Pharmacological Inhibition of the Chemokine Receptor, CX3CR1, Reduces Atherosclerosis in Mice

Lucie Poupel, Alexandre Boissonnas, Patricia Hermand, Karim Dorgham, Elodie Guyon, Constance Auvynet, Flora Saint Charles, Philippe Lesnik, Philippe Deterre, Christophe Combadière

Objective—Alterations of the chemokine receptor CX3CR1 gene were associated with a reduced risk of myocardial infarction in human and limited atherosclerosis in mice. In this study, we addressed whether CX3CR1 antagonists are potential therapeutic tools to limit acute and chronic inflammatory processes in atherosclerosis.

Approach and Results—Treatment with F1, an amino terminus–modified CX3CR1 ligand endowed with CX3CR1 antagonist activity, reduced the extent of atherosclerotic lesions in both Apoe<sup>−/−</sup> and Ldlr<sup>−/−</sup> proatherogenic mouse models. Macrophage accumulation in the aortic sinus was reduced in F1-treated Apoe<sup>−/−</sup> mice but the macrophage density of the lesions was similar in F1-treated and control mice. Both in vitro and in vivo F1 treatment reduced CX3CR1-dependent inflammatory monocyte adhesion, potentially limiting their recruitment. In addition, F1-treated Apoe<sup>−/−</sup> mice displayed reduced numbers of blood inflammatory monocytes, whereas resident monocyte numbers remained unchanged. Both in vitro and in vivo F1 treatment reduced CX3CR1-dependent inflammatory monocyte survival. Finally, F1 treatment of Apoe<sup>−/−</sup> mice with advanced atherosclerosis led to smaller lesions than untreated mice but without reverting to the initial phenotype.

Conclusions—The CX3CR1 antagonist F1 is a potent inhibitor of the progression of atherosclerotic lesions by means of its selective impact on inflammatory monocyte functions. Controlling monocyte trafficking and survival may be an alternative or complementary therapy to lipid-lowering drugs classically used in the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:2297-2305.)

Key Words: atherosclerosis ■ chemokine receptors ■ CX3CR1 protein, mouse ■ migration, cell ■ monocytes ■ receptor antagonist

Atherosclerosis is a chronic inflammatory disease of the arterial wall that develops in a setting combining lipid metabolism and immune response disturbances. The hallmark feature of atherosclerosis is the formation of atherosclerotic lesions that result from the accumulation of fatty materials, such as cholesterol and lipid-rich lipoprotein (low-density lipoprotein [LDL]), and of inflammatory leukocytes (mostly macrophages) in the subendothelial space of the vessel wall. The proposed pathological mechanisms leading to plaque formation rely on the recruitment of intimal macrophages that accumulate modified LDL and convert them into lipid-rich foam cells, releasing proinflammatory and cytotoxic molecules. The proinflammatory environment further promotes plaque progression to more advanced and complex lesions, which are prone to rupture.

Most current therapeutic drugs target the best-documented atherogenesis-promoting risk factors. Examples of this include the treatment of dyslipidemia with inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase such as statins and the regulation of high blood pressure with inhibitors of angiotensin-converting enzyme. Although statins have a pronounced anti-inflammatory activity by reducing the plasma level of the inflammation marker C-reactive protein, they are not designed to control inflammation. In fact, these drugs do not specifically or directly target recruitment of leukocytes into the lesions. Among the many different leukocyte populations that infiltrate atherosclerotic lesions, macrophages seemed to be the main cellular species. The pathogenic role of macrophages was mostly inferred from animal models. Osteopetrotic mice with spontaneous deficiency in monocyte colony-stimulating factor and circulating monocytes showed a profound reduction in atherosclerosis resulting from a marked decrease in macrophage accumulation within lesions. Similarly, monocyte depletion using toxic agents, such as clodronate and diphtheria toxin, in CD11b-diphtheria toxin receptor transgenic mice led to reduced atherosclerosis.

Two major monocyte subsets can be distinguished in the blood based on differential expression of the chemokine

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receptors CCR2 and CX3CR1. The inflammatory monocytes express high levels of CCR2 but low levels of CX3CR1 (CCR2: CX3CR110), whereas the resident monocytes have a reciprocal marker expression profile (CCR2: CX3CR19). Inflammatory monocytes rapidly enter sites of inflammation where they give rise to macrophages or antigen-presenting cells.13–14 Resident monocytes enter lymphoid and non-lymphoid organs under homeostatic conditions, and patrol the vascular endothelium in a CX3CR1-dependent manner.15 The former subset dominates hypercholesterolemia-associated monocytopsises14,16 and seems to be recruited into atherosclerotic arteries primarily through CCR2.14 The main CCR2 ligand seems to be important during early atherogenesis, whereas the unique CX3CR1 ligand (CX3CL1) is thought to be involved in the progression of the disease. Atherosclerosis is attenuated in Ccr2−/−,17 Ccl2−/−,18 Cx3cl1−/−,19 and in Cx3cr1−/− mice20,21 but combined deficiencies of these axes lead to an even more drastic reduction in atherosclerotic lesions,22,23 confirming a nonredundant role of these chemokine axes in atherosclerosis. In addition, pharmacological inhibition of CCR5, another chemokine receptor involved in leukocyte recruitment, resulted in an almost entire abrogation of atherosclerosis in Cx3cr1−/−Ccl2−/−Apoe−/− mice.22 Targeting the chemokine system to prevent or to reduce atherosclerosis seems to be of great interest. However, blocking CCR2 with antagonists was poorly efficient on aortic lesions24,25 but led to a significant reduction in monocytopsy 26,27 probably by regulating leukocyte recruitment and systemic inflammation. To date, no CX3CR1 antagonist has been tested in atherosclerosis. We recently developed an amino terminus–modified CX3CL1 (N-terminal sequence ILDNGVS replacing QHHGVT of the human CX3CL1) endowed with CX3CR1 antagonist activity reducing both CX3CR1-dependent migration and adhesion28 and evaluated its efficacy in murine models of atherosclerosis.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Pharmacological Inhibition of CX3CR1 Reduces Atherosclerotic Plaque Development
To assess the therapeutic potential of the CX3CR1 antagonist (F1) on atherosclerotic lesion development, Apoe−/− mice were fed on high-fat diet (HFD) for 15 weeks (Figure 1) and treated for the last 10 weeks of the HFD, 3× a week by intraperitoneal injection of 50 µg of F1 or saline solution. Differential interference contrast microscopy was used to reveal atherosclerotic lesions in the aorta of saline- and F1-treated Apoe−/− mice (Figure 1A). Quantitative computer-assisted image analysis showed a 40% decrease of the lesion area after F1 treatment (Figure 1B). The F1 antiatherogenic effect was similar to the atheroprotection observed in Cx3cr11flo/floApoec−/− mice with the green fluorescent protein (GFP) being expressed under the control of the CX3CR1 promoter.29 In addition, F1 effect was lost in the Cx3cr1-deficient mice. Lipid staining with oil Red O (ORO) of aortic sinus sections revealed a marked reduction in atherosclerotic lesions in mice treated with F1 compared

Pharmacological Inhibition of CX3CR1 Attenuates Macrophage Lesion Accumulation
Assuming a role of CX3CL1 on monocyte recruitment, we assessed the effect of F1 on the macrophage content of the lesions in the Apoe−/− model (Figure 2). Total macrophage accumulation, assessed by monocyte/macrophage-2 staining, was reduced in F1-treated Apoe−/− mice compared with saline-treated Apoe−/− mice (Figure 2A and 2B). The inhibition reached 40% (Figure 2C). However, the macrophage density within the lesion, evaluated by the ratio of the monocyte/macrophage-2 staining to the ORO staining, was not affected by F1 (Figure 2D). Similar results were observed using CX3CR1 staining to assess CX3CR1-positive macrophages (Figure 2E–2H) with an absolute reduction of the CX3CR1-stained surface by 40% in F1-treated mice (Figure 2E and 2F with quantification in Figure 2G) and no change when expressed as a ratio to the lesion area (Figure 2H). These results indicate that F1 limited macrophage accumulation in the aortic sinus without altering macrophage density.
CX3CR1 Antagonist Limits Atherogenesis

Both In Vitro and In Vivo F1 Treatment Inhibits CX3CR1-Dependent Monocyte Adhesion

To identify F1 cellular target in vivo, fluorescent-labeled F1 (FluoF1) was injected intravenously in Cx3cr1^gfp/+ mice. FluoF1 was selectively detected on monocytes (Figure 3A) but not on polymorphonuclear neutrophils or lymphocytes except natural killer cells (data not shown). Interestingly, FluoF1 staining was more intense on GFP<sup>-</sup>/ly6C<sup>-</sup> monocytes (mean fluorescence intensity=22.1±0.2) compared with GFP<sup>+</sup>/ly6C<sup>-</sup> monocytes (mean fluorescence intensity=41.05±3.45). FluoF1 staining faded rapidly over time and could not be detected 4 hours after injection (Figure 3B). FluoF1 could not be detected in plasma or serum. Intraperitoneal injection of FluoF1 leads to fluorescent accumulation in resident macrophages but no staining could be detected in serum, plasma, or circulating cells (data not shown). The CX3CR1 ligand is strongly produced by atherosclerotic lesions and participates in the capture of blood monocytes. Thus, a CX3CR1 antagonist may inhibit CX3CR1-positive cell adhesion to CX3CL1-expressing surfaces. As shown in Figure 3C, bone marrow cells from Cx3cr1<sup>1flo/+</sup> mice strongly adhered to a CX3CL1-coated surface. The GFP intensity was used to discriminate GFP<sup>-</sup> from GFP<sup>+</sup> adherent cells. Computer-assisted image analysis was used to quantify GFP<sup>-</sup> from GFP<sup>+</sup> adherent cells as shown in Figure 3D. Bone marrow cells from Cx3cr1<sup>1flo/+</sup> mice were treated with F1 and exposed to a CX3CL1-coated surface (Figure 3E). As expected, GFP<sup>+</sup> cells adhered more efficiently to CX3CL1-coated surfaces than GFP<sup>-</sup> cells. F1 almost abrogated CX3CR1-dependent adhesion to coated CX3CL1 of both GFP<sup>-</sup> subpopulations. Interestingly, GFP<sup>+</sup> cells from saline- or F1-treated Cx3cr1<sup>1flo/+</sup> Apo<sup>-</sup> mice adhered similarly (Figure 3F), whereas GFP<sup>-</sup> cells were ≈40% less adherent than those from saline-treated Cx3cr1<sup>1flo/+</sup> Apo<sup>-</sup> mice. We conclude that both in vitro and in vivo F1 treatments attenuated CX3CR1-dependent cell adhesion, thus limiting monocyte recruitment to the lesion site.

F1 Reduces Monocytosis in Apo<sup>-</sup> Mice

Because F1 treatment reduced monocyte adhesion, long-term treatment in mice may lead to increased monocytosis by preventing monocytes to infiltrate the atherosclerotic lesions. We, therefore, decided to investigate the F1 effect on blood monocytes (Figure 4). The inflammatory and resident monocyte subpopulations were identified by flow cytometry as low side
scatter, high forward scatter cells, expressing high level of CD11b and either high or low levels of the myeloid antigen 7/4 (Figure 4A, right). Polymorphonuclear neutrophils and natural killer cells were identified on the basis of the expression of specific markers, Ly6G and NK1.1, respectively (Figure 4A, left). Surprisingly, F1 treatment reduced by 50% the absolute numbers of 7/4 hi or inflammatory monocytes in Apoe−/− mice (Figure 4B; with 7/4 hi numbers of 12.55±1.9×10^3/mL and 6.9±1.7×10^3/mL for saline- and F1-treated Apoe−/− mice, respectively), whereas the number of 7/4 lo or resident monocytes was not altered (Figure 4C; with 7/4 lo numbers of 36.2±4.7×10^3/mL and 33.3±4.4×10^3/mL for saline- and F1-treated Apoe−/− mice, respectively). Accordingly, the frequency of 7/4 hi monocytes was reduced (P<0.05; 2.4±0.3% versus 1.3±0.2% for saline- and F1-treated Apoe−/− mice, respectively), whereas the frequency of 7/4 lo monocytes was unaltered (P=ns; 6.6±0.9% versus 5.8±0.7% for saline- and F1-treated Apoe−/− mice, respectively). Polymorphonuclear neutrophils and natural killer cell numbers were not affected by F1 treatment (Figure 4D and 4E). In the Ldlr−/− mice, similar results were observed with F1-limiting monocytosis of 7/4 lo monocytes (P<0.005; 10.4±0.1×10^3/mL versus 5.9±0.7×10^3/mL monocytes for saline- and F1-treated Ldlr−/− mice, respectively) without any significant effect on 7/4 hi monocytes (P=ns;
20.2±0.3×10^3/mL versus 16.4±0.3×10^3/mL for saline- and F1-treated Ldlr−/− mice, respectively). In addition, neither the frequency nor the number of monocyte subpopulations was significantly affected by F1 treatment in the bone marrow and in the spleen (data not shown). Taken together, our data show that F1 treatment specifically decreased the size of the blood 7/4^hi monocyte population and dissociated the monocytosis from lesion progression suggesting an antiatherogenic role of F1 through changes in monocyte functions.

**F1 Treatment Reduces CX3CR1-Dependent Monocyte Survival**

Interactions between CX3CR1 and its ligand provide an essential survival signal for monocytes and may participate in atherogenesis. We hypothesized that, in addition to blocking CX3CR1-dependent adhesion, F1 may inhibit a CX3CR1-dependent survival signal (Figure 5). Bone marrow cells taken from C57bl/6 mice were grown in FCS-free conditions and live cells were identified using flow cytometry by the exclusion of the propidium iodide (Figure 5A). CX3CL1, at 100 nmol/L, increased 7/4^high and 7/4^low monocytes survival by ≈35% (Figure 5B). In contrast, F1 reduced cell survival by ≈40% for both monocyte subpopulations. To test whether in vivo F1 treatment may alter cell survival, monocyte survival was compared in saline- and F1-treated Apoe−/− mice. The number of live 7/4^hi monocytes was reduced by ≈50% in F1-treated Apoe−/− mice compared with untreated control mice (Figure 5C). In contrast, 7/4^lo monocyte survival was similar after in saline- and F1-treated mice. Overall, our results show that both in vitro and in vivo F1 treatment reduced CX3CR1-dependent inflammatory monocyte survival, consistent with the reduction in monocytosis observed after long-term F1 treatment.

**F1 Treatment Slows Down the Development of Advanced Atherosclerotic Lesions**

Because F1 displayed strong anti-adhesion and anti-survival properties, we thought that it could represent an interesting tool for limiting the development of more advanced atherogenic lesions. To address this question, F1 treatment of Apoe−/− mice was delayed by 3 weeks (from 5 to 8 weeks) after HFD and the progression of the atherosclerotic was compared between 8 and 16 weeks of HFD (Figure 6A). ORO staining showed a significant increase in the lesion volume of the 16-week HFD...
Apoe\(^{-/-}\) mice (0.24±0.03 mm\(^3\)) compared with the 8-week HFD Apoe\(^{-/-}\) mice (0.12±0.03×10\(^3\) mm\(^3\)). In the F1-treated group, the lesion volumes did not significantly progress between 8 and 16 weeks (0.19±0.03 mm\(^3\)) and were reduced by \(\approx 20\%\) compared with the 16-week HFD saline-treated mice. As expected, F1 treatment reduced by 40% the absolute number of 7/4\(^{hi}\) monocytes in Apoe\(^{-/-}\) mice (Figure 6B; 17.8±4.6×10\(^3\)/mL versus 10.6±2.3×10\(^3\)/mL for saline- and F1-treated mice, respectively) with no effect on the 7/4\(^{lo}\) monocytes (\(P=\text{ns}\); 43.0±6.6×10\(^3\)/mL versus 43.6±12.3×10\(^3\)/mL monocytes for saline- and F1-treated mice, respectively). We conclude that F1 acts as a potent inhibitor of atherosclerosis progression probably by controlling monocytosis.

**Discussion**

Here, we have shown for the first time that pharmacological inhibition of CX3CR1 in murine models of atherogenesis led to reduced atherosclerotic lesion development and attenuated monocytosis. The antiatherogenic potency of CX3CR1 blockade was associated with reduced CX3CR1-dependent adhesion and survival of inflammatory monocytes. This work reveals that controlling monocytosis by blocking CX3CR1 may help prevent atherogenesis.

Although convincing evidence has validated the key role of chemokine receptors in promoting atherosclerosis, very few studies have used chemokine receptor antagonists to characterize their proatherogenic functions. Indeed, their role has essentially been inferred from studies using genetically deficient mice with chemokine or chemokine receptor gene deletion.\(^{31,32}\) The most studied chemokines regarding atherosclerosis are those controlling monocyte redistribution, as the prevailing model for lesion development proposed that local production of inflammatory chemokines promotes recruitment of monocytes into the intima and their differentiation into macrophages. For these reasons, mice deficient for genes encoding CCR2, CCR5, and CX3CR1 have been instrumental and showed that individual deletion of these genes resulted in a decrease in atherosclerotic lesions.\(^{17,20,21,33}\) However, none of these genetic deficiencies resulted in the abrogation of atherosclerotic lesions or of macrophage accumulation indicating that a complex network of recruitment signals is at work in atherogenesis. Indeed, multiple chemokine receptors acting in concert to orchestrate the migration of monocyte subpopulations were identified\(^{14,22,23}\) and showed that CCR2, CX3CR1, and CCR5 play independent and additive roles in atherogenesis. Accordingly, antagonists blocking CCR2\(^{25}\) and CCR5\(^{26,27}\)
only partially prevented progression of atherosclerotic lesions and macrophage accumulation. To date, no study has assessed CX3CR1 blockade in atherogenesis models.

The mechanistic role of CX3CR1 in atherosclerotic lesion progression remains highly debated and data argue for its involvement in monocyte recruitment,34 platelet–monocyte complex formation,35 dendritic cell accumulation,36 smooth muscle cell migration,37 and monocyte survival.30 We assumed that because the sole ligand of CX3CR1 is a membrane-tethered chemokine, it might have a crucial role in capturing monocytes from the blood. Indeed, monocytes exposed in vivo or in vitro to F1 have much less propensity to adhere to CX3CL1-expressing surfaces, therefore, reducing their ability to migrate into the lesions. It remained to elucidate why only adhesion of GFPlow bone marrow cells was inhibited after mice treatment and not that of GFPhigh bone marrow cells. Cellular distribution of these 2 monocyte subtypes may differ allowing selective antagonist accessibility.

Because F1 treatment reduced monocyte adhesion, preventing a portion of monocytes from infiltrating the atherosclerotic lesion, we were expecting that long-term treatment of mice would lead to increased monocytosis. In contrast, we observed that long-term F1 treatment led to reduced monocytosis, specifically affecting inflammatory monocytes. We postulated that F1 might reduce monocyte survival by blocking the antiapoptotic effect of CX3CL1, as previously reported.30 Our data indicate that only inflammatory monocytes exposed in vivo or in vitro to F1 have impaired survival capacity. In addition, Apoe−/− and Ldlr−/− long-term F1 treatment resulted in a 30% reduction in inflammatory monocyte numbers, while having no effect on resident monocytes. These results seemed to contradict those obtained in Cx3cr1−/− mice, where a 3-fold reduction in resident monocyte numbers was observed (as compared with wild type) with no alteration in the number of inflammatory monocytes.30 Such a difference in phenotype, which arises from the comparison of genetic deletion to pharmacological inhibition, is not unique and may exist for several reasons. A common cause of discrepancies is compensatory mechanisms that take place in gene-deleted mice during development. Another issue in this work is the potential existence of other sites of action of the antagonist that we have not as yet been able to identify. Future studies looking notably at the bioavailability of F1 need to be performed.

The effects of F1 treatment solely on inflammatory monocytes and not on resident monocytes proved particularly intriguing because the former express lower levels of CX3CR1 than the latter. It was expected that monocytes exhibiting strong expression of CX3CR1, the so-called resident monocytes, would be more sensitive to F1. However, previous work from Tacke et al14 has shown that (1) blood monocytosis was skewed toward an increase in inflammatory monocytes in
Apoe<sup>−/−</sup> mice fed on HFD, (2) inflammatory monocytes accumulated in atherosclerotic lesions, whereas resident monocytes entered less frequently, and (3) inflammatory monocytes unexpectedly required CX3CR1 in addition to CCR2 and CCR5 to accumulate within plaques. This study proposes CX3CR1 blockade as an effective treatment, aiming to limit recruitment of inflammatory monocytes while keeping intact CCR2-dependent functions. Our data are in perfect agreement with this study but the reasons for this skewing still remain elusive.

In conclusion, we show that F1 represents a potent inhibitor of the CX3CR1 axis and a promising compound for the prevention of atherogenesis. F1 was able to not only limit atherosclerosis progression but also prevent the development of more advanced lesions. These results suggest that agents blocking the CX3CL1-CX3CR1 interaction might help to prevent the onset and progression of atherosclerotic lesions. By limiting monocytosis, CX3CR1 inhibition may, therefore, be an alternative or complementary therapy to the more widely used lipid-lowering drugs in the treatment of atherosclerosis.

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**Disclosures**

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Supplementary Methods

Analysis of atherosclerotic lesion stage.

On 10µm thin frozen sections, the collagen composition was analyzed following Sirius Red staining (Sigma–Aldrich, Lyon, France) and blinded analysis. The terminal dUTP nick end-labeling (TUNEL) staining was performed using In Situ Cell Death detection Kit TMR Red and following manufacturer’s instructions (Roche, Rosny sous Bois, France). The specificity of antibodies was tested with their isotype controls.

Plasma lipid and lipoproteins analyses.

Blood samples were collected and analyzed as previously described. Briefly, plasma total cholesterol, free cholesterol and triglyceride concentrations were measured by enzymatic colorimetric assays respectively from Roche Diagnostics (Saint-Egrève, France), Wako Chemicals GmbH (Neuss, Germany) and Biomérieux (Marcy l’Etoile, France) using the automatic system Konelab (Thermo Scientific, Brebieres, France). Plasma lipoproteins were fractioned by gel filtration on two Superose 6 (Amersham Biosciences, Glattbrugg, Switzerland) columns connected in series using a BioLogic DuoFlow Chromatography System (BioRad, Marnes-la-Coquette, France).
Supplementary Figure I: F1 did not alter atherosclerotic lesion stage. Frozen sections from aortic sinus were stained for collagen content and apoptosis. Representative photomicrographs of Sirius red (a and b) and apoptosis (d and e) staining in saline- (a and d) and F1-treated (b and e) mice, respectively. Graphs in c and d represent the qualitative analysis of the Sirius Red staining (c) and the quantitative analysis of the TUNEL staining expressed as percent of nuclei TUNEL+/total nuclei (f). Bars represent mean±sem of 8 to 10 mice in each group. “ns” for not significant. Scale bar=300µm.
Supplementary figure II: Levels of plasma lipids and cholesterol, and lipoprotein cholesterol profiles.
Levels of plasma lipids and cholesterol (a and b), and lipoprotein cholesterol profiles (c and d) from Apoe-/- (a and c) and Ldlr-/- (b and d) mice fed on high-fat diet and treated or not with F1. Plasma lipids were total cholesterol (TC), free cholesterol (FC) and triglycerides (TG). Data averaged from 2 independent experiments for each genotype, each composed of pooled plasma from 4-6 mice per experiment are shown. Approximate elution positions of human VLDL, LDL and HDL are indicated.
Materials and Methods

Pharmacological inhibition of the chemokine receptor CX3CR1 reduces atherosclerosis in mice

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**Mouse model of atherosclerosis**

*Apoe*-deficient (*Apoe*^/-^) mice (B6-*Apoe*^tm1Unc/Crl from Charles River, Belgium) and *Ldlr*-deficient (*Ldlr*^/-^) mice (B6-*Ldlr*^tm1Her/J from Charles River, Wilmington, MA, USA), were maintained under pathogen-free conditions at the « Centre d’Exploration Fonctionnelle » animal facility (Pitié-Salpêtrière, Paris, France). At 5 weeks of age, mice were fed on high fat diet (HFD) containing 24% fat (D12451, Research Diets by Broogarden, Denmark) for the time indicated. The *Cx3cr1*^gfp/gfp* Apoe*^/-^ mice were generated by crossing *Cx3cr1*^gfp/+^ mice (B6.129P-*Cx3cr1*^tm1Litt/J from Charles River, Wilmington, MA, USA) with *Apoe*^/-^ mice. Treatment with CX3CR1 antagonist called F1 (produced as previously described \(^1\) and kindly provided by A. Proudfoot, Merck-Serono) was performed by intraperitoneal injection (50µg in phosphate buffered saline) three times a week for 10 weeks starting at 5 weeks of diet. F1 was labeled using Alexa Fluor 647 carboxylic acid, succinimidyl ester dye (Life Technology, Saint Aubin, France). The amount of F1 injected was based on previous experiments using altered chemokines \(^2\). For the advanced atherosclerotic lesion model, the mice were maintained for a longer period (8 weeks) on HFD before the beginning of the F1 treatment. Animal experiments were approved by the local institutional animal care and use committee of the “faculté de Médecine Pitié-Salpêtrière” (Paris).

**Analysis of atherosclerotic lesions**

Mice were euthanized at 20 weeks of age. Atherosclerotic lesions were quantified using oil red O (ORO) staining on 25 sections, 10µm thick, cut through the proximal aorta as previously described \(^3\). Briefly, the heart was taken out, fixed in 10% formalin for 2 hours, and placed in 10% sucrose-PBS overnight at 4°C before being embedded in Tissue–Tek OCT compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and frozen at -80°C. The extent of atherosclerotic lesions was delimited manually following ORO staining and their
surface was calculated using ImageJ software (NIH, Bethesda, MD, USA). The volume of the lesion was calculated on the length of the vessel displaying detectable ORO staining typically ranging from 400 to 600 µm. The ascending aorta and the aortic arch before the bifurcation of carotid artery were collected, quantified after en face mounting and differential interference contrast (DIC) microscopy to enhance the contrast between atherosclerotic lesions and healthy tissues. Plaque composition was determined using immunohistochemistry with monoclonal rat anti-mouse macrophage antibody (clone MOMA-2 MAB1852 Chemicon, AbCys, Paris, France) and with polyclonal rabbit anti-rat CX3CR1 (TP501, Torrey Pines Biolabs, East Orange, NJ, USA). The specificity of antibodies was tested with their isotype controls.

**Mouse organ cell isolation**

Blood was drawn via retroorbital puncture with heparin as anticoagulant. Bone marrow cells were collected by flushing thighbone and shinbone with PBS. Splenocytes were obtained by mashing the spleen on a 70µm pored cell strainer (Becton Dickison, Rungis, France). Following tissue homogenization, erythrocytes were lysed using buffer containing 0.15 M NH4Cl, 0.01 mM KHCO3 and 0.1 mM EDTA and resuspended in PBS complemented with 0.5% foetal calf serum (FCS).

**Cell Adhesion Assays**

Full length CX3CL1-His at 25 nM from R&D Systems (Lille, France) was adsorbed overnight to flat bottom 96 well microtiter plates (Nunc A/S, Roskilde, Denmark) at 4°C in 50 µL of 25 mM Tris pH=8, 150 mM NaCl. Wells were saturated for 2 h at room temperature with 1% non-fat milk in the same buffer. Bone marrow cells from Cx3cr1^{gfp/+} mice or Csf1r-Gal4VP16/UAS-ecfp (MacBlue) mice, a generous gift from D. Hume (Roslin Institute, Scotland, UK) were resuspended in calcium- and magnesium-free PBS and 5x10^5 total cells were added per well and incubated at room temperature. Before adhesion, cells from
**Apoptosis Assay**

For *in vitro* assays, mononuclear cells from the bone marrow of C57bl/6 mice were incubated for 4 hours in supplemented or FCS-free RPMI in the presence or absence of F1. In addition, *ApoE−/−* mice were treated with F1 or left untreated for 24 hours. Myeloid cells were isolated from the blood and the bone marrow and incubated for 4 hr in RPMI supplemented with 100 U/mL Penicillin, 2 mM Streptomycin, 2 mM L-glutamine, 10% FCS, 20 ng/mL GM-CSF, 50µM 2β-Mercaptoethanol. Propidium iodide (5µg/mL) was added in each sample 30 minutes before sample acquisition to identify dead cells.

**Flow cytometry**

Cells were incubated in 0.5% FCS-PBS in the presence of appropriate dilution of specific antibodies. The following panel of antibodies was used: anti- CD11b (clone M1/70), anti-Ly-6G (clone 1A8), anti-NK1.1 (clone PK136) from Becton Dickinson (Franklin lakes, NJ, USA) and anti-neutrophil (clone 7/4) from Serotec (Düsseldorf, Germany). Samples were acquired on a FACSCalibur cytometer (Becton Dickison, Franklin lakes, NJ, USA) using Cell Quest Pro and analyzed using FlowJo (Tree Star, Ashland, OR, USA) software. Calculation of absolute number was performed by using a fixed number of non-fluorescent 10µm polybead®carboxylate microspheres (Polysciences, Niles, IL, USA) as a reference.
Statistical Analysis

Graph Pad Prism 5 (GraphPad Software, San Diego, USA) was used for data analysis and graphic representation. Data are presented as the mean±sem (standard error of the mean) of each group of mice. Gaussian distribution was tested using Kolmogorov-Smirnov test. Statistical significance was determined using unpaired Student’s t tests to compare Gaussian-like distributions for flow cytometry analysis and immunohistological analysis. One-way analysis of variance with Dunnett’s multiple comparison test was performed to compare multiple conditions. Symbols used were * for p values <0.05, ** for p values <0.01, *** for p values <0.001 and ns when not significant.

References.