Transplantation of Monocyte CC-Chemokine Receptor 2–Deficient Bone Marrow Into ApoE3–Leiden Mice Inhibits Atherogenesis


Objective—To determine the role of leukocyte CC-chemokine receptor 2 (CCR2) in the early development of atherosclerosis

Methods and Results—Bone marrow cells harvested from CCR2 (−/−) and CCR2 (+/+) mice were transplanted into ApoE3–Leiden mice, a mouse strain susceptible for diet-induced atherosclerosis. Eight weeks after bone marrow transplantation, the diet of regular chow was switched to a high-cholesterol diet (1% cholesterol, 15% fat, 0.5% cholate) for another 8 weeks to induce atherosclerosis. No significant differences in serum cholesterol and triglyceride levels were observed between the CCR2 (+/+) → ApoE3–Leiden and CCR2 (−/−) → ApoE3–Leiden mice. However, the mean cross-sectional aortic root lesion area of CCR2 (−/−) → ApoE3–Leiden mice was only 2.94±1.94×10^4 μm^2 compared with 20.94±12.71×10^4 μm^2, for CCR2 (+/+) → ApoE3–Leiden mice. Thus, the absence of CCR2 on leukocytes induces a 86% reduction of aortic lesion area as compared with controls (n=10, P<0.01).

Conclusion—These results provide direct evidence that CCR2 expressed by leukocytes plays a critical role in the initiation of early atherosclerosis and that pharmacological intervention in CCR2 function represents an attractive target to inhibit atherogenesis. (Arterioscler Thromb Vasc Biol. 2003;23:447-453.)

Key Words: atherosclerosis ■ chemokines ■ bone marrow transplantation ■ transgenic animals

Over the last decade it has become clear that atherosclerosis is a chronic disease initiated by the overrecruitment of leukocytes, mainly monocytes and, to a lesser extent, T-cells. Recruitment of leukocytes requires the interaction of various classes of adhesion molecules, for example, selectins, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1, and the presence of counter receptor molecules expressed on the leukocytes/endothelial cells, in addition to the establishment of a chemotactic gradient to guide leukocytes to the source of the inflammatory signal. In atherogenesis, monocyte chemotactic protein 1 (MCP-1) has been shown to be a central mediator of monocyte adhesion and attraction into the subendothelial space. The primary trigger for cells to secrete MCP-1 is likely to be oxidized LDL and remove this substance by receptor-mediated endocytosis. In this process they can be converted into lipid-laden macrophage-foam cells and become themselves a rich source of MCP-1 secretion and further monocyte recruitment.

MCP-1 is a member of the CC family of low molecular mass (8 to 10 kDa) chemokines, chemotactic cytokines that are involved in the recruitment of leukocytes to sites of inflammation and in the process of immunosurveillance and antigen presentation. Many cells are able to produce MCP-1, including monocytes and vascular endothelial cells. In addition to oxidized LDL, MCP-1 expression is induced by inflammatory cytokines, such as tumor necrosis factor-α and interferon-γ. In vivo studies suggest that MCP-1 helps to recruit monocytes to sites of inflammation in a variety of pathological conditions, not only in atherosclerosis but also in rheumatoid arthritis, restenosis, pulmonary fibrosis, and granulomatous lung disease. The effects of chemokines are mediated by a family of closely related G-protein–coupled receptors. MCP-1 initiates signal transduction through binding to the CCR2, which can also be activated by multiple agonists, including MCP-2.
MCP-3,18,19 MCP-4,20,21 and MCP-5.22 In human, two splice variants of the CCR2 receptor have been identified, CCR2A and CCR2B, both able to mediate an in vitro response to MCP-1. There are indications that in vivo only the CCR2B variant is transported to the cell membrane and functionally active.16 One homologous receptor has been identified in mice, mediating responses to the murine MCP-1 analogue, CCR2 may be useful in inhibiting atherogenesis.

Animal Experiments of Leiden University.

Dam Center for Drug Research in accordance with the national laws. Animals were housed and bred at the animal facility provided by Dr. L.M. Havekes (TNO-PG, Leiden, The Netherlands). 35,36 The animals were housed and bred at the animal facility.

Mice with a targeted disruption of the CCR2 gene [CCR2 (−/−)] do develop normally and display no hematopoietic abnormalities. However, CCR2 (−/−) mice did fail to recruit monocytes in an experimental model of peritoneal inflammation.23,24 Furthermore, the systemic absence of CCR2 decreases atherosclerotic lesion formation markedly in apoE (−/−) mice without influencing plasma lipid or lipoprotein concentrations.25,26 These results suggest that antagonists of CCR2 may be useful in inhibiting atherogenesis.

The mechanism whereby CCR2 deficiency influences lesion formation is under dispute. Roque et al29 showed recently in a model of intimal hyperplasia after arterial injury that there was no significant difference in leukocyte accumulation in the arterial wall between CCR2 knockout mice and their wild-type littermates. It is now suggested that CCR2 plays an important role in mediating smooth muscle cell proliferation, and both Roque et al and the accompanying editorial80 suggest that bone marrow transplantation (BMT) between CCR2 knockout and wild-type mice and vice versa are needed to parse out leukocyte versus nonleukocyte contributions. To expand on these findings and to develop a model where the effect of leukocyte CCR2 deficiency could be studied under conditions of established atherosclerosis, we created mice with a specific deficiency of CCR2 in leukocytes using the technique BMT. We and others have previously shown BMT to be a very useful technique to study the role of specific monocyte/macrophage genes in atherogenesis,31–34 In addition, BMT can be performed at any stage of development of the disease. Here we report on the effect of BMT with bone marrow from CCR2 (−/−) mice in apoE3–Leiden mice and show a dramatic (86%) suppression of atherosclerotic lesion formation in comparison with animals that received CCR2 (+/+) bone marrow.

Methods

Animals

CCR2 (−/−) mice and matched controls were generated as previously described.26 Recipient ApoE3–Leiden mice were kindly provided by Dr. L.M. Havekes (TNO-PG, Leiden, The Netherlands).35,36 The animals were housed and bred at the animal facility of the Sylvius Laboratories in Leiden, The Netherlands. Mice used for BMT experiments were housed in sterilized filter-top cages and fed a sterilized regular chow diet, containing 5.7% fat (Hope Farms, Woerden, The Netherlands). Eight weeks after BMT, the diet was switched to a high-cholesterol diet (1% cholesterol, 15% fat, 0.5% cholate) for another 8 weeks to induce atherosclerosis. Drinking water was infused with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sugar. Animal procedures were performed at the Sylvius Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the national laws. All experimental protocols were approved by Ethics Committee for Animal Experiments of Leiden University.

Irradiation and BMT

To induce bone marrow aplasia, female ApoE3–Leiden mice (6 to 8 weeks of age) were exposed to a single dose of 13 Gy (0.28 Gy/min, 200 kV, 4 mA) x-ray total body irradiation using an Andrex Smart 225 (Andrex Radiation Products AS, Copenhagen, Denmark) with a 4-mm aluminum filter, 1 day before the transplantation. Bone marrow cell suspensions were isolated by flushing the femurs and tibias from either CCR2 (−/−) or CCR2 (+/+)) mice with phosphate-buffered saline. Single cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients received 1.0×10^7 bone marrow cells by intravenous injection into the tail vein, n=10 for each group.

Blood Genotyping of Recipient ApoE3–Leiden Mouse After BMT

Genomic DNA isolated from the blood of recipient ApoE3–Leiden mice, at 8 weeks after BMT and after another 8 weeks on high-cholesterol diet, was used as template DNA for polymerase chain reaction (PCR) amplification. Three primers were used together in a single reaction, CCR2 up: 5′-GATGATGGTGAGCCTTGTCA-3′, CCR2 down: 5′-CACAGCATGAAACATAGCCA-3′, plus another primer specific for pgk-neo cassette: Neo 5′-TTAAGGGCC-AGCTCATTCT-3′. Primer CCR2 up and CCR2 down flank the N-neo sequence of the coding sequence of murine CCR2 (360 bp), including the BamHI site where the pgk-neo cassette was inserted. In the presence of pgk-neo cassette, primer CCR2 down and primer Neo will generate a 290-bp fragment. PCR was conducted as follows: 94°C, 5 minutes; 94°C, 30 seconds, 95°C, 30 seconds, and 72°C, 50 seconds, for 40 cycles; 72°C, 10 minutes. Final PCR products were fractionated on a 2% agarose gel. Quantitative PCR was set up by mixing the PCR products from both wild-type and knockout animals, ranging from 0%, 20%, 40%, 60%, 80%, 90%, 95%, 98%, and up to 100% of knockout versus wild type.

Serum Cholesterol and Triglyceride Analysis

After an overnight fasting period, approximately 100 μL of blood was drawn from each individual mouse by tail bleeding. The concentrations of total cholesterol, free cholesterol, and triglycerides in the serum were determined using enzymatic procedures (Roche, Germany). Precipath (standardized serum; Roche, Germany) was used as internal standard. The distribution of cholesterol over the different lipoproteins in serum was determined by loading 30 μL of serum from each mouse onto a Superose 6 column (3.2×30 mm, Smart-system, Pharmacia, Uppsala, Sweden). Serum was fractionated at a constant flow rate of 50 μL/min, using phosphate-buffered saline. Total cholesterol content of the effluent was determined enzymatically.

Histological Analysis of Hearts and Aortas for Atherosclerosis

To analyze the development of atherosclerosis, mice were sacrificed at 16 weeks after BMT (8 weeks on regular chow diet followed by 8 weeks on high-cholesterol diet). Hearts and aortas were perfused in situ with oxygenated Krebs buffer (37°C, 100 mm Hg) for 20 to 30 minutes via a cannula in the left ventricle, followed by a postperfusion fixation with 3.7% neutral-buffered formalin (Formal-fixx, Shandon Scientific Ltd., UK) and subsequent storage in formalin. To evaluate the development of atherosclerotic lesions, the aortas were separated from the hearts. Hearts were bisected at the level of the atras and the base of the heart plus aortic root were taken for analysis. Cryostat 10-μm cross sections of the aortic root were made and stained with oil red O (Sigma). The atherosclerotic lesion area in the sections was quantified using a light microscope connected with a 24-bit full-color video camera and Leica Qwin image analysis software. Mean lesion area was calculated (in μm²) from 10 sections, starting at the appearance of the tricuspid valves as described previously,38 with 10 animals for each group.
Figure 1. Blood genotyping of recipient ApoE3–Leiden mice after BMT. Blood DNA was extracted from ApoE3–Leiden mice that received bone marrow either from CCR2 (+/+) or CCR2 (−/−) animals at 16 weeks after BMT and used as template during PCR amplification. CCR2 up and down primers generate 360-bp bands on wild-type animals and CCR2 down and Neo primer generate a 290-bp band on knockout animals. A, Lanes 1 to 4: blood DNA from ApoE3–Leiden mice received CCR2 (+/+) bone marrow; lane 5: DNA ladder; lanes 6 to 9: blood DNA from ApoE3–Leiden mice received CCR2 (−/−) bone marrow. B, Lane 1: DNA ladder; lanes 2 to 11: mixtures of PCR products ranging from 0%, 20%, 40%, 60%, 80%, 90%, 95%, 98%, 99%, and up to 100% of knockout vs wild type.

Immunostaining of CCR2 Protein Expression
Cryostat 10-μm cross sections of the aortic root were first incubated for 2 hours in blocking buffer containing 1% bovine serum albumin, 1% dry milk powder, and 0.5% Tween-20 to prevent nonspecific binding. After incubation for 1 hour at room temperature with either a goat-anti-mouse CCR2 polyclonal antibody (Santa Cruz, 1:200 dilution), a sheep-anti-mouse CCR2 polyclonal antibody (a generous gift from Dr. Mack, Medical Policlinic, University of Munich, Munich, Germany; 1:1000 dilution), a rat-anti-mouse CCR2 monoclonal antibody (a generous gift from Dr. Pieter H.E. Groot, GSK Pharmaceuticals, 1:200 dilution), a sheep-anti-mouse CCR2 polyclonal antibody (a generous gift from Dr. Mack, Medical Policlinic, University of Munich, Munich, Germany; 1:1000 dilution), sections were washed three times in phosphate-buffered saline and then incubated with donkey-anti-goat, rabbit-anti-sheep, or goat-anti-rat IgG (Santa Cruz, HRP conjugate, 1:500 dilution) respectively for another hour, then washed five times in phosphate-buffered saline for 30 minutes. CCR2 protein expression was visualized under Leica Qwin image analysis software, after adding substrate TMB/H2O2 (Amersham Pharmacia Biotech) and quenched with 2 mol/L H2SO4.

Macrophage Content of Atherosclerotic Lesions
Ten corresponding cryosections next to the ones used for oil red O staining for lesions from both groups were first incubated for 1 hour at room temperature with antibody against a macrophage-specific antigen (MOMA-2, polyclonal rat IgG, 1:50 dilution, Research Diagnostics Inc), then with goat-anti-rat IgG (alkaline phosphatase conjugate, Sigma Diagnostics, 1:200 dilution). Macrophage content from CCR2 (+/+) → ApoE3–Leiden and CCR2 (−/−) → ApoE3–Leiden mice were visualized by adding enzyme substrates 3,3′-diamino-benzidine (Sigma Diagnostics), nitro blue tetrazolium (Sigma Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Diagnostics) and calculated as macrophage positive area versus total lesion area.

Statistical Analysis
Statistical analysis of the data was performed using the unpaired Student t-test.

Results
Blood Genotyping of Recipient ApoE3–Leiden Mice After BMT
Genomic DNA from blood of CCR2 (+/+) → ApoE3–Leiden mice displayed a single 360-bp band. In contrast, among the CCR2 (−/−) → ApoE3–Leiden mice, a single 290-bp band could be observed (Figure 1A), whereas wild-type 360-bp bands are only faintly detectable among some of the CCR2 (−/−) → ApoE3–Leiden mice. Semiquantitative PCR demonstrates that about 98% of the hematopoietic systems of the recipient ApoE3–Leiden mice were replaced by CCR2 (−/−) bone marrow, indicating a nearly complete replacement of donor bone marrow origin (Figure 1B).

Effect of Leukocyte CCR2 Deficiency on Total Cholesterol and Triglyceride Levels
During the weeks after BMT, the total serum cholesterol levels were repeatedly determined. No significant differences between CCR2 (+/+) → ApoE3–Leiden mice and CCR2 (−/−) → ApoE3–Leiden animals could be observed at 5 and 8 weeks after BMT and levels were not significantly different from those measured in week 0, before BMT (Figure 2A). To induce atherosclerotic lesion formation, the transplanted ApoE3–Leiden mice were fed a high-cholesterol diet contain-
ing 1% cholesterol, 15% fat, and 0.5% cholate starting at 8 weeks after BMT. As a result, the total serum cholesterol levels in both the control and experimental groups increased approximately 6-fold (Figure 2A) but no differences were seen between the CCR2 (+/+ → ApoE3–Leiden mice and CCR2 (–/–) → ApoE3–Leiden animals. Although there is more variation of the serum triglyceride levels (Figure 2B) in both groups before BMT and at the different time points after BMT, no significant difference could be found either.

The distribution of cholesterol among serum lipoproteins was analyzed by liquid chromatography. The lipoprotein profiles of the two groups were essentially identical on standard chow diet (Figure 3A, week 8 after BMT) and high-cholesterol diet (Figure 3B, week 16 after BMT). After the switch to high-cholesterol diet, cholesterol in the VLDL and IDL/LDL fraction increased about 10-fold, and there was no significant change of cholesterol in the HDL fraction.

**Effect of Leukocyte CCR2 Deficiency on Atherosclerosis**

To determine the effects of leukocyte CCR2 deficiency on the formation of atherosclerotic lesions, the hearts and aortas of the ApoE3–Leiden mice transplanted with either CCR2 (+/+ or CCR2 (–/–) bone marrow were perfused and fixed at 16 weeks after BMT. Representative photomicrographs of the aortic root of control transplanted mice and the mice transplanted with CCR2 (–/–) bone marrow are shown in Figure 4A.

The mean atherosclerotic lesion area of CCR2 (–/–) → ApoE3–Leiden mice was only 2.94 ± 1.94 × 10^4 μm^2, which is 7-fold smaller compared with 20.94 ± 12.71 × 10^4 μm^2 for CCR2 (+/+ → ApoE3–Leiden mice (Figure 4B). Thus, deficiency of CCR2 in leukocytes induces a 7-fold or 86% reduction in aortic atherosclerotic lesion area (n = 10 for each group, P < 0.01). No correlation between the individual serum cholesterol levels and the mean lesion area could be observed (data not shown).

**CCR2 Protein Expression Within the Atherosclerotic Lesions**

To address the extent to which CCR2 protein was expressed among the lesions, after the bone marrow reconstitution from CCR2 (+/+ and CCR2 (–/–) into ApoE3–Leiden mice, cryosections were stained with a goat-anti-mouse CCR2 polyclonal antibody, a sheep-anti-mouse CCR2 polyclonal antibody, or a rat-anti-mouse monoclonal antibody. Surprisingly, no detectable CCR2 protein expression could be found among both of the CCR2 (–/–) → ApoE3–Leiden and the CCR2 (+/+ → ApoE3–Leiden mice.

**Macrophage Content of Atherosclerotic Lesions**

Macrophage content of both CCR2 (+/+ → ApoE3–Leiden and CCR2 (–/–) → ApoE3–Leiden mice were quantified by applying a macrophage specific antigen (MOMA-2). For CCR2 (+/+ → ApoE3–Leiden transplanted mice, the macrophage positive area over total lesion area was 56.3 ± 4.8% and for CCR2 (–/–) → ApoE3–Leiden mice, a relative MOMA-2-positive area of 49.7 ± 6.5% was observed (n = 10 for each group). This indicates that the majority of the lesions were composed of monocytic origin. These values were not significantly different, suggesting that the cellular composition of the lesion is similar for both groups of transplanted animals.

**Discussion**

Chemokines or chemotactic cytokines represent an expanding family of structurally related small molecular weight proteins that are responsible for leukocyte trafficking and activation. The role of MCP-1 and its receptor CCR2 in atherosclerosis have been extensively investigated. The first evidence came from immunohistochemical studies in which expression of MCP-1 was found in macrophage-rich areas of human and
rabbit atherosclerotic lesions.10 Strong supporting evidence subsequently came from transgenic and knockout mouse studies. Deletion of the MCP-1 gene in mice provided protection against macrophage recruitment and atherosclerotic lesion formation in both LDLR (−/−) and apoB transgenic mice.37,38 Furthermore, local overexpression of MCP-1 in the vessel wall induced infiltration of macrophages and the formation of atherosclerotic lesions.39 Another approach showed that transfecting an N-terminal deletion mutant of the human MCP-1 gene into a remote organ (skeletal muscle) in apoE knockout mice effectively blocked MCP-1 activity and inhibited the formation of atherosclerotic lesions but had no effect on serum lipid concentrations.40 At the same time, most compelling evidence of the role of CCR2 in atherosclerosis has emerged from recent studies using CCR2 knockout animals. Boring et al41 found, in addition to impaired monocyte migration, a reduced type 1 (Th1) cytokine response in CCR2 (−/−) mice and CCR2 (−/−) animals have a dramatically decrease in the level of interferon-γ, which also plays a role in atherosclerosis.42,43 CCR2 has also been reported to transduce a variety of intracellular signals associated with cell growth and migration, including mobilization of intracellular Ca2+,44 activation of p42/44 mitogen-activated protein kinase,45 and Janus kinase 2.46 Furthermore, CCR2 has been found on endothelial cells47 and has recently been implicated in smooth muscle cell proliferation and intimal hyperplasia.49 Thus, by using knockout animals, the systemic absence of CCR2 makes it impossible to discriminate between the leukocyte and nonleukocyte contributions to atherosclerotic lesion formation.

Therefore, we used BMT to develop a model that selectively addresses the effect of the absence of CCR2 in leukocytes in the initiation of atherosclerosis. Bone marrow
cells from CCR2 (+/+) and CCR2 (−/−) mice were transplanted into ApoE3–Leiden mice, a well-characterized transgenic mouse model for diet-induced atherosclerosis. These ApoE3–Leiden mice carry a dysfunctional human ApoE variant, ApoE3–Leiden, and have a delayed clearance of VLDL remnants resulting in high levels of cholesterol and triglycerides when fed lipid-enriched diets.35,36 Here we show that absence of CCR2 in leukocytes in ApoE3–Leiden mice is sufficient to have a dramatic protective effect on atherosclerotic lesion development. The mean cross-sectional aortic root lesion area of CCR2 (−/−) → ApoE3–Leiden was 7-fold smaller as compared with CCR2 (+/+ → ApoE3–Leiden mice. This effect of leukocyte CCR2 deficiency on atherosclerosis could not be explained by the effects on plasma lipids. No significant difference of cholesterol and triglyceride levels could be observed at any time during the study. Also, the lipoprotein profiles of the two groups were essentially identical both on standard Chow diet and on the atherogenic diet, indicating that CCR2 deficiency in leukocytes has no effect on the distribution of serum lipoprotein cholesterol. Chimerism of hematopoietic system could be used by semiquantitative PCR, which reveals that about 98% of hematopoietic system of the ApoE3–Leiden mice was replaced by CCR2 (−/−) bone marrow cells. In the atherosclerotic plaques we tried to confirm the presence or absence of CCR2 expression on protein level by immunohistochemical staining. No detectable CCR2 protein expression could be found among both the CCR2 (−/−) → ApoE3–Leiden and the CCR2 (+/+ → ApoE3–Leiden mice. This might be explained by an effect of monocyte differentiation into matured macrophages inside the plaque and the subsequent downregulation of CCR2 expression as part of a feedback regulatory system. In vitro studies by Wong et al34 and Fantuzzi et al49 have indicated the nearly complete loss of leukocyte CCR2 deficiency among the blood monocytes after they differentiate into macrophages.

The cellular composition of the plaques was analyzed by measuring the macrophage-positive area versus total lesion area. It appears that for both the CCR2 (+/+) → ApoE3–Leiden and the CCR2 (−/−) → ApoE3–Leiden mice, the lesions are relatively macrophage rich (≈50% of total lesion area occupied by macrophages), indicating a similar cellular macrophage component for both groups.

Our present results confirm and extend previous findings by Boring et al27 and Dawson et al28 demonstrating a suppression of atherogenesis in CCR2 (−/−) × apoE (−/−) without affecting plasma lipoprotein levels. However, our study allowed us also to conclude that the antiatherosclerotic effect of CCR2 deficiency could most likely be explained by the lack of CCR2 on leukocytes, especially monocytes. It is of interest to note that the magnitude of the effect of selective leukocyte CCR2 deficiency on lesion development seems to be larger in the present study (86% suppression) than seen before in the models of systemic CCR2 deficiency (about 50%). A recent editorial by Gerszten30 mentions that it should be important to dissect out the leukocyte versus nonleukocyte contributions by applying BMT studies. Our study does provide the desired direct evidence that CCR2 expressed by leukocytes plays a critical role in the initiation of atherosclerosis.

Blood monocytes are the precursors of the lipid-laden foam cells that are the hallmark of early atherosclerotic lesions. Understanding the roles of monocyte/macrophage-derived chemokines and cytokines in atherogenesis may provide a basis for the development of future therapeutic agents aimed at interrupting monocyte recruitment and activation. Our result clearly shows the important role of the CCR2 leukocytes in the early development of atherosclerotic lesions. Whether inhibition of CCR2 would suppress lesion development and/or stability in established atherosclerosis is presently unknown, but the protocol as used here may be useful to address these questions. Resolution of the latter question will be the next challenge and will determine if CCR2 is a valuable target for antiatherosclerotic therapy.

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References


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