The CD16+ Monocyte Subset Is More Permissive to Infection and Preferentially Harbors HIV-1 In Vivo


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The CD16⁺ Monocyte Subset Is More Permissive to Infection and Preferentially Harbors HIV-1 In Vivo


HIV-1 persists in peripheral blood monocytes in individuals receiving highly active antiretroviral therapy (HAART) with viral suppression, despite these cells being poorly susceptible to infection in vitro. Because very few monocytes harbor HIV-1 in vivo, we considered whether a subset of monocytes might be more permissive to infection. We show that a minor CD16⁺ monocyte subset preferentially harbors HIV-1 in infected individuals on HAART when compared with the majority of monocytes (CD14⁺/CD16⁻). We confirmed this by in vitro experiments showing that CD16⁺ monocytes were more susceptible to CCR5-using strains of HIV-1, a finding that is associated with higher CCR5 expression on these cells. CD16⁺ monocytes were also more permissive to infection with a vesicular stomatitis virus G protein-pseudotyped reporter strain of HIV-1 than the majority of monocytes, suggesting that they are better able to support HIV-1 replication after entry. Consistent with this observation, high molecular mass complexes of apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) were observed in monocytes, suggesting that they are permissive for HIV-1 infection. This is in part attributed to the presence of anatomical and cellular sanctuary sites where HIV-1 can persist due to a combination of poor drug penetration, viral latency, and low-level ongoing replication [1–3].

Resting memory T cells are the major cellular HIV-1 reservoir (1, 3, 4). However, monocytes and macrophages are thought to be underestimated sources of HIV-1 (5), and genetic studies during remission, and strains of HIV-1 that exclusively use CXCR4 can also infect cells of the macrophage lineage (20–23). The Journal of Immunology, 2007, 178: 6581–6589.
Because <0.1% of monocytes harbor HIV-1 in vivo (13), this raises the possibility that a subset of monocytes may be more permissive to HIV-1 infection. A minor proportion of monocytes express CD16 (FcγRIII) and variable levels of CD14 on their surface (CD16+ monocytes) and are distinguishable from the majority of monocytes that do not express CD16 and have high CD14 expression (CD14highCD16+). The CD16+ monocyte subset is a heterogeneous population of cells that may represent a transitional stage between monocytes and macrophages (28–30) or dendritic cells (28, 31–35). Based on observations using an in vitro model of transendothelial migration, Randolph et al. proposed that CD16+ monocytes contain dendritic cell precursors that transiently migrate through tissue and to lymph nodes (36). CD16+ monocytes have recently been shown to increase the susceptibility of resting T cells to HIV-1 infection by producing high levels of CCL24 and CCL2 chemokines (37).

In healthy individuals the CD16+ monocyte subset represents ~10% of circulating monocytes, but the proportion of this subset is reportedly increased in a number of pathological conditions including sepsis (38, 39), atherosclerosis (40), and reactive arthritis (30). The proportion of CD16+ monocytes is also reportedly elevated in HIV-1-infected individuals with advanced disease (29, 41, 42) or HIV-associated dementia (41). Our laboratory recently showed that the number of CD16+ monocytes was elevated only in HIV-1-infected individuals not currently receiving antiretroviral therapy. We concluded that HIV-1 infection in the setting of HAART is associated with normal monocyte function and phenotype (43). Nevertheless, the role of these cells in the pathogenesis of HIV-1 disease is unknown.

In this study we compare phenotypically defined CD16+ monocytes to CD14highCD16+ monocytes in their ability to support HIV-1 infection in vivo and in vitro. Our data show that CD16+ monocytes are more permissive to productive HIV-1 infection in vivo and in vitro than the majority of blood monocytes and that this relative permissiveness is due to enhanced viral entry and intracellular replication. CD16+ monocytes provide a continuing source of HIV-1 persistence in patients receiving HAART.

Materials and Methods

Human research ethics

All human blood samples used in this study were collected with informed consent and approval from The Alfred Hospital Human Research Ethics Committee (Melbourne, Victoria, Australia).

Isolation of monocyte subsets

Peripheral blood was collected in K3EDTA tubes from HIV-infected volunteers receiving antiretroviral therapy attending the Alfred Hospital Infectious Diseases Clinic (Melbourne, Victoria, Australia). PBMC were isolated by Ficoll (Amersham Pharmacia) density gradient centrifugation. Monocyte subsets were subsequently isolated by either flow cytometric sorting or magnetic bead technology using either the Dynabead (Dynal Biotech) or the MACS (Miltenyi Biotec) system.

Flow cytometric sorting.

For semiquantitative PCR detection of HIV-1 DNA, T cells were first depleted from PBMC by using the MACS monocyte isolation kit II and LD column as per the manufacturer’s instructions. CD16+ monocytes were then positively enriched using anti-CD16 mAb (3G8 hybridoma supernatant) and pan-mouse Dynabeads. Finally, CD14highCD16+ monocytes were positively enriched using anti-CD14-Dynabeads. Alternatively, monocytes were negatively selected using the MACS monocyte isolation kit II and LD column as per the manufacturer’s protocol (Miltenyi Biotec), resulting in >99% pure total monocytes while maintaining CD14highCD16+ and CD16+ monocyte subsets. In selected experiments, monocytes were stained with CD56. Staining routinely showed <1% NK cell contamination (not shown). Anti-CD16 microbeads (Miltenyi Biotec) were used to positively enrich (>95%) CD16+ monocytes from the total monocyte pool, and the negative fraction (CD14highCD16+ monocytes) was also collected. Monocyte subsets were subsequently adhered to plastic tissue culture plates in Iscove’s modified DMEM (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated human serum (Sydney Red Cross Blood Service, Sydney, Australia), 2 mM l-glutamine (Invitrogen Life Technologies), and 50 μg/ml gentamicin (Delta West) (HI10) and washed with PBS to remove residual nonadherent lymphocytes before the lysates were prepared to further ensure the purity of the cell isolation. In three test experiments (using separate donors to those providing blood for the subset experiments), total monocyte populations purified using this method were assessed for T cell contamination by RT-PCR for TCR mRNA as previously described (13, 44). TCR mRNA was below detection in the equivalent of 1 105 monocytes in all three experiments, suggesting <0.01% T cell contamination.

Cell lysates

Purified monocyte subsets and CD4+HLADR CD45RO memory T cells were lysed in 1 × PCR buffer (Qiagen) with 0.5% (v/v) Triton-X100, 0.5% (v/v) Nonidet P-40, and 75 μg/ml proteinase K (PCR lysis buffer; Roche) and incubated at 60°C for 1 h before proteinase K was inactivated at 95°C for 15 min.

Virus stocks

HIV-1 reverse transcriptase activity of viral stocks was quantified as previously described (45). HIV-1NL4-3 was amplified in PHA-stimulated PBMC and treated with 10 U/ml RNase-free DNase (Roche) for 1 h at 37°C (46, 47). Single-round HIV-1 recombinant luciferase reporter viruses were generated by the transfection of 293T cells using methods modified from those previously described (48–50). Reporter viruses expressing CCR5- and CXCR4-tropic HIV-1 envelopes were produced by cotransfection of the pNL4-3mid luc plasmid containing the pSVIII plasmid carrying the env gene of HIV-1ADA or HIV-1KSU2 by using the calcium phosphate precipitation method. Reporter HIV-1-pseudotyped viruses were made by cotransfection of pNL4-3env Luc with pSVIII-VSV-G (vesicular stomatitis virus envelope glycoprotein) or pSVIIIΔKsenv (a nonfunctional envelope glycoprotein) using the Lipofectamine transfection method (Invitrogen Life Technologies). Culture supernatants containing reporter virus were clarified by centrifugation at 10,000 g for 10 min and filtered through 0.45-μm pore-sized syringe-top filters (Sartorius). VSVG and AKS viruses were concentrated by ultracentrifugation through a 20% sucrose cushion in an L-90 ultracentrifuge (Beckman Coulter) at 93,000 × g at 4°C for 1 h.

Viral entry assays

Monocytes were enriched from buffy coats from HIV-seronegative donors by Ficoll density gradient centrifugation followed by countercurrent elutriation and then incubation with anti-CD14-PE and anti-CD16-FITC mAb (BD Biosciences) for 30 min. Cells were washed and fixed overnight with 3% methanol-free formaldehde (Polysciences) in magnesium- and calcium-free PBS and then monocyte subsets were sorted (FACSStarTM; BD Biosciences). For real-time PCR analysis of HIV-1 DNA copy numbers, PBMC were incubated with anti-CD14-PE, anti-CD16-PE, anti-CD4-FITC, anti-CD45RO-PE-Texas red and anti-HLA DR-allophycocyanin mAbs (BD Biosciences) for 30 min on ice, washed, and resuspended in PBS (Invitrogen Life Technologies) with 1% FBS (FACS wash buffer) and 2 mM EDTA. Monocyte subsets were sorted based on light scatter characteristics and CD14 and CD16 expression by using a FACSAria high-speed cell sorter (BD Biosciences). Resting memory T cells were defined as CD4+HLA DR+ or CD45RO+. Twenty milliliters of patient blood typically allowed the isolation of ~1 × 106 CD14highCD16+ monocytes and 1 × 106 CD16+ monocytes by using this method.

Magnetic bead isolation.

T cells and NK cells were first depleted from patient PBMC with anti-CD3 Dynabeads and anti-CD56 mAb (BD Biosciences) followed by anti-mouse Dynabeads according to the manufacturer’s instructions. CD16+ monocytes were then positively enriched using anti-CD16 mAb (3G8 hybridoma supernatant) and pan-mouse Dynabeads. Finally, CD14highCD16+ monocytes were positively enriched using anti-CD14-Dynabeads. Alternatively, monocytes were negatively selected using the MACS monocyte isolation kit II and LD column as per the manufacturer’s protocol (Miltenyi Biotec), resulting in >99% pure total monocytes while maintaining CD14highCD16+ and CD16+ monocyte subsets. In selected experiments, monocytes were stained with CD56. Staining routinely showed <1% NK cell contamination (not shown). Anti-CD16 microbeads (Miltenyi Biotec) were used to positively enrich (>95%) CD16+ monocytes from the total monocyte pool, and the negative fraction (CD14highCD16+ monocytes) was also collected. Monocyte subsets were subsequently adhered to plastic tissue culture plates in Iscove’s modified DMEM (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated human serum (Sydney Red Cross Blood Service, Sydney, Australia), 2 mM l-glutamine (Invitrogen Life Technologies), and 50 μg/ml gentamicin (Delta West) (HI10) and washed with PBS to remove residual nonadherent lymphocytes before the lysates were prepared to further ensure the purity of the cell isolation. In three test experiments (using separate donors to those providing blood for the subset experiments), total monocyte populations purified using this method were assessed for T cell contamination by RT-PCR for TCR mRNA as previously described (13, 44). TCR mRNA was below detection in the equivalent of 1 105 monocytes in all three experiments, suggesting <0.01% T cell contamination.
recombinant luciferase reporter viruses (4 RTU/cell) in IH10 by spinocu-
lation to enhance infection (51, 52). Cells were exposed to virus for 2 h at
37°C before being washed as described above and cultured for a further 5
days. Cells were then lysed in a cell culture lysis reagent (Promega) and
assayed following the addition of 10 µl of cell lysate to 50 µl of a luci-
ferase assay reagent (Promega) using a TD-20/20 luminometer (Turner
Designs).

Detection of HIV-1 DNA

For each experiment, cell numbers were standardized by real-time PCR for
the CCR5 gene using the primers LK46 and LK47 and the beacon LK155
as previously described (53) or iQ SYBR Green Supermix (Bio-Rad) in
selected experiments. Real-time PCR quantification of HIV-1 DNA was
used for two of six in vitro infection experiments with HIV-1gag and for
the comparison of HIV-1 copy numbers in cell populations by amplification of
a conserved region of HIV-1 gag-5’LTR using the primers SL19 and SL20
and the beacon SL30 as described previously (53, 54). Standard curves were
generated using known numbers of ACH2 cells, which contain one integrated
HIV-1 provirus per cell in the HIV-1-negative PBMC background.

Semi-quantitative PCR was used for the detection of HIV-1 DNA in monocyte
subsets for all other experiments. Briefly, a conserved region of
HIV-1 gag was amplified from monocyte lysates and standardized by real-
time PCR as described above in a 25-µl reaction containing 200 nM primers
A2B2 and HotStar Taq (Qiagen) (10). Products were resolved on 2% agarose
gels containing ethidium bromide and visualized under UV light.

The assay had a lower detection limit of <10 10^6 cells (one proviral copy
per cell). Densitometry (Fuji Image Gauge version 3.3) of PCR products
was used to estimate HIV-1 gag expression levels in monocyte subsets
by comparison with the 8E5 standard. HIV-1 DNA was always detected at a
higher level in monocytes cultured at 37°C compared with control cells
kept on ice for the infection period. Despite DNase treatment, trace levels
of contaminating viral DNA persisted in HIV-1Ba-L stocks in some exper-
iments. In these cases, the level of background viral DNA in control sam-
iples was subtracted from the matched sample cultured at 37°C.

HIV-1 receptor expression

Peripheral blood was collected in K3EDTA tubes from HIV-infected and
uninfected volunteers and prepared for flow cytometric analysis within 6 h.
Briefly, 250 µl of whole blood was washed with FACS wash and then
incubated with anti-CD14-PE, anti-CD14-CyChrome, anti-CD4-FITC or
anti-CCR5-FITC, and anti-CXCR4-allophycocyanin mAbs or isotype-
matched control mAbs (BD Biosciences) for 30 min on ice. Erythrocytes
were lysed (FACS lysis solution; BD Biosciences) and the cells were then
washed with FACS wash buffer, fixed in 200 µl of 3% formaldehyde in
PBS, and analyzed by flow cytometry (FACSCalibur; BD Biosciences). A
monocyte gate was established using light scatter characteristics, and then
monocyte subset gates were established using CD14 and CD16 fluores-
cence. CD14^highCD16^ and CD14^highCD16^, and CD14^highCD16^ monocytes
were gated as shown in Fig. 3. Net mean fluorescence intensity of CD4, CCR5,
and CXCR4 were calculated on monocyte subsets by subtracting
the background fluorescence of isotype control stained cells from the spe-
cific staining of cells from each donor.

APOBEC3G analysis

Monocytes were enriched from PBMC by countercurrent elutriation. Con-
taminating lymphocytes were depleted using anti-CD2, anti-CD3, and anti-
CD20 mAbs (BD Biosciences) followed by anti-mouse IgG MACS beads
(Miltenyi Biotec). Monocyte subsets were separated by positively selecting
for CD16 expression as described above. Cells were separated using an
autoMACS (Miltenyi Biotec). Cells were snap frozen in liquid nitrogen and
then lysed and analyzed for APOBEC3G by fast protein liquid chroma-
tography and Western blotting as previously described (24).

Statistical analysis

Flow cytometric data of HIV-1 receptors on monocyte subsets were ana-
lyzed using the Mann-Whitney U test. Infection of monocyte subsets in
vitro was analyzed by a paired two-tailed t test. The detection of HIV-1
DNA in monocyte subsets isolated from HIV-infected individuals by con-
ventional PCR was analyzed using the Fisher’s exact test. Wilcoxon signed
rank test was used to analyze HIV-1 DNA by real-time PCR in
CD14^highCD16^, CD16^, and CD4 memory T cells isolated from HIV-
infected individuals. Correlation was determined by the Spearman rank
order correlation test. p < 0.05 was considered statistically significant.

Results

CD16^ monocytes preferentially harbor HIV-1 in infected
individuals

Initially, 17 patients receiving HAART were studied, 12 of whom
showed incomplete viral suppression as evident by viral loads of
>50 copies/ml on more than one occasion (Table I). One patient
(patient 1) had maintained a viral load below detectable limits for
>5 years (n = 33 assays) before sampling for this study. Analysis
of the other 16 patients studied (patients 2–17) showed that their viral
load decreased by a mean of 4.96 log_{10} from their pretherapy or the
highest recorded viral load attributable to antiretroviral therapy.

<table>
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<tr>
<th>Patient No.</th>
<th>CD14^highCD16</th>
<th>CD16</th>
<th>CD4 Count (cells/µl)</th>
<th>Nadir CD4 (cells/µl)</th>
<th>Viral Load (copies/ml)</th>
<th>Pretherapy Viral Load (copies/ml)</th>
<th>Therapy Regimen</th>
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<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>259</td>
<td>149</td>
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<td>Undetectable</td>
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<td>-</td>
<td>+</td>
<td>1842</td>
<td>230</td>
<td>50</td>
<td>900</td>
<td>d4T, IDV</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>152</td>
<td>130</td>
<td>15,500</td>
<td>28,500</td>
<td>3TC, d4T, NVP</td>
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<td>4</td>
<td>-</td>
<td>+</td>
<td>178</td>
<td>2</td>
<td>&lt;50</td>
<td>&gt;100,000</td>
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<td>+</td>
<td>426</td>
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<td>600</td>
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<td>+</td>
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<td>510</td>
<td>&lt;50</td>
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<td>757</td>
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<td>201</td>
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<td>+</td>
<td>+</td>
<td>520</td>
<td>10</td>
<td>300</td>
<td>&gt;100,000</td>
<td>AZT, ddl, NVP, RTV</td>
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</tbody>
</table>
We compared the detection of HIV-1 DNA in highly purified CD14<sup>high</sup>CD16<sup>−</sup> and CD16<sup>+</sup> monocyte subsets from these 17 patients. Purified monocyte subsets contained <0.01% total T cell contamination (Fig. 1A) as determined by the quantification of TCR mRNA in selected experiments (13, 44). HIV-1 DNA was detected by semiquantitative PCR using primers that amplify a highly conserved region of gag against gag-TCR RT-PCR. HIV-1 DNA was detectable but below a quantifiable threshold of 10 copies per 10<sup>6</sup> cells (n = 11; limit of detection 10 copies per 1 × 10<sup>6</sup> cells).

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** CD16<sup>+</sup> monocytes preferentially harbor HIV-1 in patients receiving HAART. *A*, Three monocyte isolation preparations (lanes marked A, B, and C) were tested for T cell contamination by an in-house TCR RT-PCR. *B*, Detection of HIV-1 DNA in 1 × 10<sup>6</sup> CD16<sup>−</sup> and CD14<sup>high</sup>CD16<sup>−</sup> monocytes of patients (n = 17) by PCR against gag (limit of detection ≤10 copies) and CD16<sup>−</sup> monocytes (0–535 copies) compared with resting memory T cells (0–3280 copies) per 1 × 10<sup>6</sup> cells (n = 11; limit of detection 10 copies per 1 × 10<sup>6</sup> cells).

A CD14<sup>high</sup>CD16<sup>−</sup> monocyte preparation had a median range of 0.3% (0.1–1.1%) CD4<sup>+</sup> T cell contamination and the CD16<sup>+</sup> monocyte had a median range CD4<sup>+</sup> T cell contamination of 0.6% (0.2–1.4%). HIV-1 DNA was detectable but below a quantifiable threshold of 10 copies per 1 × 10<sup>6</sup> cells.

Areal T cell contamination (Fig. 1A) as determined by the quantification of TCR mRNA in selected experiments (13, 44). HIV-1 DNA was detected by semiquantitative PCR using primers that amplify a highly conserved region of gag against gag-TCR RT-PCR. HIV-1 DNA was detectable but below a quantifiable threshold of 10 copies per 10<sup>6</sup> cells (n = 11; limit of detection 10 copies per 1 × 10<sup>6</sup> cells).

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** CD16<sup>+</sup> monocytes were more permissive to infection with HIV-1 in vitro. *A* and *B*, CD14<sup>high</sup>CD16<sup>−</sup> and CD16<sup>+</sup> monocyte subsets were isolated by flow cytometric sorting from six HIV-seronegative buffy coats on the day of venepuncture. Equal numbers of monocyte subsets were immediately exposed to DNase-treated HIV-1Ba-L at an multiplicity of infection of ~0.06 infectious units and cultured for a further 36 h at 37°C. *C* and *D*, Real-time PCR using gag-5'LTR primers (donors 29 and 30) or semiquantitative conventional PCR using gag primers (donors 31–34) (C) and densitometric comparison to an 8E5 cell standard curve (D, left panel) was used to detect infection. An example of the detection of HIV-1 DNA in monocyte subsets cultured at 37°C or on ice (0°C) by conventional PCR is also shown (D, right panel).
in the CD16⁺ monocyte subset, although the difference did not achieve significance (p = 0.13). There was no correlation between the percentage of CD16⁺ monocytes and the amount of HIV-1 DNA detected within this population (r = -0.41) in this data set.

**CD16⁺ monocytes are more permissive to HIV-1 infection in vitro than the majority of monocytes**

To confirm the above findings, monocyte subsets were evaluated for susceptibility to HIV-1 infection in vitro. CD14<sup>high</sup>CD16⁻ and CD16⁺ monocyte subsets isolated from six HIV-seronegative donors by high speed flow cytometric sorting (Figs. 2, A and B) were infected within 1 h of isolation with CCR5-using HIV-1<sub>Ba-L</sub> (4 RTU/cell; multiplicity of infection of 0.11). Three subsets of monocytes, CD14<sup>high</sup>CD16⁻, CD14<sup>high</sup>CD16⁺, and CD14<sup>low</sup>CD16⁺, were identified and further examined for CD4, CXCR4, and CCR5 expression (Table III). The figures are derived from a representative HIV-seronegative donor.

**HIV-1 coreceptors are differentially expressed on monocyte subsets**

To determine whether the susceptibility to infection in monocyte subsets correlated with receptor expression, we examined the expression of the major HIV-1 receptors CD4, CCR5, and CXCR4 on monocyte subsets of HIV-1-infected and uninfected volunteers using a whole blood flow cytometric assay (56, 57). Monocytes from HIV-1-infected individuals were differentially gated from other leukocytes by size (forward light scatter) and granularity (side light scatter) (Fig. 3A). Three monocyte subsets were defined using CD14CD16 expression patterns, namely CD14<sup>high</sup>CD16⁻ monocytes, CD14<sup>high</sup>CD16⁺ monocytes, and CD14<sup>low</sup>CD16⁺ monocytes (Fig. 3B). Compared with CD14<sup>high</sup>CD16⁻ and CD14<sup>low</sup>CD16⁺ monocyte subsets, CD14<sup>high</sup>CD16⁺ monocytes expressed ~20–40% higher levels of CD4 (n = 5; both p < 0.01) and 2- to 5-fold higher levels of CCR5 (n = 14; p = 0.05 and 0.09 respectively; Table III) in terms of both the percentage positive and the mean fluorescence intensity. Conversely, CD14<sup>low</sup>CD16⁻ monocytes expressed 4-fold higher CXCR4 compared with CD14<sup>low</sup>CD16⁻ monocytes and ~2-fold higher CXCR4 than CD14<sup>high</sup>CD16⁻ monocytes (n = 6; p = 0.03 and 0.005 respectively; Table III). The expression of CD4 and CCR5 on monocytes was similar in HIV-1-infected and HIV-1-seronegative individuals. Although there was no significant alteration in mean fluorescence intensity of CXCR4 expression on CD14<sup>high</sup>CD16⁻ monocytes (p = 0.07), CD14<sup>high</sup>CD16⁺ monocytes (p = 0.09), and CD14<sup>low</sup>CD16⁻ monocytes were more susceptible to infection by CCR5 using reporter strains and are inherently more permissive for HIV-1 replication than CD14<sup>high</sup>CD16⁻ monocytes. CD14<sup>high</sup>CD16⁻ monocytes were exposed to reporter strains of HIV-1 carrying CCR5-using (HIV-1<sub>ADA</sub>) or CXCR4-using (HIV-1<sub>HXB2</sub>) envelopes or the envelope of VSV, which permits entry independent of CD4 and coreceptors. Infection was detected by the luciferase activity in cell lysates at 36 h. A reporter HIV-1-pseudotyped virus with a nonfunctional envelope (HIV-1<sub>KS</sub>) was used as a negative control to determine the background levels of luciferase activity.

**FIGURE 3.** Gating of monocyte subsets for analysis of HIV-1 receptor expression. Monocyte subsets present in whole blood were identified using light scatter characteristics (left panel, Monocyte Gate) and CD14/CD16 expression patterns (right panel). Three subsets of monocytes, CD14<sup>high</sup>CD16⁻, CD14<sup>high</sup>CD16⁺, and CD14<sup>low</sup>CD16⁺, were identified and further examined for CD4, CXCR4, and CCR5 expression (Table III). The figures are derived from a representative HIV-seronegative donor.

**FIGURE 4.** CD16⁺ monocytes were more susceptible to infection by CCR5 using reporter strains and are inherently more permissive for HIV-1 replication than CD14<sup>high</sup>CD16⁻ monocytes. CD14<sup>high</sup>CD16⁺-sorted (gray bars) and CD16⁻-sorted (filled bars) monocyte populations were exposed to reporter strains of HIV-1 carrying CCR5-using (HIV-1<sub>ADA</sub>) or CXCR4-using (HIV-1<sub>HXB2</sub>) envelopes or the envelope of VSV, which permits entry independent of CD4 and coreceptors. Infection was detected by the luciferase activity in cell lysates at 36 h. A reporter HIV-1-pseudotyped virus with a nonfunctional envelope (HIV-1<sub>KS</sub>) was used as a negative control to determine the background levels of luciferase activity.

**Table III. HIV receptor expression on the surface of monocyte subsets**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Donor Type</th>
<th>n</th>
<th>Data Type</th>
<th>CD14&lt;sup&gt;high&lt;/sup&gt;CD16⁻</th>
<th>CD14&lt;sup&gt;high&lt;/sup&gt;CD16⁺</th>
<th>CD14&lt;sup&gt;low&lt;/sup&gt;CD16⁻</th>
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<tr>
<td>CD4</td>
<td>HIV⁻</td>
<td>5</td>
<td>Percentage</td>
<td>92.6 ± 1.2</td>
<td>91.1 ± 1.9</td>
<td>89.7 ± 1.8</td>
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<td></td>
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<td>MFI</td>
<td>21.3 ± 2.8</td>
<td>27.5 ± 3.6</td>
<td>17.0 ± 3.8</td>
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<tr>
<td>CCR5</td>
<td>HIV⁻</td>
<td>14</td>
<td>Percentage</td>
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<td>42.9 ± 7.9</td>
<td>18.8 ± 5.7</td>
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<td>CXCR4</td>
<td>HIV⁻</td>
<td>6</td>
<td>Percentage</td>
<td>52.3 ± 13.8</td>
<td>35.6 ± 13.8</td>
<td>20.2 ± 8.7*</td>
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<td>HIV⁺</td>
<td>10</td>
<td>Percentage</td>
<td>86.8 ± 4.6</td>
<td>67.6 ± 7.1</td>
<td>49.8 ± 8.7*</td>
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<td>MFI</td>
<td>20.7 ± 3.1</td>
<td>13.9 ± 3.6</td>
<td>7.2 ± 2.3</td>
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*Whole blood from non-infected and HIV-infected patients was stained for HIV-1 coreceptor percent expression and mean fluorescent intensity (MFI).

Monocytes were gated according to CD14 and CD16 expression.

*Significant difference (p = 0.04) was found between the percentage of CD14<sup>low</sup>CD16⁻ monocytes that express CXCR4 from HIV-1-infected donors compared with uninfected donors.
CD16+ monocytes ($p = 0.12$) of HIV-1-infected ($n = 10$) compared with HIV-1-seronegative individuals ($n = 6$), there was a significant difference between infected and uninfected individuals in their percentages of CXCR4+ cells within the CD14^{low}CD16+ subset ($p = 0.04$; Table III), indicating a trend toward increased CXCR4 on all monocyte subsets from HIV-1 infected individuals on therapy.

**Mechanism of enhanced susceptibility of CD16+ monocytes**

To further investigate the mechanisms underlying the differences in the susceptibility of the monocyte subsets to HIV-1 infection, we assessed the effect of specific viral envelopes on the ability of HIV-1 to enter and initiate productive infection of these cells. Previously, we (13) and others (58, 59) have found that monocytes preferentially harbor CCR5-using strains of HIV-1 in vivo, consistent with the results of our in vitro infection experiments with HIV-1_{Ba-L} reported in this article. However, recently it has been shown that monocytes may also harbor CXCR4-using strains of HIV-1 (59). We therefore compared the infection of CD14^{high}CD16+ and CD16+ monocyte subsets from three HIV-1-seronegative donors with recombinant reporter strains of HIV-1, which have the advantage of an interchangeable envelope (i.e., CXCR4- or CCR5-using). In this system, luciferase reporter production requires proviral transcription and therefore indicates productive infection. All reporter viruses were standardized by reverse transcriptase activity and initially tested for infectivity in PHA-stimulated PBMC (data not shown).

A CCR5-using HIV-1_{ADA} enveloped virus initiated the infection of CD16+ monocytes, but not CD14^{high}CD16+ monocytes, in all three donors examined (Fig. 4), confirming results from our initial in vitro infection experiments with HIV-1_{Ba-L}. The CXCR4-using HIV-1_{HXB2} enveloped virus did not efficiently infect either monocyte...
subset in any of the three donors, although low levels of luciferase activity were detected in CD16+ monocytes (Fig. 4). These experiments suggest that HIV-1 strains using CCR5 (HIV-1Lai, and HIV-1ADADA), preferentially infect CD16+ monocytes and that the infection of CD14<sup>hi</sup>CD16<sup>+</sup> monocytes may be restricted at viral entry.

To determine whether the infection of CD14<sup>hi</sup>CD16<sup>+</sup> monocytes was also restricted after coreceptor binding and entry, we used a VSV-G-pseudotyped reporter virus to bypass the requirement for CD4 and coreceptors (60). In contrast to receptor-dependent (HIV-1<sub>ADA</sub> and HIV-1Kb) virions, infection by a VSV-G-pseudotyped virus was evident in CD14<sup>hi</sup>CD16<sup>+</sup> monocytes as well as the CD16<sup>+</sup> monocyte subset, confirming the restriction at the level of HIV-1 entry. However, the luciferase activity of CD16<sup>+</sup> monocytes following infection with a VSV-G-pseudotyped virus was almost double that seen in CD14<sup>hi</sup>CD16<sup>+</sup> monocytes (n = 6; p = 0.036; Fig. 4), suggesting that other intracellular restrictions to HIV-1 infection is mediated by both entry and intracellular mechanisms. Our molecular analyses, together with our previous finding that these cells can act as a source of viral persistence in individuals, is highly permissive to HIV-1 infection, also expressed predominantly high (and some intermediate) molecular mass complexes (Fig. 5). The H9 T cell line, which is very highly permissive to HIV-1 infection, also expressed predominantly high (and some intermediate) molecular mass complexes (Fig. 5C) as previously shown (24). These data suggest that APOBEC3G activity may also regulate the susceptibility of monocyte subsets to HIV-1 infection. The observed donor variability of the level of the different APOBEC3G complexes in monocyte subsets correlates with our observed variability in the permissiveness of CD16<sup>+</sup> monocytes to HIV-1 in vitro and in vivo.

**Discussion**

In this study, we have shown that the minor CD16<sup>+</sup> subset of monocytes from HIV-1-infected individuals receiving HAART preferentially harbors HIV-1 compared with the major CD14<sup>hi</sup>CD16<sup>+</sup> monocyte subset. Of note, HIV-1 was identified within the CD16<sup>+</sup> monocyte subset of patients with undetectable viral loads, showing these cells to be a source of viral persistence. These findings are supported by our in vitro studies that also demonstrate that CD16<sup>+</sup> monocytes are more permissive to HIV-1 infection than CD14<sup>hi</sup>CD16<sup>+</sup> monocytes and that the increased susceptibility to infection is mediated by both entry and intracellular mechanisms. Our molecular analyses, together with our previous finding that monocytes harbor a replication-competent virus (13), confirm that these cells can act as a source of viral persistence in individuals receiving HAART.

In our relatively small patient cohort, all of whom are likely to be subtype B, the detection of HIV-1 in monocyte subsets did not correlate with plasma viral load, CD4 count, nadir CD4 count, or the antiretroviral therapy regimen at the time the sample was taken. Our data are supported by a study of a cohort of HIV-1-infected, untreated pregnant women in Malawi whose CD16<sup>+</sup> monocytes were also shown to be preferentially infected with HIV-1 (most likely subtype C) (A. Jaworowski, accepted for publication). We and others have previously established that the persistence of HIV-1 in monocytes is not due to mutations associated with antiretroviral resistance (12, 13, 17). The detection of HIV-1 DNA within monocyte subsets was unlikely to be due to the presence of infected T cells contaminating the monocyte preparations, as selected experiments showed <0.01% T cell contamination.

The CD4<sup>+</sup>CD45RO<sup>+</sup> HLA DR<sup>+</sup> resting memory T cell is recognized as a major HIV-1 reservoir (1–4, 61–63). We found that CD16<sup>+</sup> monocytes had about half the number of HIV-1 DNA copies contained within memory T cells with this sample size (n = 11), although there was no significant difference between these cell populations. Although the ontogeny of CD16<sup>+</sup> monocytes and their differentiation into mature cells is still controversial, these cells indisputably have a much shorter half-life than memory T cells (14, 61) and, thus, the detection of a significant amount of HIV-1 suggests a high level of ongoing infection within this monocyte compartment. Furthermore, because HIV-1 is noncytopathic to monocytes/macrophages (64, 65), these cells have the potential to constitutively produce virions, albeit at very low levels, for the duration of their lifespan and to transfer infection to other susceptible cells.

Despite the relatively high CXCR4 expression on all monocyte subsets, HIV-1 appears to preferentially use CCR5 for entry into monocytes as we (13) and others (58, 59) have shown previously. However, one recent study has identified HIV-1 genotypes that predict a CXCR4-using virus as a minor variant in monocytes from some patients (59). Although CD14<sup>hi</sup>CD16<sup>+</sup> monocytes expressed more CXCR4 than CD14<sup>hi</sup>CD16<sup>-</sup> or CD14<sup>+</sup>CD16<sup>-</sup> monocytes, both CD14<sup>hi</sup>CD16<sup>-</sup> and CD16<sup>-</sup> monocytes were relatively refractory to a virus using the CXCR4-tropic envelope of HIV-1Kb (25). Low levels of CXCR4-mediated infection were detected in CD16<sup>-</sup> monocytes even though they expressed less CXCR4 than CD14<sup>hi</sup>CD16<sup>+</sup> monocytes. This suggests that co-receptor expression is not the only limiting factor to infection of the major CD14<sup>hi</sup>CD16<sup>-</sup> monocyte subset. We show here that monocytes that coexpress CD16 and CD14 at high levels also express higher levels of CCR5 than CD14<sup>hi</sup>CD16<sup>-</sup> monocytes. As described previously (66), CCR5 expression is not altered in HIV-1-infected patients on HAART whereas we found that CXCR4 tends to be increased on monocytes from HIV-1-infected patients when compared with noninfected donors.

Our experiments with CCR5-using strains (HIV-1Lai, and a reporter virus expressing the HIV-1<sub>ADA</sub> envelope) showed that CD14<sup>hi</sup>CD16<sup>-</sup> monocytes are resistant to HIV-1 infection at the level of viral entry when compared with CD16<sup>+</sup> monocytes. Using a VSV-G-pseudotyped reporter HIV-1, bypassing the entry requirement for CD4 and coreceptors, we found that both monocyte subsets demonstrated productive infection. However, significantly higher levels of luciferase activity were detected in the CD16<sup>-</sup> monocytes, providing evidence for the resistance to infection of CD14<sup>hi</sup>CD16<sup>-</sup> monocytes at and beyond the level of viral entry. Recently, Triques and Stevenson have shown that β-lactamase reporter VSV-G-pseudotyped HIV-1 fuses very efficiently with monocytes as we (13) and others (58, 59) have shown previously. These authors show a block at the level of reverse transcription in total monocyte populations at a step in the replication cycle following attachment, fusion, and uncoating (48), confirming early work from our laboratory in which viral replication in freshly isolated monocytes was shown to be inhibited during or before complete reverse transcription (11). They suggest that differentiation-dependent cofactors of reverse transcription are rate limiting in monocytes (48). These data are further supported by our findings that an enzymatically active LMM form of the deoxyadenosine deaminase APOBEC3G restricts HIV-1 replication and blocks late reverse transcription in...
CD16+ MONOCYTE SUBSET HARBORS HIV-1

Disclosures
The authors have no financial conflict of interest.

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31: 13 –17.
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