SUPPLEMENTAL METHODS

Populations studied

HIV-infected subjects were recruited from an ongoing clinic-based cohort (SCOPE) based at the University of California, San Francisco. From this cohort, we recruited individuals who were either off antiretroviral therapy for at least one year or on a stable antiretroviral regimen for at least one year. We excluded individuals who had experienced an acute infection or vaccination within the prior eight weeks or who were receiving ongoing treatment with an immune-based therapy such as interferon-alpha. Carotid intima-media thickness (IMT) measurements were available for 29 previously published individuals (8) that were separated in two groups: one group of 16 subjects with a low IMT (below 1 mm) and one group of 13 subjects with a high IMT (over 1 mm) at baseline. Control subjects were selected mainly from those answering advertisements to participate in clinical studies who were of similar age and gender to the HIV-infected participants. Eligible individuals underwent an extensive interview regarding prior cardiovascular risk factors and an in-depth assessment including a detailed interview and structured questionnaire covering HIV disease history (for the HIV-infected subjects), medication exposure, and cardiac risk factors. Total and HDL cholesterol, triglycerides, and glucose were measured from blood obtained in the fasting state. HIV RNA levels were measured using branched chain DNA method (Quantiplex HIV RNA, ChironVersion3.0: Chiron Corporation, Emeryville, California, USA). Antibodies (IgG) against CMV were measured using enzyme immunoassay (Quest Diagnostics, Nichols Institute, San Juan Capistrano, California, USA). All HIV-infected subjects tested in this study were CMV-positive. The University of California, San
Francisco Committee on Human Research approved the study and all subjects provided written informed consent.

**Carotid Intima-Media Thickness measurements**

Carotid IMT was measured by high resolution ultrasound with the GE VividSeven Imaging System and a 10MHz linear array probe, as described previously (1). Briefly, carotid IMT was measured in 12 predefined segments (six segments per side) using the standardized protocol of the Atherosclerosis Risk in Communities (ARIC) Study, which includes measurements of the near and far wall of the common carotid, the carotid bifurcation, and the internal carotid (13). The mean value of 12 segments was calculated. All scans were performed and measurements obtained on digital images using manual calipers by a single experienced vascular technician who was blinded to the subject’s clinical status. The measurement reproducibility in our laboratory has been described previously and is greater than 0.9 (1). Baseline carotid IMT measurements were obtained within one month of the immunophenotyping analysis. Each subject had a mean of 3.8 ± 1.3 measurements for a mean follow-up of 3.6 ± 1.6 years. IMT progression was calculated as the mean IMT at follow up – mean IMT at baseline / duration of follow up (in years).

**Flow cytometry**

The panels of antibodies used for phenotypic detection and intracellular cytokine detection are described in Table S1. Cytokine assays were performed *in vitro* on 5 × 10^5 cells after no stimulation or stimulation with media or anti-CD3 (1 µg/ml) – anti-
CD28 (2.5 µg/ml) antibodies (BD Pharmingen) for 6 hours at 37°C in the presence of brefeldin A (GolgiPlug, BD Pharmingen). A pool of 138 peptide sequences (15-mers) spanning the entire amino acid sequence of the CMV pp65 protein, overlapping by 11 amino acid residues, was used for testing the CMV specificity of T cells (final concentration: 2 µg/ml). Cytokine detection and phenotyping were performed by cell surface staining and subsequent intracellular staining following the manufacturer’s instruction. FACS analysis was performed on one four-laser BD LSR-II flow cytometers and data were analyzed with FlowJo software v8-6 (Treestar) and transferred into analysis and graphic software including Excel (Windows), and/or GraphPad Prism4. All analyses were carried out without knowledge of the subject’s clinical status, including IMT measurements.

**Enzyme-linked immunosorbent assay**

Soluble CX3CL1 was detected in culture supernatants with a commercially available enzyme-linked immunosorbent assay (ELISA) detection kit (R&D Systems), performed according to the standard protocol. All samples were prepared in duplicate and graphed as the mean and standard deviation.

**Cell culture**

Human artery endothelial cells (HAEC) were purchased from Clonetics (BioWhittaker) and maintained with EGM-2-MV BulletKit (Clonetics). Cells from passages 2–4 were used for experiments. The purity of endothelial cells was verified in each passage by immunofluorescence staining of paraformaldehyde-fixed slide cultures with rabbit
polyclonal anti-human Von Willebrand Factor (Abcam). To form polarized monolayers, the cells were seeded on 12-mm-diameter permeable filters (3 µm pore size, Transwell; Corning) coated with fibronectin (10 µg/cm²; Sigma) and maintained for 2–4 weeks. The formation of confluent monolayers was monitored by staining the cells with mouse anti-human MAb to VE-cadherin (R&D systems).

**In vitro CMV infection**

VR1814, an endothelial cell–tropic clinical strain of CMV isolated from the cervix, was adapted for growth in HAEC (14). HAEC were infected (1 PFU/cell) in EBM Basal Medium (Clonetics) containing 1% fetal bovine serum (HyClone) for 2 hours; the inoculum was then removed, replaced with fresh medium, and the cells were monitored daily. CMV infection was identified by production of its characteristic cytopathic effects (CPE) and immunostaining for CMV immediate early proteins (Millipore).

**Co-cultures of PBMC or subset populations with HAEC monolayers**

Co-cultures were set up with resting confluent primary HAEC monolayers that had been infected or not by CMV and that were overlaid with either $10^6$ PBMC or CD4+ T cells. CMV infection of HAEC was assessed when more than 50% of the cells showed CPE. The CD4+ T cells were depleted from PBMCs according to the manufacturer’s instructions by using a positive selection MidiMACS system and LD immunomagnetic columns (Miltenyi Biotech). Co-cultured cells were incubated in a humidified incubator overnight at 37°C in 5% CO₂. Co-cultures were maintained in a 50:50 mix of RPMI and EGM-2 medium (Clonetics) to support both cell types.
Neutralization assays

Neutralization assays with antibodies specific for TNFα and IFNγ were carried out by adding the specific antibodies at the time of the co-cultivations of PBMC with CMV-infected HAEC. Commercially available antibodies used in the neutralization assays were anti-IFNγ monoclonal antibody B27 (BD Pharmingen), anti-TNFα monoclonal antibody MABTNF-A5 (BD Pharmingen), and purified mouse IgG1 antibody clone 107.3 and mouse IgG2a,K as isotype controls (BD Pharmingen). All antibodies were prepared without azide. The manufacturer’s recommended concentrations were used for neutralization assays.

Transendothelial migration assay

The transwell inserts used were 12 mm in diameter and had 3 µm pore size polycarbonate membranes in 12-well polystyrene plates (Corning). HAEC were seeded on the membrane to form polarized monolayers. CD4⁺ T cells were enriched from PBMC populations according to the manufacturer’s instructions by using a negative selection MidiMACS system and LS immunomagnetic columns (Miltenyi Biotech). Recombinant human CX3CL1 or control media were loaded into lower well chambers using a volume of 1ml. The upper insert was placed into the well, and enriched CD4⁺ T cells (10^5) in a 250 µl volume of control media were placed into the upper-chamber well inserts with the lower compartment containing varying concentrations (0, 10, 50 and 200 ng/ml) of human recombinant CX3CL1. Transwell assays were placed in a 5% CO₂/37°C humidified incubator for four hours. The number and percentage of input CD4⁺ T cells and of CD4⁺CX3CR1⁺ T cells deposited in the upper compartment were determined by
flow cytometric analysis. For each assay, three replicate wells were set up, and the
transmigrated populations were counted from each well independently. Results are
expressed as the ratio and percentage of migrated cells for each dilution of chemottractant
and control media.

**Immunohistochemistry assay**

Specimens of coronary arteries were collected from autopsies (NCI AIDS and Cancer
Specimen Resource, UCSF). They were fixed in buffered formalin, embedded in paraffin,
sectioned, and stained with hematoxylin and eosin. Sections were examined by
hematoxylin–eosin stain, and classified into minimal atherosclerotic and diffuse
atherosclerotic coronary arteries. Five μm-thick sections were prepared for staining.
Tissue sections were rehydrated, incubated in a pressure cooker with EDTA buffer for
antigen retrieval, and treated with 3% H₂O₂ to block endogenous peroxidase activity and
with TBS with 1% BSA to block nonspecific conjugation. Slides were incubated with a
mouse monoclonal antibody to CD4 (clone 1F6, 2 hours; Thermoscientific), a mouse
monoclonal antibody to CD3 (clone PS1, 1 hour; Thermoscientific), and a rabbit
polyclonal antibody to CX3CR1 (1 hour; Thermoscientific) at room temperature.
Detection of primary antibodies was performed with horseradish peroxidase polymer
(DAKO Envision kit)–conjugated antibodies to mouse or rabbit and developed with 3,3′-
diaminobenzidine. Counterstains were done with Mayer’s hematoxylin. Paraffin sections
of tonsils served as positive control. Negative controls included replacement of the
primary antibody by TBS/BSA.
Statistical analyses

Exact nonparametric two-tailed tests were used. The nonparametric Mann-Whitney test was used to compare continuous variables. The Fisher’s exact test was used to compare dichotomous variables. The Spearman rank correlation test was used to determine correlations between variables, with \( r \) being the Spearman correlation coefficient. Statistical analysis was performed with GraphPad Prism 5.01 software. Multiple linear regression was used to assess the strength of association between pairs of variables while adjusting for confounders (e.g., age). P values of <0.05 were considered statistically significant.
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Flow cytometry gating strategy

Gating strategy by flow cytometry for the detection of T cells [singlet (forward scatter A (FSC-A) × FSC-H diagonal), live [Aqua]-, CD3^+ cells]), B cells (singlet, live, CD19^+ cells), CD4^+ T cells (singlet, live, CD3^+CD4^+ cells), CD8^+ T cells (singlet, live, CD3^+CD8^+ cells), monocytes (singlet, live, CD3^-CD19^-CD14^+ cells), and NK cells (singlet, live, CD3^-CD19^-CD16^+ cells).

Figure S2. HIV viral load is not correlated with CD4^+CXCR1^+ T cells or with IMT and IMT is not associated with protease inhibitor treatment

(A) Absence of correlation between the frequency of CD4^+ T cells expressing CX3CR1 and HIV RNA measurement in plasma (log10 copies/ml) at baseline. (B) Comparison of the HIV RNA measurement in plasma (log10 copies/ml) between HIV-infected subjects separated into two groups: one with low (<1 mm) and another with high (≥1 mm) IMT. (C) No statistical difference in baseline IMT measurement (mm) between HIV-infected subjects treated (PI, protease inhibitor) or not (no PI) with a protease inhibitor. ART refers to antiretroviral therapy.

Figure S3. CD4^+CX3CR1^+ T cells are CD57^+, CCR7^-, and PD-1^+

(A) Flow cytometric analysis of CD57 (top), CCR7 (middle), and PD-1 (bottom) expression on CD4^+CX3CR1^- T cells (left) and on CD4^+CX3CR1^+ T cells (right) from a representative subject. (B) Statistical comparison of the expression of CD57 (top), CCR7 (middle), and PD-1 (bottom) on CD4^+ T cells that express and that do not express CX3CR1. The analysis was carried out on PBMCs from 16 HIV-infected subjects.
**Figure S4. CMV-pp65-specific CD4^+ T cells**

Flow cytometric analysis of IFN\(\gamma\) and TNF\(\alpha\) production after CMV-pp65 stimulation of CD4^+ T cells (left panel) from a representative subject. Mean production of IFN\(\gamma\) and TNF\(\alpha\) (right panel) by CD4^+ T cells upon CMV-pp65 stimulation, as analyzed in 16 HIV-infected subjects.

**Figure S5. Immunohistochemical detection of CX3CR1 at a late stage of atherosclerotic disease.**

Immunohistochemical staining of CD3, CD4, and CX3CR1 in adjacent sections of the same coronary artery showing late atheromatous lesions from an HIV^+^ subject. CX3CR1^+^ cells (x20, bottom) are present in the lumen of a small blood vessel running in the adventitia of the artery. No CD3^+^ (x20, top) or CD4^+^ immunoreactive cells (x20, middle) were detected.

**Figure S6. A model for CMV-induced T cell immunopathology in HIV-associated atherosclerosis**

Immune dysfunction associated with HIV infection is associated with: (1) an increased frequency of circulating CD4^+CX3CR1^+ T cells, some of which are specific for CMV, (2) increased presentation of CMV by endothelial cells to T cells, (3) increased production of TNF\(\alpha\) and IFN\(\gamma\) by CMV-stimulated CD4^+ T cells, which then induce production of the ligand CX3CL1 in the endothelium, and (4) migration of pro-inflammatory CD4^+CX3CR1^+ T cells through the endothelium.
Table S1. Panels of antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>Dye</th>
<th>Antigen</th>
<th>Clone</th>
<th>Manufacture</th>
<th>Dilution</th>
<th>Antigen</th>
<th>Clone</th>
<th>Manufacture</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>CX3CR1</td>
<td>2A9-1</td>
<td>MBL</td>
<td>25</td>
<td>CX3CR1</td>
<td>2A9-1</td>
<td>MBL</td>
<td>25</td>
</tr>
<tr>
<td>PerCP</td>
<td>CD4</td>
<td>SK3</td>
<td>BD</td>
<td>20</td>
<td>CD4</td>
<td>SK3</td>
<td>BD</td>
<td>20</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>HLA-DR</td>
<td>L243</td>
<td>BD</td>
<td>250</td>
<td>TNFα</td>
<td>Mab11</td>
<td>BD</td>
<td>100</td>
</tr>
<tr>
<td>PE-TR</td>
<td>CD14</td>
<td>RM-052</td>
<td>Coulter</td>
<td>50</td>
<td>CD45RA</td>
<td>2H4</td>
<td>Coulter</td>
<td>50</td>
</tr>
<tr>
<td>Al700</td>
<td>CD19</td>
<td>HIB19</td>
<td>BD</td>
<td>50</td>
<td>CD27</td>
<td>O323</td>
<td>eBioscience</td>
<td>25</td>
</tr>
<tr>
<td>APC</td>
<td>CD8</td>
<td>RPA-T8</td>
<td>BD</td>
<td>50</td>
<td>CD8</td>
<td>RPA-T8</td>
<td>BD</td>
<td>50</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>CD3</td>
<td>SP34-2</td>
<td>BD</td>
<td>25</td>
<td>CD3</td>
<td>SP34-2</td>
<td>BD</td>
<td>25</td>
</tr>
<tr>
<td>PB</td>
<td>CD16</td>
<td>3G8</td>
<td>BD</td>
<td>50</td>
<td>IFNγ</td>
<td>4SB3</td>
<td>eBioscience</td>
<td>100</td>
</tr>
<tr>
<td>Amcyan</td>
<td>Live/Dead</td>
<td>Invitrogen</td>
<td>250</td>
<td>Live/Dead</td>
<td>Invitrogen</td>
<td>250</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CMV-pp65 stimulation

% CMV-specific CD4+ T cells

IFNγ  |  TNFα
CMV-specific T cells

CD4^+CX3CR1^+ T cells

CMV-antigen presentation

TNFα, IFNγ

CX3CL1

Transendothelial migration

Endothelium

Arterial wall