Combined Inhibition of CCL2, CX3CR1, and CCR5 Abrogates Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo} Monocytosis and Almost Abolishes Atherosclerosis in Hypercholesterolemic Mice

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Background—Monocytes are critical mediators of atherogenesis. Deletion of individual chemokines or chemokine receptors leads to significant but only partial inhibition of lesion development, whereas deficiency in other signals such as CXCL16 or CCR1 accelerates atherosclerosis. Evidence that particular chemokine pathways may cooperate to promote monocyte accumulation into inflamed tissues, particularly atherosclerotic arteries, is still lacking.

Methods and Results—Here, we show that chemokine-mediated signals critically determine the frequency of monocytes in the blood and bone marrow under both noninflammatory and atherosclerotic conditions. Particularly, CCL2-, CX3CR1-, and CCR5-dependent signals differentially alter CD11b\textsuperscript{+} Ly6G\textsuperscript{7/4\textsuperscript{hi}} (also known as Ly6C\textsuperscript{hi}) and CD11b\textsuperscript{+} Ly6G\textsuperscript{7/4\textsuperscript{lo}} (Ly6C\textsuperscript{lo}) monocytosis. Combined inhibition of CCL2, CX3CR1, and CCR5 in hypercholesterolemic, atherosclerosis-susceptible apolipoprotein E–deficient mice leads to abrogation of bone marrow monocytosis and to additive reduction in circulating monocytes despite persistent hypercholesterolemia. These effects are associated with a marked and additive 90% reduction in atherosclerosis. Interestingly, lesion size highly correlates with the number of circulating monocytes, particularly the CD11b\textsuperscript{+} Ly6G\textsuperscript{7/4\textsuperscript{lo}} subset.

Conclusions—CCL2, CX3CR1, and CCR5 play independent and additive roles in atherogenesis. Signals mediated through these pathways critically determine the frequency of circulating monocyte subsets and thereby account for almost all macrophage accumulation into atherosclerotic arteries. (Circulation. 2008;117:1649-1657.)

Key Words: atherosclerosis ▪ chemokines ▪ inflammation ▪ leukocytes ▪ monocytes

Vascular inflammation caused by the accumulation of modified lipids induces the recruitment of leukocytes into the subendothelial space and initiates atherosclerosis.\textsuperscript{1–4} Monocytes in particular are critical mediators of this process. Osteopetrotic mice with spontaneous deficiency in monocyte colony-stimulating factor (M-CSF) show a profound reduction in atherosclerosis resulting from a marked decrease in macrophage accumulation within the lesions.\textsuperscript{5} Two major monocyte subsets show differential expression of CCR2 and CX3CR1 receptors. Ly6C\textsuperscript{lo} monocytes represent the inflammatory subtype. They express high levels of CCR2 but low levels of CX3CR1 (Ly6C\textsuperscript{lo} CCR2\textsuperscript{hi} CX3CR1\textsuperscript{lo}) and are actively recruited to inflamed tissues, where they give rise to macrophages or antigen-presenting cells.\textsuperscript{6–9} This subset dominates hypercholesterolemia-associated monocytosis\textsuperscript{10,11} and appears to be recruited into atherosclerotic arteries primarily through CCR2.\textsuperscript{11} However, atherosclerosis is not abrogated in CCR2-deficient (CCR2\textsuperscript{−/−}) mice.\textsuperscript{12,13} Two potential nonexclusive explanations may be offered for this finding. The first is that, in addition to CCR2, other chemokine receptors significantly contribute to the recruitment of Ly6C\textsuperscript{lo} CCR2\textsuperscript{+} CX3CR1\textsuperscript{lo} monocytes into atherosclerotic arteries; this explanation is suggested by a recent report showing that CX3CR1 may contribute to their recruitment.\textsuperscript{11} CX3CR1\textsuperscript{−/−} or CX3CL1\textsuperscript{−/−} mice show a significant reduction in atherosclerosis. However, whether combined CCR2 and CX3CR1 inhibition has additive effects on the trafficking of Ly6C\textsuperscript{lo} CCR2\textsuperscript{+} CX3CR1\textsuperscript{lo} monocytes in atherosclerosis remains unknown. The other hypothesis is that other monocyte subset(s) significantly contribute to atherogenesis. Ly6C\textsuperscript{lo} monocytes represent the noninflammatory subtype. They express high levels of CX3CR1 (Ly6C\textsuperscript{lo} CCR2\textsuperscript{−} CX3CR1\textsuperscript{lo}) and give rise to resident macrophages and myeloid dendritic cells in noninflamed tissues, including liver, spleen, lung, and...
Although CX3CR1 is required for the accumulation of Ly6C<sup>hi</sup> CCR2<sup>−</sup> CX3CR1<sup>−</sup> monocytes in noninflammatory tissues,<sup>6</sup> recent data unexpectedly indicated that CX3CR1 was dispensable for their recruitment into atherosclerotic arteries,<sup>11</sup> suggesting that the mechanisms of the CX3CL1/ CX3CR1 pathway to promote lesional macrophage accumulation have yet to be clearly defined. In addition, whatever the monocyte subset and its response to specific chemokines, the current prevailing paradigm accounting for macrophage accumulation in atherosclerosis highlights the role of chemokines in the promotion of local monocyte trafficking between the blood and vessel wall. Yet, it remains elusive whether particular chemokine signals could mediate atherosclerosis-associated Ly6C<sup>hi</sup> and/or Ly6C<sup>lo</sup> monocyteosis and thereby promote lesion development. In the present study, we hypothesized that different chemokine pathways cooperate to promote the accumulation of different monocyte subsets in atherosclerosis, possibly through the modulation of both local trafficking and systemic monocytes.

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**Methods**

**Mice**

CX3CR1-deficient mice (CX3CR1<sup>−/−</sup>), apolipoprotein E/CX3CR1-deficient (Apo<sup>e−/−</sup>/CX3CR1<sup>−/−</sup>) mice, and CCL2-deficient (CCL2<sup>−/−</sup>) mice were generated as previously described. All deficient mice were bred onto the C57BL/6 background (6 to 8 backcrosses). The CX3CR1<sup>−/−</sup>/CCL2<sup>−/−</sup> mice were generated by crossing CX3CR1<sup>−/−</sup> with CCL2<sup>−/−</sup> mice and by subsequent intercrossing of heterozygous F1 animals; the Apo<sup>e−/−</sup>/CX3CR1<sup>−/−</sup>/CCL2<sup>−/−</sup> mice were generated by crossing CX3CR1<sup>−/−</sup>/CCL2<sup>−/−</sup> mice with Apo<sup>e−/−</sup>/CX3CR1<sup>−/−</sup> mice and by subsequent intercrossing of heterozygous F1 animals. The mice were maintained at the Centre d’Exploration Fonctionnelle animal facility (Pitié-Salpêtrière, Paris, France) under pathogen-free conditions with food and water available ad libitum. Only male mice were analyzed in this study. Treatment with Met-CCL5 was performed by intraperitoneal injection (100 µg in PBS) twice a week for 12 weeks as previously described starting at 8 weeks of age. Plasma cholesterol and high-density lipoprotein levels were measured with a commercial cholesterol kit (bioMerieux, Marcy l’Etoile, France). The heart was taken out, fixed in 4% paraformaldehyde for 2 hours, and placed in a PBS sucrose 30% solution overnight at 4°C before being included in a cutting medium and frozen at −70°C. Successive 10-µm transversal sections of aortic sinus were obtained. Lipids were detected with Oil Red O as previously described. Plaque composition was determined by use of a monoclonal rat anti-mouse macrophage antibody (clone MOMA-2 MAB1852 Chemicon, AbCys, Paris, France), a polyclonal anti-CCL2 antibody (M-50, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), or a polyclonal anti-CCR5 antibody (Santa Cruz). The specificity of the last 2 antibodies was tested in tissue recovered from CCR2<sup>−/−</sup> (Figure 1 of the online-only Data Supplement) or CCR5<sup>−/−</sup> mice. 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. The thoracic aorta was available from some animals for analysis of the extent of lipid accumulation with Oil Red O staining as previously described.<sup>18</sup>

**Peritoneal Inflammation**

Peritonitis was induced by intraperitoneal injection of sterile thiglycrolate (3% wt/vol in 1 mL sterile saline, Sigma-Aldrich, St Louis, Mo). Cells were quantified by flow cytometric analysis of the peritoneal lavage 72 hours after injection.

**Cells**

Blood was drawn via retroorbital puncture with heparin as an anticoagulant. Bone marrow cells were collected from femurs and tibias by insertion of a needle into the bone and flushing with Hanks’ buffered salt solution supplemented with 0.2% BSA and 1% FCS as previously described. Total viable leukocyte number was determined with the trypan-blue exclusion method. Leukocyte subpopulation numbers were calculated as total leukocytes multiplied by percent cells within the selected population gated by flow cytometry analysis.

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical significance was determined by use of 2- or 3-factor (monocyte number) ANOVA. A value of P<0.05 (Bonferroni test) was considered statistically significant. The relation between circulating monocyte number and lesion size was determined through a simple linear regression analysis.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**CCL2 and CX3CR1 Play Nonredundant Roles in Atherosclerosis**

We first examined whether simultaneous deletion in CCL2/ CCR2 and CX3CL1/CX3CR1 has independent and additive protective effects in atherosclerosis. To this end, we generated Apo<sup>e−/−</sup>/CCL2<sup>−/−</sup>/CX3CR1<sup>−/−</sup> triple-knockout mice and compared their susceptibility to atherosclerosis with Apo<sup>e−/−</sup>/CCL2<sup>−/−</sup> and Apo<sup>e−/−</sup>/CX3CR1<sup>−/−</sup> littermates. The mice were maintained on chow diet, allowing the development of spontaneous moderate hypercholesterolemia, and were euthanized at 25 weeks, an age at which Apo<sup>e−/−</sup> mice develop advanced lesions in the aortic sinuses.<sup>20</sup> The 4 groups of mice had comparable weight (data not shown). Total plasma cholesterol levels were similar between Apo<sup>e−/−</sup>, Apo<sup>e−/−</sup>/CCL2<sup>−/−</sup>, and Apo<sup>e−/−</sup>/CX3CR1<sup>−/−</sup> mice (Figure 1). However, unexpectedly, Apo<sup>e−/−</sup>/CCL2<sup>−/−</sup>/CX3CR1<sup>−/−</sup> mice showed an ∼75% increase of total but not high-density lipoprotein
cholesterol levels (P < 0.0001) compared with the other groups (Figure 1). Lesion size was significantly different among the studied groups (P < 0.0001). Lesion size was significantly altered by CCL2 (P < 0.0001) or CX3CR1 deficiency (P = 0.0009), and no interaction was found between these 2 variables (P = 0.86), indicating independent effects of CCL2 and CX3CR1 on lesion size. Interestingly, despite important hypercholesterolemia, triple-knockout mice exhibited a profound 67% decrease in lesion size at the level of the aortic sinus (95 777 ± 17 047 versus 287 999 ± 20 113 µm² in Apoe⁻/⁻/CCL2⁻/⁻/CX3CR1⁻/⁻ and Apoe⁻/⁻ mice, respectively; P < 0.0001; Figure 1). This marked inhibition of plaque development was significantly more pronounced than the 28%, 36%, or 48% reduction in lesion size observed in Apoe⁻/⁻/CX3CR1⁻/⁻ (P = 0.0012 versus Apoe⁻/⁻/CCL2⁻/⁻/CX3CR1⁻/⁻ and P = 0.039 versus Apoe⁻/⁻), Apoe⁻/⁻/CCL2⁻/⁻ (P = 0.0082 versus Apoe⁻/⁻/CCL2⁻/⁻/CX3CR1⁻/⁻ and P = 0.0072 versus Apoe⁻/⁻), or Apoe⁻/⁻/CCL2⁻/⁻/CX3CR1⁻/⁻ mice (P = 0.024 versus Apoe⁻/⁻/CCL2⁻/⁻/CX3CR1⁻/⁻ and P < 0.0001 versus Apoe⁻/⁻), respectively. These results clearly suggest that CX3CR1 and CCL2 play independent and complementary roles in atherosclerosis.

Modulation of Local Monocyte/Macrophage Accumulation by CCL2 and CX3CR1

We next examined potential atheroprotective mechanisms associated with CCL2 and/or CX3CR1 deficiency. We found significant and similar inhibition of macrophage accumulation in lesions of Apoe⁻/⁻/CCL2⁻/⁻ and Apoe⁻/⁻/CX3CR1⁻/⁻ mice compared with Apoe⁻/⁻ mice (Figure 2A and 2B). Reduced macrophage accumulation in Apoe⁻/⁻/CCL2⁻/⁻ mice was associated with a marked decrease in the accumulation of CCR2⁺ macrophages within the lesions (Figure 2C). Unexpectedly, but in agreement with the results of 1 recent study, we observed a reduction in the accumulation of CCR2⁺ macrophages in lesions of Apoe⁻/⁻/CX3CR1⁻/⁻ mice (Figure 2C). However, accumulation of CCR2⁺ macrophages was less affected by CX3CR1 compared with CCL2, and combined CCL2 and CX3CR1 deficiency showed no additive effect (Figure 2C). These results suggest a predominant role for CCL2/CCR2 pathway in the recruitment of CCR2⁺ monocytes into atherosclerotic arteries compared with CX3CR1. Interestingly, lesional macrophage accumulation was lowest in Apoe⁻/⁻/CCL2⁻/⁻/CX3CR1⁻/⁻ mice (Figure 2A and 2B) despite higher cholesterol levels (Figure 1C). This could result from an additive reduction in the recruitment of the other major monocyte subset (ie, CCR2⁺ CX3CR1⁺ Ly6C⁶⁰). However, a recent study clearly showed that neither CCR2, the only CCL2 receptor, nor CX3CR1 contributes to recruitment of CCR2⁺ CX3CR1⁺ Ly6C⁶⁰ monocytes in atherosclerosis. Thus, we hypothesized that the additive protection from lesion development conferred by the simultaneous deletion of CCL2 and CX3CR1 may be related to systemic rather than local effects of chemokine signaling, which may affect the numbers of monocytes in the bloodstream and their interaction with the vessel wall.
A recent study suggested that in response to bacterial infection, CCR2-mediated signals in bone marrow determine the emigration of immature Ly6C\(^{hi}\) monocytes into the circulation rather than promoting monocyte trafficking between the blood and peripheral tissue.\(^{21}\) We therefore examined the effect of CCL2 and/or CX3CR1 deficiency on the frequency of the 2 major monocyte subsets in the bone marrow and bloodstream.

**Differential Modulation of Bone Marrow and Circulating Monocyte Subsets by CCL2 and CX3CR1**

In the present study, we defined monocytes as side scatter–low, forward scatter–high cells expressing the myeloid antigen 7/4 (high and low populations) and high levels of CD11b but showing no expression for the neutrophil marker Ly6G (Figure 3A). In addition, side scatter–low, forward scatter–high, CD11b–high, 7/4–high cells showed almost no staining for NK cell marker NK1.1, and <20% of 7/4–low cells were contaminated by NK cells (online-only Data Supplement Figure II). CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\) cells correspond to Ly6C\(^{hi}\) monocytes, and CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{−}\) cells correspond to Ly6C\(^{lo}\) monocytes (online-only Data Supplement Figure III). Monocyte number and phenotypes were significantly different among the various groups (P<0.001). ApoE background and CCL2 deficiency significantly affected monocyte number (P<0.001). In agreement with recent data,\(^{10}\) we observed increased bone marrow and blood monocytosis in Apoe\(^{−/−}\) mice (Figures 3 and 4, solid bars) and a shift toward the CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\) subset in the blood compared with Apoe\(^{−/−}\) mice (Figure 3, open bars). However, in the present study, both CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\) and CD11b\(^{−}\) Ly6G\(^{−}\) 7/4\(^{−}\) subsets contributed to blood monocytosis under moderate hypercholesterolemia (Figure 3B). The difference between these studies may be due in part to differences in the phenotyping of the 7/4\(^{+}\) monocyte subset. We observed a significant reduction in the total number of circulating monocytes in CCL2\(^{−/−}\) mice under normocholesterolemic conditions (ApoE\(^{−/−}\) background) because of a reduction in both CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\) and CD11b\(^{−}\) Ly6G\(^{−}\) 7/4\(^{−}\) monocytes (Figure 3B), supporting similar findings reported in CCR2\(^{−/−}\) animals.\(^{21,22}\) The significant reduction in circulating monocyte numbers was maintained under the hypercholesterolemic and atherosclerotic ApoE\(^{−/−}\) background (ApoE\(^{−/−}/CCL2\(^{−/−}\) compared with Apoe\(^{−/−}\) mice in Figure 3B). However, in contrast to CCR2 deficiency, which led to accumulation of Ly6C\(^{hi}\) (here, CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\)) monocytes in the bone marrow,\(^{21,22}\) CCL2 deficiency did not, and it even led to a reduction in bone marrow monocyte number under the ApoE\(^{−/−}\) background (Figure 4B), suggesting that other chemokines compensate for the absence of CCL2 to mediate emigration of CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\) monocytes into the circulation.\(^{22}\) Short-term administration of CCL2 to C57Bl/6 mice led to an increase in monocyte number in the blood and bone marrow (online-only Data Supplement Figure IV), suggesting that CCL2 may control monocyte number. Whether this effect requires CCR2 signaling in monocytes is currently unknown. Our results clearly show that CCL2 deficiency inhibits bone marrow CD11b\(^{+}\) Ly6G\(^{−}\) 7/4\(^{+}\) monocytosis in hypercholesterolemic mice and controls circulating
monocyte number, which may have contributed, at least in part, to the marked inhibition of CCR2+ macrophage accumulation in the atherosclerotic lesions of Apoε−/−/CCL2−/− mice (Figure 2).

CX3CR1 deficiency was associated with a nonsignificant trend toward a lower number of monocytes in the bone marrow (Figure 4) but did not affect total blood monocyte number (Figure 3). Our observation that CX3CR1 deficiency resulted in reduced accumulation of CCR2+ cells within the lesions without affecting the number of CD11b+ Ly6G−/7/4hi (CCR2+) monocytes in the circulating blood suggests a role for CX3CR1 signaling in the recruitment of this monocyte subset from the blood into the atherosclerotic lesions, which is in agreement with previous findings.11 Interestingly, CX3CR1 deficiency induced a specific reduction in the number of circulating CD11b+ Ly6G−/7/4hi (Figure 3C), consistent with the high level of CX3CR1 expression on this monocyte subset in Apoε−/− and Apoε−/− mice. Furthermore, combined CCL2 and CX3CR1 deficiency in the Apoε−/− background resulted in a significant reduction in bone marrow and circulating monocytes as a result of the additive reduction in CD11b+ Ly6G−/7/4hi and CD11b+ Ly6G−/7/4lo subsets (Figures 3 and 4). Of note, individual deficiency of either CCL2 or CX3CR1 was not sufficient to inhibit the ability of mice to increase total monocyte number in the bone marrow and circulating blood when switched from a normo-

cholesterolemic nonatherosclerotic Apoε−/− to a hypercholes-

terolemic atherosclerotic Apoε−/− background (Figures 3 and 4). However, combined CCL2 and CX3CR1 deficiency totally abrogated Apoε−/−-associated bone marrow and blood monocytosis, which returned to levels observed in Apoε−/− mice, despite higher levels of plasma cholesterol (Figures 3 and 4). We found no effects of CCL2 and/or CX3CR1 deficiency on neutrophil, CD4+, or CD8+ lymphocyte count in the Apoε−/− background (data not shown). These results identify critical, independent, and complementary roles for CCL2- and CX3CR1-mediated signals in bone marrow and blood (CD11b+ Ly6G−/7/4hi and CD11b+ Ly6G−/7/4lo) monocytosis, which could explain the additive roles of these pathways in promoting lesion development in Apoε−/− mice. In contrast to its role in atherosclerosis, we found that specific inhibition of the recruitment of CD11b+ Ly6G−/7/4hi monocytes in CX3CR1−/− mice was not sufficient to alter monocyctic peritonitis in response to intraperitoneal thioglycollate (online-only Data Supplement Figure V), confirming the prominent role of CD11b+ Ly6G−/7/4hi monocyte recruitment through CCL2/CCR2+ in this setting.

Combined Inhibition of CCL2, CX3CR1, and CCR5 Almost Abolishes Atherosclerosis

Finally, we wanted to identify the chemokine pathway(s) responsible for the residual atherogenesis that showed resistance
CCL5 treatment did not affect blood monocyte number in Apoe−/− mice (data not shown), ruling out any toxic effect of Met-CCL5 on blood monocytes. Together, these findings confirm that CCL2 and CX3CR1 act in unison to recruit monocytes to sites of lesion formation but play no additive role in promoting arterial leukocyte accumulation at a given atherosclerosis-prone site.

When we initiated the present study >3 years ago, we hypothesized that 2 particular pathways, CCL2/CXCR2 and CX3CL1/CX3CR1, could play independent, complementary roles to promote a high level of macrophage accumulation within the atherosclerotic artery and to accelerate plaque development. Our hypothesis was based on several observations. Deletion of either the CCL2/CXCR2 or CX3CL1/CX3CR1 pathway reduces monocyte accumulation within the vascular lesions and leads to reduced susceptibility to atherosclerosis in murine models. CXCR3 play differential roles during atherogenesis, with CXCR2 promoting lesion development within the aortic root and CX3CR3 affecting lesion formation within the abdominal aorta. Thus, CXCR2 and CX3CR3 differentially influence the site of lesion formation but play no additive role in promoting arterial leukocyte accumulation at a given atherosclerosis-prone site.

**Discussion**

The present study presents 3 major findings: (1) Combined activation of CCL2, CX3CR1, and CCR5 pathways accounts for most of the macrophage accumulation within atherosclerotic lesions; (2) CCL2, CX3CR1, and CCR5 markedly and differentially alter the number of monocyte subsets within the bone marrow and the circulating blood and are required for blood and bone marrow monocytosis in hypercholesterolemic atherosclerotic mice; and (3) control of systemic monocyte number by these chemokine pathways is highly correlated with their ability to modulate atherogenesis.

Taken individually, several chemokines and chemokine receptors play a significant role in the development of atherosclerosis. However, direct evidence of additive roles for these pathways in experimental models of the disease is still lacking. A recent study examined the effect of combined inhibition of CCR2 and CX3CR3 signaling on lesion formation in Apoe−/− mice. The authors showed that CCR2 and CX3CR3 play differential roles during atherogenesis, with CCR2 promoting lesion development within the aortic root and CX3CR3 affecting lesion formation within the abdominal aorta. Thus, CCR2 and CX3CR3 differentially influence the site of lesion formation but play no additive role in promoting arterial leukocyte accumulation at a given atherosclerosis-prone site.

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we observed a trend toward a more profound inhibition of atherosclerosis in the thoracic aorta after combined deletion of CCL2 and CX3CR1 (online-only Data Supplement Figure VI) despite higher plasma cholesterol levels. In addition, in the complementary work by Saederup et al\textsuperscript{13} (see the companion article), the authors nicely show an additive reduction in atherosclerotic lesion size (within both the thoracic aorta and the aortic root) after combined deficiency in CCR2 and CX3CL1 using Apoe\textsuperscript{−/−} mice fed a Western diet. Taken together, these 2 studies clearly show for the first time that specific chemokine pathways, here CCL2/CCR2 and CX3CL1/CX3CR1, play nonredundant, complementary roles in vivo in a chronic inflammatory disease, here atherosclerosis.

Interestingly, we found that macrophage accumulation within atherosclerotic arteries was lowest in Apoe\textsuperscript{−/−}/CCL2\textsuperscript{−/−}/CX3CR1\textsuperscript{−/−} mice but was not significantly different from that observed in Apoe\textsuperscript{−/−}/CCL2\textsuperscript{−/−} or Apoe\textsuperscript{−/−}/CX3CR1\textsuperscript{−/−} mice. It could be argued that this suggests no substantial additive effect of combined inhibition of CCL2 and CX3CR1 on macrophage accumulation within the vessel wall. However, it is important to note that Apoe\textsuperscript{−/−}/CCL2\textsuperscript{−/−}/CX3CR1\textsuperscript{−/−} mice showed the smallest reduction in lesion size despite a very important and significant increase in plasma cholesterol levels, a major atherogenic stimulus. Indeed, lesion size was significantly correlated with plasma cholesterol levels in the triple-knockout mice (online-only Data Supplement Figure VII), suggesting that if plasma cholesterol levels had been equivalent to those found in the other groups of mice, the reduction in lesion size would have been much more profound in the triple-knockout animals. Thus, taken together, these results clearly reveal a major inhibitory role of combined CCL2 and CX3CR1 deficiency on arterial inflammation.

An important finding in the present study is that the major role of chemokines and chemokine receptors in atherosclerosis may relate to their role in the modulation of monocyte number in both bone marrow and circulating blood. Our results clearly show that deletion of CCL2 and CX3CR1 abolishes hypercholesterolemia-associated blood monocytosis, identifying a critical role for chemokine signaling in this process. In addition, we found that chemokine pathways differentially affect the number of monocyte subsets, with CCL2 having major impact on both Ly6\textsuperscript{C\textsuperscript{hi}} and Ly6\textsuperscript{C\textsuperscript{lo}} monocytes and CX3CR1 specifically affecting Ly6\textsuperscript{C\textsuperscript{lo}} monocytosis. Unexpectedly, the control of circulating monocyte number by CCL2 and CX3CR1 was associated with a reduction in, not accumulation of, monocytes within the bone marrow, suggesting that their role in the modulation of monocytosis goes beyond the control of monocyte emigration.
from the bone marrow into the circulating blood, as recently suggested for CCR2-mediated signaling.21,22 It will be important in future studies to identify in detail the precise signals and mechanisms responsible for the chemokine-dependent increase in monocyte number, which may include increased monocyte differentiation, proliferation, and/or survival. A previous study10 suggested a predominant role for Ly6C\textsuperscript{hi} monocytes in atherogenesis under high-fat feeding. However, given the strong correlation between the number of Ly6C\textsuperscript{hi} monocytes and lesion size reported in the present work, we believe that this monocyte subset may significantly contribute to lesion formation, at least under moderate hypercholesterolemia.

An intriguing finding in the present study is that inhibition of CX3CR1 signaling resulted in a relative increase in the accumulation of CCR5\textsuperscript{+} cells within the lesions, suggesting an exclusive interaction between these 2 pathways in the recruitment of CCR5\textsuperscript{+} leukocytes. The latter may include macrophages, T cells, and smooth muscle cells. However, CCR5\textsuperscript{+} staining most likely represented macrophages given the very small size of T cells (relative to macrophages) and the absence of smooth muscle cells within the lesions of the triple-knockout mice (data not shown). Interestingly, inhibition of CCR5 signaling in CCL2/CX3CR1-deficient mice almost abrogated arterial macrophage accumulation, identifying 3 major and complementary pathways that control lesion development in ApoE\textsuperscript{-/-} mice. Again, inhibition of CCR5 signaling led to a marked reduction in the number of circulating monocytes, further supporting an important role for chemokines in the modulation of systemic monocyte number. The effect of Met-CCL5 on atherosclerosis could be related, at least in part, to inhibition of CCR5 signaling in T cells. However, it is remarkable that the reduction in lesion size in mice with defective CCL2, CX3CR1, and/or CCR5 signaling strongly correlated with the reduction in circulating monocyte number. Similar results were reported by Smith et al\textsuperscript{5} in ApoE\textsuperscript{-/-} mice with osteopetrotic mutation. It will be important to examine in future studies whether chemokine-mediated regulation of bone marrow and blood monocyte number requires an intact M-CSF pathway. Finally, it could be argued that modulation of bone marrow and blood monocytoysis is not a primary effect of chemokine inhibition but is secondary to atherosclerosis reduction. However, our results show that short-term administration of CCL2 enhances bone marrow and circulating monocyte number, suggesting a direct effect of chemokines on monocytoysis. In addition, the 99% reduction in atherosclerosis in severely hypercholesterolemic ldlr\textsuperscript{-/-} mice with heterozygous osteopetrotic mutation was not associated with significant changes in the percentage of circulating monocytes,24 suggesting that a reduction in atherosclerosis is not a prerequisite for a reduction in circulating monocyte number.

The mechanisms responsible for the increase in total and non–high-density lipoprotein plasma cholesterol levels in ApoE\textsuperscript{-/-}/CCL2\textsuperscript{-/-}/CX3CR1\textsuperscript{-/-} mice were not explored. This increase could occur as a direct or indirect consequence of combined CCL2 and CX3CR1 deletion. Interestingly, Smith et al\textsuperscript{5} reported similar findings in ApoE\textsuperscript{-/-}/M-csf\textsuperscript{-/-} mice that showed reduced monocyte number, suggesting a potential role for monocytes/macrophages in the modulation of lipid metabolism.

Conclusions

In addition to their roles in leukocyte recruitment from the blood into the vessel wall, which is the prevailing paradigm to explain the proatherogenic effects of several chemokine/chemokine receptor pathways, we show that CCL2-, CX3CR1-, and CCR5-dependent signals differentially alter CD11b\textsuperscript{+} Ly6G\textsuperscript{7/4hi} and CD11b\textsuperscript{+} Ly6G\textsuperscript{7/4lo} monocytoysis in the blood and bone marrow and cooperate to promote full macrophage accumulation within atherosclerotic vessels. These results identify critical independent and complementary chemokine pathways in atherosclerosis and shed new light on the mechanisms operated by chemokines to enhance monocyte accumulation into inflamed tissues.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Monocytes are critical mediators of atherogenesis and are recruited into atherosclerotic arteries in response to chemokine/chemokine receptor signaling. Evidence that particular chemokine pathways may cooperate to promote monocye accumulation into inflamed tissues, particularly atherosclerotic arteries, is still lacking. Here, we show that combined inhibition of CCL2, CX3CR1, and CCR5 in apolipoprotein E−deficient mice almost abolishes lesion formation, indicating that these pathways play independent and additive roles in atherosclerosis. Another important finding is that the major role of chemokines and chemokine receptors in atherosclerosis may relate to their role in the modulation of monocyte number in both the bone marrow and circulating blood. Our results clearly show that inhibition of CCL2, CX3CR1, and CCR5 abolishes hypercholesterolemia-associated blood monocyte monocytosis, identifying a critical role for chemokine signaling in this process. It is remarkable that the reduction in lesion size in mice with defective CCL2, CX3CR1, and/or CCR5 signaling strongly correlated with the reduction in circulating monocyte number, particularly the CD11b+ Ly6G−/Ly6C+ subset. Thus, in addition to their roles in leukocyte recruitment from the blood into the vessel wall, which is the prevailing paradigm to explain the proatherogenic effects of several chemokine/chemokine receptor pathways, signals mediated through CCL2, CX3CR1, and CCR5 critically determine the frequency of circulating monocyte subsets and cooperate to promote full macrophage accumulation within atherosclerotic vessels. It will be important to examine in future studies whether particular blood monocyte subsets are associated with the extent of atherosclerosis in humans.
Combined Inhibition of CCL2, CX3CR1, and CCR5 Abrogates Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo} Monocytosis and Almost Abolishes Atherosclerosis in Hypercholesterolemic Mice

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Supplementary Figure 1 online. Staining of spleen sections with the anti-CCR2 antibody (M-50, #K1705; Santa Cruz). a and b) As expected, positive staining is shown in spleen sections of Ldlr knockout (a) or C57Bl/6 (b) CCR2+/+ animals; b) No staining was detected in spleen sections of CCR2−/− mice, indicating that the antibody is specific for CCR2. Original magnifications, a and c X40; b X 200.
CD11b high 7/4 high population is not contaminated by NK cells (the CD115 neg cells are neutrophils, see Figure 2). Less than 20% of CD11b high, 7/4 low population are NK cells.
Supplementary Figure 3 online. The intensity of staining with the myeloid marker 7/4 (7/4\textsuperscript{hi} or 7/4\textsuperscript{lo}) in CD11b\textsuperscript{+} Ly6G\textsuperscript{−} monocytes corresponds to the previously described Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo} monocyte subsets.
Supplementary Figure 4 online. Number of bone marrow and blood monocytes in C57Bl/6 mice 7 days after intraperitoneal administration of CCL2 or PBS at day 0, 2 and 4 (n=4/group).
Supplementary Figure 5 online. CCL2 and CX3CR1 differentially alter monocytic peritonitis. Quantitative analysis of the infiltration of total and individual macrophage subsets in the peritoneum after the induction of monocytic peritonitis using thioglycollate. Values represent mean values ± s.e.m of 10 mice per group under Apoe^{+/+} background; single asterisk, P < 0.05; double asterisk, P < 0.01 (compared with wild type mice); ns indicates P not significant.
Supplementary Figure 6 online. Combined inhibition of CCL2, CX3CR1 and CCR5 pathways markedly inhibits atherosclerotic lesion development in the thoracic aorta of Apoe<sup>−/−</sup> mice. Values represent percentage of thoracic aorta covered by Oil red O-positive staining. P values are in comparison with Apoe<sup>−/−</sup> mice. *Lesion size in this group is also significantly different from lesion size in Apoe<sup>−/−</sup>/CCL2<sup>−/−</sup>/CX3CR1<sup>−/−</sup> mice (P=0.009).
Supplementary Figure 7 online. Correlation between lesion size in the aortic sinus and plasma total cholesterol levels in Apoe<sup>−/−</sup>/CCL2<sup>−/−</sup>/CX3CR1<sup>−/−</sup> mice; r=0.66; P=0.0076.