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

Running title: Cipriani et al.; Maraviroc Prevents the RTV-induced Atherosclerosis

Sabrina Cipriani, PhD1; Daniela Francisci, MD1; Andrea Mencarelli, PhD2; Barbara Renga, PhD2; Elisabetta Schiaroli, MD1; Claudio D’Amore, PhD2; Franco Baldelli, MD1*; Stefano Fiorucci, MD2*

1Dipartimento di Medicina Sperimentale e Scienze Biochimiche; 2Dipartimento di Medicina Clinica e Sperimentale, University of Perugia, Perugia, Italy

*Contributed equally to this work

Address for Correspondence:
Franco Baldelli, MD
Clinica Malattie Infettive
Ospedale S. Maria della Misericordia
Piazzale Menghini 1
06129 Perugia, Italy
Tel: +39 075 578 4374
Fax: +39 075 578 4346
E-mail: franco.baldelli@unipg.it

Abstract:

**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 M-tropic HIV infections patients at high risk for cardiovascular diseases.

**Methods and Results**—Exposure to maraviroc limits the evolution and the associated systemic inflammation of ritonavir-induced atherosclerotic in an ApoE<sup>−/−</sup> 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 the plaque areas and macrophage infiltration, down-regulated the local expression of VCAM-1, ICAM-1, MCP-1 and IL-17A and reduced TNF-α, RANTES. Moreover, maraviroc counter-regulated the ritonavir-induced lipatrophy and IL-6 gene expression in epididymal fat, along with the splenic pro-inflammatory profile and expression of CD36 in 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. Yet, 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 pro-inflammatory profile. Being so, 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. In order to determine these benefits, large clinical studies are needed.

**Key words**: AIDS, APOE, atherosclerosis, inflammation, chemoreceptor
CCR5, a G-protein coupled receptor, first identified as the receptor for CCL3 / MIP-1α, CCL4 / MIP-1β and CCL5/RANTES, regulates the migrations of monocytes, NK cells and Th1 cells into the inflammation sites and is involved in the progression of atherosclerosis\(^1,2\). This chemokine receptor is expressed on monocyte/macrophage and T cells\(^3\), on both endothelial and vascular smooth muscle cells\(^4,5\) and is best known for its role as co-receptor for macrophage tropic (R5) HIV-1\(^6,7\). A polymorphism in CCR5, a 32-nucleotide deletion known as CCR5 delta32, has been described in subjects who remain uninfected, despite extensive exposure to HIV-1\(^8,9\). This evidence has led to the successful search for CCR5 antagonists as antiretroviral drugs and the approval of maraviroc for both treatment-experienced and treatment-naïve HIV infected subjects.

Nevertheless, over the last decade, the involvement of CCR5 in the development of atherosclerosis and coronary artery disease (CAD) has been recognized. Some investigations on CCR5, as a target in atherosclerosis in dyslipidemic mouse models, have been performed, demonstrating its capacity to reduce both the atherosclerotic burden\(^10,12\) and systemic secretion of pro-inflammatory Th1- cytokines\(^11\). Of relevance, the above mentioned delta 32 deletion in the CCR5 gene, besides being a resistant factor to HIV-1 infection, associates with a reduced risk for severe CAD\(^13\) and myocardial infarction in the general population\(^14\), as well as a lower cardiovascular disease risk 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 the atherosclerotic, inflammatory, disease and influences the evolution of HIV infection. RANTES expression has been detected on atherosclerotic plaques, on myofibroblasts and endothelial cells\(^17,18\) and is mostly up-regulated in late stages of
atherosclerosis, both in murine and in humans plaques. Moreover, CCL5/RANTES is released by stimulated, degranulating, platelets so that it can trigger accumulation of shear-resistant monocyte on atherosclerotic, inflamed endothelium. Further, its expression and levels seem to be correlated with accelerated, high risk atherosclerosis. The presence of CCL5/RANTES in has been linked to an unstable phenotype. Therefore, CCR5, through its ability to link either RANTES chemokine or the macrophage tropic HIV-1 gp120, has a pathogenetic role in fostering atherosclerosis and ischemic cardiovascular diseases, as well as the progression of HIV infection, i.e a clinical setting where there is an increased burden of cardiovascular disease due to several factors including systemic inflammation and/or toxicity due to exposure to ritonavir (RTV)-boosted protease inhibitors.

We have previously shown that RTV induces inflammation in adipose tissue, increasing the levels of pro-inflammatory cytokines and accelerates the extension of atherosclerotic aortic plaques as well as a Sterol Regulatory Element binding protein (SREBP)1c-mediated CD36 over-expression on circulating monocytes. Therefore, in an early atherogenic model we sought to verify the extension of the RTV-induced associated pro-atherogenic and pro-inflammatory profile and the efficacy of maraviroc, the only validated CCR5 antagonist available as antiretroviral drug, in reducing the atherosclerotic plaque evolution, as well as the local and systemic inflammation. Moreover, because investigations on the role of maraviroc on atherosclerosis might have a broad interest, we also investigated whether the CCR5-antagonist attenuated plaques development in a late phase of spontaneous atherogenesis, where dyslipidemia plays a major pathogenic role.

Methods

Animals and Treatments
Male ApoE−/− on a C57BL6/J background were from Harlan Nossan (Udine) and supplied by the animal centre of the University of Perugia, housed under pathogen-free conditions, controlled temperatures (22°C) and photoperiods (12:12-hour light/dark cycle), allowed ad libitum access to standard mouse chow and water. Protocols had been approved by the University of Perugia Animal Care Committee according to the Italian guideline for care and use of laboratory animals. The ID for this project is #98/2010-B. The authorization had been released to Prof. Stefano Fiorucci, as a principal investigator.

By using a murine model of genetic dyslipidemia (ApoE−/− mice) we have investigated whether: a) maraviroc counteracted the early, RTV-induced, progression to atherosclerosis in young mice, and, b) interfered with the spontaneous plaque progression due to the underlying dyslipidemia in old mice.

**Early, RTV-induced, Atherosclerosis**

Littermate 8-week-old male Apo E−/− mice were randomized into three different groups: group 1 (n=8), vehicle (saline); group 2 (n=10), intraperitoneally administration of RTV (5 mg/kg) alone; and group 3 (n=10), received RTV 5mg/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 latest administration and 2 h later were sacrificed with a lethal dose of pentothal. Blood, aortas, spleens and epididymal fat were collected. Blood was collected for flow cytometry and biochemicals analyses. Aortas were snap frozen for protein isolation, Bioplex analysis and histopathology and immunohistochemistry analyses. Spleens were removed for lymphocyte isolation. Epidydimal fat was removed and weighted, then frozen and collected for rt-PCR. The epidydimal fat ratio was calculated using the following formula: epidydimal ratio = (epidydimal weight/body weight) x 100.
Late, Spontaneous, Dyslipidemic Atherosclerosis

Littermate 39-week-old male Apo E\(^{-/-}\) mice were randomized into two different groups: group 1 (n=13), vehicle (saline); group 2 (n=13), maraviroc (50 mg/kg by gavage). Treatment was daily administered during 13 weeks. At the end of the experiment, animals received the latest administration and 2 h later were sacrificed with a lethal dose of pentothal. Blood, aortas, and spleens were collected and analyzed. Blood was utilized for subsequent biochemicals 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 three valve cusps which are the junctions of the aorta to the heart. Coronal sections were evaluated for fatty streak lesions after staining with hematoxylin and eosin (H&E). The mean lesion area was quantified from ten digitally captured sections per mice. Area measurements (expressed in pixels) were done using the free software Image J 1.33u (Rasband W., National Institutes of Health; Bethesda, MD) by individuals who were blind to treatment.

En-face Staining

Aortas (n=7-8 for group) were dissected for quantification of atherosclerotic plaques. Aortas were washed with phosphate-buffered saline followed by fixative (4% formaldehyde, 5% sucrose, 20 mM 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 min in PBS followed by 5 min in 70% ethanol and then stained in freshly filtered Sudan IV for 6 min with occasional agitation. The stained aortas were then differentiated in 80% ethanol for 3 min and finally washed in PBS for 3 min. Images of the aortas were captured, and plaque area measurements (expressed in % of total area) were done using the free software Image J by individuals who were blind to treatment.

**Immunohistochemistry**

The mouse aorta samples were removed and fixed in 10% buffered formalin phosphate, 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 min, and then incubated with 5% bovine serum albumin in PBS with Triton X-100 (0,1%) for 30 min. 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% bovine serum albumin, at RT for 1 h. The sections were incubated with biotinylated anti-rat and anti rabbit IgG 1:200 (Vector) and then processed or 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 using ImageJ software as the percentage of the stained area compared to lesion area.

**Blood Biochemistry**

Serum content of total cholesterol, high density lipoprotein, low density lipoprotein, triacylglycerol and aspartate aminotransferase were 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 IL-17A, IFN-γ, TNF-α and 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 expression of cytokines and chemokines in aortic arch homogenates (pg/500μg 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, stained by anti-CD11b-PE antibody (Milteny Biotech; Germany) and Alexa Fluor488-anti-CD36 antibody or Alexa Fluor488-anti-CD195 (CCR5) antibody (Biolegend, San Diego). The analysis was made by a flow cytometer (Beckman Coulter, Fullerton, CA).

Quantitative Real-time PCR

Fat samples for RNA isolation were immediately snap frozen in liquid nitrogen. Quantization of IL-6 gene expression was performed by quantitative real-time PCR (qRT-PCR). One μl of the RNA was incubated with DNase I and reverse-transcribed with Superscript II (Invitrogen, Italy) according to manufacturer specifications. For real-time PCR, 1μl of template was used in a 25-μl reaction containing a 0.2 μM concentration of each primer and 12.5 μl of 2× SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA). PCR primers were designed using the software PRIMER3-OUTPUT using published sequence data obtained from the NCBI database.

mIL6: cccggagagacttcacag and tcaegatttcccagagaac

mGAPDH: ctgagatatgtgaggctac and gtggggtgaggcaggatgttg
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 (Miltenyi Biotec, Germany). After isolation, lymphocytes 2 X 10⁶/ml were cultured in a 24 well plate for 36 hours alone or in combination with 2 μg/ml concanavalin A (ConA). At the end of incubation the supernatants were collected for cytokine determinations by Bioplex.

Monocyte-endothelial Cell Adhesion Assay

HUVECs (Human Umbilical Vein Endothelial Cells), obtained from Gibco (Invitrogen), were maintained in supplemented Medium 200 according to the instructions of manufacturer at 37°C in a humidified atmosphere of 5% CO2 in air. HUVEC at the fifth passage were plated on 24 wells plates, starved for 6 hours and then treated with maraviroc (5 μM) or vehicle. Two hours later, cells were exposed to 500 nM or 1μM RTV or 10 ng/ml TNFα. Cells were incubated for 16 hours. Two set of experiments were made in triplicate.

U937 monocyte-like cells were maintained in RPMI supplemented with 10% FBS (fetal bovine serum) with 100 U/ml penicillin and 100 μg/ml streptomycin. U937 cells (2.5 x 10⁵ /ml) were layered over 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 four high power fields per well under microscope and expressed as number of adherent U937 cells per high power field ²⁹.

Statistical Analysis

Data are expressed as mean ± SE. Statistical significance was determined for comparison of more than two groups by one-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 two 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, USA).

**Results**

**Maraviroc Attenuates Atherosclerotic Plaque Progression in ApoE$^{-/-}$ Mice Exposed to Ritonavir**

Since we had previously shown that RTV accelerates the progression of atherosclerotic plaques in ApoE$^{-/-}$ mice\(^{28}\), in the present study we investigated whether treating ApoE$^{-/-}$ mice with maraviroc would protect from progression of atherosclerosis caused by this HIV protease inhibitor. For this purpose 8 week old ApoE$^{-/-}$ mice were treated for 13 weeks with RTV alone or in combination with maraviroc and the extent of aortic plaques measured in H&E stained coronal sections of aortic arches. After a 13 week treatment with RTV, the plaque area increased by approximately 2-folds in comparison with control mice. Administering mice with maraviroc effectively protected against aortic plaque progression (Figure 1 A-D; n=6-7 mice per group, $P \leq 0.05$). Treating ApoE$^{-/-}$ mice with RTV caused a robust increase in macrophages 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 %. Co-treating mice with maraviroc effectively reduced this effect (from 48.5 ± 9.5% to 21.3 ± 2.9 %; Figure 1 E-H, n=3-5 mice per group, $P < 0.05$). Further, while treating mice with RTV increased the staining for CCR5, this effect was reversed by maraviroc (Figure 1 I-M). Exposure of ApoE$^{-/-}$ mice to RTV increased total cholesterol, LDL and triacylglycerol levels. This effect persisted even in maraviroc co-treated mice (Figure 1 N-R, n= 5 mice per group, $P \leq 0.05$).

**Maraviroc Counter-regulation of Ritonavir-induced Plaque Inflammation**
Because these data indicate that beneficial effects exerted by maraviroc were dissociated from its effects on lipid metabolism, we have then investigated whether CCR5 antagonism modulated inflammatory pathways in this model. As shown in Figure 2, while RTV boosted the aortic content of ICAM-1, VCAM-1, MCP-1 and IL-17A, co-treating mice with maraviroc reversed this pattern. Further, maraviroc effectively reduced the aortic content of RANTES/CCL5 and TNF-α, i.e. two mediators that were not affected significantly by exposure to RTV and reduced IL-6, though in a non significant manner (Figure 2 A-H; n= 6 mice per group, P ≤ 0.05).

Maraviroc Inhibits Monocyte Adhesion to RTV or TNF-α Activated Endothelial Cells

To evaluate whether exposure of endothelial cells to RTV induced their monocyte interaction and to investigate the efficacy of maraviroc in counteracting this event, we exposed HUVEC untreated or pre-treated with maraviroc to RTV, using TNF-α as a validated control. The exposures of endothelial cells to RTV increased the monocyte adhesion by ≈8-fold and to TNF-α ≈17-fold. As shown in Figure 3 A-B, this endothelial-monocyte interaction was robustly attenuated by maraviroc (>60% in all settings; P ≤ 0.05).

Maraviroc Counter-regulation of Ritonavir-induced Systemic Inflammation

As shown in Figure 4, exposure to RTV increased significantly the expressions of CD36 and CCR5 on blood monocytes. The effect on CD36 was reversed by co-treating mice with maraviroc (Figure 4 A-B, n=5-6 mice per group P ≤ 0.05). In contrast, both agents failed to modulate the levels of circulating CRP (Figure 4 C, n=5 mice per group).

Activation of adipose tissue is marker of systemic inflammation. Interestingly, we found that RTV induced lipoatrophy and increased IL-6 mRNA expression on epididymal fat. Co-treating mice with maraviroc counter-regulated the lipoatrophic effect of RTV and down-regulated the IL-6 mRNA expression (Figure 4D, n=6-7 mice per group, P ≤ 0.05; and Figure 4E...
n=5 mice per group, P≤0.05).

Given that systemic inflammation and immune activation are correlated to a higher risk of cardiovascular diseases, we have then investigated whether RTV induced a pro-inflammatory 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 Bioplex platform. No difference were observed in basal cytokine secretion. In contrast, ConA stimulation of spleen-derived lymphocytes increased IL-17A secretion from RTV treated mice. This effect was reversed by in vivo co-treatment with maraviroc. Moreover, maraviroc interfered with the ConA stimulation reversing TNF-α and IFN-γ secretion and increasing TGF-β production (Figure 5 A-D; n= 6-7 mice per group P≤0.05).

Maraviroc Inhibits 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 have then investigated whether maraviroc attenuated progression of atherosclerotic plaques in 39-week old ApoE⁻/⁻ mice, where dyslipidemia is the only pathogenic factor.

As illustrated in Figure 6 A-C, exposure of 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≤0.05). Noteworthy, 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 6 D-F, maraviroc reduced the extent of macrophages infiltration as assessed by measuring the surface expression of MAC-3 (from 55.8% ± 5.8% to 22.7% ± 3.9%) and CCR5 staining (Figure 6 G-H). 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 a reducing trend for TNF-α as well as a rising one for TGF-β was seen (Figure 7A-I; n=4-5 mice per group, P ≤ 0.05). Unlike the early model, no reductions were observed in the IL-17A, and MCP-1 levels. Additionally, exposure to MVC effectively decreased CD36 and CCR5 expression on circulating monocytes (Figure 7L-M; n=7-8 mice per group, P≤0.05). In contrast to the early model in which the inflammation had been boosted by RTV, treating ApoE−/− mice with maraviroc had no effect on cytokine release from spleen-derived lymphocytes stimulated by ConA (Supplementary Figure 1).

Discussion

It has been recently suggested 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 added advantage to persons with a M-tropic HIV infection and an increased cardiovascular risk, the latter which is usually associated with a high systemic pro-inflammatory profile.

We have previously shown that exposure of genetically dyslipidemic ApoE−/− mice to RTV induces inflammation in adipose tissue, characterized by an increase in pro-inflammatory mediators such as TNF-α and a reduction in the expression of counter-regulatory nuclear receptors such as PPARγ. This exposure accelerates the atherosclerotic progression, as demonstrated by a robust increase in the extension of aortic plaques and a SREBP1c-mediated CD36 over-expression on circulating monocytes, with a consequential higher uptake of oxLDL.
In the present study we have extended on this model obtaining evidence that the CCR5 antagonist maraviroc protects against detrimental effects caused by RTV on plaque progression and systemic inflammation in ApoE<sup>−/−</sup> mice. Moreover, we have also observed in the late stage of this model, in which the dyslipidemia is the major driving factor, that MVC inhibits the plaque evolution interfering with the recruitment of inflammatory cells without modifying the metabolic profile.

Exposure to RTV worsened the pro-atherogenic lipid profile of ApoE<sup>−/−</sup>, accelerated the progression of atherosclerotic plaques by favoring an extensive monocyte/macrophages 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 HUVEC to both RTV and TNF-α, increased the ability of HUVEC to bind to human monocytes. A consequence of exposing ApoE<sup>−/−</sup> mice to RTV was the development of a “low-grade” inflammation, as illustrated by a trend toward an increase of CRP, which is a valid marker of systemic inflammation, and a diffused activation of innate and adaptive immunity cells. Exposure to RTV increased the expression of CD36 and CCR5 on circulating monocytes and IL-17A release from spleen lymphocytes stimulated by ConA and boosted a 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 RTV on both endothelial cells<sup>33-35</sup> and human adipocytes<sup>36</sup> as well as with recent observations in humans<sup>37</sup>. Lefèvre et al. observed that long-term exposure to ritonavir of human coronary artery endothelial cells progressively induced endothelial dysfunction with an increased secretion of MCP-1, IL-6, ICAM and VCAM<sup>35</sup>. Lagathu et al., reported that, in human adipocytes, RTV increased reactive oxygen species as well as MCP-1 and IL-6<sup>36</sup>. Additionally, in virologically
suppressed patients, Martinez et al. observed that a switching from RTV-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\textsuperscript{37}.  

An important finding of our study was that co-treating ApoE\textsuperscript{−/−} 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, since maraviroc failed to counteract the pro-dyslipidemic effects of RTV. 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 expression of VCAM-1 and ICAM-1, TNF-α, IL-17, MCP-1 and RANTES. The activity of maraviroc was probably exerted by reducing not only the recruitment of inflammatory cells but also the activation of endothelial cells. In fact, the pre-treatment of HUVEC with maraviroc, decreasing the ritonavir or TNF-α observed binding to human monocytes, suggests a direct effect of the drug on endothelial cells.

Furthermore, exposure to maraviroc resulted in a robust down-regulation of CD36 on blood monocytes not associated with reduced levels of low density lipoproteins which induce, via PPAR-γ, the expression of this scavenger receptor\textsuperscript{38}.  

An important observation we made was that exposure to maraviroc effectively counteracted systemic effects caused by RTV 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, the latter promoting inflammation not only in adipose tissue, but also in endothelial and liver cells\textsuperscript{39}. 

15
This RTV-induced lipoatrophy in our experimental model cannot be compared to findings in the human setting. Indeed, exposure to protease inhibitors usually causes lipohypertrophy in HIV infected persons, even though RTV 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 adipocitokine 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 dilation and IL-6. It is widely held that flow-mediated dilation 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, IFN-γ and inducing TGF-β. Indeed, RTV exposure increases the immune activation of lymphocytes and maraviroc dampens their pro-inflammatory response, leading to a higher therapeutic potency which goes beyond blocking chemokine/receptor interaction. These are interesting findings because a persistent (“low grade”) inflammatory state is postulated to contribute to the observed high rate of CAD 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 MW 513.67, while Met-RANTES is 7.8 kD. This significant difference may contribute to different pharmacokinetic distribution profiles and different receptor affinities, leading to activation of different signal transduction pathways.
The anti-atherogenic activity of maraviroc was confirmed in the intermediate-late stage of spontaneous atherosclerosis. In this model we observed that treating old ApoE<sup>−/−</sup> mice with maraviroc inhibits the progression of spontaneous atherosclerosis. The molecular mechanism that supports this effect was associated to the ability of the CCR5 antagonist to modulate the expression/function of biomarkers of inflammation including expression of adhesion molecules and the RANTES while no effect was detected on the lipid profile of ApoE<sup>−/−</sup> mice.

The evolution of plaque was inhibited, while the macrophage and CCR5 cell infiltration, as well as the expressions of ICAM-1, VCAM-1, RANTES and IL-2 were reduced. The other pro-inflammatory cytokine secretions were not down-regulated. Indeed, only a trend in lower TNF-α and higher levels of the regulatory cytokine TGF-β expression were seen. Noteworthy though, significant down-regulations of CD36 and CCR5 expressions on blood monocytes were seen. Still, in spleen lymphocytes, only a mild counter-regulation of systemic inflammation was observed, which involved, in a non-significant manner, IL-17A and TGF-β. No down-regulations of IFN-γ and TNF-α were observed.

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

In conclusion, maraviroc reduced atherosclerotic progression in our murine models by interfering with recruitment of inflammatory cells. Moreover, in the RTV-induced inflammatory model, we found that maraviroc modulated the inflammatory plaque profile as well as systemic inflammation. These results need to be confirmed in clinical trials where the role of maraviroc in protecting HIV positive patients at higher risk of acute coronary disease against CAD is investigated.
Funding Sources: This study was in part supported by an unrestricted grant by ViiV Healthcare. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Acknowledgements: We would like to thank Mr. Thomas Charles Kilcline for his important editorial assistance.

Conflict of Interest Disclosures: None.

References:


**Figure Legends:**

**Figure 1.** Maraviroc attenuates atherosclerotic plaque progression in RTV exposed ApoE<sup>-/-</sup> mice.

Eight weeks old ApoE<sup>-/-</sup> mice were treated for 13 weeks with ritonavir alone or ritonavir in combination with maraviroc (see materials and Methods). In each panel data are mean ± SE of 6-7 mice per group unless stated differently. **Panel A-D.** The plaque area was calculated by assessing the H/E stained surface in coronal sections of aortic arches. Quantitative analysis of plaque size by Image J showed a protective effect of MVC on aortic plaque progression, **P<0.01, *P<0.05** (magnification 40 X). **Panel E-M.** Effect of maraviroc on macrophages infiltration caused by RTV in ApoE<sup>-/-</sup> mice. The plaque macrophage recruitment was evaluated by assessing MAC 3 and CCR5 immunostaining (see Materials and Methods). **P<0.01,**
*P<0.05 (magnification 10 X). ** Panel N-R. Effect of RTV and MVC on serum biochemistry. n=5 mice per group, ** P<0.01, *P <0.05 versus naive.

**Figure 2.** Maravirocin counter-regulation of Ritonavir-induced plaque inflammation. Protein levels adhesion molecules, cytokines and chemokines were measured by using the BioPlex platform. Data are expressed as mean ± SE of 6 animals per group, ** P<0.01, *P <0.05.

**Figure 3.** Maravirocin inhibits U937 cell adhesion to Ritonavir or TNF-α activated endothelial cells. MVC inhibited U937 cells adhesion to ritonavir (500 nM or 1 μM) or TNF-α (10 ng/ml) activated HUVEC. HUVECs were pre-incubated with MVC (5 μM) for 2 h and then stimulated with RTV or TNF-α for 6 h. U937 cells were layered over HUVEC monolayer and incubated for 2 h at 37° C. Adherent cells were counted under a light microscope. Data are mean ± SE of results from two different independent experiments made in triplicate; ** P<0.01, * p ≤ 0.05.

**Figure 4.** Effects of maravirocin on circulating monocytes, CRP and adipose tissue. Panel A-B. maravirocin attenuates CD36 up-regulation caused by ritonavir on blood monocytes from ApoE−−/− mice. Data are mean ± SE of 5-6 animals. ** P<0.01, *P <0.05. ** Panel C. Maravirocin and RTV failed to modulate CRP. n=5 mice per group. Panel D-E. RTV induces lipoatrophy on the epididymal fat and increases IL-6 mRNA expression. The epididymal fat ratio is calculated using the following formula: epididymal ratio= (epididymal weight/body weight) x 100 n=5 mice per group, MVC counter-regulates this lipoatrophic effect. n=6-7 mice per group. ** P<0.01, *P<0.05 versus naive.
Figure 5. Anti-inflammatory Activity of Maraviroc on Cytokine Secretion by Splenic Lymphocytes

Cytokine level from supernatants of spleen lymphocytes were evaluated by Bioplex platform. CD5+ cells were stimulated with ConA as indicated in Material and Methods. Data are mean ± SE. n=6-7 mice per group. Statistic was as following: **p<0.01,*p<0.05, ConA RTV vs ConA Ctrl; ##p<0.01 #p<0.05, ConA RTV-maraviroc vs ConA RTV; ¤p<0.01, ¤p<0.05 ConA RTV-maraviroc vs ConA ctrl.

Figure 6. Maraviroc Inhibits The Spontaneous Atherosclerosis Progression.

Panel A-C: en-face staining of aortas isolated from 52-week-old mice treated with maraviroc (area lesion varies from 16.6 ± 3.3 % in control animals to 7.1 ± 1.4 % in the MVC group) (n=7-8 mice per group,* p<0.05). Panel D-F: Maraviroc reduces the macrophage infiltration (from 55.8% ± 5.8% to 22.7% ± 3.9% as evaluated by MAC-3 staining) (n=4 mice per group, * p<0.05) (magnification 10 X), and the CCR5 staining (Panel G,H) (magnification 10 X).

Figure 7. Adhesion Molecules And Inflammatory Cytokines in The Atherosclerotic Aortic Plaques and Blood Monocyte CD36 and CCR5. Protein levels of aortic adhesion molecules, cytokines and chemokines were measured with Bio-Plex. The protein levels of aortic ICAM-1, VCAM-1, IL-2 and RANTES are reduced in MVC treated mice. A rising trend of the anti-inflammatory cytokine TGF-β is observed. No significant reductions in IL-17A, TNF-α and MCP-1 levels are seen (Panel A-I, n=4-5 mice per group, * p<0.05 ). Maraviroc decreases CD36 and CCR5 monocyte expression (Panel L-M, n=7-8 mice per group, P≤0.05). Data are expressed as mean ± SE.
Figure 1
Figure 2
Figure 3

(A) Number of adherent monocytes (cells/optical field) for different treatments: Naive, Ritonavir 500nM, Ritonavir 1μM, Maraviroc, and their respective combinations.

(B) Number of adherent monocytes (cells/optical field) for treatments: Naive, TNFα, TNFα + Maraviroc, and Maraviroc alone.
Figure 4
Figure 5
Figure 6
Figure 7
Efficacy of CCR5 Antagonist Maraviroc in Reducing the Early, Ritonavir Induced, Atherogenesis and the Advanced Plaque Progression in Mice
Sabrina Cipriani, Daniela Francisci, Andrea Mencarelli, Barbara Renga, Elisabetta Schiaroli, Claudio D'Amore, Franco Baldelli and Stefano Fiorucci

Circulation. published online April 30, 2013;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2013/04/30/CIRCULATIONAHA.113.001278

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/04/30/CIRCULATIONAHA.113.001278.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
Supplemental material
Supplementary Figure 1
Legend to supplementary figure

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.