mice, numerous ($> 50/\times 100$ microscope field) large, well-circumscribed granulomas had developed throughout the liver parenchyma (data not shown). These were composed mainly of epithelioid histiocytes with occasional polymorphonuclear cells (Fig. 5A). In contrast, the livers of CCR2-deficient mice did not show these large granulomas, but instead had fewer ($< 10/\times 100$ microscopic field) small cellular foci consisting of only a few histiocytes and polymorphonuclear cells. In addition to these small foci, the CCR2-deficient mice also had occasional (1–2/ $\times 100$ microscopic field), larger necrotising gran-

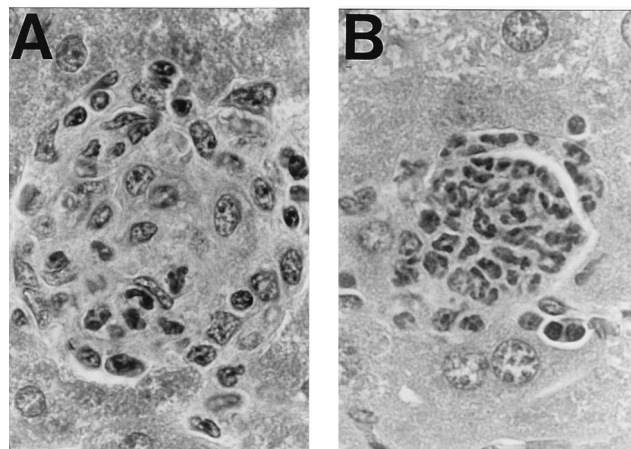


FIG. 5. Differences in glucan-induced hepatic granuloma formation in wild-type and CCR2-deficient mice. (A) A typical large, well-defined granuloma consisting predominantly of epithelioid histiocytes and a few polymorphonuclear cells found in the livers of wild-type but not CCR2^{-/-} mice after intravenous glucan injection. (B) One of the small number of well-formed necrotising granulomas consisting of polymorphonuclear cells found in the livers of glucan-injected CCR2-deficient mice. ($\times 330$)

ulomas composed primarily of polymorphonuclear cells (Fig. 5B).

DISCUSSION

Blocking MCP-1 binding to CCR2 has been proposed as a possible intervention to reduce the pathological sequelae caused by the accumulation of macrophages and T cells that occurs during the course of some diseases. Mice deficient in CCR2 provide a means of determining whether this strategy can provide therapeutic benefits and can help ascertain the potential negative effects of this strategy on normal immune processes. We find that CCR2 deficiency has no observable effects on growth, development, or fertility, and that it does not result in significant reductions in constitutive accumulation of peritoneal macrophages (Fig. 4), Kupffer cells, and alveolar macrophages (R. Alam and W.A.K., unpublished observations). However, the biological consequences of CCR2 deficiency in mice become clear when the animals are challenged with pro-inflammatory stimuli.

Our results with intravital microscopy indicate that CCR2 is not required for the rolling of leukocytes in the presence of MCP-1. However, it is important for firm adhesion to the endothelium and subsequent diapedesis. The finding that both processes are similarly reduced suggests that the reduction in leukocyte passage through the endothelium is only a reflection of the reduced firm adhesion and that the ability of CCR2-negative adherent cells to transmigrate is not impaired.

The dramatic reductions in macrophage accumulation in the peritonitis model and hepatic granuloma assay clearly demonstrate that CCR2 is important for induced macrophage trafficking, although we cannot exclude the presence of a low level of induced macrophage migration into the peritoneal cavity or liver that is CCR2 independent. For example, the small, histiocyte-containing cell foci in the livers of our glucan-injected CCR2-deficient mice might have arisen from a small subpopulation of CCR2-independent macrophages.

Which of the MCPs is the major stimulus for CCR2-dependent macrophage accumulation in the peritonitis model and granuloma assay is not yet established, but our current data make MCP-1 a strong candidate. Neutrophils and eosinophils have the ability to produce MCP-1 (52–54), and they accumulate in the peritoneal cavity of normal animals treated with thioglycollate prior to macrophage recruitment. High peritoneal MCP-1 concentrations generated by these cells would likely lead to basophil degranulation (9–11), contributing to the observed steady reduction in basophilic cells in control animals. The absence of this reduction in basophils in the CCR2^{-/-} animals would then be understood.

Phagocytosis of intact neutrophils and eosinophils that have accumulated at inflammatory sites and the efficient resolution of inflammatory responses are important macrophage functions (55). The prolonged or excessive presence of activated neutrophils and eosinophils can contribute to the damage of normal tissues through the release of oxygen intermediates, proteolytic enzymes and pro-inflammatory cytokines (56–58). Several modes of inflammatory cell clearance have been identified that would avoid the release of these substances, including apoptosis followed by macrophage engulfment (59, 60), phagocytosis without apoptosis (61), and emigration out of the inflammatory site (62). We observed the characteristic features of apoptotic granulocytes (63, 64) in the cytoplasts of freshly harvested peritoneal exudate cells from both wild-type and CCR2-deficient mice at all time points after thioglycollate injection (data not shown), although the proportion of cells showing these features was low. The time course data in Fig. 4B show that most of the accumulated neutrophils and eosinophils have disappeared by 5 days irrespective of the strain, yet the CCR2-deficient mice have relatively few resident peritoneal macrophages and do not recruit large numbers of new

macrophages. Possibly, therefore, emigration may be largely responsible for the sharp drop in granulocytes that occurs between 36 and 72 h in the mutant animals. However, our data do not exclude the contribution of multiple clearance mechanisms contributing to resolving the peritonitis.

In conclusion, we have found that CCR2-deficient mice have no obvious phenotypic defects and are not severely immunocompromised. The mutant mice are not significantly different from wild-type animals in leukocyte rolling and constitutive macrophage accumulation, but they do exhibit dramatic reductions in induced leukocyte firm adhesion, extravasation, and macrophage accumulation *in vivo*. Our results support the idea that specific blockers of CCR2 might provide significant therapeutic benefit without severe side effects in pathological conditions characterized by MCP-1 production and subsequent mononuclear cell infiltration (65). The CCR2-deficient mouse should provide a valuable tool for studying the pathogenesis of inflammatory diseases and for determining which conditions might improve or be exacerbated by antagonists of CCR2 ligands.

We acknowledge Kim Kluckman, John Hagaman, Kelli Czarra, O. Shi, Chris Knouff, and Yuchan Zhou for technical assistance; Robert Reddick for help with evaluating tissue sections; Don Cook, Sarah Bronson, Suzanne Kirby, and Randy Goldblum for advice and discussions; Thomas Tedder and Sergio Lira for comments on the manuscript; and Phil Tucker for temporary lab space. This work was supported by Public Health Service Grants GM37567 (N.M.), GM20069 (O.S.), and HL 58108 (K.L.), a grant from the State of Texas (W.A.K.), and a Special Opportunity Award from the Whitaker Foundation (K.L.).

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