Molecular Medicine

Genetic Deletion of Chemokine Receptor Ccr6 Decreases Atherogenesis in ApoE-Deficient Mice

Wuzhou Wan, Jean K. Lim, Michail S. Lionakis, Aymeric Rivollier, David H. McDermott, Brian L. Kelsall, Joshua M. Farber, Philip M. Murphy

Rationale: The chemokine receptor Ccr6 is a G-protein–coupled receptor expressed on various types of leukocytes identified in mouse atherosclerotic lesions. Recent evidence suggests that both CCR6 and its ligand CCL20 are also present in human atheroma; however, their functional roles in atherogenesis remain undeфинsd.

Objective: Our objective was to delineate the role of Ccr6 in atherogenesis in the apolipoprotein E–deficient (ApoE−/−) mouse model of atherosclerosis.

Methods and Results: Both Ccr6 and Ccl20 are expressed in atherosclerotic aorta from ApoE−/− mice. Aortic lesion area in Ccr6−/−ApoE−/− mice was ~40% and ~30% smaller than in Ccr6+/+ApoE−/− mice at 16 and 24 weeks of age, respectively. Transplantation of bone marrow from Ccr6−/− mice into ApoE−/− mice resulted in ~40% less atherosclerotic lesion area than for bone marrow from Ccr6+/+ mice; lesions in Ccr6−/−ApoE−/− mice had 34% less macrophage content than lesions in Ccr6+/+ApoE−/− mice. Ccr6 was expressed on a subset of primary mouse monocytes. Accordingly, Ccl20 induced chemotaxis of primary monocytes from wild-type but not Ccr6−/− mice; moreover, Ccl20 induced mononcytosis in ApoE−/− mice. Consistent with this, we observed 30% fewer monocytes in circulating blood of Ccr6−/−ApoE−/− mice, mainly because of fewer CD11b+Ly6C hi inflammatory monocytes.

Conclusions: Ccr6 promotes atherosclerosis in ApoE-deficient mice, which may be due in part to Ccr6 support of normal monocyte levels in blood, as well as direct Ccr6-dependent monocyte migration. (Circ Res. 2011;109:374-381.)

Key Words: atherosclerosis ■ inflammation ■ monocytes ■ aorta

Atherosclerosis is a chronic inflammatory disease and the leading cause of mortality in the developed world. Many different inflammatory cell types have been shown to accumulate in atherosclerotic plaques, including monocytes/macrophages, dendritic cells, T cells, B cells, and neutrophils. Of these, monocytes/macrophages are particularly important, because they are recruited in the largest numbers to atherosclerotic sites, where they ingest oxidized LDL and produce inflammatory mediators. Selective depletion of macrophages has been reported to inhibit early atherogenesis, which suggests a critical role for monocyte recruitment during atherogenesis.

The chemokine family coordinates directional movement of leukocytes to inflammatory sites by signaling through G-protein–coupled receptors. A subset of chemokines can induce monocyte chemotaxis, whereas others may selectively trigger monocyte arrest on inflamed endothelium. In vivo, gene targeting studies have revealed that specific chemokines (Ccl2, Cc5, and Cx3c11) and chemokine receptors (Ccr2, Ccr5, Cx3cr1, and Cxcr6) play nonredundant roles in promoting atherogenesis through macrophage accumulation in plaque.

Ccr6 is the sole receptor for macrophage inflammatory protein-3α (Ccl20) and is expressed on various cell types that have been identified in atherosclerosis, including CD4+ T cells, CD8+ T cells, natural killer T cells, natural killer cells, B cells, dendritic cells, and neutrophils. With regard to monocytes, CCL20 has been shown to induce human monocyte chemotaxis in vitro. Ex vivo, interleukin (IL)-1β has been reported to stimulate synoviocytes from inflamed joints of rheumatoid arthritis patients to recruit CCR6+ human monocytes in a CCL20-dependent manner, and in vivo, monocytes may accumulate via Ccr6 and Ccl20 in a mouse model of dermatitis. In humans, both CCR6 and CCL20 have been detected in carotid plaques; however, their functional role in atherogenesis is undefined. Here, we address this question using Ccr6−/− mice in the apolipoprotein E–deficient (ApoE−/−) mouse model of atherosclerosis.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. Ccr6−/− mice were generated as described previously. Ccr6−/−ApoE−/− mice were obtained by crossing ApoE−/− mice on a C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME) with Ccr6−/− mice on a C57BL/6N background (NCI-DCT). Female littermate Ccr6+/+ mice were used as controls.

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ApoE−/− and Ccr6−/− ApoE−/− mice were weaned at 6 weeks, fed a Western diet (TD88137; Harlan Teklad, Madison, WI) for an additional 10 or 18 weeks, and then euthanized for analysis. Female mice euthanized at 16 weeks of age were subjected to all the analyses detailed below. All mice were kept in pathogen-free conditions, and animal study protocols were approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Results
Atherogenesis Is Reduced in Ccr6−/− ApoE−/− Mice
Ccr6+/− ApoE−/− and Ccr6−/− ApoE−/− mice had similar lesion distribution, with the highest density occurring in the lesser curvature of the aortic arch in both groups of mice (Figure 1A); however, female Ccr6−/− ApoE−/− mice had approximately 40% and 30% less total lesion area than Ccr6+/− ApoE−/− mice at age 16 and 24 weeks, respectively (Figure 1B). Atherosclerotic lesion size in the aortic root was decreased by 43% in Ccr6+/− ApoE−/− mice relative to Ccr6+/− ApoE−/− mice (Figures 1C and 1D), which was similar to the lesion area reduction in the whole aorta. Male mice were also studied, and Ccr6−/− ApoE−/− mice had an ∼30% reduction in total lesion area at age 19 weeks (13 weeks on Western diet; Online Figure I). No significant difference was found between the 2 genotypes in (13 weeks on Western diet; Online Figure I). No significant difference was found between the 2 genotypes in

Macrophage Content in the Aortic Root Is Reduced Significantly in Ccr6−/− ApoE−/− Mice
Circulating monocytes can be recruited to atherosclerosis-prone arteries at an early stage, where they may then differentiate into lipid-laden macrophages known as foam cells.2 To determine whether Ccr6 may affect monocyte recruitment and foam cell accumulation, we examined macrophage content in the aortic root by MOMA-2 (monocyte plus macrophage) staining. Compared with Ccr6+/− ApoE−/− mice, Ccr6−/− ApoE−/− mice had 44% less macrophage content in the aortic root area, whereas the content of T cells or dendritic cells was not affected (Figures 2A and 2B and data not shown). Masson’s trichrome staining was performed to compare aortic root collagen content, which may affect the stability of atherosclerotic plaques. Although a trend toward an increase in collagen content (Figure 2C, blue area) in the aortic root of Ccr6−/− ApoE−/− mice was observed, it was not statistically significant (Figures 2C and 2D).

Circulating Monocyte Counts Are Reduced Significantly in Ccr6−/− ApoE−/− Mice
Interpretation of the macrophage deficit that we observed in the aorta of Ccr6−/− ApoE−/− mice relative to Ccr6+/− ApoE−/− mice must take into account the monocyte level in the blood of the 2 strains. Therefore, we next quantified the numbers and percentages of monocytes and other leukocyte subsets in the blood by complete blood count. We found that circulating monocyte counts were reduced by 30% in Ccr6−/− ApoE−/− mice compared with Ccr6+/− ApoE−/− mice (Figure 3A), whereas no significant difference was observed in the number of total white blood cells, peripheral blood lymphocytes, neutrophils, eosinophils, or basophils (Online Table I). In addition, no differences in these lipids were observed between wild-type C57BL/6 mice and Ccr6−/− mice (data not shown).

Figure 1. Ccr6 deficiency reduces atherogenesis in the ApoE−/− mouse model of atherosclerosis. Female Ccr6+/− ApoE−/− and Ccr6−/− ApoE−/− mice were fed a high-fat Western diet and analyzed for atherosclerosis development in whole aorta at age 16 weeks (A, B) and 24 weeks (B) and in aortic root at age 16 weeks (C, D). A, Representative photographs of Sudan IV–stained mouse aortas (red indicates positive staining). B, Quantification of atherosclerotic lesions (red area) in Figure 1A, shown as percentage of the whole aorta. (**P<0.001 for 16 weeks and *P<0.05 for 24 weeks). C, Representative frozen aortic root sections stained with oil red O. D, Aortic root lesion size (mean area) was quantified by IVision software (n=8 in each group; **P=0.009). E, D, each symbol represents data from a single mouse, and each data set is summarized as mean±SEM.
Ccr6\textsuperscript{+/+}ApoE\textsuperscript{−/−} mice and Ccr6\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice (data not shown). In contrast, there was no significant difference in the distribution of CD11b\textsuperscript{+}Ly6C\textsuperscript{high} monocytes (gated on Ly6G-negative leukocytes) in the blood and bone marrow of Ccr6\textsuperscript{+/+}ApoE\textsuperscript{−/−} mice and Ccr6\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice (Figure 3D), which indicates that the monocytopenia in Ccr6\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice was caused mainly by reduction of the CD11b\textsuperscript{+}Ly6C\textsuperscript{high} cell subset. In addition, we found that bone marrow transplantation of Ccr6\textsuperscript{−/−} cells into irradiated ApoE\textsuperscript{−/−} mice resulted in significantly less atherosclerotic lesion area in the aorta than when Ccr6\textsuperscript{+/+} bone marrow cells were transplanted (Figure 3E). Body weight and serum levels of total cholesterol, HDL, and LDL/VLDL were similar for the 2 transplanted groups of mice (data not shown). Thus, the Ccr6\textsuperscript{−} cells affecting atherogenesis in this model appeared to be bone marrow derived.

**Ccr6 Is Expressed on Primary Mouse Monocytes, and Ccl20 Is Expressed on Lesional Macrophages**

Ccr6 has been shown to be expressed on many cell types, including T cells, B cells, natural killer cells, natural killer T cells, immature dendritic cells, and neutrophils, and it may also be expressed on monocytes,\textsuperscript{10,11} which we examined in greater detail by fluorescence-activated cell sorter analysis. We checked the expression of Ccr6 on CD11b\textsuperscript{+}Gr1\textsuperscript{−} cells and CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells using C57BL/6 mice and Ccr6 EGFP (enhanced green fluorescent protein) knock-in mice.\textsuperscript{19} We found that a subset of splenic CD11b\textsuperscript{+}Gr1\textsuperscript{−} cells were Ccr6\textsuperscript{+}, whereas CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells were Ccr6\textsuperscript{−} (Figures 4A and 4B; Online Figure IV, A). Because Gr1 antibody may interact with both Ly6C and Ly6G, we then used specific antibodies against CD11b, Ly6C, and Ly6G to further differentiate inflammatory monocytes (CD11b\textsuperscript{+}Ly6C\textsuperscript{high}), resident monocytes (CD11b\textsuperscript{+}Ly6C\textsuperscript{low}), and neutrophils (CD11b\textsuperscript{+}Ly6G\textsuperscript{+}), as reported previously.\textsuperscript{7,20} Approximately 12\% of spleen CD11b\textsuperscript{+}Ly6C\textsuperscript{high} inflammatory monocytes (11.80±3.10\%, n=6, ApoE\textsuperscript{−/−} mice) stained positive for Ccr6, and Ccr6 was also found on a subset of spleen CD11b\textsuperscript{+}Ly6C\textsuperscript{low} monocytes and CD11b\textsuperscript{+}Ly6C\textsuperscript{low} neutrophils in both Ccr6\textsuperscript{+/+}ApoE\textsuperscript{−/−} mice and C57BL/6 mice (Figure 4C; Online Figure IV, B and C; and data not shown). In addition, Ccr6 was found on a small subset of blood monocytes and bone marrow–derived macrophages in Ccr6 EGFP knock-in mice, C57BL/6 mice, and Ccr6\textsuperscript{+/+}ApoE\textsuperscript{−/−} mice, whereas no Ccr6\textsuperscript{−} cells could be detected in monocytes from Ccr6\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice (Online Figure IV, D; data not shown). Fluorescence-activated cell sorter analysis of spleen cells from Cx3cr1-GFP mouse showed that ~20\% CD11b\textsuperscript{+}Ly6C\textsuperscript{high} cells were Cx3cr1\textsuperscript{+}, whereas CD11b\textsuperscript{+}Ly6C\textsuperscript{low} cells were almost all Cx3cr1\textsuperscript{−} (data not shown). Both Ccr6 and Ccl20 were found to be expressed in the whole aorta of Ccr6\textsuperscript{+/+}ApoE\textsuperscript{−/−} mice as measured by real-time polymerase chain reaction (Online Figure V). Expression was confirmed at the protein level by immunofluorescence, and Ccl20 was found to be colocalized with macrophages and smooth muscle cells/endothelial cells in atherosclerotic lesions (Figure 4D; Online Figure VI; and data not shown), which is consistent with previous reports.\textsuperscript{11} We also attempted to stain Ccr6 in these sections, but all commercially available antibodies showed high nonspecific reactivity when tested on Ccr6\textsuperscript{−/−} tissue.

**Ccr6 Expression on Monocyte/Macrophages Is Up-Regulated by Interferon-γ, and Ccl20 Expression Is Significantly Reduced in Ccr6-Deficient Mice**

We next studied how the proinflammatory cytokines interferon-γ (IFN-γ), tumor necrosis factor-α, IL-1β, and IL-6, which have all been implicated in atherogenesis,\textsuperscript{2} may regulate Ccr6 expression. For this, we used RAW 264.7 cells, a mouse monocyte/macrophage cell line. As with primary monocytes (Figure 4), we found that RAW 264.7 cells constitutively expressed Ccr6 on the cell surface, and IFN-γ induced a 3-fold and 6-fold increase of Ccr6 mRNA expression on purified primary mouse monocytes and RAW 264.7 cells, respectively (Figure 5A; Online Figure VII), which was further confirmed by surface expression analysis (Figure 5B), whereas the other cytokines (tumor necrosis factor-α, IL-1β, and IL-6) had no effect on Ccr6 expression in either cell type (data not shown). To further characterize differences between Ccr6\textsuperscript{+/+}ApoE\textsuperscript{−/−} mice and Ccr6\textsuperscript{−/−}ApoE\textsuperscript{−/−} mice that might contribute to the difference in lesion progression, we measured RNA expression in whole aorta for other chemokines and chemokine receptors (Ccl2-25, Ccr1-10, Cxcl11-16, Cxcr1-7, Cx3c11, and Cx3cr1), as well as several hallmark inflammatory cytokines, eg, IFN-γ (Th1), IL-4 (Th2), IL-10...
(Treg), IL-17A, IL-17F, IL-22 (Th17), and tumor necrosis factor-α. No significant difference was observed for any of the cytokines/chemokines tested except for Ccl20, which showed an 80% mRNA reduction in the Ccr6<sup>−/−</sup> ApoE<sup>−/−</sup> mice (Figure 5C; Online Figure VIII; and data not shown). The level of Ccl20 protein in the aorta of Ccr6<sup>−/−</sup> ApoE<sup>−/−</sup> mice was also significantly reduced as measured by ELISA (Figure 5D), but the magnitude of the effect was less than for Ccl20 mRNA. In addition, Ccr6<sup>−/−</sup> mice showed 70% less Ccl20 mRNA than C57BL/6 mice in the whole aorta (Figure 5E). We also measured serum levels of Ccl20, but no significant difference was found between Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice and Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice or between wild-type C57BL/6 mice and Ccr6<sup>−/−</sup> mice (data not shown).

**Ccr6 Is Functional on Mouse Monocytes, and Ccl20 Induces Blood Monocytosis In Vivo**

Consistent with the expression of Ccr6 on RAW 264.7 cells and primary monocytes, we found that RAW 264.7 cells were chemotactically responsive to Ccl20 in a dose-dependent manner, and Ccl20-induced chemotaxis of these cells was increased by 50% after Ccr6 up-regulation by IFN-γ stimulation (Figures 6A and 6B). In addition, Ccl20 could induce chemotaxis of purified splenic monocytes from wild-type but not Ccr6<sup>−/−</sup> mice, which indicates that Ccl20 signaling on this cell type involves Ccr6 (Figure 6C). Consistent with this,
in vivo we found that tail-vein injection of Ccl20 was able to induce peripheral blood monocytosis in ApoE\(^{-/-}\) mice (Figure 6D), and this reflected a selective egress of the CD11b\(^{+}\)Ly6C\(^{hi}\) monocyte subset (data not shown). The number of circulating neutrophils was also increased at the same time point, but the increase was not significant (Online Figure IX, A). Because spleen has recently been identified as a reservoir of undifferentiated monocytes,21 we also tested whether Ccl20 treatment affected spleen monocyte content. We found that the total number of splenic monocytes was reduced after intravenous injection of Ccl20, but the reduction was not significant (Online Figure IX, B).

**Discussion**

In the present study, we have demonstrated that genetic deficiency of Ccr6 significantly reduces lesion development in the ApoE\(^{-/-}\) mouse model of atherosclerosis. The reduction was apparent throughout the aorta and the aortic root, was large at both of 2 time points measured over 6 months of age, and was not attributable to changes in cholesterol or triglyceride levels. At the cellular level, protection was associated with a major reduction in macrophage accumulation in plaque in Ccr6\(^{-/-}\)-ApoE\(^{-/-}\) mice compared with Ccr6\(^{+/+}\)-ApoE\(^{-/-}\) mice. Transplantation studies indicated that protection was mediated by a Ccr6\(^{+}\) bone marrow–derived cell(s).

Consistent with a direct effect of monocyte/macrophage Ccr6 signaling on macrophage accumulation in the vessel wall in the model, we found that (1) both Ccr6 and its ligand Ccl20 were expressed in atherosclerotic aorta in this model; (2) Ccr6 was expressed on a subset of primary mouse monocytes and the mouse monocyte/macrophage cell line RAW 264.7; (3) Ccr6 mediated chemotactic responses of both primary mouse monocytes and RAW 264.7 cells to Ccl20 in vitro; (4) Ccr6\(^{-/-}\)-ApoE\(^{-/-}\) mice were monocyteogenic compared with Ccr6\(^{+/+}\)-ApoE\(^{-/-}\) mice; and (5) Ccl20 could induce monocytes when injected into Ccr6\(^{+/+}\)-ApoE\(^{-/-}\) mice. Taken together, we propose that the mechanism by which Ccr6 deficiency protects against atherogenesis in this model involves Ccr6-dependent monocyte trafficking into the vessel wall due to reduced monocyte levels in the blood or reduced migration capacity into atherosclerotic lesions.

The magnitude of protection in the absence of Ccr6 (40%) was comparable to what has been reported previously for Ccr2\(^{-/-}\) (36%), Cx3cr1\(^{-/-}\) (28%), and Ccr5\(^{-/-}\) (50%) mice in the same ApoE\(^{-/-}\) mouse model that we used.
Importantly, Ccr6 deficiency in ApoE<sup>−/−</sup> mice did not affect the expression of other chemokine receptors (eg. Ccr2, Cx3cr1, or Ccr5) or chemokines in the atherosclerotic aorta (Online Figure VIII), which suggests that these receptors may have nonredundant roles in atherogenesis. This could reflect action at different stages of atherogenesis or on different subsets in the monocyte migration process. For example, although all of these receptors are known to mediate monocyte/macrophage migration, Cx3cr1 may be more important as an adhesion receptor fostering interactions of foam cells with each other and with smooth muscle cells once they migrate into the vessel wall. The fact that Ccr6 deficiency results in a level of protection similar to these other monocyte/macrophage receptors may at first seem surprising, because it is expressed on only a small subset of monocytes. As a potential mechanism, Ccr6 may operate at 2 steps important in macrophage accumulation: control of blood monocyte levels and direct recruitment of the cells into vessel wall. Dual action could greatly amplify the overall effect on pathogenesis beyond what might be expected simply on the basis of the frequency of Ccr6<sup>−/−</sup> cells. Additional work will be needed to define temporal and spatial expression of these ligands and receptors in the model to gain further insight into different mechanisms of monocyte recruitment. All of these receptors (Ccr2, Ccr5, Cx3cr1, and Ccr6) are also expressed on other leukocyte subsets represented in lesions in the model, which could also be contributing to pathogenesis through their recruitment and function. In particular, Ccr6 is expressed on neutrophils, dendritic cells, natural killer T cells, B cells, and subsets of CD4<sup>+</sup> T lymphocytes, and it is known that depletion of any of these cell types results in reduced atherogenesis in the model. However, the major leukocyte subtype present in atherosclerotic lesions by far is the foam cell, derived from blood monocytes.

We found that Ccr6 deletion in ApoE<sup>−/−</sup> mice significantly reduced not only the percentage of monocytes among peripheral blood leukocytes but also the total absolute monocyte counts in the blood, whereas other cell subsets were unaffected (Figure 3). Monocyte levels did not appear to depend on Ccr6 in ApoE<sup>+/−</sup> mice, which were maintained under the same conditions as ApoE<sup>−/−</sup> mice (Online Figure II). The monocytopenia in Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice was caused by a significant reduction of Ly6C<sub>high</sub> inflammatory monocytes in the blood. At the same time, there was a significant increase of Ly6C<sub>high</sub> inflammatory monocytes in the bone marrow of Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice, which suggests that Ccr6 likely affects the bone marrow egress of these cells. This is reminiscent of a previously identified role of Ccr2 in controlling monocyte mobilization from the bone marrow. It has been reported that Ccr6<sup>−/−</sup> mice did not exhibit gross abnormalities in any major organ, but they have increased numbers of T cells in the intestinal mucosa. Also, it has been shown that Ccr6<sup>−/−</sup> mice have impaired development of M cells and underdeveloped Peyer’s patches, with a 2-fold decrease of total leukocytes in the intestinal mucosa; however, this is the first report of Ccr6 regulation of blood monocytosis.

Monocytosis is an independent predictor of atherosclerotic plaque progression in acute myocardial infarction. In the mouse model of atherosclerosis, it has also been reported that monocytes accumulate continuously in the aorta during atheroma formation. In particular, total blood Ly6C<sub>high</sub> monocytes increase dramatically in hypercholesterolemic ApoE<sup>−/−</sup> mice fed a high-fat diet compared with wild-type mice, which is consistent with our findings in the present study (Online Figure II). We found that both Ly6C<sub>high</sub> and Ly6C<sub>low</sub> monocytes expressed low levels of Ccr6, but only Ly6C<sub>high</sub> monocytes were dysregulated in Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice, which may reflect the different characteristics of these 2 subsets, eg, Ly6C<sub>high</sub> monocytes have been shown to preferentially adhere to activated endothelium and accumulate in atherosclerotic plaques compared with Ly6C<sub>low</sub> monocytes. Thus, we propose that the significant reduction of circulating monocyte counts in Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice may directly reduce the monocytes available for recruitment to atherosclerotic sites, as an explanation for reduced macrophage content and atherosclerotic lesion size in these mice. Future studies comparing the effects of transferring Ccr6<sup>+</sup> versus Ccr6<sup>−/−</sup> monocytes or effects resulting from monocyte-specific deletion of Ccr6 will be needed to further refine this conclusion.

We found that Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice had an 80% reduction of Ccl20 expression in the aorta compared with Ccr6<sup>+/+</sup>ApoE<sup>−/−</sup> mice, although the systemic serum level of Ccl20 in these 2 groups was similar (Figure 5). This suggests that Ccr6 and Ccl20 may form a local positive feedback loop in the vessel wall. A CCL20/CCR6 positive feedback loop has been needed to explain reduced macrophage accumulation. Previous studies showed that IL-17A deficiency does not alter plaque burden in ApoE<sup>−/−</sup> mice, which indicates that more studies will be needed to clarify the role of IL-17 and Th17 cells in atherogenesis.

The present data on Ccr6 and previously reported results connecting other chemokines and chemokine receptors to outcome in ApoE<sup>−/−</sup> mice are consistent with the inflammation theory of atherogenesis. In this regard, Ccr6 and Ccl20 have been linked to multiple other mouse models of chronic inflammatory disease, including psoriasis, inflammatory bowel disease, rheumatoid arthritis, and experimental auto-immune encephalomyelitis. Recently, a triallelic dinucleotide polymorphism of CCR6 was correlated with the expression level of CCR6 and was associated with susceptibility to rheumatoid arthritis, Graves’ disease, and Crohn’s disease; however, its association with atherosclerosis has not been defined.

In conclusion, Ccr6 deficiency in ApoE<sup>−/−</sup> mice causes significant reduction of circulating blood monocytes and reduces progression of atherosclerosis. Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice had markedly less Ccl20 in the aorta, which suggests a local positive feedback loop. Given the atherogenic effect of Ccr6 in this mouse model, Ccr6 should be further considered in the molecular pathogenesis and therapeutic targeting of this disease.
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Disclosures
None.

References
Novelty and Significance

What Is Known?

- Ccr6 is a G-protein–coupled receptor expressed on T cells, B cells, and dendritic cells.
- CCR6 and its sole ligand, CCL20, have been detected in human carotid plaques.
- Selective depletion of monocytes/macrophages inhibits early atherogenesis.

What New Information Does This Article Contribute?

- Ccr6 deficiency in ApoE<sup>-/-</sup> mice reduced atherogenesis and macrophage content in aortic roots.
- Ccr6 deficiency decreased circulating inflammatory monocytes and increased bone marrow inflammatory monocytes in ApoE<sup>-/-</sup> mice.
- Ccr6 functions on a subset of mouse monocytes.

CCR6 is a chemokine receptor that plays a pathogenic role in several autoimmune diseases. It has been detected in human atherosclerotic plaques, but its functional role in atherogenesis is still unknown. We determined that genetic deletion of Ccr6 in ApoE<sup>-/-</sup> mice significantly reduced atherosclerosis development and macrophage accumulation in aortas. Transplantation studies suggested that Ccr6 deficiency in bone marrow–derived cell(s) caused this phenotype. Ccr6 deficiency significantly reduced the numbers of circulating blood monocytes in ApoE<sup>-/-</sup> mice, and this was associated with increased inflammatory monocytes in the bone marrow, which suggests that Ccr6 may control egress of monocytes from the bone marrow into the blood. Also, we showed that Ccr6 is expressed on a small subset of mouse monocytes and is functional, because Ccl20 induced migration of mouse monocytes both in vitro and in vivo. Our findings provide the first in vivo evidence that Ccr6 is a mediator of atherogenesis. The data suggest that the mechanism may involve direct recruitment of inflammatory monocytes to the vessel wall and/or regulation of monocyte egress from bone marrow to blood, among other possibilities. These preclinical results support further study of CCR6 as a potential target for therapeutic development in atherosclerosis.
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Genetic Deletion of Chemokine Receptor Ccr6

Decreases Atherogenesis in ApoE-deficient Mice

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SUPPLEMENTAL MATERIAL
Detailed Methods

Animals
Ccr6-/- mice were generated as previously described.1 Ccr6-/-ApoE-/- mice were obtained by crossing ApoE-/- mice on a C57BL/6J background (Jackson Labs, Bar Harbor, ME) with Ccr6-/- mice on a C57BL/6N background (Division of Cancer Therapy, NCI). Female littermate Ccr6+/+ApoE-/- and Ccr6-/-ApoE-/- mice were weaned at 6 weeks, fed a Western diet (TD88137; Harlan Teklad, Madison, WI) for an additional 10 or 18 weeks, and then sacrificed for analysis. Female mice sacrificed at 16 weeks of age were subjected to all the analyses detailed below. Female mice sacrificed at 24 weeks of age and male littermates sacrificed at 19 weeks of age were subjected only to analysis of lesion area in the aorta. All mice were kept in pathogen-free conditions and animal study protocols were approved by the Animal Care and Use Committee of the NIAID, NIH. Blood counts were analyzed by the Department of Laboratory Medicine, Clinical Center (NIH, Bethesda, MD) using an automated hematology analyzer (Cell Dyn 3700, Abbott Laboratories).

Atherosclerotic Lesion Analysis
The extent of atherosclerosis was assessed by quantifying lesion size in whole aortas and aortic roots, as previously described.2 Briefly, mice were anesthetized and perfused with PBS/0.2 mM EDTA prior to the removal of hearts for sectioning. Aortas were stained with Sudan IV or frozen for RNA extraction. Hearts were bisected transversely and snap frozen in OCT with the cut side facing down. Relevant sections were captured by cutting the frozen blocks at 100 µm increments until the valves appeared. The sections were cut at 10 µm thickness starting from the appearance of the first aortic sinus valve leaflet. Six consecutive sections 50 µm apart (with 3 leaflets of the aortic valve) were stained with 0.5% Oil Red O solution and counterstained with hematoxylin (Histoserv Inc., Germantown, MD). Images of the entire aorta were captured with Leica AF6000 LX microscope (Mannheim, Germany) and analyzed by Image J (NIH), whereas images of the aortic root were captured with a Zeiss light microscope (Jena, Germany) and analyzed by IVision software (Biovision Inc., Exton, PA).

Lipid Analysis
Serum samples were obtained after mice had fasted for four hours. Total cholesterol and HDL, LDL/VLDL levels were analyzed by EnzyChrom Kit (BioAssay Systems, Hayward, CA), and triglyceride level was determined by Stanbio Triglyceride LiquiColor assay (Stanbio Lab., Boerne, TX).

Immunohistochemistry
Frozen aortic root sections were stained with MOMA-2 (MCA519G; Serotec, Raleigh, NC) as primary antibody and goat anti-rat Alexa Fluor 488 as secondary antibody (Molecular Probes, Carlsbad, CA) for macrophage quantification, as described previously.2 For Ccl20 staining, sections were fixed in acetone for 10 minutes and then incubated with blocking buffer (2% goat serum in 1% BSA/PBS) for 30 minutes. After incubating with primary mAb against Ccl20 (R&D, Minneapolis, MN) for 60 minutes, goat anti-rat Alexa Fluor 568 (Molecular Probes, Carlsbad, CA) was applied for visualization. Masson’s trichrome staining for collagen was carried out by Histoserv Inc. (Germantown, MD). Images were captured using a Zeiss light microscope (Jena, Germany) and analyzed by IVision software (Biovision Inc., Exton, PA).

Cell Isolation and Flow Cytometry
Primary leukocytes were harvested from mouse peripheral blood, spleen and bone marrow. Anti-coagulated peripheral blood was treated with lysing buffer (BD Biosciences, San Jose, CA) to remove erythrocytes. Splenocytes were released into HBSS by gentle homogenization or liberase digestion (Roche Applied Science, Indianapolis, IN) and filtered through 100 µm nylon mesh (BD Biosciences, San Jose, CA). Bone marrow was flushed from tibia and femur with HBSS/1% FCS/10 mM HEPES, and then cultured in RPMI1640 with 40 ng/ml M-CSF to obtain bone marrow-derived macrophages. Cells
were first stained with a Live/Dead marker (Invitrogen, Carlsbad, CA) in PBS for 20 minutes and then washed with FACS buffer (PBS, 1% BSA, 0.1% sodium azide) and incubated with rat anti-mouse CD16/32 for 15 minutes to block Fc receptors. Then cells were incubated at 4°C for 30 minutes with the following mouse-specific fluorochrome-conjugated antibodies: CD45-PerCP (BD Biosciences, Cat: 557235), CD45-APCCy7 (BD Biosciences, Cat: 557659), CD3-FITC (BD Biosciences, Cat: 555274), CD4-APC Cy7 (eBioscience, Cat: 47-0042-82), CD11b-APC (BD Biosciences, Cat: 553312), CD11b-PerCP-Cy5.5 (BD Biosciences, Cat: 550999), Gr1-APC (eBioscience, Cat: 17-5931-82), Ly6C-FITC (BD Biosciences, Cat: 553942), Ly6G-APC-Cy7 (BD Biosciences, Cat: 560600), 7/4-Alexa Fluor 647 (AbD Serotec., Cat: MCA771A647), MHCII-Pacific Blue (Biolegend, Cat: 116422), CD11b-PE (BD Biosciences, Cat: 553311), F4/80-APC (AbD Serotec, Cat: MCA497APC) Ccr6-PE (R&D Systems, Cat: FAB590P), CD4-APC (eBioscience, Cat: 17-0041-83), CD8α-APC (eBioscience, Cat: 17-0081-82), CD19-APC (eBioscience, Cat: 17-0193-82), CD117-APC (eBioscience, Cat: 17-1171-82), CD11c-APC (eBioscience, Cat: 17-0114-82), NK1.1-APC (eBioscience, Cat: 17-5941-82), CD115-biotinylated (eBioscience, Cat: 13-1152-82), SA-PE (eBioscience, Cat: 12-4317-87), CD11b-APCCy7 (eBioscience, Cat: 47-0112-82), and F4/80-PECy7 (eBioscience, Cat: 25-4801-82). Cells were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (version 7.5.5; Treestar, Ashland, OR). Mouse inflammatory monocytes were defined as Lin−CD115biot/SA+CD11b+ F4/80+Ly6Chigh (Lin: CD4/CD8α/CD19/CD117/CD11c/NK1.1) cells and sorted using a FACSAria-blue instrument (BD Biosciences, San Jose, CA). Antibodies used for sorting included CD4-APC, CD8α-APC, CD19-APC, CD117-APC, CD11c-APC, NK1.1-APC, CD115-biotinylated, SA-PE, CD11b-APCCy7, F4/80-PECy7 (eBioscience, San Diego, CA) and Ly6C-FITC (BD Biosciences, San Jose, CA).

**Bone Marrow Transplantation**

Female *ApoE−/−* mice (8 weeks old) were irradiated with a dose of 900 rads and transplanted with 5 × 10^6 bone marrow cells from either wild-type or *Ccr6−/−* mice. The bone marrow cells were obtained by flushing the femurs and tibias of female donor mice with PBS and 2 mM EDTA. After bone marrow reconstitution, mice were maintained on a chow diet for 4 weeks to recover and then switched to a Western Diet for an additional 10 weeks, followed by the analysis of atherosclerotic lesions in the whole aorta.

**Intravenous Injection of Ccl20**

Sterile PBS or mouse recombinant Ccl20 (2 µg or 20 µg, Pepro Tech, Rocky Hill, NJ) were injected into the tail vein of *ApoE−/−* mice (female, 9 weeks old, Jackson Labs, Bar Harbor, ME) in a volume of 100 µl. Mice were killed after 15 hours and blood counts were analyzed using an automated hematology analyzer (Cell Dyn 3700, Abbott Laboratories).

**Chemotaxis Assay**

Chemotaxis was measured using a Boyden Chamber assay. Briefly, purified mouse monocytes or RAW 264.7 cell suspensions (30 µl, 3×10^6 cells/ml) were added to the upper chamber and mouse Ccl20 was added to the lower chamber separated by a membrane containing 8 µm diameter pores (Neuro Probe Inc., Gaithersburg, MD). Following a 5 h incubation in a 37°C, 5% CO₂ incubator, chemotaxis was assessed by counting the number of migrated cells in three random fields per well (400× magnification). Chemotaxis index was calculated by comparing the number of cells migrating toward Ccl20 and the number of cells migrating toward control media.

**mRNA Expression Analysis**

Total aorta RNA was isolated by homogenization in Trizol (Invitrogen, Carlsbad, CA), and total RAW 264.7 cell RNA was prepared using RNeasy kit (Qiagen, Valencia, CA). One microgram of total RNA was converted into cDNA using reagents from Promega Corp. (Madison, WI). Real-time PCR (ABI Prism 7900HT, Applied Biosystems) was used to determine mRNA levels using either SYBR Green.
(Supplemental Table II) or Taqman primers (Applied Biosystems, Carlsbad, CA). All samples were normalized to GAPDH (Ccr6 knockout did not affect GAPDH expression in ApoE<sup>-/-</sup> mice) and relative changes in expression levels were determined by the ΔΔCT methods, displayed as either gene amplicons or mRNA fold change.

**Cytokine Assays**
Mouse serum was frozen at -80°C and thawed prior to measurement. Ccl20 protein levels were determined using murine Quantikine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

**Statistical Analysis**
The data were analyzed using unpaired t tests (two-tailed) or Two-way ANOVA tests with Prism 5.0 software (GraphPad Software, San Diego, CA) and data are presented as the mean ± SEM. The cutoff for statistical significance was defined as \( P<0.05 \).

**Supplemental Tables**

**Supplemental Table I.** Ccr6 deficiency does not affect lipid profiles or body weight in the ApoE<sup>-/-</sup> mouse model of atherosclerosis. Mice (n=7 - 10) were 16 weeks old and had been on a Western Diet for 10 weeks at the time of euthanasia. Data are the mean ± SEM. Lipid values are in mg/dL.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total cholesterol</th>
<th>HDL cholesterol</th>
<th>LDL/VLDL cholesterol</th>
<th>Triglyceride</th>
<th>Body weight (g)</th>
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<tbody>
<tr>
<td>Ccr6&lt;sup&gt;+/+&lt;/sup&gt;ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1492 ± 50</td>
<td>82 ± 22</td>
<td>1375 ± 63</td>
<td>145 ± 3</td>
<td>22.7 ± 1.9</td>
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<tr>
<td>Ccr6&lt;sup&gt;-/-&lt;/sup&gt;ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>1609 ± 24</td>
<td>84 ± 5</td>
<td>1281 ± 28</td>
<td>137 ± 5</td>
<td>24.1 ± 2.4</td>
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**Supplemental Table II.** Sequences of the primers used for qPCR with SYBR Green.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Primer name&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Primer sequence (5' → 3')</th>
<th>Amplicon length (bp)</th>
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<td></td>
<td>GAPDH R</td>
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</table>

<sup>*</sup> F, forward; R, reverse.
Supplemental Figure I: Ccr6 knockout decreases atherosclerosis for males in the ApoE⁻/⁻ mouse model of atherosclerosis. Male Ccr6⁺/+ApoE⁻/⁻ and Ccr6⁻/⁻ApoE⁻/⁻ mice were fed a high-fat Western diet for 13 weeks (19 weeks of age) and then analyzed for atherosclerosis development in whole aorta. Quantification of the atherosclerotic lesions was reported as percentage of the whole aorta. Eleven mice were used in each group (**)P=0.009). Each symbol represents data from a single mouse, and each data set is summarized as mean ± SEM.
Supplemental Figure II: Ccr6 deficiency does not change the number of total white blood cells, peripheral blood lymphocytes, neutrophils, eosinophils or basophils in either wild type C57BL/6 mice or ApoE<sup>−/−</sup> mice (female, 16 weeks old). Absolute numbers of different blood cell subsets in C57BL/6 mice, Ccr6<sup>−/−</sup> mice, Ccr6<sup>+/+</sup>ApoE<sup>−/−</sup> mice and Ccr6<sup>−/−</sup>ApoE<sup>−/−</sup> mice were determined by complete blood count (CBC), as described in Methods. Data are summarized as mean ± SEM (n=9 mice in each group, *P=0.011 for monocytes).
Supplemental Figure III: Ccr6 deficiency does not affect the differentiation of bone marrow derived monocytes/macrophages. Bone marrow derived macrophages from wild-type and Ccr6/- mice were stimulated 18 hrs with either 25 ng/ml IFNγ and 100 ng/ml LPS or 10 ng/ml IL-4 to derive M1/M2 macrophages. Real-time PCR was used to analyze the mRNA levels of IL12α, iNOS, TNFα (M1 markers) and Arg1, Fizz1, IL-10 (M2 markers) both before (A) and after (B) stimulation. All samples were normalized to β-actin.
Supplemental Figure IV: Ccr6 is expressed on primary mouse splenic monocytes. A, Leukocytes from the spleen of Ccr6 EGFP knock-in mice were stained with antibodies to CD11b and Gr1 and gated on CD11b\(^+\)Gr1\(^-\), CD11b\(^+\)Gr1\(^+\) and CD11b\(^-\)Gr1\(^+\) cells. The percentage of the total gated population is indicated within the box. B, Representative flow cytometry staining of CD11b\(^+\)Ly6\(^{\text{high}}\) monocytes (CD11b\(^+\)Ly6C\(^{\text{high}}\)7/4\(^{\text{bri}}\)Ly6G\(^-\) cells), CD11b\(^+\)Ly6C\(^{\text{low}}\) monocytes (CD11b\(^+\)Ly6C\(^{\text{low}}\)Ly6G\(^-\)MHCII\(^{-}\)F4/80\(^{-}\) cells) and CD11b\(^+\)Ly6G\(^+\) neutrophils (CD11b\(^+\)Ly6G\(^+\)Ly6C\(^{\text{low}}\)MHCII\(^{-}\)7/4\(^{\text{bri}}\)F4/80\(^{-}\) cells) from the spleen of ApoE\(^{-}\) mice.
Supplemental Figure IV (cont.): Ccr6 is expressed on primary mouse splenic monocytes. C, Representative flow cytometry staining of either isotype control (upper panel) or Ccr6 specific antibody (lower panel) on CD11b$^+$Ly6C$^{\text{hi}}$ monocytes (left column), CD11b$^+$Ly6C$^{\text{low}}$ monocytes (middle column) and CD11b$^+$Ly6G$^+$ neutrophils (right column) from the spleen of ApoE$^{-/-}$ mice. D, Ccr6 expression on blood CD11b$^+$Gr1$^-$, CD11b$^+$Gr1$^+$, CD11b$^-$Gr1$^+$ cells was examined by GFP expression and cell gates were made as in A.
Supplemental Figure V: Ccl20 and Ccr6 are expressed in the aorta of ApoE⁻/⁻ mice. Real-time PCR analysis of chemokines and chemokine receptors involved in atherosclerosis in aortas from wild type C57BL/6 mice and ApoE⁻/⁻ mice were shown (n=4 mice in each group).
Supplemental Figure VI: Representative immunofluorescence photomicrographs of aortic root sections from $\text{Ccr6}^{+/+}\text{ApoE}^{-/-}$ mice stained with rat anti-mouse Ccl20 monoclonal antibody and rat IgG1 isotype control. Goat anti-rat Alexa Fluor 568 was used as secondary antibody for visualization.
Supplemental Figure VII: IFNγ induced production of Ccr6 mRNA in primary monocytes. Purified primary mouse monocytes from the bone marrow of C57BL/6 mice were stimulated with different doses of IFNγ for 24 hrs and then mRNA levels of Ccr6 were examined by real-time PCR.
Supplemental Figure VIII: Ccr6 deficiency does not affect the expression of other chemokine receptors and chemokines (Ccr2, Ccr5, Cx3cr1 and Ccl2, Ccl5, Cx3cl1) involved in atherosclerosis. Real-time PCR was used to analyze the RNA expression in aortas from Ccr6<sup>+/+</sup>ApoE<sup>-/-</sup> and Ccr6<sup>-/-</sup>ApoE<sup>-/-</sup> mice (n=3 mice in each group).
Supplemental Figure IX: Circulating neutrophil levels and total number of spleen monocytes do not change after tail vein injection of Ccl20 in ApoE−/− mice. Blood neutrophil levels and spleen monocyte numbers were quantified 15 hours post injection (n=3-4 in each group). The experiment was repeated two times and representative data are shown as mean ± SEM.