# Role of Bone Marrow–Derived CC-Chemokine Receptor 5 in the Development of Atherosclerosis of Low-Density Lipoprotein Receptor Knockout Mice

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***Objective***—CC chemokine receptor CCR5 is expressed by atheroma-associated cells and could mediate leukocyte attraction into developing lesions. We examined the role of bone marrow-derived CCR5 in the development of atherosclerotic lesions after 8, 12, or 35 weeks of high-fat diet.

***Methods and Results***—Low-density lipoprotein-receptor (LDLr)-deficient mice were lethally irradiated and transplanted with $CCR5^{+/+}$ or $CCR5^{-/-}$ bone marrow. After 8 weeks of fat diet, CCR5 deficiency in leukocytes led to 30% decrease of macrophage accumulation within the fatty streak ($P<0.05$), with no change in lesion size. After 12 weeks of fat diet, CCR5 deficiency also resulted in 30% decrease of plaque-macrophage accumulation ($P<0.005$), associated with 16% reduction in lesion size in the aortic sinus ($P=0.13$), despite a significant increase in total cholesterol levels ($P=0.03$). Lesions with CCR5 deficiency showed 52% reduction in matrix metalloproteinase (MMP)-9 expression ($P=0.02$) and 2-fold increase in collagen accumulation ($P<0.0001$). These changes were associated with a significant increase of interleukin (IL)-10 mRNA expression in spleens of $CCR5^{-/-}$ mice compared with $CCR5^{+/+}$ controls. In addition, we found enhanced IL-10 production by CCR5-deficient peritoneal macrophages and decreased tumor necrosis factor (TNF)-$\alpha$ production by $CCR5^{-/-}$ T cells in comparison with $CCR5^{+/+}$ controls. $CCR5^{-/-}$ and $CCR5^{+/+}$ reconstituted animals showed no differences in plaque size or composition after 35 weeks of high-fat diet despite the persistent absence of CCR5 in plaques of mice reconstituted with $CCR5^{-/-}$ bone marrow.

***Conclusion***—Bone marrow-derived CCR5 favors the development of an inflammatory and collagen-poor plaque phenotype in association with decreased macrophage-derived IL-10 and enhanced T cell-derived TNF-$\alpha$. These effects are not sustained in the very advanced stages of atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2006;26:1858-1863.)**

**Key Words:** atherosclerosis ■ chemokines ■ cytokines ■ inflammation



Atherosclerosis is a chronic inflammatory disease of the arterial wall, characterized by the accumulation of leukocytes, especially macrophages and T cells.[1–4] These cells first interact with the endothelial layer and then migrate to the subendothelial space where monocytes become lipid-loaded macrophages and interact with T cells and vascular cells. These mechanisms rely on the expression of adhesion and chemotactic molecules that are produced within the vascular wall and facilitate cell migration while amplifying local immune responses.[5–7] The importance of chemokines in atherosclerosis was demonstrated by gene disruption experiments. As a result, various chemokines and chemokine receptors have already been reported to be expressed in atherosclerotic arteries and to play key roles in the development of atherosclerosis.[8–14] The chemokine receptor CCR5 is expressed on cells that contribute to disease progression (macrophages, T cells, and smooth muscle cells), and has several ligands (RANTES, MIP-1$\alpha$, MIP-1$\beta$) detected in plaques.[15–19] In vitro studies revealed the role of CCR5 in mediating leukocyte attraction,[20] a step critical to atherosclerosis initiation, and the ability of its ligands to induce tissue factor[17] or MMP expression,[21] which contribute to plaque complications and thrombotic processes. Moreover, genetic screening demonstrated that a human 32-basepair deletion in CCR5 protects against early myocardial infarction and severe coronary artery disease.[22,23] Nevertheless, the role of this chemokine receptor in atherosclerosis is still not well understood. A recent study showed that CCR5 does not affect lesion formation in the early stages of atherogenesis in apolipoprotein E (apoE)/CCR5 double knockout mice.[20] However, differences in genetic background may have confounded the results and the authors acknowledged the need to explore the direct role of CCR5 in advanced stages of atherosclerosis and examine its effect on plaque inflammation

Original received December 13, 2005; final version accepted May 18, 2006.

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*Arterioscler Thromb Vasc Biol.* **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000231527.22762.71**

and composition. In this study, we used the irradiation/bone marrow transplantation model to address this question. The mice were fed a pro-atherogenic diet and were analyzed at 3 different times during lesion formation.

## Materials and Methods

### Generation of Chimeric Mice

Ten C57Bl/6 low-density lipoprotein receptor (LDLr)-deficient male mice and 30 C57Bl/6 LDLr-deficient female mice 15 weeks old (provided by Genfit, Lille, France) were subjected to 9.5 Gray lethal total body irradiation. The day after, mice were reconstituted intravenously with $2.5\times10^6$ bone marrow cells isolated from femurs and tibias of age and sex-matched C57Bl/6 wild-type or CCR5-deficient mice (purchased from Jackson Laboratory and backcrossed 9 times into C57Bl/6 background). $LDLr^{-/-}$ mice reconstituted with $CCR5^{-/-}$ bone marrow were designated $CCR5^{-/-}$ mice (n=20) comparatively to the control group reconstituted with wild-type bone marrow that was designated $CCR5^{+/+}$ (n=20). After 4 weeks of recovery, mice were fed a pro-atherogenic diet containing 15% fat, 1.25% cholesterol, 0% cholate, during 8 weeks (n=5 males in each group), 12 weeks (n=10 females in each group), or 35 weeks (n=5 females in each group). We used male mice put on a high-fat diet for 8 weeks to examine leukocyte CCR5 deficiency on the development of early lesions in the context of severe hypercholesterolemia (males develop smaller lesions than females do at the same age). Mice were housed under conventional conditions.

### Analysis of the Extent and Composition of Atherosclerotic Lesions

Mice were anesthetized with isoflurane before euthanization. Plasma cholesterol and high-density lipoprotein levels were measured using a commercial cholesterol kit (Sigma). The heart and aorta, including the brachiocephalic artery, were taken off, fixed in 4% paraformaldehyde for 2 hours; the heart was then placed in a phosphate-buffered saline sucrose 30% solution overnight at 4°C, before being included in a cutting medium and frozen at −70°C. Successive 10 $\mu$m transversal sections of aortic sinus were obtained. Lipids and collagen were detected using Oil-red O and Sirius Red coloration, respectively. Plaque composition was determined by use of a monoclonal rat anti-mouse macrophage antibody (clone MOMA-2 MAB1852 Chemicon, AbCys; Paris, France), a polyclonal goat anti-CD3 antibody (Santa Cruz, Tebu; le Perray-en-Yuelines, France), a monoclonal alkaline phosphatase-conjugated anti $\alpha$-smooth muscle actin antibody (clone 1A4, Sigma), a polyclonal anti-CCR5 antibody (Santa Cruz), or an anti-MMP-9 antibody (Santa Cruz). At least 4 sections per mouse were inspected for each immunostaining and appropriate negative controls were used. Lesion size in aortic sinus represents the whole intimal surface. Lesion extent in the thoracic aorta represents the percentage of Oil red O staining.

### Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis of IL-10 and IFN-$\gamma$

Transcipt level of IL-10 was measured by real-time reverse-transcription polymerase chain reaction. Total spleen RNA from mice after 12 weeks of fat diet was extracted using standard procedure. RNA were quantified and their integrity was tested by a 1% Agarose gel analysis. Oligo-dT first-strand cDNA was synthesized from 1 $\mu$g total RNA in a 20 $\mu$L total volume, containing 1 $\mu$L of random hexamer primers (300 $\mu$g/$\mu$L), 1 $\mu$L of DNTP (10 mmol/L), and 7 $\mu$L of Superscript III reverse transcriptase mix, following the manufacturer's instructions (InVitrogen, Cergy Pontoise, France). Real-time polymerase chain reaction amplification reactions were performed in triplicate in a total reaction volume of 25 $\mu$L per aliquot, in a 96-well plate on a ABI Prism 5700 Sequence Detection System with cDNA signal detection using the SYBR Green technology (InVitrogen). After 2-minute activation of *Taq* polymerase, amplification was allowed to proceed for 40 cycles, each consisting of denaturation at 95°C for 15 seconds, annealing at 57°C (for IL-10) or 59°C (for IFN-$\gamma$) for 15 seconds and extension at 72°C for 20 seconds. Sequences of IL-10 primers are: forward: 5′-TTTGAATTCCCTGGGTGAGAA-3′; reverse: 5′-ACAGGGGAGAAATCGATGACA-3′ (Invitrogen). Sequences of IFN-$\gamma$ primers are: forward: 5′-AGCAACAGCAAGGCGAAAA-3′; reverse: 5′- CTGGACCTGTGGGTTGTTGA-3′ (Invitrogen). Results were normalized to GAPDH transcription.

### RANTES Binding Studies

Binding experiments were carried out using 0.2 nM $^{125}$I- CCL4 (specific activity=2200 Ci/mmol protein) purchased from Amersham (Saclay, France). One million splenocytes from mice reconstituted with $CCR5^{+/+}$ or $CCR5^{-/-}$ (12 weeks of high fat diet) were incubated in duplicate with of $^{125}$I-labeled chemokine in the presence or absence of a 1000-fold excess of unlabeled recombinant chemokine (PeproTech, Rocky Hill, NJ) in binding medium (HBSS with 1 mg/mL bovine serum albumin (BSA) and 0.01% azide, pH 7.4) in a total volume of 200 $\mu$L. After incubation for 2 hour at room temperature, unbound chemokine was separated from cells by washing with 1 mL of binding medium containing 0.5 mol/L NaCl. Gamma emissions were then counted in the cell pellet.

### Cytokine Profile of Stimulated Splenocytes

Mouse splenocytes ($2\times$ to $4\times10^6$/mL) were cultured in presence of concanavalin A (5 $\mu$g/mL), which promotes T cell activation, and brefeldin A (5 $\mu$g/mL) overnight at 37°C. Cell surface antigens and intracellular cytokine content were characterized with a standard staining method with the following monoclonal antibodies (BD Biosciences Pharmingen, Le Pont de Claix, France): fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3, phycoerythrin (PE)-conjugated anti-mouse IFN-$\gamma$ or IL-10, peridinin chlorophyll-a protein cyanine 5.5-conjugated anti-mouse CD8 (clone Ly-2), allophycocyanin-conjugated anti-mouse CD4 (clone L3T4), phycoerythrin cyanine 7 (PEcy7)-conjugated anti-mouse tumor necrosis factor (TNF)-$\alpha$. Cell suspensions were incubated with appropriate fluorochrome-conjugated monoclonal antibodies and run for 5-color fluorescence staining on a cytofluorometer (LSRII, Becton Dickinson) and analyzed with DIVA software.

### Cytokine Production and Metalloproteinase Activity of Stimulated Peritoneal Macrophages

Peritoneal macrophages were isolated from $CCR5^{-/-}$ (n=5) or $CCR5^{+/+}$ (n=5), 3 days after a single thioglycollate intraperitoneal injection. Macrophages were placed in a culture medium (RPMI +1% FCS supplemented with antibiotics) for 3 hours before they were stimulated with lipopolysaccharide (LPS) or with various concentrations of CCR5 ligands (MIP-1$\beta$: 50, 100 ng/mL and RANTES 50, 100, 150, and 200 ng/mL) for 48 hours. Sandwich enzyme-linked immunosorbent assays (ELISAs) were used to measure, IL-10 (BD Biosciences) and IL-12 (BD Biosciences) in the supernatants, according to manufacturer's instructions. Plates were read on an ELISA reader at a wavelength of 405 nm after color development (Labsystems iEMS reader). Protein extracts from cells and concentrated supernatants were obtained by adjunction of 100 $\mu$L of lysis buffer (0.5% triton X100, 0.5% NP-40, 17% NaCl 5 mol/L, 5% Tris 1 mol/L, supplemented with antiproteases) followed by one centrifugation (10 minutes, 12 000*g*, 4°C). Electrophoresis was performed on a 9% SDS-PAGE gel containing 0.1% gelatin. Gels were washed twice in a 2.5% triton solution and incubated in a 50 mmol/L Tris/10 mmol/L CaCl2 solution overnight. The zymography was revealed with 0.12% Coomassie blue coloration in a 30% ethanol/10% acetic acid solution. Similar results were obtained whether macrophages came from mice on chow diet (data not shown), or from mice after 8 (data not shown) or 12 weeks of high-fat diet (data presented here).

**TABLE 1. Weights, Plasma Total Cholesterol, HDL Cholesterol, Atherosclerotic Lesion Size, and Composition in CCR5$^{+/+}$ and CCR5$^{-/-}$ Mice After 8 Weeks of High-Fat Diet**

| | CCR5$^{+/+}$ (n=5) | CCR5$^{-/-}$ (n=5) | *P* |
|---|---|---|---|
| Weights, g | 26.4±0.7 | 26.8±1.0 | NS |
| Total cholesterol, g/L | 10.6±1.0 | 10.8±0.7 | NS |
| HDL cholesterol, g/L | 1.2±0.09 | 0.9±0.17 | NS |
| Lesion size in thoracic aorta, % | 13.1±0.9 | 12.2±2.0 | NS |
| Lesion size in aortic sinus, $\mu m^2$ | 30 543±2309 | 32 298±5213 | NS |
| MOMA-2, % of plaque area | 47.5±3.6 | 33.6±3.2 | 0.026 |

Data are means ±SE.
HDL indicates high-density lipoprotein.

## Statistical Analysis

Data were expressed as mean±SEM. Statistical significance was determined by use of ANOVA. A value of $P<0.05$ was considered as statistically significant.

# Results

## Effect of CCR5 Deficiency in Bone Marrow Cells on Atherosclerotic Lesion Size

After 8 weeks of atherogenic diet, the 2 groups of mice had comparable weight and similar total and high-density lipoprotein cholesterol levels (Table 1). We did not observe any difference in fatty streak size, neither in the aortic sinus, nor in the thoracic aorta between mice deficient for CCR5 and controls (Table 1).

After 12 weeks of atherogenic diet, the extent of lesions in the thoracic aorta was comparable between the 2 groups (Table 2). We observed a nonsignificant trend toward a modest decrease in lesion size in the aortic sinus of mice transplanted with CCR5-deficient bone marrow in comparison with controls (16.3% reduction, $P=0.12$) (Table 2; supplemental Figure I, available online at http://atvb.ahajournals.org). This occurred despite significantly higher cholesterol levels in the CCR5-deficient mice ($P<0.05$) (Table 2). When mice with comparable total cholesterol levels were examined (13.4±0.5 g/L in CCR5 KO mice, n=7 versus 12.2±0.7 g/L in controls, n=10, $P=0.22$), mice transplanted with a CCR5-deficient bone marrow showed a significant but modest 25.4% decrease in lesion size compared with controls (362 455±33 838 $\mu m^2$ versus 486 125±34 740 $\mu m^2$, respectively; $P=0.03$).

**TABLE 2. Weights, Plasma Total Cholesterol, HDL Cholesterol, Atherosclerotic Lesion Size, and Composition in CCR5$^{+/+}$ and CCR5$^{-/-}$ Mice After 12 Weeks of High-Fat Diet**

| | CCR5$^{+/+}$ (n=10) | CCR5$^{-/-}$ (n=10) | *P* |
|---|---|---|---|
| Weights, g | 22.4±0.8 | 24.2±1.5 | NS |
| Total cholesterol, g/L | 12.2±0.7 | 14.0±0.5 | 0.046 |
| HDL cholesterol, g/L | 0.24±0.05 | 0.20±0.05 | NS |
| Lesion size in thoracic aorta, % | 21.6±1.1 | 23.8±1.6 | NS |
| Lesion size in aortic sinus, $\mu m^2$ | 486 125±34 740 | 407 058±35 068 | 0.12 |
| MOMA-2, % of plaque area | 49.6±3.0 | 35.1±2.6 | 0.002 |
| Sirius red, % of plaque area | 15.5±2.1 | 34.7±2.8 | <0.0001 |
| $\alpha$-actin, % of plaque area | 6.7±1.2 | 8.2±1.3 | NS |
| CD3, nb/mm$^2$ of plaque area | 121.6±26.3 | 125.7±24.5 | NS |

Data are means±SE.

After 35 weeks of high-fat diet, lipid deposition in the thoracic aorta was still comparable between the 2 groups (data not shown). Lesion size in the aortic sinus was no longer different between the 2 groups (supplemental Table I). Lesion size measured at the level of the brachiocephalic artery was also similar between CCR5-deficient mice and controls (supplemental Table I). Overall, these results indicate that the CCR5 deficiency in leukocytes has, at most, a transient and very modest effect on the extent of atherosclerotic lesions.

## Effect of CCR5 Deficiency in Bone Marrow Cells on Atherosclerotic Lesion Composition

As expected, CCR5 was mainly detected in macrophage-rich plaque areas of CCR5$^{+/+}$ transplanted animals and in inflammatory cells of the adventitia (Figure 1). No expression was detectable in the plaques or in the adventitia of CCR5$^{-/-}$ mice, even 35 weeks after recovery from bone marrow transplantation. In addition, CCR5 was also undetectable in plaque smooth muscle cells of mice reconstituted with CCR5$^{-/-}$ bone marrow.

After 8 weeks of atherogenic diet, CCR5 deficiency was associated with a significant 30% decrease in MOMA-2–positive area, indicating a lower accumulation of macrophages in the very early lesions of atherosclerosis (Table 1). Staining for sirius red and $\alpha$-actin positive smooth muscle cells was barely detectable and was similar between the two groups of mice at this very early stage of lesion development (data not shown).

After 12 weeks of high-fat diet, CCR5 deficiency was still associated with a significant 30% reduction in macrophage content ($P=0.002$) (Table 2) (Figure 1), suggesting an important contribution of CCR5 to the infiltration of monocytes within developing lesions beyond the early stage. We found no statistical differences in plaque T cell or smooth muscle cell accumulation between CCR5$^{-/-}$ and CCR5$^{+/+}$ mice (Table 2). Interestingly, the reduction in macrophage infiltration in CCR5$^{-/-}$ plaques was associated with a doubling in collagen density ($P<0.0001$) (Table 2, Figure 1), suggesting a better plaque healing in the absence of CCR5 expression.

After 35 weeks of high-fat diet, plaque macrophage accumulation was no longer different between the 2 groups (supplemental Table I). Moreover, plaques of CCR5$^{-/-}$ mice showed substantially lower accumulation of smooth muscle cells ($P=0.004$) (supplemental Table I). These changes may account for the reduction in collagen content of CCR5-deficient plaques between week 12 and week 35 (supplemental Table I).

## Potential Mechanisms Contributing to Reduced Plaque Inflammation and Increased Collagen Accumulation in the Absence of CCR5 Expression

We examined the role of CCR5 deficiency in the modulation of the immuno-inflammatory response. IL-10 and IL-12 play cross-regulatory roles in macrophages.[24,25] As shown in Figure 2, supernatants of peritoneal macrophages from

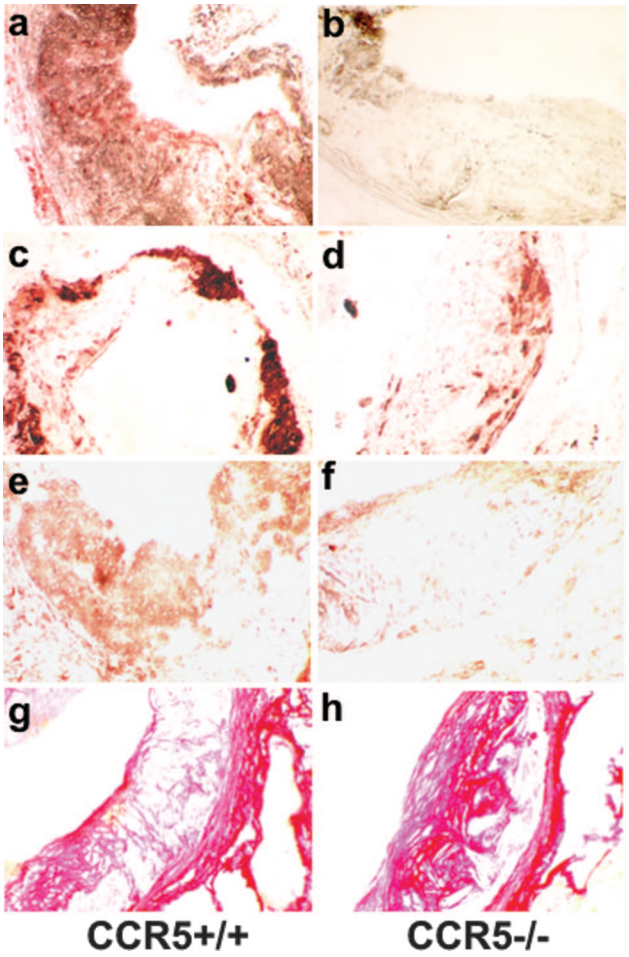


**Figure 1.** Immunohistochemi cal studies on cryostat sections (aortic sinus) from CCR5$^{+/+}$ CCR5$^{-/-}$ mice after 12 weeks of high-fat diet. Representative stainings shown in this figure include CCR5 in a and b, MOMA-2 (to detect macrophages) in c and d, MMP-9 in e and f, and Sirius red (to detect collagen) in g and h. Original magnifications: ×200.

CCR5$^{-/-}$ mice showed a 4-fold increase in IL-10 production ($P$=0.017) (Figure 2) in response to LPS stimulation compared with macrophages of CCR5$^{+/+}$ transplanted mice. However, LPS-stimulated IL-12 production was similar between CCR5$^{-/-}$ and CCR5$^{+/+}$ mice (1.74±0.96 ng/mL versus 2.1±0.35 ng/mL, respectively). T cell-derived IL-10 and IFN-γ cross-regulate lesion development and inflammation,[25] and recent studies suggest that CCR5 deficiency in T cells may affect IL-10 and IFN-γ production.[26] T lymphocytes from CCR5$^{-/-}$ mice showed similar conA-stimulated IL-10 and IFN-γ profile in vitro as CCR5$^{+/+}$ lymphocytes (intracellular IFN-γ/IL-10 ratio in CD4 T cells: 2.6±0.2 versus 3.3±0.5, respectively, n=4 per group, $P$=0.23; and intracellular IFN-γ/IL-10 ratio in CD8 T cells: 4.4±0.3 versus 3.8±0.3, respectively, n=4 per group, $P$=0.2). Quantitative analysis of in vivo mRNA cytokine expression revealed a marked 2-fold increase in IL-10 expression in CCR5$^{-/-}$ spleens compared with controls (Figure 3), and no difference in IFN-γ expression (Figure 3). Taken together, these results suggest that increased IL-10 production in vivo in CCR5$^{-/-}$ mice most likely derives from macrophages and other related

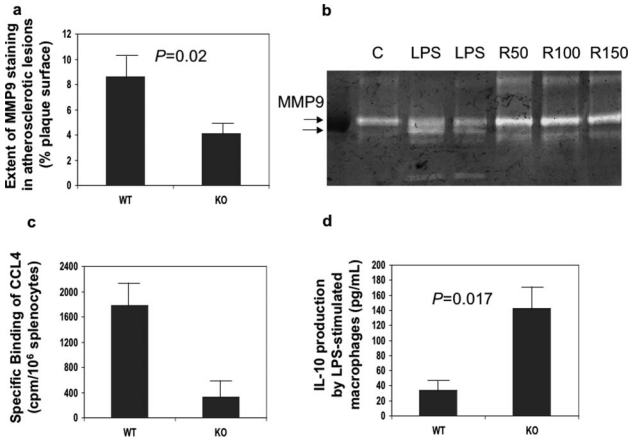


**Figure 2.** In a, quantification of the extent of MMP9 staining (using Histolab software) in atherosclerotic lesions of LDLR$^{-/-}$ mice reconstituted with CCR5$^{+/+}$ (WT) or CCR5$^{-/-}$ (KO) bone marrow. In b, gelatin zymography on protein extracts of CCR5$^{-/-}$ peritoneal macrophages (from LDLR$^{-/-}$ mice reconstituted with CCR5 WT or KO bone marrow, after 12 weeks of high-fat diet) stimulated in vitro with LPS or increasing concentrations (50 ng/mL; 100 ng/mL and 150 ng/mL) of RANTES. Unstimulated macrophages denoted in C. Arrows point to pro- and active MMP-9. In c, quantification of the binding of splenocytes from CCR5$^{+/+}$ and CCR5 $^{-/-}$ mice to CCL4, $P$<0.01. Quantification of IL-10 production (ELISA) by LPS-stimulated peritoneal macrophages from LDLR KO mice transplanted with CCR5 wild-type (WT) or CCR5-deficient (KO) bone marrow, after 12 weeks of high-fat diet.

antigen-presenting cells. We also assessed TNF-α production by spleen-derived T cells, because this pro-inflammatory cytokine is produced by pro-atherogenic Th1 cells.[25] Interestingly, we found a 3-fold decrease in TNF-α production by CD4 or CD8 T cells after stimulation with conA (Figure 4).

In addition to the modulation of the systemic immunoinflammatory response, we observed a significant 52% decrease in MMP-9 expression in CCR5$^{-/-}$ plaques compared with controls ($P$=0.02) (Figures 1 and 2) (supplemental Table I), which may have contributed to enhanced collagen accumulation. Mouse peritoneal macrophages (or bone marrow-derived macrophages, not shown) from CCR5$^{+/+}$

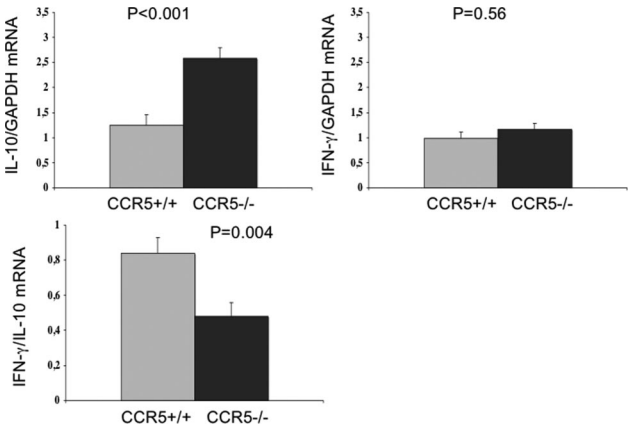


**Figure 3.** Quantitative reverse-transcription polymerase chain reaction to assess the in vivo expression of IL-10 and IFN-γ mRNAs in the spleens of LDLr$^{-/-}$ mice reconstituted with CCR5$^{+/+}$ (n=6) or CCR5$^{-/-}$ (n=5) bone marrow and put on a high -at diet for after 12 weeks.

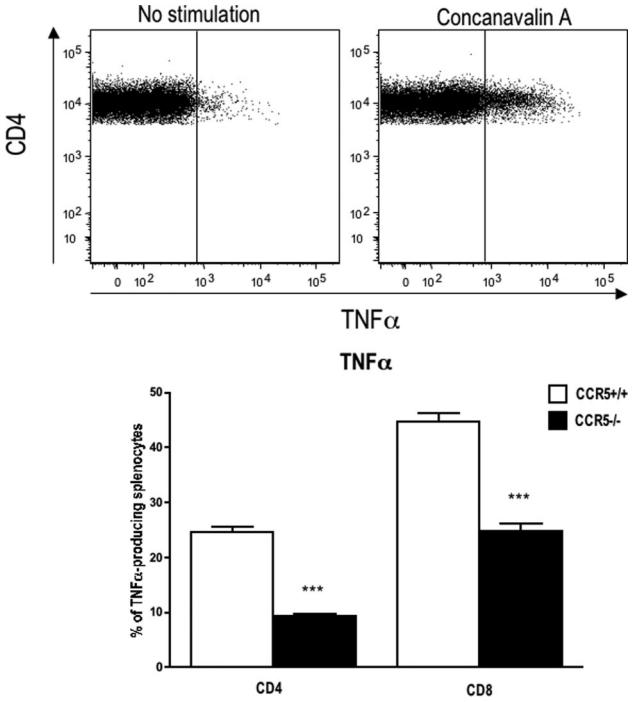


**Figure 4.** Quantitative determination of TNF-$\alpha$ production in spleen-derived T cells using flow cytometry. Top panel, Example of flow cytometric analysis of TNF-$\alpha$ expression by CD4 T cells before and after stimulation by concanavalin A and brefeldin A. Bottom panel, Quantitative analysis of intracellular TNF-$\alpha$ expression by spleen-derived CD4 and CD8 T cells of CCR5$^{+/+}$ and CCR5$^{-/-}$ mice.

mice showed a significant increase in MMP-9 activity (gelatin zymography on cell or supernatant protein extracts) after stimulation with various concentrations of LPS, RANTES, and/or MIP-1$\beta$. Surprisingly, the increase in gelatinase activity was also observed in the CCR5-deficient cells, whether these cells were recovered from mice on chow diet (not shown) or from mice on high-fat diet (Figure 2), suggesting no specific role for CCR5 in this process. Type I procollagen production by human SMC, evaluated by Western blot, was not affected after incubation with various concentrations of RANTES or MIP-1$\beta$ (data not shown).

## Discussion

The available published data suggest that chemokines may have specific and complementary roles in atherosclerosis.[27,28] For instance, IL-8 strongly participates to the firm adhesion of leukocytes to the endothelium via its receptor CXCR2 whereas MCP-1 seems to be involved in leukocytes infiltration via CCR2.[9,10,18,29] More recently, we and others showed that the chemokine receptor CX3CR1 plays a critical role in the development of atherosclerosis.[12,14] Other chemokine receptors may be involved in some specific steps during lesion development and/or progression. A recent report showed that partial lack of human CCR5 expression was associated with reduced risk of early myocardial infarction and severe coronary artery disease, suggesting a potential role for CCR5 in atherosclerosis. Kuziel et al examined Apoe/CCR5 double knockout mice and found that CCR5 deficiency was not protective in the early stages of atherosclerosis.[20] No information was available regarding the development and composition of advanced lesions. In a more recent study, Veillard et al reported a marked decrease in plaque size and inflammation both in the aortic sinus and the thoracoabdominal aorta of LDLr$^{-/-}$ mice treated with Met-RANTES.[30] Among the potential explanations for this observation, the authors suggested a possible role for RANTES/CCR5 pathway in the development of atherosclerosis. In our present study, we examined the effect of bone marrow CCR5 deficiency on the development of atherosclerosis at 3 time points: the early fatty streak stage, the mature lesion stage, and the very advanced stage. We found that bone marrow CCR5 deficiency led to a moderate, nonsignificant, and transient reduction in lesion size at the level of the aortic sinus with no effect on lesion development in the thoracic aorta or the brachiocephalic artery. These results suggest that, in comparison to other chemokine receptors such as CCR2, CXCR2, or CX3CR1, CCR5 has, at most, a minor effect on the extent of atherosclerosis.

Besides plaque size, analysis of plaque composition, particularly smooth muscle cell, macrophage, and collagen contents, appears to be of considerable interest.[27,31] Indeed, smooth muscle cells provide an essential structural component of the plaque, and greatly contribute to the healing process. Smooth muscle cells provide the major part of the plaque extracellular matrix, including collagen, which is known to play a pivotal role in plaque stability. In addition, increased release of pro-inflammatory mediators and metalloproteinases by infiltrating T cells and macrophages contributes to smooth muscle cell apoptosis, induces collagen degradation in the fibrous cap and inhibits its synthesis, thus promoting plaque instability.[32] In our study, we showed that after either 8 or 12 weeks of high-fat diet, CCR5 deficiency led to a significant 30% decrease in macrophage accumulation. These results are consistent with the role of CCR5 in leukocyte migration, a crucial process during plaque development and progression. The reduction in macrophage accumulation was associated with a significant reduction in MMP9 expression and an increase in collagen content at the stage of mature plaques. Because CCR5 expression by macrophages had no influence on MMP9 activity in vitro, we suggest that the decrease in plaque MMP9 expression in CCR5$^{-/-}$ animals reflected the significant reduction in macrophage infiltration, which may have contributed to a reduction in collagen degradation.

Activation of CCR5 on smooth muscle cells in vitro did not affect collagen production. In addition, we found no differences in IFN-$\gamma$ production, a potent inhibitor of collagen synthesis, between CCR5$^{-/-}$ and CCR5$^{+/+}$ mice. However, we found a marked and significant increase in spleen IL-10 mRNA expression in vivo in the absence of CCR5, and a very reproducible and unexpected increase in IL-10 production by CCR5-deficient LPS-stimulated peritoneal macrophages in vitro. This was associated with a profound reduction in TNF-$\alpha$ production by spleen-derived T cells. Overall, our results suggest that the absence of CCR5 expression allowed the development of a systemic and pronounced anti-inflammatory immune response, which may have contributed to the reduction in plaque inflammation and to the promotion

of plaque healing during the formation of mature plaques. These CCR5 effects on plaque composition were not observed in the very advanced stages of atherosclerosis, suggesting that other chemokine receptors or inflammatory pathways may efficiently substitute for CCR5 overtime. The analysis of lesion size/composition at a time point between 12 and 35 weeks would have been necessary for a detailed and more precise understanding of the role of CCR5 in the dynamics of lesion development. Interestingly, our results are very consistent with a clinical observation in humans showing that CCR5 polymorphism is associated with a lower risk of an early episode of myocardial infarction (in patients younger than 55 years) compared with the risk in older patients (older than 60 years).[22] Taken together, the experimental and clinical results suggest that CCR5 expression could accelerate the development of inflammatory plaques and modulate the age at onset of myocardial infarction.

In conclusion, we showed that CCR5 deficiency in bone marrow cells did not significantly affect atherosclerotic plaque size. However, it significantly affected the systemic immuno-inflammatory response and led to profound changes in lesion composition during the initial stages of plaque development. This occurred through reduction in macrophage infiltration, allowing substantial collagen accumulation within the plaques. These effects were not sustained overtime, suggesting that other mechanisms prevailed during the very advanced stages of atherosclerosis.

## Acknowledgments

We are indebted to Saveria Casanova (Inserm U543, Paris, France) for her help in cytokine determination.

## Sources of Funding

This work was supported by the Nouvelle Société Française d'Athérosclérose (S.P.) and the Fondation de France (C.C.).

## Disclosures

None.

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