Replication of Apolipoprotein E Knockout Mice With CCR2-Deficient Bone Marrow Progenitor Cells Does Not Inhibit Ongoing Atherosclerotic Lesion Development


Objective—Using bone marrow transplantation, we have previously demonstrated the critical role that hematopoietic CCR2 plays in early atherogenesis. Reconstitution of irradiated apolipoprotein (apo) E3-Leiden mice with CCR2-deficient bone marrow progenitor cells resulted in 86% reduction on overall atherosclerotic lesion development. However, no data on CCR2 in the cause of established atherosclerosis have been reported so far.

Methods and Results—To study the role of CCR2 in established atherosclerotic lesions, bone marrow progenitor cells harvested from apoE-/-/ and apoE-/-/CCR2-/- mice were transplanted into lethally irradiated 16-week-old apoE-/- mice with established atherosclerotic lesions. No significant differences were found in serum total cholesterol and triglycerides levels at different time points after transplantation. At age 16 weeks, lesion size in control apoE-/- mice was 3.28±1.06×10^5 μm². At 9 weeks after transplantation, apoE-/-/→apoE-/- and apoE-/-/CCR2-/-→apoE-/- mice had developed significantly larger atherosclerotic lesions (4.49±0.92×10^5 μm², P<0.02 and 4.15±0.62×10^5 μm², P<0.04 compared with controls, respectively). However, no significant effect of disruption of hematopoietic CCR2 was observed on the progression of lesions. Furthermore, the macrophage positive area (78±4% versus 72±9%) and collagen content (11±6% versus 15±3%) of the lesions were similar as well.

Conclusion—In contrast to the critical role of CCR2 in the initiation of atherogenesis, bone marrow progenitor cell-derived CCR2 does not influence the progression of established atherosclerotic lesions, pointing to additional mechanisms for recruitment of monocytes at later stages of lesion development. (Arterioscler Thromb Vasc Biol. 2005;25:1014-1019.)

Key Words: bone marrow transplantation ■ CCR2 ■ regression of atherosclerosis

The recruitment of monocytes and the migration, growth, and activation of macrophages, T lymphocytes, endothelial cells, and smooth muscle cells during the development of atherosclerotic plaques are critical features of the chronic inflammatory response. Early studies by Nelken et al showed that the monocyte chemoattractant protein 1 (MCP-1) is abundantly expressed in macrophage-rich areas of atherosclerotic plaques, suggesting that MCP-1 may be important for monocyte extravasation and for the formation of atherosclerotic lesions. Further evidence implicating MCP-1 in the pathogenesis of atherosclerosis has come from transgenic mouse models. Atherosclerosis-susceptible transgenic mice overexpressing human apolipoprotein (apo) B become resistant to lesion formation even on a high-fat atherogenic diet. Furthermore, low-density lipoprotein (LDL) receptor/MCP-1-defective mice on a high-cholesterol diet had 83% less lipid deposition throughout their arteries than controls. Disruption of the receptor for MCP-1, CCR2, results in a very similar phenotype. CCR2 deficiency results in a >50% suppression of lesion formation against the atherosclerosis-susceptible apoE knockout background, indicating that CCR2 is also associated with the development of early atherosclerosis. Furthermore, by using bone marrow transplantation (BMT), we recently demonstrated that absence of CCR2 on bone marrow progenitor cells resulted in an 86% reduction in atherosclerotic lesion development in apoE3 Leiden transgenic mice. Although these studies established a central role for the MCP-1/CCR2 pathway in early atherogenesis, no data have been reported so far on CCR2 in the cause of established atherosclerosis. Such data are eagerly awaited because CCR2 has been considered as a therapeutic target for atherosclerosis in humans.

In the present study, we transplanted bone marrow progenitor cells from apoE-/- and apoE-/-/CCR2-/- mice into apoE-/- mice with established lesions to address the effect of...
leukocyte-derived CCR2 on progression and remodeling of atherosclerotic lesions.

Materials and Methods

Animals

CCR2−/− mice were generated as previously described.7 CCR2−/− mice were interbred with apoE−/− mice (Jackson Laboratories) to generate the apoE and CCR2 double-knockout mice, apoE−/−/CCR2−/− (back-crossed >8 generations). The animals were housed and bred at the animal facility of the Gorlaeus 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 4.3% fat and no added cholesterol (RM3; Special Diet Services, Witham, UK). Drinking water was supplied 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 Gorlaeus 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 apoE−/− mice at the age of 16 weeks were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) X-ray total body irradiation using an Andrex Smart 225 (YXLON International, Copenhagen, Denmark) with a 6-mm aluminum filter and connected to an Inovision Therapy Dosimeter model 35040 1 day before the transplantation. Bone marrow progenitor cell suspensions were isolated by flushing the femurs and tibias from either apoE−/− or apoE−/−/CCR2−/− mice at the age of 6 weeks with phosphate-buffered saline. Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients received total unfractionated bone marrow progenitor cells (1.0×107) by intravenous injection into the tail vein. Another 10 μL bone marrow progenitor cells at 9 weeks after BMT was used as template DNA for PCR with phosphate-buffered saline and stained for the surface markers F4/80 (macrophage marker), CD3 (T-cell marker), CD19 (B-cell marker), and/or CCR2 (MC21) (0.5 μg Ab/300 000 cells). All antibodies, except MC21, were purchased from BioScience (San Diego, Calif). Samples were analyzed by flow cytometry. All data were acquired by fluorescence-activated cell sorting analysis on a FACS Calibur and 10 000 to 50 000 leukocyte events were analyzed with CELLQuest software (BD Biosciences).

Histological Analysis of Hearts and Aortas for Atherosclerosis

To analyze the development of atherosclerosis, mice were euthanized at 9 weeks after BMT. Hearts and aortas were perfused in situ with phosphate-buffered saline for 20 to 30 minutes via a cannula in the left ventricle, followed by a perfusion-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. The hearts were bisected at the level of the atria 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-Aldrich Chemie Gmbh, Steinheim, Germany). 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.8

Macrophage Recruitment in Response to Thioglycollate

Eight apoE−/− mice underwent transplantation with bone marrow progenitor cells derived from either apoE−/− mice or apoE−/−/CCR2−/− mice (n=4 for each group). Six weeks after BMT, the mice were injected with 3% (weight/volume) thioglycollate solution intraperitoneally. Five days after injection, the total number of recruited peritoneal cells were harvested, counted, and calculated as the mean±SD of each group.

Analysis of Immune Profile in Peritoneal Macrophages and Lymph Nodes

Eight apoE−/− mice were transplanted with bone marrow progenitor cells derived from either apoE−/− mice or apoE−/−/CCR2−/− mice (n=4 for each group, different from thioglycollate groups). Six weeks after BMT, the mice were euthanized and the lymph nodes were harvested. Single-cell suspensions were prepared by passing the lymph nodes through a 30-μm nylon gauze. Cell suspensions from peritoneal fluid (from thioglycollate experiment) and lymph nodes were incubated with 1% normal mouse serum in phosphate-buffered saline and stained for the surface markers F4/80 (macrophage marker), CD3 (T-cell marker), CD19 (B-cell marker), and/or CCR2 (MC21) (0.5 μg Ab/300 000 cells). All antibodies, except MC21, were purchased from BioScience (San Diego, Calif). Samples were analyzed by flow cytometry. All data were acquired by fluorescence-activated cell sorting analysis on a FACS Calibur and 10 000 to 50 000 leukocyte events were analyzed with CELLQuest software (BD Biosciences).

Macrophage and Collagen Content of Atherosclerotic Lesions

Macrophage infiltration into the atherosclerotic lesions was determined at 9 weeks after BMT by immunolocalization of CD68. Formaldehyde-fixed cryostat sections were incubated for 1 hour in blocking buffer containing 5% milk powder in phosphate-buffered saline. Subsequently, sections were incubated with rabbit anti-mouse CD68 (kindly provided by S. Gordon, Sir William Dunn School of Pathology, University of Oxford, UK) for 2 hours at room temperature. After washing, the sections were successively exposed to biotinylated goat anti-rabbit IgG (DAKOCytomation, Glostrup, Denmark) and ABC complex of streptavidin and biotinylated horseradish peroxidase (DAKO). MCP-1 staining was performed using goat anti-MCP-1 polyclonal antibody M-18 (Santa Cruz Biotechnology, Calif) and subsequently a biotinylated mouse anti-goat secondary antibody (Santa Cruz). Finally, CD68 and MCP-1 staining was visualized by incubation with Nova RED substrate (Vector, Calif). As negative controls, rabbit-IgG or goat-IgG (Santa Cruz) was used. Masson’s trichrome (Sigma) staining was used to determine the collagen content. The CD68-positive area and collagen content of lesions were subsequently calculated as the percentage of mean cholesterol and triglyceride in the serum were determined using enzymatic procedures according to the manufacturer’s instructions (Roche, Germany). Precipact (standardized serum; Roche) was used as the internal standard.
positive area versus mean total lesion area using 5 sections per mouse and 10 mice per group.

Results

Chimerism Analysis of Recipient ApoE⁻/⁻ Mice After BMT

To study the role of CCR2 in established atherosclerotic lesions, bone marrow progenitor cells harvested from apoE⁺/⁻ and apoE⁺/⁻CCR2⁻/⁻ mice were transplanted into lethally irradiated apoE⁺/⁻ mice at the age of 16 weeks. At 9 weeks after transplantation, genomic DNA from white blood cells of apoE⁺/⁻ mice received apoE⁺/⁻CCR2⁻/⁻ bone marrow; lane 7: DNA Ladder; lane 8 to 13: blood DNA from apoE⁺/⁻ mice received apoE⁺/⁻CCR2⁻/⁻ bone marrow. B, Lane 1 to 10: mixtures of PCR products ranging from 0%, 10%, 50%, 70%, 80%, 90%, 95%, 98%, 99%, and 100% of knockout versus wild-type.

Peritoneal Macrophage Recruitment After Thioglycollate Treatment

To determine the effect of hematopoietic CCR2 deficiency on leukocyte recruitment, apoE⁺/⁻ → apoE⁺/⁻ mice and apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice received a thioglycollate injection 6 weeks after BMT. The number of cells recruited to the peritoneal cavity 5 days after thioglycollate treatment was 18.8±5.9×10⁶ in the apoE⁺/⁻ → apoE⁺/⁻ mice versus 4.0±1.4×10⁶ in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice (n=4 for each group, P<0.003) as shown in Figure 2A). Similar results were obtained in C57Bl6 versus CCR2 single knockout mice, in which the number of recruited cells in the peritoneal cavity after thioglycollate-induced peritonitis revealed 16.9×10⁶ cells and 5.5×10⁶ cells, respectively.

Interestingly, 45±4% of the peritoneal macrophages was CCR2-positive in the apoE⁺/⁻ → apoE⁺/⁻ mice versus only 4±1% in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice (P<0.0001) (Figure 2B), demonstrating severe loss of CCR2-positive cells after the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ BMT.

Immune Profile of Lymphocytes in the Lymph Nodes

T-cell and B-cell distribution was analyzed in the lymph nodes 6 weeks after BMT (Figure 3A). The percentage of CD3-positive T cells was 16±7% in the lymph nodes of apoE⁺/⁻ → apoE⁺/⁻ mice and was reduced to 6±5% in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice (P<0.007). The percentage of CD19-positive B cells was 34±19% in the apoE⁺/⁻ → apoE⁺/⁻ mice versus 11±8% in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice (P<0.008). The T-cell and B-cell populations were thus affected by CCR2 deficiency in the lymph nodes. In addition, the percentage of CCR2-positive cells in the lymph nodes decreased from 29±19% in the apoE⁺/⁻ → apoE⁺/⁻ mice to 10±7% in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice (P<0.02) (Figure 3B).

Total Cholesterol and Triglyceride Levels

During the weeks after BMT, the total serum cholesterol levels were repeatedly determined. No significant differences

Statistical Analysis

Statistical analysis of the data were performed using the unpaired Student t test or Mann–Whitney test when appropriate.

Figure 1. Chimerism analysis of recipient apoE⁻/⁻ mice after BMT. White blood cell DNA was extracted from apoE⁻/⁻ mice that received bone marrow progenitor cells either from apoE⁺/⁻ or apoE⁺/⁻CCR2⁻/⁻ animals at 9 weeks after BMT and subsequently used as template during PCR amplification. CCR2 up and down primers generate 360-bp bands for wild-type mice; CCR2 down and Neo primer generate a 290-bp band for knockout mice. A, Lane 1 to 6: blood DNA from apoE⁻/⁻ mice received apoE⁺/⁻CCR2⁻/⁻ bone marrow; lane 7: DNA Ladder; lane 8 to 13: blood DNA from apoE⁺/⁻ mice received apoE⁺/⁻CCR2⁻/⁻ bone marrow. B, Lane 1 to 10: mixtures of PCR products ranging from 0%, 10%, 50%, 70%, 80%, 90%, 95%, 98%, 99%, and 100% of knockout versus wild-type.

Figure 2. Macrophage recruitment after thioglycollate-induced peritonitis. The number of cells recruited into the peritoneal cavity 5 days after thioglycollate treatment is decreased in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice compared with the apoE⁺/⁻ → apoE⁺/⁻ mice (A). Moreover, the percentage of F4/80/CCR2 double-positive macrophages is diminished in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice (Figure 2B). Values are means ±SD of 4 mice for each group. *P<0.003. **P<0.0002.

Figure 3. Immune profile of T cells and B cells. The percentage of both the CD3-positive T cells and the CD19-positive B cells are diminished in the apoE⁺/⁻CCR2⁻/⁻ → apoE⁺/⁻ mice compared with the apoE⁺/⁻ → apoE⁺/⁻ (Figure 3A). Yet, the ratio between the 2 cell types remained similar. In addition, a reduction in the percentage of CCR2-positive cells is observed (Figure 3B). *P<0.008; **P<0.02.
between apoE<sup>−/−</sup>→ apoE<sup>+/−</sup> and apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> animals were measured at different time points either before or after BMT (Figure 1A, available online at http://atvb.ahajournals.org). Although there were variations of the serum triglyceride levels in both groups before BMT and at the different time points after BMT, no significant differences could be found either (Figure 1B). Both the serum total cholesterol and triglycerides levels decreased slightly after BMT, but still no significant difference was observed compared with week 0 before BMT.

**Progression of Atherosclerosis**

To determine the effects of bone marrow progenitor cell-derived CCR2-deficiency on the progression of established atherosclerotic lesions, the hearts of the apoE<sup>−/−</sup> mice transplanted with either apoE<sup>−/−</sup> or apoE<sup>−/−</sup>/CCR2<sup>−/−</sup> bone marrow progenitor cells were perfused and fixed at 9 weeks after BMT. Another 10 apoE<sup>−/−</sup> mice at the age of 16 weeks were used to determine control lesion development at baseline. Representative photomicrographs of the aortic roots of control apoE<sup>−/−</sup> mice at the age of 16 weeks, apoE<sup>−/−</sup>→ apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> mice are shown in Figure 4A.

The control apoE<sup>−/−</sup> mice had developed intermediate atherosclerotic lesions of 3.28±1.06×10<sup>5</sup> μm<sup>2</sup> at the age of 16 weeks. Compared with the lesion development in control apoE<sup>−/−</sup> mice, reconstitution of apoE<sup>−/−</sup> recipients with either apoE<sup>−/−</sup> or apoE<sup>−/−</sup>/CCR2<sup>−/−</sup> bone marrow progenitor cells and an additional period of 9 weeks after BMT resulted in a significant increase in lesion size up to 4.49±0.92×10<sup>5</sup> μm<sup>2</sup> (P<0.014) and 4.15±0.62×10<sup>5</sup> μm<sup>2</sup>, respectively (P<0.038; Figure 4B). No statistically significant difference could be found between the apoE<sup>−/−</sup>→ apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> mice (n=10 for each group, P=0.34), indicating that leukocyte CCR2 does not influence the progression of established atherosclerotic lesions.

To further substantiate our findings, we also analyzed atherosclerotic lesion development of 6-month-old apoE knockout mice and apoE/CCR2 double-knockout mice at the aortic root (Figure 4C). Here, we again observed advanced lesions in both groups of mice, which did not differ significantly in size. Lesion size in the apoE knockout mice was 4.02±0.96×10<sup>5</sup> μm<sup>2</sup> versus 3.23±0.77×10<sup>5</sup> μm<sup>2</sup> for the apoE/CCR2 double-knockout mice (n=10 for apoE<sup>−/−</sup> and n=7 for apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>; P=0.13).

**Collagen and Macrophage Content of Atherosclerotic Lesions**

The collagen content of atherosclerotic lesions in 16-week-old control apoE<sup>−/−</sup> mice and 25-week-old apoE<sup>−/−</sup>→ apoE<sup>−/−</sup> and apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> mice was analyzed by Masson’s trichrome staining. The macrophage-positive area was determined by immunohistochemical staining for the macrophage-specific antigen CD68. Control apoE<sup>−/−</sup> mice at the age of 16 weeks displayed no detectable collagen staining and overall macrophage-positive areas within the lesions, characterizing the atherosclerotic lesion development in early stage (Figure 5). Collagen content (blue) in the lesions of apoE<sup>−/−</sup>→ apoE<sup>−/−</sup> transplanted mice was 11±6% as compared with 15±3% in the lesions of apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> mice (upper panel). Macrophage-positive area (dark brown) in the apoE<sup>−/−</sup>→ apoE<sup>−/−</sup> transplanted mice was 78±4% of the lesion area, whereas in apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> mice a similar area of 72±9% was observed. This indicates that in both groups the majority of the lesion was composed of cells from monocyctic origin (lower panel). Furthermore, these values were not significantly different (P>0.05), suggesting that the cellular composition of the lesions was similar for both groups of transplanted mice.

In addition, the expression of the CCR2 ligand MCP-1 was analyzed in the atherosclerotic lesions of both apoE<sup>−/−</sup>→ apoE<sup>−/−</sup> mice and apoE<sup>−/−</sup>/CCR2<sup>−/−</sup>→ apoE<sup>−/−</sup> mice (Figure II, available online at http://atvb.ahajournals.org). In both groups of mice, MCP-1 staining (brown) was similar and primarily detected in the matrix at the luminal surface of the lesion, where a layer of macrophages (CD68 positive in brown) formed a cap on the advanced lesion. This indicates that both in the presence and absence of CCR2, MCP-1 expression is not a limiting factor and is available to mediate the influx of monocytes to advanced lesions.
Atherosclerosis is a complex chronic inflammatory disease and is the principal cause of death in the USA, Europe, and part of Asia. Early atherosclerotic lesions contain monocyte-derived macrophages that have left the circulation and penetrated the subendothelial layer of the arterial wall. Within the past few years, much attention was focused on the role of MCP-1/CCR2 in early development of atherosclerotic lesions. CCR2 has been demonstrated by others and us to play a central role in the recruitment of monocytes/macrophages in the initiation of atherosclerosis. However, the role of CCR2 in the progression of established atherosclerotic lesions remains unknown.

We applied BMT to investigate the effect of silencing of CCR2 in bone marrow progenitor cells on established atherosclerosis, using 16-week-old apoE−/− mice as recipients of apoE+/+ or apoE+/−/CCR2−/− donor bone marrow. After an additional period of 9 weeks of chow diet feeding, the white blood cell population of the apoE+/−/CCR2−/−→apoE−/− mice was nearly completely replaced by white blood cells of CCR2-deficient donor origin. In addition, a reduction in the percentage of CCR2-positive macrophages was observed in the thioglycollate-induced peritonitis model.

Absence of bone marrow progenitor cell-derived CCR2 did not influence the serum total cholesterol and triglycerides levels, which corresponds to our previous findings. Because of the extra 9 weeks of chow diet feeding, both apoE+/−→apoE+/+ and apoE+/−/CCR2−/−→apoE−/− had developed significantly larger atherosclerotic lesions, compared with the control apoE−/− mice at the age of 16 weeks. Lesion composition in these mice has shifted slightly toward more advanced lesions, ie, collagen deposition was observed in addition to an increase in macrophage-rich area. However, in contrast to previous findings on the crucial role of CCR2 in early atherogenesis, no significant difference could be detected between apoE−/−→apoE−/− and apoE+/−/CCR2−/−→apoE−/− mice regarding atherosclerotic lesion development or macrophage/collagen content of the lesions. Thus, the absence of hematopoietic CCR2 does not result in a regression of established atherosclerosis. Based on our earlier major inhibitory effect of the absence of macrophage CCR2 on initial lesion formation (86% reduction), we should have observed only a marginal further increase in the magnitude of the lesions if CCR2 should play a similar decisive role in more advanced lesions. Moreover, 6-month-old apoE/CCR2 double-knockout mice also had developed advanced lesions, which did not differ significantly in size from the 6-month-old control apoE−/− mice. It can be concluded that the difference in lesion size after 6 months is at most 20%, whereas in our previous study a reduction in initial lesion formation of 86% was found.

Atherogenesis starts with the excessive recruitment of circulating monocytes into the arterial wall, and CCR2 and MCP-1 have been intimately implicated in the formation of the fatty streak in early atherosclerotic lesions. Fatty streaks can progress into complex atherosclerotic lesions over time and these advanced atherosclerotic lesions are characterized by a necrotic core consisting of acellular lipid gruel, covered by a cap of proliferated vascular smooth muscle cells and endothelial cells. It is generally thought that monocytes/macrophages also play a role in the formation of the complex lesion and in plaque rupture, which leads to thrombosis and the acute manifestations of atherosclerosis. Despite the reduced macrophage recruitment in the thioglycollate-induced peritonitis model in apoE−/−/CCR2−/−→apoE−/− mice, our study demonstrates that CCR2 on bone marrow progenitor cells, albeit critical in early atherogenesis, does not appear to be so important in the progression and/or mobilization of monocytes to established atherosclerotic lesions.

Immunohistochemical staining for the CCR2 ligand MCP-1 was similar in lesions of apoE−/−/CCR2−/−→apoE−/− mice and apoE−/−→apoE−/− mice, and MCP-1 was primarily detected in the matrix at the luminal surface of the lesion where a layer of CD68-positive macrophages formed a cap on the advanced lesion. Whether MCP-1 can bind to another chemokine receptor in the absence of CCR2 or other chemokines are expressed in advanced lesions is now open for future research, because it is clear that new influx or proliferation of monocytes/macrophages is still possible in apoE−/−/CCR2−/−→apoE−/− mice or apoE/CCR2 double-knockout mice. Candidate chemokines that regulate monocyte influx and that could be responsible for progression of the atherosclerotic lesions in apoE−/−/CCR2−/−→apoE−/− mice or apoE/CCR2 double-knockout mice are KC (murine homologue of IL-8/Gro-α) or fractalkine and their receptors, CXCR2 and CX3CR1, respectively. IL-8 and Gro-α were long described as chemoattractant proteins for neutrophils. Recently, it has been shown that these chemokines are also potent attractants for monocytes and regulate monocyte adhesion to endothelium. Further evidence for
involvement of these chemokines in atherosclerosis is summarized in a recent review by Boisvert.14

However, apart from other chemokines, there may be a different process responsible for the observed lesion formation in apoE−/−/CCR2−/− → apoE−/− mice or apoE/CCR2 double-knockout mice. Recently, Bruhl et al.15 studied the role of CCR2 in the initiation and progression phase of collagen-induced arthritis. A murine model for arthritis consists of 2 phases whereby the initial phase involves a humoral and cellular immune response, whereas during the progression phase inflammatory cells migrate into the joints and arthritis becomes clinically apparent. It appears that blockade of CCR2 by a monoclonal antibody during the initial phase markedly improved clinical signs of arthritis and histological scores measuring leukocyte infiltration.16 Surprisingly, CCR2 blockade during the progression phase aggravated clinical scores measuring leukocyte infiltration.16 Interestingly, CCR2-positive T cells appear to downregulate an inflammatory response in this model, so that in the absence of CCR2 this regulatory activity becomes clinically apparent. It appears that blockade of CCR2 by a monoclonal antibody during the initial phase markedly improved clinical signs of arthritis and histological signs measuring leukocyte infiltration.16 Surprisingly, CCR2-positive T cells appear to downregulate an inflammatory response in this model, so that in the absence of CCR2 this regulatory activity is absent. Also, in atherosclerosis a function for regulatory T cells has been defined,16 and it is feasible that CCR2-positive regulatory T cells might modulate specifically the progression phase of atherosclerosis. We observed a significant lowering of the CCR2-positive cell population in the lymph nodes of the apoE−/−/CCR2−/− → apoE−/− mice. Such a function would point to a dual role of CCR2, not only in arthritis but also in the initiation and progression of atherosclerotic lesion development.

In conclusion, our present experiments clearly indicate that for the progression of established atherosclerotic lesions, CCR2 expression is absolutely less critical than during the initiation phase of atherosclerosis, pointing to additional mechanisms for recruitment of monocytes at later stages of atherosclerotic lesion development.

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References
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Figure I. Serum total cholesterol and triglyceride levels in apoE(-/-) mice transplanted with apoE (-/-) or apoE (-/-)/CCR2(-/-) bone marrow. Serum total cholesterol levels (Fig IA) and triglyceride levels (Fig IB) were measured at the indicated time points before and after transplantation with apoE(-/-) or apoE(-/-)/CCR2(-/-) bone marrow progenitor cells into apoE (-/-) mice. No significant differences in total cholesterol and triglyceride values between the apoE (-/-) -> apoE(-/-) mice and apoE(-/-)/CCR2(-/-) -> apoE(-/-) mice were observed at the different time points. Values are means ±SD of 10 mice for both groups.
Figure II. MCP-1 present in atherosclerotic lesions. CCR2 ligand MCP-1 (brown) is abundantly present in lesions of both apoE(-/-) -> apoE(-/-) and apoE(-/-)/CCR2(-/-) -> apoE(-/-) mice (magnification x100 and box x400). Localization of CD68 (brown) indicates macrophage presence in close proximity to MCP-1 in the advanced lesions of apoE(-/-)/CCR2(-/-) -> apoE(-/-) mice as well as in the apoE(-/-) -> apoE(-/-) group.