Efficacy of the CCR5 Antagonist Maraviroc in Reducing Early, Ritonavir-Induced Atherogenesis and Advanced Plaque Progression in Mice

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Background—CCR5 plays an important role in atherosclerosis and ischemic cardiovascular diseases, as well as in HIV replication and diffusion. HIV infection is characterized by a high burden of cardiovascular diseases, particularly in subjects exposed to ritonavir-boosted protease inhibitors. Maraviroc, a CCR5 antagonist antiretroviral drug, might provide benefit for patients with M-tropic HIV infections at high risk for cardiovascular diseases.

Methods and Results—Exposure to maraviroc limits the evolution and associated systemic inflammation of ritonavir-induced atherosclerotic in ApoE−/− mice and inhibits plaques development in a late model of atherosclerosis in which dyslipidemia plays the main pathogenic role. In ritonavir-treated mice, maraviroc reduced plaque areas and macrophage infiltration; downregulated the local expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and interleukin-17A; and reduced tumor necrosis factor-α and RANTES (regulated on activation, normal T cell expressed, and secreted). Moreover, maraviroc counterregulated ritonavir-induced lipoatrophy and interleukin-6 gene expression in epididymal fat, along with the splenic proinflammatory profile and expression of CD36 on blood monocytes. In the late model, maraviroc inhibited atherosclerotic progression by reducing macrophage infiltration and lowering the expression of adhesion molecules and RANTES inside the plaques. However, limited systemic inflammation was observed.

Conclusions—In a mouse model of genetic dyslipidemia, maraviroc reduced the atherosclerotic progression by interfering with inflammatory cell recruitment into plaques. Moreover, in mice characterized by a general ritonavir-induced inflammation, maraviroc reversed the proinflammatory profile. Therefore, maraviroc could benefit HIV-positive patients with residual chronic inflammation who are at a high risk of acute coronary disease despite a suppressive antiretroviral therapy. To determine these benefits, large clinical studies are needed. (Circulation. 2013;127:2114-2124.)

Key Words: acquired immunodeficiency syndrome ■ atherosclerosis ■ inflammation ■ maraviroc ■ receptors, CCR5

Maraviroc for both treatment-experienced and treatment-naïve HIV-infected subjects.

Clinical Perspective on p 2124

Nevertheless, over the last decade, the involvement of CCR5 in the development of atherosclerosis and coronary artery disease has been recognized. Some investigations into CCR5 as a target in atherosclerosis in dyslipidemic mouse models have been performed, demonstrating its capacity to reduce both the atherosclerotic burden and the systemic secretion of proinflammatory Th1 cytokines. Of relevance, the above-mentioned Δ32 deletion in the CCR5 gene, besides being a resistant factor to HIV-1 infection, is associated with a reduced risk for severe coronary artery disease and myocardial infarction in the general population, as well as a...
lower risk for cardiovascular disease and mortality in patients with renal failure and rheumatoid arthritis.15,16

The interaction of CCL5/RANTES with CCR5 plays a pivotal role in the development and progression of atherosclerotic inflammatory disease and influences the evolution of HIV infection. RANTES expression has been detected on atherosclerotic plaques, myofibroblasts, and endothelial cells17,18 and is mostly upregulated in late stages of atherosclerosis in both murine19 and human plaques.17 Moreover, CCL5/RANTES is released by stimulated degranulating platelets so that it can trigger accumulation of shear-resistant monocyte on atherosclerotic, inflamed endothelium.20 Furthermore, its expression and levels seem to be correlated with accelerated, high-risk atherosclerosis.21 The presence of CCL5/RANTES in plaques has been linked to an unstable phenotype.22 Therefore, CCR5, through its ability to link either the RANTES chemokine or the macrophage tropic HIV-1 gp120, has a pathogenic role in fostering atherosclerosis and ischemic cardiovascular diseases, as well as in the progression of HIV infection, that is, a clinical setting in which there is an increased burden of cardiovascular disease as a result of several factors, including systemic inflammation or toxicity23–25 resulting from exposure to ritonavir-boosted protease inhibitors.26

We have previously shown that ritonavir induces inflammation in adipose tissue, increasing the levels of proinflammatory cytokines,27 and accelerates the extension of atherosclerotic aortic plaques and sterol regulatory element binding protein (SREBP) 1c–mediated CD36 overexpression on circulating adipocytes,28,29 as well as in the progression of HIV infection, that is, a clinical setting in which there is an increased burden of cardiovascular disease as a result of several factors, including systemic inflammation or toxicity23–25 resulting from exposure to ritonavir-boosted protease inhibitors.26

Animals and Treatments
Male ApoE−/− mice on a C57BL/6j background from Harlan Nossan (Udine) and supplied by the animal center of the University of Perugia were housed under pathogen-free conditions in controlled temperatures (22°C) and photoperiods (12:12-hour light/dark cycle) and allowed ad libitum access to standard mouse chow and water. Protocols were approved by the University of Perugia Animal Care Committee according to the Italian guideline for care and use of laboratory animals. The identification for this project is 98/2010-B. The authorization was released to a principal investigator (S.F.).

Immunohistochemistry
By using a murine model of genetic dyslipidemia (ApoE−/− mice), we investigated whether maraviroc counteracted the early, ritonavir-induced progression of atherosclerosis in young mice and interfered with spontaneous plaque progression resulting from the underlying dyslipidemia in old mice.

Early, Ritonavir-Induced Atherosclerosis
Littermate 8-week-old male Apo E−/− mice were randomized into 3 different groups: group 1 (n=8), vehicle (saline); group 2 (n=10), intraperitoneal administration of ritonavir (5 mg/kg) alone; and group 3 (n=10), ritonavir 5 mg/kg in combination with maraviroc (50 mg/kg by gavage). Treatment was administered daily for 13 weeks. At the end of the experiment, animals received the last administration and 2 hours later were euthanized with a lethal dose of pentothal. Blood, aortas, spleens, and epididymal fat were collected. Blood was collected for flow cytometry and biochemical analyses. Aortas were snap-frozen for protein isolation and Bio-Plex, histopathology, and immunohistochemistry analyses. Spleens were removed for lymphocyte isolation. Epididymal fat was removed, weighed, frozen, and collected for real-time polymerase chain reaction (PCR). The epididymal fat ratio was calculated from the following formula: epididymal ratio=(epididymal weight/body weight)×100.

Late, Spontaneous, Dyslipidemic Atherosclerosis
Littermate 39-week-old male Apo E−/− mice were randomized into 2 different groups: group 1 (n=13), vehicle (saline); and group 2 (n=13), maraviroc (50 mg/kg by gavage). Treatment was administered daily for 13 weeks. At the end of the experiment, animals received the last administration and were euthanized 2 hours later with a lethal dose of pentothal. Blood, aortas, and spleens were collected and analyzed. Blood was used for subsequent biochemical analysis and flow cytometry. Aortas were frozen for protein isolation and paraffin embedded for immunohistochemistry or were removed for en face atherosclerotic plaques staining. Spleens were removed for lymphocyte isolation.

Quantification of Atherosclerotic Plaques
The distal portion of the aortic sinus was recognized by the 3 valve cusps that are the junctions of the aorta to the heart. Coronal sections were evaluated for fatty streak lesions after staining with hematoxylin and eosin. The mean lesion area was quantified from 10 digitally captured sections per mice. Area measurements (expressed in pixels) were done with the free Image J 1.33a software (W. Rasband, National Institutes of Health, Bethesda, MD) by individuals who were blinded to treatment.

En Face Staining
Aortas (n=7–8 for group) were dissected for quantification of atherosclerotic plaques. Aortas were washed with PBS followed by fixative (4% formaldehyde, 5% sucrose, 20 mmol/L EDTA, pH 7.4). With the major branching vessels attached, aortas were opened longitudinally from the iliac arteries to the aortic root. Aortas (from the iliac bifurcation to a point equidistant between the aortic valve and the brachiocephalic artery) were removed and pinned flat onto a paraffin wax board. Sudan IV stain solution 0.5% (wt/vol) was dissolved in equal parts 70% ethanol and 100% acetone. The mounted aortas were stained in the following manner: The fixative was rinsed out for 3 minutes in PBS followed by 5 minutes in 70% ethanol and then stained in freshly filtered Sudan IV for 6 minutes with occasional agitation. The stained aortas were then differentiated in 80% ethanol for 3 minutes and finally washed in PBS for 3 minutes. Images of the aortas were captured, and plaque area measurements (expressed in percent of total area) were done with the free Image J software by individuals who were blinded to treatment.

Immunohistochemistry
The mouse aorta samples were removed, fixed in 10% buffered formalin phosphate, and embedded in paraffin, and sections (7-μm thickness) were processed for immunohistochemistry. Briefly, sections were deparaffinized and washed in PBS, soaked in 3% H2O2 for 8 minutes, and then incubated with 5% BSA in PBS with Triton X-100 (0.1%) for 30 minutes. Sections were then incubated with rat anti–MAC-3 (BD Pharmingen, Italy) or with rabbit anti-CCR5 (Novus Biological, UK) in PBS with 0.3% Triton X-100 and 1% BSA at room temperature for 1 hour. The sections were incubated with biotinylated anti-rat and anti-rabbit IgG 1:200 (Vector) and then processed by the avidin-biotin-peroxidase method with Vectastain ABC kit (Vector, UK) with diaminobenzidine as chromogen or by the avidin-biotin-alkaline phosphatase method with Vectastain Universal
ABC-AP KIT (Vector, UK) with Vector Blue Alkaline Phosphatase Substrate as chromogen (Vector, UK). The amount of macrophage infiltrate was evaluated with Image J software as the percentage of the stained area compared with the lesion area.

**Blood Biochemistry**

Serum content of total cholesterol, high-density lipoprotein, low-density lipoprotein, triacylglycerol, and aspartate aminotransferase was measured by enzymatic assays (Wako Chemicals, Osaka, Japan).

**Cytokine Analysis**

Frozen aortas were lysed for protein extraction and processed for cytokine determinations. Supernatants from lymphocyte culture were collected for interleukin (IL)-17A, interferon-γ, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) evaluations. The Bio-Plex mouse cytokine custom Plex panel was used with the Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA) to profile the expression of cytokines and chemokines in aortic arch homogenates (pg/500 µg protein) and to evaluate the secretion from spleen lymphocytes (pg/mL).

**Flow Cytometry Quantification of CD36 Expression on Monocytes**

Blood monocytes were collected from ApoE−/− mice. The cells were treated with ACK solution to eliminate red blood cells and stained by anti-CD11b-PE antibody (Milenyi Biotech; Germany) and Alexa Fluor488–anti-CD36 antibody or Alexa Fluor488–anti-CD195 (CCR5) antibody (Biolegend; San Diego, CA). The analysis was done by a flow cytometer (Beckman Coulter, Fullerton, CA).

**Quantitative Real-Time PCR**

Fat samples for RNA isolation were immediately snap-frozen in liquid nitrogen. Quantification of IL-6 gene expression was performed by quantitative real-time PCR. One microliter of the RNA was incubated with DNase I and reverse transcribed with Superscript II (Invitrogen, Italy) according to the manufacturer’s specifications. For real-time PCR, a 1-µL template was used in a 25-µL reaction containing a 0.2-µmol/L concentration of each primer and 12.5 µL of 2× SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA). PCR primers were designed with the PRIMER3-OUTPUT software using published sequence data obtained from the NCBI database: mouse IL6, ccgagagagagctcag and tcagcatttcccagac; and mouse GAPDH, ctgtagctgtgaggtcct and tgttgctggctcgtcatg.

**Isolation and Culture of Spleen Lymphocytes**

Mouse immune cells were obtained from mice spleens. Lymphocytes were isolated by CD5 microbeads according to the manufacturer’s instructions (Milenyi Biotech, Germany). After isolation, lymphocytes 2×10^6/mL were cultured in a 24-well plate for 36 hours alone or in combination with 2 µg/mL concanavalin A. At the end of the incubation, the supernatants were collected for cytokine determination by Bio-Plex.

**Monocyte–Endothelial Cell Adhesion Assay**

Human umbilical vein endothelial cells (HUVECs) obtained from Gibco (Invitrogen) were maintained in supplemented Medium 200 according to the manufacturer’s instructions at 37°C in a humidified atmosphere of 5% CO₂ in air. HUVECs at the fifth passage were plated onto 24-well plates, starved for 6 hours, and then treated with maraviroc (5 µmol/L) or vehicle. Two hours later, cells were exposed to 500 nmol/L or 1 µmol/L ritonavir or 10 ng/mL TNF-α. Cells were incubated for 16 hours. Two sets of experiments were made in triplicate.

U937 monocyte-like cells were maintained in RPMI supplemented with 10% FBS with 100 U/mL penicillin and 100 µg/mL streptomycin. U937 cells (2.5×10³/mL) were layered over a treated HUVEC monolayer and incubated for 2 hours. The cells were then washed with PBS and fixed in 4% paraformaldehyde in PBS. The adherent U937 cells were counted in 4 high-powered fields per well under a microscope and expressed as number of adherent U937 cells per high-powered field.

**Statistical Analysis**

Data are expressed as mean±SE. Statistical significance was determined for comparison of ≥2 groups by 1-way ANOVA followed by the Tukey test or Kruskal-Wallis test for the analysis of plaque areas. The Mann-Whitney test was used to compare 2 groups of data. A value of P<0.05 was considered significant. GraphPad Prism version 3.0 was used for graphics and statistical analyses (GraphPad Software, San Diego, CA).

**Results**

**Maraviroc Attenuates Atherosclerotic Plaque Progression in ApoE−/− Mice Exposed to Ritonavir**

Because we had previously shown that ritonavir accelerates the progression of atherosclerotic plaques in ApoE−/− mice, in the present study, we investigated whether treating ApoE−/− mice with maraviroc would protect against the progression of atherosclerosis caused by this HIV protease inhibitor. For this purpose, 8-week-old ApoE−/− mice were treated for 13 weeks with ritonavir alone or in combination with maraviroc, and the extent of aortic plaques was measured in hematoxylin and eosin–stained coronal sections of aortic arches. After a 13-week treatment with ritonavir, the plaque area increased by 2-fold compared with control mice. Administering maraviroc to mice effectively protected against aortic plaque progression (n=6–7 mice per group; P=0.05; Figure 1A–1D). Treating ApoE−/− mice with ritonavir caused a robust increase in macrophage accumulation in the aortic wall. Thus, the extent of aortic plaques that stained positively for MAC-3 increased from 10.4±5.3% to 48.5±9.5%. Cotreating mice with maraviroc effectively reduced this effect (from 48.5±9.5% to 21.3±2.9%; n=3–5 mice per group; P<0.05; Figure 1E–1H). Furthermore, although treating mice with ritonavir increased the staining for CCR5, this effect was reversed by maraviroc (Figure 1I–1K). Exposure of ApoE−/− mice to ritonavir increased total cholesterol, low-density lipoprotein, and triacylglycerol levels. This effect persisted even in maraviroc-cotreated mice (n=5 mice per group; P≤0.05; Figure 1L–1P).

**Maraviroc Counterregulation of Ritonavir-Induced Plaque Inflammation**

Because these data indicate that the beneficial effects exerted by maraviroc were dissociated from its effects on lipid metabolism, we then investigated whether CCR5 antagonism modulated inflammatory pathways in this model. As shown in Figure 2, although ritonavir boosted the aortic content of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), and IL-17A, cotreating mice with maraviroc reversed this pattern. Furthermore, maraviroc effectively reduced the aortic content of RANTES/CCL5 and TNF-α, that is, 2 mediators that were not affected significantly.
by exposure to ritonavir and reduced IL-6, although in a nonsignificant manner (n=6 mice per group; \( P \leq 0.05 \); Figure 2A–2H).

**Maraviroc Inhibits Monocyte Adhesion to Ritonavir- or TNF-\( \alpha \)-Activated Endothelial Cells**

To evaluate whether exposure of endothelial cells to ritonavir induced their monocyte interaction and to investigate the efficacy of maraviroc in counteracting this event, we exposed HUVECs untreated or pretreated with maraviroc to ritonavir using TNF-\( \alpha \) as a validated control. The exposure of endothelial cells to ritonavir increased the monocyte adhesion by \( \approx 8 \)-fold and to TNF-\( \alpha \) \( \approx 17 \)-fold. As shown in Figure 3A and 3B, this endothelial cell–monocyte interaction was robustly attenuated by maraviroc (>60% in all settings; \( P \leq 0.05 \)).

**Maraviroc Counterregulation of Ritonavir-Induced Systemic Inflammation**

As shown in Figure 4, exposure to ritonavir significantly increased the expression of CD36 and CCR5 on blood monocytes. The effect on CD36 was reversed by cotreating mice with maraviroc (n=5–6 mice per group; \( P \leq 0.05 \); Figure 4A and 4B). In contrast, both agents failed to modulate the levels of circulating C-reactive protein (n=5 mice per group; Figure 4C).

Activation of adipose tissue is a marker of systemic inflammation. Interestingly, we found that ritonavir induced lipolysis and increased IL-6 mRNA expression on epididymal fat. Cotreating mice with maraviroc counterregulated the lipatrophic effect of ritonavir and downregulated IL-6 mRNA expression (n=6–7 mice per group; \( P \leq 0.05 \); Figure 4D; and n=5 mice per group; \( P \leq 0.05 \); Figure 4E).

Given that systemic inflammation and immune activation are correlated with a higher risk of cardiovascular diseases, we then investigated whether ritonavir induced a proinflammatory profile on spleen-derived CD5\( ^+ \) lymphocytes and whether maraviroc reversed this effect. Specifically, cytokines from supernatants of lymphocytes obtained from mice spleen were evaluated by the Bio-Plex platform. No differences were observed in basal cytokine secretion. In contrast, concanavalin A stimulation of spleen-derived lymphocytes increased IL-17A secretion from ritonavir-treated mice. This effect was reversed.
by in vivo cotreatment with maraviroc. Moreover, maraviroc interfered with the concanavalin A stimulation, reversing TNF-α and interferon-γ secretion and increasing TGF-β production (n=6–7 mice per group; P≤0.05; Figure 5A–5D).

Maraviroc Inhibits the Development of Spontaneous Atherosclerosis in Old ApoE−/− Mice

Given the different pathogenic role for CCL5/CCR5 interaction and T-cell subsets in early and late atherosclerosis,30–32 we

Figure 2. Maraviroc counterregulation of ritonavir-induced plaque inflammation. Protein levels in adhesion molecules, cytokines, and chemokines were measured with the Bio-Plex platform. A through H, ICAM, VCAM, TNF-α, MCP-1, RANTES, IL-6, IL-17A, IFNγ levels. Data are expressed as mean±SE of 6 animals per group. **P<0.01, *P<0.05. ICAM indicates intercellular adhesion molecule; IFNγ, interferon-γ; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation, normal T cell expressed, and secreted; TNF α, tumor necrosis factor-α; and VCAM, vascular cell adhesion molecule.

Figure 3. Maraviroc inhibits U937 cell adhesion to ritonavir or tumor necrosis factor-α (TNF-α)-activated endothelial cells. A-B, Maraviroc inhibited U937 cell adhesion to ritonavir- (500 nmol/L or 1 μmol/L) or TNF-α (10 ng/mL)-activated human umbilical vein endothelial cells (HUVECs). HUVECs were preincubated with maraviroc (5 μmol/L) for 2 hours and then stimulated with ritonavir or TNF-α for 16 hours. U937 cells were layered over an HUVEC monolayer and incubated for 2 hours at 37°C. Adherent cells were counted under a light microscope. Data are mean±SE of results from 2 different independent experiments made in triplicate. **P<0.01, *P<0.05.
then investigated whether maraviroc attenuated the progression of atherosclerotic plaques in 39-week-old ApoE−/− mice in which dyslipidemia is the only pathogenic factor.

As illustrated in Figure 6A–6C, exposing these mice to maraviroc for 13 weeks attenuated the spontaneous progression of aortic plaques, as shown by en face staining of aortas isolated from 52-week-old mice from 16.6±3.3% to 7.1±1.4% (n=7–8 mice per group; \( P \leq 0.05 \)). It is noteworthy that the efficacy of maraviroc was in inhibiting this spontaneous progression because the plaque area extension was equal to that of 39-week-old mice (data not shown). Moreover, as shown in Figure 6D–6F, maraviroc reduced the extent of macrophage infiltration as assessed by measurement of the surface expression of MAC-3 (from 55.8±5.8% to 22.7±3.9%) and CCR5 staining (Figure 6G and 6H). As seen in the early model, treating ApoE−/− mice with maraviroc had no effect on lipid biochemistry (data not shown).

Maraviroc treatment effectively reduced the aortic content of ICAM-1, VCAM-1, IL-2, and RANTES/CCL5, and trends for a reduction in TNF-α and an increase in TGF-β were seen (n=4–5 mice per group; \( P \leq 0.05 \); Figure 7A–7I). Unlike the early model, no reductions were observed in the IL-17A and MCP-1 levels. Additionally, exposure to maraviroc effectively decreased CD36 and CCR5 expression on circulating monocytes (n=7–8 mice per group; \( P \leq 0.05 \); Figure 7J–7K). In contrast to the early model in which inflammation was boosted by ritonavir, treating ApoE−/− mice with maraviroc had no effect on cytokine release from spleen-derived lymphocytes stimulated by concanavalin A (Figure I in the online-only Data Supplement).

**Discussion**

It has been suggested recently that CCR5 plays an important role in atherosclerosis and ischemic cardiovascular diseases such as in HIV replication and diffusion; the availability of maraviroc, a small, potent CCR5 antagonist molecule, could provide an additional advantage to persons with a M-tropic HIV infection and an increased cardiovascular risk, which usually is associated with a high systemic proinflammatory profile.

We have previously shown that exposure of genetically dyslipidemic ApoE−/− mice to ritonavir induces inflammation in adipose tissue, characterized by an increase in proinflammatory mediators such as TNF-α and a reduction in the expression of counterregulatory nuclear receptors such as peroxisome proliferator–activated receptor-γ.\(^{27} \) This exposure accelerates atherosclerotic progression, as demonstrated by a robust increase in the extension of aortic plaques and a SREBP1c-mediated CD36 overexpression on circulating monocytes, with a consequential higher uptake of oxidized low-density lipoprotein.\(^ {28} \)

In the present study, we have extended this model, obtaining evidence that the CCR5 antagonist maraviroc protects against detrimental effects caused by ritonavir on plaque progression and systemic inflammation in ApoE−/− mice. Moreover, we have also observed in the late stage of this...
model, in which the dyslipidemia is the major driving factor, that maraviroc inhibits plaque evolution, interfering with the recruitment of inflammatory cells without modifying the metabolic profile.

Exposure to ritonavir worsened the proatherogenic lipid profile of ApoE−/−, accelerated the progression of atherosclerotic plaques by favoring extensive monocyte/macrophage infiltration in the aortic wall, and brought about a robust activation of those inflammatory mediators involved in monocytes and endothelial cells biology, including MCP-1, ICAM-1, and VCAM-1. Furthermore, the exposure of HUVECs to both ritonavir and TNF-α increased the ability of HUVECs to bind to human monocytes. A consequence of exposing ApoE−/− mice to ritonavir was the development of a low-grade inflammation, as illustrated by a trend toward an increase in C-reactive protein, which is a valid marker of systemic inflammation, and diffused activation of innate and adaptive immunity cells. Exposure to ritonavir increased the expression of CD36 and CCR5 on circulating monocytes and IL-17A release from spleen lymphocytes stimulated by concanavalin A and boosted the severe lipoatrophy of the epididymal fat with increased expression of IL-6. These results were consistent with several in vitro and ex vivo studies reporting direct toxicity of ritonavir on both endothelial cells and human adipocytes, ritonavir increased reactive oxygen species, MCP-1, and IL-6. Additionally, in virologically suppressed patients, Martinez et al37 observed that switching from ritonavir-boosted protease inhibitors to raltegravir ameliorated cardiovascular biomarkers (TNF-α, MCP-1, ICAM-1, VCAM-1) by a mechanism not completely explained by modification of the lipid pattern.

An important finding of our study was that cotreating ApoE−/− mice with maraviroc, a CCR5 antagonist, reduced the extent of plaque area by 70%. This clinical effect could not be explained by any change in the lipid profile because maraviroc failed to counteract the prodyslipidemic effects of ritonavir. Instead, the protection exerted by maraviroc was supported by a robust attenuation of monocyte/macrophage infiltration (∼50%) and CCR5 expression in the aortic plaques. The immune-regulatory activity of the CCR5 antagonist extended to the regulation of a variety of inflammatory biomarkers, including the expression of VCAM-1, ICAM-1, TNF-α, and RANTES. The activity of MVC was probably exerted by reducing not only the recruitment of inflammatory cells on aortic plaques but also the activation of endothelial cells. In fact, the pretreatment of HUVEC with MVC decreased either RTV or TNF-α observed bindings to human monocytes, suggesting a direct effect of maraviroc on endothelial cells.

Furthermore, exposure to maraviroc resulted in a robust downregulation of CD36 on blood monocytes not associated with reduced levels of low-density lipoproteins, which induce, via peroxisome proliferator–activated receptor-γ, the expression of this scavenger receptor.38
An important observation here was that exposure to maraviroc effectively counteracted systemic effects caused by ritonavir on epididymal fat and splenic lymphocytes. In fact, the CCR5 antagonist counteracted the epididymal lipoatrophy caused by this protease inhibitor and attenuated the expression of IL-6. Adipose tissue is a significant source of circulating IL-6, which promotes inflammation not only in adipose tissue but also in endothelial and liver cells.

This ritonavir-induced lipoatrophy in our experimental model cannot be compared with findings in the human setting. Indeed, exposure to protease inhibitors usually causes lipohypertrophy in HIV-infected people, even though ritonavir seems to exacerbate the lipoatrophic effects of reverse-transcriptase inhibitors. Nevertheless, our principal aim was to evaluate the inflammation of visceral fat and therefore the adipocytokine IL-6 gene expression. In fact, higher levels of IL-6 in HIV-infected patients with lipodystrophy, having both isolated lipoatrophy and mixed lipodystrophy, have already been reported, as well as an inverse correlation between flow-mediated dilatation and IL-6. It is widely held that flow-mediated dilatation is related to the prevalence and severity of coronary atherosclerosis and is a predictor of future cardiovascular events. Moreover, results from our investigation carried out on CD5+ cells, a mix of T and B lymphocytes isolated from the spleen and then stimulated with concanavalin A, demonstrate that in vivo treatment with maraviroc modulates a number of T-cell effector functions in an anti-inflammatory sense, decreasing IL-17A, TNF-α, and interferon-γ and inducing TGF-β. Indeed, ritonavir exposure increases the immune activation of lymphocytes, and maraviroc dampens their proinflammatory response, leading to a higher therapeutic potency that goes beyond blocking chemokine/receptor interaction. These findings are interesting because a persistent (low-grade) inflammatory state is postulated to contribute to the observed high rate of coronary artery disease in treated HIV patients and in other clinical settings, including systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel diseases. Maraviroc, the only CCR5 antagonist available in clinical practice, is a small molecule with a molecular weight of 513.67, whereas Met-RANTES is 7.8 kDa. This significant difference may contribute to different pharmacokinetic distribution profiles and different receptor affinities, leading to activation of different signal transduction pathways.

The antiatherogenic activity of maraviroc was confirmed in the intermediate to late stage of spontaneous atherosclerosis. In this model, we observed that treating old ApoE−/− mice with maraviroc inhibits the progression of spontaneous atherosclerosis. The molecular mechanism that supports this effect was associated with the ability of the CCR5 antagonist to modulate the expression/function of biomarkers of inflammation, including the expression of adhesion molecules and RANTES, whereas no effect was detected on the lipid profile of ApoE−/− mice.

The evolution of plaque was inhibited, whereas macrophage and CCR5 cell infiltration and the expression of ICAM-1, VCAM-1, RANTES, and IL-2 were reduced. The other proinflammatory cytokine secretions were not downregulated. Indeed, only trends toward a lower TNF-α level and a higher level of the regulatory cytokine TGF-β expression were seen. It is noteworthy, however, that significant downregulation of CD36 and CCR5 expression on blood monocytes was seen. Still, in spleen lymphocytes, only a mild counterregulation

![Figure 6. Maraviroc inhibits spontaneous atherosclerosis progression.](http://circ.ahajournals.org/)}
of systemic inflammation was observed, which involved, in a nonsignificant manner, IL-17A and TGF-β. No downregulation of interferon-γ and TNF-α was observed.

This experimental study has limitations linked to the number of animals used. However, we assessed a number of different biochemical and clinical parameters, and the coherence of the experimental results supports a robust anti-inflammatory effect for maraviroc in this model.

Conclusions
Maraviroc reduced atherosclerotic progression in our murine models by interfering with the recruitment of inflammatory cells. Moreover, in the ritonavir-induced inflammatory model, we found that maraviroc modulated the inflammatory plaque profile and systemic inflammation. These results need to be confirmed in clinical trials in which the role of maraviroc in protecting HIV-positive patients at higher risk of acute coronary disease against coronary artery disease is investigated.

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Disclosures
None.

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CLINICAL PERSPECTIVE
Maraviroc is a small-molecule CCR5 antagonist, the only one available in clinical practice for treating R5 tropic HIV infection, and it is known to be associated with mild side effects. Our studies found that, in a murine model, maraviroc reduced atherosclerotic progression by modulating not only inflammatory cell plaque recruitment but also systemic inflammation. Reports from clinical studies in HIV-infected patients indicate that maraviroc possesses anti-inflammatory properties, and in a recent in vitro study on human adipocytes, it caused a significant decrease in the gene expression and release of interleukin-6, the proinflammatory cytokine that is known to be associated with the severity of coronary artery disease. The efficacy of maraviroc has been reported in other non-HIV clinical settings: graft versus host disease and concomitant inflammatory pathologies. Persistent low-grade chronic inflammation is associated with early aging and the development of cancer and ischemic cardiovascular diseases. Therefore, the antiatherosclerotic efficacy of maraviroc should be evaluated in the HIV clinical setting in patients with a high inflammatory profile and elevated risk of coronary artery disease, as well as in other chronic inflammatory diseases associated with a high risk of coronary artery disease.
Efficacy of the CCR5 Antagonist Maraviroc in Reducing Early, Ritonavir-Induced Atherogenesis and Advanced Plaque Progression in Mice
Sabrina Cipriani, Daniela Francisci, Andrea Mencarelli, Barbara Renga, Elisabetta Schiaroli, Claudio D’Amore, Franco Baldelli and Stefano Fiorucci

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Supplementary Figure 1
Supplementary Figure 1: Cytokine Secretion from Splenic Lymphocytes

Cytokines and chemokines from supernatants of spleen lymphocytes, with and without Concanavallin A (ConA) stimulation, were evaluated by Bioplex. No difference was observed comparing the various experimental groups. Panel A-D, n=7 - 8 mice per group. Data are mean ± SE.