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Cenicriviroc Inhibits Trans-endothelial Passage of Monocytes and is Associated with Impaired E-Selectin Expression

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Abstract

Incidences of cardiovascular diseases (CVD) are high among virologically suppressed HIV-infected individuals. Monocyte activation and trafficking are key mechanisms in the evolution of CVD. We studied the ability of cenicriviroc, a dual C-C chemokine receptor type 2 (CCR2) and CCR5 antagonist, to influence the migration of monocytes from HIV-infected individuals on anti-retroviral therapy (ART). Monocytes were derived from 23 ART-suppressed HIV-infected and 16 HIV-uninfected donors. In a trans-endothelial migration model, monocytes and human aortic endothelial cells (HAoECs) were exposed to cenicriviroc and migrated monocytes, quantified. Expression of CCR2 and CCR5 on monocytes and adhesion molecules (E-selectin, ICAM-1, VCAM-1, PECAM-1 and CD99) on HAoECs were measured. The single antagonists, BMS-22 (CCR2) and maraviroc (CCR5), served as controls. When both HAoECs and monocytes together were exposed to the antagonists, cenicriviroc led to a greater decrease in monocyte migration compared to BMS-22 or vehicle in both HIV-infected and HIV-uninfected groups ($p < 0.05$), with maraviroc having no inhibitory effect. Cenicriviroc treatment of HAoECs alone decreased monocyte migration in the HIV-infected group when compared to vehicle ($p < 0.01$). Inhibition of

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Authorship

MLD contributed to acquisition and interpretation of the data, to study design and wrote the manuscript. YB contributed to acquisition and interpretation of the data, to study design and revised the manuscript. LCN contributed to interpretation of the data, and revised the manuscript. BIM SM and MMB contributed to acquisition and interpretation of the data, as well as manuscript review. DOA, DC, NNM, WAB, EL and CMS aided in the manuscript revision.

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Conflict of Interest Disclosure

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migration was not evident when monocytes alone were exposed to cenicriviroc, BMS-22 or maraviroc. Incubation of HAoECs with cenicriviroc decreased E-selectin expression ($p=0.045$) but had limited effects on the other adhesion molecules. Cenicriviroc inhibits monocyte trans-endothelial migration more effectively than single chemokine receptor blockade, which may be mediated via disruption of monocyte-endothelial tethering through reduced E-selectin expression. Cenicriviroc should be considered as a therapeutic intervention to reduce detrimental monocyte trafficking.

Summary:

Cenicriviroc (CVC), a dual CCR2 and CCR5 antagonist, inhibits monocyte trafficking in an *in vitro* trans-endothelial migration assay more effectively than a single chemokine receptor blockade.

Graphical abstract

Cenicriviroc (CVC), a dual CCR2 and CCR5 antagonist, inhibits trans-endothelial migration of monocytes derived from HIV-infected individuals more effectively than single chemokine receptor blockade and is associated with decreased expression of E-selectin on endothelial cells, a major receptor for monocyte recruitment.

Keywords

Monocytes; chemokine receptors; HIV; selectins; migration; endothelial cells

Introduction

Although current HIV combination antiretroviral therapies (ART) have prolonged life expectancy in HIV-infected individuals, the prevalence of non-AIDS-related chronic complications persists, such as HIV-associated neurocognitive disorders (HAND)[1, 2] and cardiovascular diseases (CVD).[3, 4] CVD risk is increased in HIV, as HIV-infected persons are 2 fold more likely to develop CVD compared to uninfected individuals.[5–7] While the underlying mechanisms leading to the clinical observations of CVD are likely multifactorial[8–13], immune activation and inflammation, driven in part by monocytes and macrophages, are believed to be a key contributor to the unique pathophysiology of CVD that develops in HIV-infected individuals.[14–19]

During atherosclerosis development and progression, monocytes migrate through the endothelium into the subendothelial space and differentiate into macrophages.[20–22] There, the accumulation of macrophages leads to foam cell formation and secretion of pro-atherogenic factors such as chemokines and cytokines.[20, 23, 24] The process of trans-endothelial migration is a multistep process: monocytes first tether and loosely roll along the vessel by binding to selectins on the surface of the endothelium.[25–27] Firm adhesions are then created at the vascular surface between integrin expressing monocytes and vascular cell adhesion molecule 1 (VCAM-1) and intercellular (I)CAM-1 on the endothelial layer.[28–30] Diapedesis finally follows, where adhesion molecules such as platelet endothelial cell adhesion molecule (PECAM-1) and CD99 are involved.[31–34] Whether or not HIV-infection of monocytes/macrophages affects one or more of the steps from rolling to diapedesis has not been clearly defined.

Atherosclerosis and HIV studies utilizing pharmacological inhibitors that antagonize chemokine receptors (CKRs) have not only highlighted pathways that are important in monocyte migration, but have also demonstrated that CKR blockades are an effective strategy to improve disease state. In an apolipoprotein E (ApoE) knockout mouse model, the use of C-C chemokine receptor type 2 (CCR2) pharmacological inhibitors, GSK1344386B[35] and TLK-19705[36], decreased macrophage infiltration and atherosclerotic lesion size, respectively. Antagonizing CCR5 has also been successful in reducing atherosclerosis development and progression by blocking inflammatory cell infiltration in both a low density lipoprotein receptor deficient murine model and in a ritonavir atherosclerosis murine model by treating the animals with TAK-779[37] and maraviroc (an approved HIV ART drug)[38], respectively. Clinical trials involving CCR5 antagonism have yielded variable results. We and others have reported that maraviroc has positive effects in the setting of HAND, as evidenced by improvements in neurocognitive testing performance scores.[39, 40] These effects were likely due, in part, to the ability of maraviroc to inhibit of HIV entry into cells, and to reduce monocyte migration and immune inflammation, including monocyte activation.[39, 41–43] Other studies however have described either no changes in inflammatory parameters such as T cell activation[44, 45] or increases in inflammation, namely T cell and monocyte activation with maraviroc intensification.[45, 46]

Given the findings of single blockade of either CCR2 or CCR5 as well as the redundancy and compensatory mechanisms between the various CKRs and chemokine ligands[47], targeting both CKRs may be more effective than antagonizing a single CKR to inhibit migration and inflammation, and potentially impede HIV-associated CVD development and/or progression. As proof of concept of the benefit of targeting multiple CKRs, administering the CCR5 antagonist, Met-CCR5, to an atherosclerosis mouse model with combined deficiencies of ApoE, MCP-1 and CX3CR1 led to less atherosclerotic lesion development and monocyte/macrophage migration compared to single chemokine receptor targeting.[48]

Cenicriviroc (CVC) is an oral, dual CCR2 and CCR5 antagonist with nanomolar potency and long plasma half-life.[49–51] CVC has completed Phase 2b development in HIV (NCT01338883)[52] where it was shown to decrease HIV viremia and sCD14 levels (marker

of monocyte/macrophage activation) in ART naïve HIV-infected individuals. CVC has also been shown, *ex vivo*, to decrease HIV entry in peripheral blood mononuclear cells (PBMCs). [53] Additionally, CVC is currently in Phase 3 evaluation for treatment of liver fibrosis in adults with nonalcoholic steatohepatitis (NASH; NCT03028740). The results of the Year 1 primary analysis of the Phase 2b CENTAUR study 652–2-203 (NCT02217475[54]) showed that CVC was well tolerated and resulted in twice as many subjects achieving 1 stage improvement in fibrosis and no worsening of steatohepatitis versus placebo in the intention to treat population.[55] CVC has also demonstrated anti-fibrotic activity in animal models of liver disease, which was largely attributed to decreased monocyte/macrophage infiltration in the liver following hepatocyte injury.[56–58] We hypothesize that blocking a combination of CKRs will inhibit monocyte activation and trafficking, and ultimately provide a therapeutic strategy to decrease HIV-related CVD.

Materials and Methods

Cohort Description

This *ex vivo* study utilized freshly isolated PBMCs from 23 chronically HIV-infected individuals on virally suppressive combination ART who participated in the Hawaii Aging with HIV - CVD cohort study. Ninety one percent of the participants were male, with a median age of 55 years (interquartile range (IQR) 25th-75th percentile [51, 57], a median CD4 count of 689 cells/mm³ [519, 782] and all had a HIV viral load of <20 copies /ml. Of the 23 participants, 100% were on nucleoside reverse transcriptase inhibitors (NRTIs), 43% on non-nucleoside reverse transcriptase inhibitors (NNRTIs), 22% were on protease inhibitors (PIs) and 48% were on integrase inhibitors (INSTI). More specifically, 10 were on dual NRTIs (either tenofovir/emtricitabine or abacavir/emtricitabine) and one INSTI (dolutegravir, elvitegravir or raltegravir); 6 were on dual NRTIs (tenofovir/emtricitabine or abacavir/lamivudine) and one NNRTI (efavirenz or rilpivirine); 2 were on dual NRTIs (tenofovir/emtricitabine) and dual PIs (atazanavir/ritonavir). The other 5 participants were on less common regimens consisting of a triple NRTIs (tenofovir, emtricitabine, abacavir) plus a PI (efavirenz); triple NRTIs (tenofovir, emtricitabine, abacavir) plus an INSTI (raltegravir); dual NRTIs (tenofovir, emtricitabine) plus dual PIs (darunavir, ritonavir) plus an NNRTI (etravirine); single NRTI (emtricitabine), single NNRTI (etravirine) and dual PIs (darunavir and ritonavir); single NRTI (abacavir), single NNRTI (efavirenz) and single PI (saquinavir). No participants were taking chemokine receptor antagonists (e.g. maraviroc). Sixteen HIV-uninfected participants were used as controls (56% male, age, 30 [28, 40]). HIV-infected participants were older compared to uninfected participants (p<0.001). The study was approved by the University of Hawaii Manoa Committee on Human Studies and participants gave informed consent.

Antagonists

Cenicriviroc, the CCR2 and CCR5 dual antagonist, was obtained from Allergan plc (South San Francisco, USA). The single antagonist for CCR2, BMS-22, was purchased from Tocris (Bristol, United Kingdom). The single antagonist for CCR5 was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Maraviroc (Cat #11580).

Cell Culture and Isolation for Trans-endothelial Migration Assay

Primary human aortic endothelial cells (HAoECs) were cultured, up to passage 8, using the Endothelial Cell Growth Media (ECGM) MV2 and split kit according to manufacturer's instructions (PromoCell, Germany). Human PBMCs were isolated from freshly drawn whole blood by Ficoll-Hypaque density gradient centrifugation (Pfizer, New York, NY). Monocytes were isolated using the EasySep™ Human Monocyte Enrichment Kit without CD16 depletion and the RoboSep automated cell separator (StemCell Technologies, Cambridge, USA) as previously described.[39]

HAoEC Barrier Integrity

VE-Cadherin Immunofluorescence—HAoECs were cultured till confluent or incubated with vehicle (DMSO), BMS-22, MVC or CVC for 24h at 0.1; 1 or 10 μ M. After 24h of incubation, cells were washed with HEPES and fixed for 10 min at room temperature using 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS). After blocking with 2% bovine serum albumin (BSA) in PBS, permeabilizing using 0.1% TritonX-100/PBS and blocking with 2% BSA/PBS, endothelial cells were incubated with a primary antibody against VE-cadherin (ab7047, Abcam, Cambridge, USA). VE-cadherin was visualized using a cy3 labeled anti-mouse secondary antibody (Abcam) and the actin cytoskeleton was labeled using a phalloidin probe (Alexa488; Invitrogen, Carlsbad, USA). Immunofluorescence images were acquired using an Axiovert microscope (Zeiss, Germany).

Electric Cell-substrate Impedance Sensing (ECIS)—An electric cell substrate impedance-sensing instrument (ECIS Z, Applied BioPhysics Inc, Troy, USA) was used to detect trans-endothelial electrical resistance (TER) of HAoECs. HAoECs were seeded on ECIS arrays and reached confluency within 5–7 days. Cells were treated with vehicle, BMS-22, MVC or CVC (0.1; 1 or 10 μ M) for 24h and changes in endothelial barrier integrity were assessed by ongoing measurements of TER at 4,000 Hz. Data were analyzed using ECIS and Excel software. Data are presented as a percentage of the sample to the baseline resistance values.

Trans-Endothelial Migration Assay

HAoECs, which were not activated or stimulated, were seeded on the top of 24-well 5 μ m transwell filters and allowed to reach confluency (5–7 days). Antagonist treatment was performed in 3 different ways: a) treatment of the HAoEC monolayer only (Supplemental Figure 1A), b) treatment of monocytes only (Supplemental Figure 1B) and c) treatment of both HAoECs and monocytes (Supplemental Figure 1C). Treatment of endothelial monolayer: Confluent HAoECs were treated for 4h at 37°C with vehicle or antagonist (BMS-22, MVC or CVC; 1 μ M, a concentration similar to pharmacokinetic data reported in CVC-treated patients[52, 59]). Unbound inhibitor was removed by carefully washing the endothelium with ECGM MV2 before 0.2×10^6 live monocytes, as assessed by trypan blue exclusion, were added to the upper compartment on top of the HAoEC monolayer in a 1:1 mixture of ECGM MV:AIM-V media (Invitrogen). Of note, the 4-hour antagonist treatment did not affect cell viability (data not shown). Treatment of monocytes: Monocytes were treated for 4h at 37°C in AIM-V media with vehicle or antagonist (BMS-22, MVC or CVC;

1 μ M). Unbound inhibitor was removed before 0.2×10^6 live monocytes were added to the upper compartment on top of the confluent HAoEC monolayer in a 1:1 mixture of ECGM MV2: AIM-V media. Treatment of HAoEC and monocytes: 0.2×10^6 live monocytes were added to the upper compartment on top of the confluent HAoEC monolayer in a 1:1 mixture of ECGM MV2: AIM-V media. Cells were treated with vehicle or antagonist (BMS-22, MVC or CVC; 1 μ M). Monocytes were then allowed to transmigrate toward the lower compartment containing 40ng/ml MCP-1 (R&D systems, Minneapolis, USA) for 16h (Supplemental Figure 1). The concentration of 40ng/ml MCP-1 was chosen to mitigate any possible effects of MCP-1 on endothelial barrier integrity as previously described.[60] Transmigrated monocytes were collected, stained with 1 ng/ml calcein (Invitrogen) and counted using a FACSCalibur flow cytometer (BD Biosciences, San Jose, USA). Data are presented as a migratory factor (percentage of migrated monocytes) by dividing the number of migrated monocytes by the number of monocytes pre-migration and expressing that value as a percentage.

CCR2 and CCR5 Expression on HAoECs

Flow Cytometry—HAoECs were grown to confluency as described above. HAoECs were then scrapped off the tissue culture plate and stained with Live/Dead® Fixable Yellow Dead Cell Stain (YARD) for 15 min at room temperature followed by two, 10 min sequential incubations with anti-CCR2 (PE) and CCR5 (AlexaFluor700) at 37 °C and lastly a 30 min room temperature incubation.[61] Cells were fixed with 1% PFA and data were acquired on a custom 4-laser BD LSRFortessa (BD Bioscience, San Jose, USA). Compensation and gating analyses were performed using FlowJo (FlowJo LCC, Ashland, USA). All antibodies were purchased from Biolegend (San Diego, USA), except for Live/Dead Stain (Invitrogen).

Western Blot: HAoECs were grown to confluency as described above. Cell lysates were obtained using RIPA buffer with addition of protease and phosphatase inhibitors. Fifteen ug of protein was loaded onto an 10% SDS-PAGE gel (Novex NuPAGE, Invitrogen) and transferred to a low fluorescence-PVDF membrane (EMD Millipore, Temecula, USA). Blocking was carried out using the Odyssey Blocking Buffer (Licor, Lincoln, NE). The membranes were incubated with antibodies for CCR2 (ab202391; 1:1000 dilution; Abcam), CCR5 (ab64620; 1:1000 dilution; Abcam) and β -actin (A2228; dilution 1:2000; Sigma, St-Louis, USA) in Odyssey Blocking Buffer and 0.1% Tween. The membranes were washed with PBS + 0.1% Tween (PBST) prior to the addition of IRDye® 800 and 680 secondary antibodies (dilution 1:10,000 in Odyssey Blocking Buffer, both Licor). The membranes were washed again with PBST, and the protein bands were visualized and quantitated using the Licor's ImageStudio software (Licor).

CCR2 and CCR5 Expression on Monocytes by Flow Cytometry

Cryopreserved PBMCs were placed in 96 well polypropylene round bottom plates and stained with Live/Dead® Fixable Red Dead Cell Stain for 15 min at room temperature followed by two, 10 min sequential incubations with anti-CCR2 (AlexaFluor647) and CCR5 (PE) at 37 °C.[61] Cells were then stained at room temperature for 30 min with anti-CD3 (Brilliant Violet (BV) 711), CD14 (AlexaFluor700), CD16 (BV421), CD56 (PE-Cy7), CD19 (PE-Cy7), CD20 (PE-Cy7), CD11b (AlexaFluor488) and HLA-DR (APC-H7). Cells were

fixed with 1% PFA and data were acquired on a custom 4-laser BD LSRFortessa (BD Bioscience). Compensation and gating analyses were performed using FlowJo (FlowJo LCC). All antibodies were purchased from BD Bioscience, except for Live/Dead Stain (Invitrogen), and CD3-BV711 and CD16-BV421 (BioLegend, San Diego, USA).

The gating strategy for identification of monocytes, their subsets, and receptor expression is shown in Supplemental Figure 4. Monocytes were identified from the PBMC population by the exclusion of dead cells, lymphocytes (CD3 positive cells), natural killer (NK) and B cells (CD56, CD19 or CD20 positive cells). Monocytes, positive for HLA-DR and CD11b, were subset by CD14 and CD16 expression: Classical monocytes (CD14⁺⁺CD16⁻), intermediate monocytes (CD14⁺⁺CD16⁺) and non-classical monocytes (CD14⁺CD16⁺). Information regarding the cohort description can be found in the Supplemental Materials and Methods and Supplemental Table 1.

HAoEC Surface Adhesion Molecule Levels

Receptors involved in trans-endothelial migration were measured on HAoECs. Confluent HAoECs were incubated with CVC for 1h at 37°C, followed by MCP-1 for 1h at 37°C. One million PBMCs were also added to certain samples during the last 30 min of the MCP-1 incubation to determine the effects of cell to cell engagement. HAoECs were harvested using DetachKit (Promocell) according to manufacturer's instructions and stained with a viability marker (YARD) followed by monoclonal antibodies for E-selectin (PE; eBioscience, San Diego, USA), VCAM-1 (PE-Cy7; eBioscience), ICAM-1 (APC; BD Biosciences), PECAM-1 (APC-Cy7; Biolegend) and CD99 (FITC; Biolegend). Cells were fixed with 1% PFA and data were acquired on a custom 4-laser BD LSRFortessa (BD Bioscience). Compensation and gating analyses were performed using FlowJo (FlowJo LCC). Histograms showing adhesion molecule geometric mean fluorescence (GMF) values are shown in Supplemental Figure 2. Data are presented as a percentage of the geometric mean fluorescence (GMF) of the baseline control (unstimulated HAoECs).

Statistical Analysis

Clinical data are presented as the median and interquartile range (IQR) 25th-75th percentile. *Ex vivo* experimental data are expressed as the mean $\pm$ standard deviation (Std Dev). Non-parametric statistical tests were employed for clinical data and all lab assays except HAoEC transmigration receptor levels (cell line data employed parametric statistical tests). Statistical analyses were performed in Prism version 6 (GraphPad, La Jolla, USA) for Mac OS X.

Results

Direct effects of BMS-22, MVC or CVC on endothelial cell barrier integrity

Endothelial barrier integrity was assessed to ensure that differences in migratory rates between antagonists were not due to disruption of the endothelial monolayer. Inter-endothelial adherence junctions, visualized by VE-cadherin immunofluorescence, were not compromised by any of the antagonist treatments (Figure 1A). Endothelial barrier integrity, assessed by Electric Cell-substrate Impedance Sensing (ECIS), was also not altered due to antagonist treatment, even at concentrations 10 times higher than the experimentally used

concentration of 1 μ M (Figure 1B). These experiments demonstrate that observed changes in trans-endothelial migration after treatment with CCR2/CCR5 antagonists were not due to disruption of the endothelial monolayer.

CVC decreases monocyte trans-endothelial migration in both HIV-infected and -uninfected donors

The trans-endothelial migration studies were performed in 3 different ways to assess the independent effects of CKR blockade on the various cell populations in the assay: A) treatment of HAoEC monolayer only (Figure 2A), B) treatment of monocytes only (Figure 2B) or C) simultaneous treatment of HAoECs and monocytes (Figure 2C). The data are also shown in Supplemental Figure 1 to better highlight the comparison between HIV-infected and HIV-uninfected migratory rates. In the absence of antagonist (vehicle alone), monocytes from HIV-infected individuals migrated at significantly higher rates than monocytes from HIV-uninfected persons (migratory factor of 1.44% vs 0.86%; Supplemental Figure 1). These data indicate that atherosclerosis development may occur at greater rates in HIV-infection due to increased monocyte trans-endothelial migration.

When CVC was added to the endothelial monolayer only, there was a significant inhibition of monocyte transmigration compared to control in HIV-infected individuals (migratory factor of 0.79% compared to 1.44%; Figure 2A). In uninfected individuals this CVC-induced inhibition could not be observed (migratory factor of 0.68% for CVC vs. 0.86% for control; Figure 2A). No significant changes were observed in monocyte transmigration when BMS-22 and MVC were added the endothelial monolayer in either the HIV-infected individuals or the HIV-uninfected group (Figure 2A). Addition of the antagonists BMS-22, MVC or CVC or vehicle alone to monocytes led to no significant inhibition of trans-endothelial migration in both HIV-uninfected and HIV-infected groups (Figure 2B).

However, treatment of both HAoECs and the monocytes with the antagonists, led to significant decreases in monocyte transmigration in HIV-infected as well as HIV-uninfected individuals with BMS-22 (0.86% and 0.52%, respectively; Figure 2C) and CVC (0.68% and 0.40%, respectively; Figure 2C) when compared to their respective controls (1.44% and 0.86%; Figure 2C). Additionally, CVC significantly inhibited monocyte migration more efficiently than BMS-22, as the migratory factor for CVC was significantly lower than for BMS-22 (HIV+: 0.68% vs 0.86%; HIV-: 0.40% vs 0.52%; Figure 2C). Moreover, it should be noted that CVC decreased trans-endothelial migration rates of the HIV-infected group to the levels observed in the HIV-uninfected group treated with vehicle (Figure 2C). MVC did not alter monocyte trans-endothelial migration (Figure 2C). These data indicate that: 1) the inhibitory effect of CVC on the endothelium appears critical in modulating trans-endothelial monocyte migration and 2) dual CKR blockade is more efficient in inhibiting trans-endothelial monocyte migration than single CKR targeting.

Expression levels of CCR2 and CCR5 on primary human aortic endothelial cells and monocytes

To confirm the presence of CVC targets (CCR2 and CCR5) on HAoECs, protein levels for both CKRs were assessed by flow cytometry and Western Blotting (Figure 3A). CCR2 and

CCR5 were detected in the HAoECs samples albeit at relatively low levels (Figure 3A). To assess whether the differences in migratory rates between HIV-infected and HIV-uninfected persons were due to varying CCR2 and CCR5 levels on monocytes, we measured CCR2 cell surface expression by flow cytometry (Figure 3B). CCR2 cell surface expression was significantly lower on non-classical monocytes from HIV-infected individuals compared to HIV-uninfected controls ($p=0.005$, Figure 3B). There were no significant differences in CCR2 levels on classical or intermediate monocytes nor were there any differences in CCR5 on all 3 monocyte subsets (Figure 3B). These data suggest that higher migratory rates observed in the HIV-infected groups were not due to higher CCR2 levels.

CVC treatment selectively decreases E-selectin and PECAM levels on HAoECs

To investigate the possible mechanisms involved in the observed reduction of trans-endothelial migration of monocytes after treatment of HAoECs with CVC, cell surface expression of adhesion molecules involved in adherence and transmigration were measured on HAoECs by flow cytometry (Figure 4 and Supplemental Figure 2). Treatment of HAoECs with CVC before the addition of only MCP-1 resulted in significantly decreased E-selectin levels (96.9% to 83.4%; Figure 4) whereas no changes in ICAM-1, VCAM-1, CD99 and PECAM-1 were observed (Figure 4 and Supplemental Figure 2). CVC treatment prior to MCP-1 and PBMCs addition resulted in significant reductions in E-selectin (93.1% to 82.5%) and PECAM-1 (89.8% to 85.7%) cell surface expression (Figure 4). Removal of CVC from the cultures prior to addition of MCP-1 and PBMCs restored E-selectin and PECAM-1 levels (96.0 and 93.4, respectively; Figure 4). No significant changes in adhesion molecule expression levels were noted when CVC was added to the HAoECs prior to incubation with PBMCs only (Supplemental Figure 2).

The addition of BMS-22, MVC or a combination of both BMS-22 and MVC to HAoECs prior to MCP-1 stimulation did not alter E-selectin, ICAM-1 or CD99 cell surface expression, suggesting this is an effect specific to CVC (Supplemental Figure 3). Incubation of HAoECs with BMS-22 before MCP-1 significantly increased VCAM-1 and PECAM-1 cell surface levels (from 72.8% to 160% and from 97.2% and 127%, respectively; Supplemental Figure 3). These observed increases in adhesion molecules may explain why BMS-22, in contrast to CVC, does not inhibit monocyte trans-endothelial migration.

CVC decreases monocyte migration from HIV-infected donors in a chemotaxis assay

Given that we did not observe inhibition of monocyte migration in the trans-endothelial assay, we investigated whether CVC had the capacity to inhibit monocyte mobilization in a chemotaxis assay. For this study, we used a Boyden chamber with a porous membrane devoid of endothelial cells (for details see Supplemental Materials and Methods). Under MCP-1 pressure, we examined monocyte migration from both virally suppressed, chronically HIV-infected individuals and demographically matched HIV-uninfected controls (Supplemental Table 1). Classical monocytes, which express relatively high levels of CCR2 (Figure 3), had the highest migratory rates towards the CCR2 gradient (Supplemental Figure 5B). Non-classical monocytes (low CCR2 expressers; Figure 3) mobilized, albeit less, towards MCP-1 and intermediate monocytes (high CCR2 expressing cells) migrated very little (Supplemental Figure 5C). In HIV-infected participants, CVC significantly inhibited

total and classical monocyte migration compared to their respective vehicle controls (migratory factor for total monocytes: 2.34% vs. 3.88%; $p<0.0001$ and migratory factor for classical monocytes: 0.33% vs. 0.46%; $p=0.003$), with no effect on intermediate or non-classical monocyte migration (Supplemental Figure 5). In HIV-infected donors, BMS-22 and MVC did not inhibit MCP-1-driven migration (Supplemental Figure 5). In fact, when examining total monocyte migratory factors, monocytes incubated with CVC migrated significantly less than cells incubated with BMS-22 (migratory factor: 4.45%; $p=0.0013$ or MVC migratory factor: 4.84%; $p=0.0002$; Supplemental Figure 5A). In HIV-uninfected controls, monocyte migration was not inhibited by any of the CCR antagonists (Supplemental Figure 5). These data suggest that CVC has the potential to inhibit monocyte mobilization.

Discussion

Cardiovascular disease (CVD) is reportedly the second highest cause of death in the aging HIV-infected population.[62, 63] Understanding the mechanism and finding novel treatment strategies to prevent or improve CVD are thus warranted. We sought to assess the use of the dual CCR2/CCR5 antagonist, CVC, on inhibiting monocyte mobilization towards a CCR2 chemokine gradient in a trans-endothelial migration model, using the single CCR2 and CCR5 antagonists, BMS-22 and MVC, as controls. To our knowledge, this is the first study, in the context of HIV, to utilize HAoECs which is paramount to mimic as closely as possible cellular transmigration that occurs in clinically-relevant anatomical sites of atherosclerosis. We chose MCP-1 as the chemotactic agent because this chemokine is a central monocyte chemoattractant and because it is important in cardiovascular disease outcome in HIV-infection as plasma MCP-1 levels are elevated in HIV-associated atherosclerosis.[64, 65] The main finding of this trans-endothelial migration study is that CVC inhibited monocyte transmigration better than BMS-22 or MVC, indicating that blocking CCR2 and CCR5 simultaneously is more effective than a single CCR2 or CCR5 blockade.

Surprisingly, in the trans-endothelial migration model, CVC inhibited monocyte migration when CCR2 and CCR5 were targeted exclusively on the HAoECs. Our results suggest this effect was mediated by decreasing E-selectin cell surface expression, which we speculate may be beneficial in the context of atherogenesis as E-selectin has been detected in human atherosclerosis-prone endothelial cells as well as on the surface of fibrous and lipid-containing human plaques.[66] Therapeutic targeting of E-selectin in the context of inhibiting metastasis is being explored and this strategy could potentially be employed in the setting of atherosclerosis. Glycomimetic E-selectin inhibitors (GMI-1271 and GMI-1359 [a dual E-selectin and CXCR4 inhibitor]), in combination with standard chemotherapy, have been shown to reduce metastatic lesions and improve survival in xenograft mouse models of cancers.[67–70] Moreover, the E-selectin inhibitor, GMI-1271, which has been shown to be well tolerated in healthy individuals[71], is currently being evaluated in combination with standard agents to treat acute myeloid leukemia in a clinical phase 1 trial (NCT02306291). Although CVC does not bind E-selectin *per se*, diminishing its cell surface expression appears to have similar benefits to targeting E-selectin directly in preventing trans-endothelial migration. Access to and further evaluation of E-selectin inhibitors in the context of HIV-related CVD warrants additional studies.

The mechanism by which CVC influences E-selectin expression is unclear. Inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) or interleukin (IL)-1 β , can transiently activate the endothelium and induce cell surface expression of E-selectin.[72] Although in our cell culture system MCP-1 did not significantly increase E-selectin levels, blocking MCP-1/CCR2 interactions may have inhibited signaling that maintains E-selectin levels at the HAoEC surface. Decreases in cell surface E-selectin could have potentially been due to shedding from the cell membrane[72, 73] or endocytosis of the receptor, where it localizes in lysosomal compartments and is degraded.[72, 74, 75] We measured soluble E-selectin in the supernatant of the HAoEC culture wells using ELISA but levels were below the limit of detection of the assay (data not shown). Given that TNF- α stimulation of HUVECs can trigger E-selectin internalization in a matter of hours[72, 74, 75]; it is conceivable that endocytosis is occurring in our model. Moreover, the interaction of E-selectin with monocytes occurs via P-selectin glycoprotein ligand-1 (PSGL-1), specifically through the carbohydrate epitope cutaneous lymphocyte antigen (CLA) found on classical monocytes, but not through carbohydrate modification 6-sulfo LacNAc (SLAN) found on intermediate and non-classical monocytes.[76–79] Although PSGL-1 on total monocytes was not different between HIV-infected and –uninfected individuals (data not shown), focusing on changes in CLA expression may provide additional insights in future studies. It also remains to be explored whether the observed decreases in PECAM-1 may be due to variations in the level of the molecules involved in upstream events such as loose tethering or by direct effects of CVC inhibiting an interaction between PBMCs and HAoECs which downregulates PECAM-1.

Dissimilarities in inhibition of monocyte trafficking by CVC in the trans-endothelial migration and chemotaxis assays could be due to the fact that CVC differentially affects the migration of the 3 monocyte subsets and it is unknown in the trans-endothelial migration assay how the monocyte subpopulations interact with and preferentially cross the endothelial layer. We did not phenotype the monocytes in this trans-endothelial migration assay as their CD14/CD16 profile was altered after 16h in culture, making it difficult to delineate the 3 subsets (data not shown).

Our trans-endothelial migration data are similar to reports that monocytes, infected with HIV *in vitro*, cross *in vitro* blood brain barrier models more readily than uninfected monocytes.[80, 81] This is in contrast with another trans-endothelial migration model where Westhorpe *et al* reported no difference in migration between monocytes infected with HIV *in vitro* and uninfected monocytes.[82] The discrepancies in results may be due to the Westhorpe study utilizing human umbilical vein endothelial cells compared to HAoECs in our case.[82] Our study offers more physiologically-relevant insight into HIV-associated atherosclerosis as firstly, the monocytes from this model were isolated from HIV-infected individuals rather than being infected *in vitro* and secondly, the source of the endothelial monolayer was from human aorta. Taken together our data may suggest that one of the first steps of atherosclerosis development, namely monocyte trans-endothelial migration, is altered in HIV-infected individuals and therefore could explain the accelerated atherosclerosis development in HIV. Given that our HIV-uninfected group was younger, we must consider age may have also been a factor.[83]

Although *in vitro* HIV-infection of monocytes has been shown to increase CCR2 cell surface expression and enhance the capacity of the monocytes to transmigrate across a BBB model towards MCP-1[80, 84], we did not observe that virally suppressed, chronically HIV-infected individuals compared to HIV-uninfected controls had greater monocyte migratory rates due to higher CCR2 cell surface expression. Unaltered expression of CCR2 in HIV may be due to the fact that the majority of peripheral monocytes are most likely not infected with HIV in aviremic individuals or perhaps ART itself is altering CCR2 expression. We have also hypothesized in a study examining neurocognitive impairment in antiretroviral therapy-naïve HIV-infected Thais that lower CCR2 levels on non-classical monocytes may be due to the fact that the higher CCR2 expressing non-classical monocytes may have migrated into tissues.[85] Further studies are needed to characterize the dynamics of CCR2 expression during the course of HIV progression and treatment. A potential explanation for why monocytes from HIV-infected individuals may migrate more than HIV-uninfected participants includes the effect of the overall inflammatory state in HIV[86], which may give rise to priming, stimulation or activation of the monocytes.

Maraviroc has been previously reported to inhibit monocyte migration towards an MCP-1 gradient[41], whereas in our assays MVC did not inhibit monocyte mobilization. Differences in experimental conditions may account for the differences in results, with the Rossi *et al* study using a 10 fold less concentration of MCP-1.[41] Interestingly in a more recent study of atherosclerotic burden in aviremic HIV-infected individuals, MVC intensification decreased indices of endothelial cell activation, namely soluble VCAM-1 and soluble ICAM-1.[87] Whether CVC has the ability to decrease endothelial cell activation in individuals with CVD should be investigated.

Limitations to this study include small sample size and randomly selected individuals without attention to the presence of CVD or traditional risk factors for CVD. Another limitation of these trans-endothelial migration studies is the age difference between HIV-infected and uninfected participants. Moreover, being able to phenotype the monocytes and examining other chemokine receptors such as CX3CR1 would offer additional insight into the effect of CVC.

The main findings of this trans-endothelial migration study are that dual CCR2 and CCR5 inhibition is more effective than a single CCR2 or CCR5 blockade in inhibiting trans-endothelial migration and that favorable alterations in the endothelium contribute to this inhibition. Given that HIV and CVD are complex pathologies in which several chemokine/CKR axes contribute to disease, targeting multiple CKRs may ultimately yield better disease outcome. In support of this, we found in a new single arm open-label trial in virally suppressed HIV-infected adults on stable ART, CVC intensification improved neuropsychological testing performance and reduced monocyte activation (in press).[88] Overall our trans-endothelial migration study highlights the potential anti-atherosclerotic effects of CVC, in both HIV and non-HIV settings, by potentially limiting monocyte/macrophage inflammation and decreasing monocyte infiltration into atherosclerotic plaques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

ApoE	Apolipoprotein E
BSA	Bovine serum albumin
CVD	Cardiovascular disease
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 2
CVC	Conciviroc
CKRs	Chemokine receptors
ART	Combination antiretroviral therapies
ECIS	Electric cell substrate impedance-sensing instrument
ECGM	Endothelial Cell Growth Media
HAND	HIV-associated neurocognitive disorders
HAoEC	Human aortic endothelial cells
ICAM-1	Intercellular cell adhesion molecule 1
YARD	Live/Dead® Fixable Yellow Dead Cell Stain
MCP-1	Monocyte chemoattractant protein 1
NASH	Non-alcoholic steatohepatitis
PFA	Paraformaldehyde
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline with Tween
PECAM-1	Platelet endothelial cell adhesion molecule
PVDF	Polyvinylidene Fluoride
RIPA	Radioimmunoprecipitation assay
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TER	Trans-endothelial electrical resistance
VCAM-1	Vascular cell adhesion molecule 1

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Figure 1. Human aortic endothelial cell barrier integrity.

A: VE-cadherin immunofluorescence for vehicle, BMS-22, maraviroc (MVC) and cenicriviroc (CVC) (1 μ M). B: Resistance, expressed as a percentage of baseline, for BMS-22 (top), MVC (middle) and CVC (bottom) at 0.1; 1 and 10 μ M.

Figure 2. Trans-endothelial monocyte migration.

Pre-migration treatment with antagonist under 3 conditions: human aortic endothelial cells (HAoECs) only (A), monocytes only (B) or HAoECs and monocytes (C). MCP-1 induced monocyte migration, presented as a monocyte migration factor by dividing the number of migrated monocytes by the number of monocytes pre-migration and expressing it as a percentage, is shown for HIV-uninfected (white circles) and HIV-infected (grey circles) for vehicle, BMS-22, maraviroc (MVC) and cenicriviroc (CVC). The hash mark indicates the mean and error bars are the standard deviations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3. CCR2 and CCR5 expression on A) human aortic endothelial cells (HAoECs).

Left: Flow cytometry histogram plots for CCR2 and CCR5 showing chemokine receptor (black line) and isotype control (grey line). Right: Representative Western Blot bands for CCR2, CCR5 and β -actin from HAoECs, detected at 42, 41 and 42 kDa, respectively and the graphical representation of the quantification of the fluorescence intensity of CCR2 and CCR5 from HAoECs and peripheral blood mononuclear cells (PBMCs; internal control). Data is presented as the ratio of CCR2 and CCR5 intensity as detected using ImageJ software relative to the samples β -actin to correct for protein loading. Values represent mean $\pm$ SEM of single values from independent experiments (n=3 for HAoECs and n=1 for PBMCs).

B) on the 3 monocytes subsets (classical, intermediate and non-classical monocytes) expressed as geometric mean fluorescence intensity (GMF). The hash mark indicates the median and error bars are the interquartile ranges (25th and 75th).

Figure 4. The effect of cenicriviroc (CVC) on adhesion molecule expression on human aortic endothelial cells (HAoECs) after stimulation with MCP-1 only (left panels) or MCP-1 and peripheral blood mononuclear cells (PBMCs) (right panels).

Cell surface expression of E-selectin (involved in loose tethering; top row), CD99 (involved in diapedesis; middle row) and PECAM-1 (involved in diapedesis; bottom row), presented as the percentage of the geometric mean fluorescence intensity (GMF) of the baseline control (unstimulated HAoECs). The hash mark indicates the mean and error bars are the standard deviations.