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

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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^{high}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 CD16$^+$ monocytes that were similar to those observed in highly permissive T cells. In contrast, CD14^{high}CD16$^-$ monocytes contained low molecular mass active APOBEC3G, suggesting this is a mechanism of resistance to HIV-1 infection in these cells. Collectively, these data show that CD16$^+$ monocytes are preferentially susceptible to HIV-1 entry, more permissive for replication, and constitute a continuing source of viral persistence during HAART. *The Journal of Immunology,* **2007, 178: 6581–6589.**

Highly active antiretroviral therapy (HAART)[4] has greatly reduced the morbidity and mortality of HIV-1 infection and many treated individuals maintain very low levels of HIV-1 in plasma and normal CD4$^+$ T cell numbers for prolonged periods. However, current HAART regimens do not eradicate 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 rebound viremia detected another unidentified source of the virus distinct from T cells (6). Cells of the macrophage lineage at varying stages of differentiation are known targets of HIV-1 infection (7–9). Compared with more differentiated cells, monocytes are relatively refractory to infection in vitro (10, 11). It is well established that a replication-competent virus may be recovered from circulating peripheral blood monocytes of HIV-1-infected individuals, including those receiving HAART and who have maintained viral loads below detectable limits (<50 HIV-1 RNA copies per milliliter of plasma) for prolonged periods (12, 13). The fact that monocytes remain in circulation for up to 3 days (14) suggests ongoing recent infection of these cells or their precursors (5); this is supported by the detection of labile unintegrated circularized forms of viral DNA (2-long terminal repeat (LTR) circles) (13, 15, 16), multiply spliced viral mRNA species in freshly isolated monocytes (13), and viral evolution within this compartment (17). Strains of HIV-1 that infect cells of the macrophage lineage typically use CCR5 as a coreceptor for viral entry, and increasing CCR5 expression on monocytes during differentiation correlates with the permissiveness of these cells to infection (18, 19). However, CCR5 usage of HIV-1 is neither necessary nor sufficient to confer macrophage tropism, and strains of HIV-1 that exclusively use CXCR4 can also infect cells of the macrophage lineage (20–23).

Recently it was demonstrated that intracellular apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) has a role in blocking HIV-1 replication in quiescent CD4$^+$ T cells and monocytes (24). In activated CD4$^+$ T cells and monocyte-derived macrophages APOBEC3G is present as a high molecular mass (HMM) inactive ribonucleoprotein complex that correlates with these cells being permissive for HIV-1 replication. Resting T cells and monocytes contain low molecular mass (LMM) APOBEC3G, which has antiretroviral activity (24). Upon activation or maturation, LMM APOBEC3G is recruited into a HMM ribonucleoprotein complex comprised of APOBEC3G, Staufen-containing RNA-transporting granules, Ro ribonucleoproteins, and Alu and hY retroelements. (25).

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[4] Abbreviations used in this paper: HAART, highly active antiretroviral therapy; APOBEC3G, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; HMM, high molecular mass; LMM, low molecular mass; LTR, long terminal repeat; RTU, reverse transcriptase unit; VSV-G, vesicular stomatitis virus envelope glycoprotein.

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 (CD14^{high}CD16$^-$) (26, 27). 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 septicemia (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 CD14^{high}CD16$^-$ 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 K_3EDTA 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 using anti-CD3 Dynabeads. The remaining cells were incubated on ice with anti-CD14-PE and anti-CD16-FITC mAb (BD Biosciences) for 30 min. Cells were washed and fixed overnight with 3% methanol-free formaldehyde (Polysciences) in magnesium- and calcium-free PBS and then monocyte subsets were sorted (FACStar^{Plus}; BD Biosciences). For real-time PCR analysis of HIV-1 DNA copy numbers, PBMC were incubated with anti-CD14-PE, anti-CD16-PC5, 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. Monocytes 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 $\times$ 10^6 CD14^{high}CD16$^-$ monocytes and 1 $\times$ 10^5 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, CD14^{high}CD16$^-$ 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 >95% pure total monocytes while maintaining CD14^{high}CD16$^-$ 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 (CD14^{high}CD16$^-$ 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) (IH10) 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 $\times$ 10^4 monocytes in all three experiments, suggesting <0.01% T cell contamination.

Cell lysates

Purified monocyte subsets and CD4$^+$HLA DR$^-$CD45RO$^+$ memory T cells were lysed in 1 $\times$ 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-1$_{Ba\text{-}L}$ 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 pNL4-3*env*$^-$Luc plasmid with the pSVIII plasmid containing the *env* of HIV-1$_{ADA}$ or HIV-1$_{HXB2}$ 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 pSVIII-ΔKS*env* (a nonfunctional envelope glycoprotein) using the Lipofectamine transfection method (Invitrogen Life Technologies). Culture supernatants containing reporter virus were clarified by centrifugation at 1460 $\times$ *g* for 10 min and filtered through 0.45-μm pore-sized syringe-top filters (Sartorius). VSV-G and ΔKS viruses were concentrated by ultracentrifugation through a 20% sucrose cushion in an L-90 ultracentrifuge (Beckman Coulter) at 93,000 $\times$ *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; subsets were sorted to >95% purity by high speed flow cytometry. Monocyte subsets (2 $\times$ 10^5 to 1 $\times$ 10^6 cells) were immediately exposed to DNase-treated HIV-1$_{Ba\text{-}L}$ (4 reverse transcriptase units (RTU) per cell) in IH10, adhered to plastic tissue culture plates for 2 h at 37°C, and were then washed with PBS containing 5 mM EDTA and 0.1% (v/v) FBS to remove unbound virus and cultured for a further 36 h. To determine the efficiency of DNase treatment of virus stocks, monocyte subsets from each donor were also exposed to a virus and kept on ice for 36 h to inhibit viral entry and subsequent reverse transcription. Lysates of infected monocyte subsets were made in PCR lysis buffer, treated with proteinase K as described above, and analyzed by PCR as described below.

Single-round infection assays

Monocyte subsets were isolated from nine HIV-seronegative donors using MACS beads as described above. Equal numbers of monocyte subsets (1.75–5 $\times$ 10^5 cells) were immediately infected in tissue culture plates with

Table I. *Details of HIV-infected individuals from whom monocyte subsets were examined for HIV-1 DNA*

Patient No.	Detection of HIV-1		CD4 Count (cells /μl)	Nadir CD4 (cells/μl)	Viral Load (copies/ml)	Pretherapy Viral Load (copies/ml)	Therapy Regimen[b]
	$CD14^{high}CD16^{-}$[a]	$CD16^{+}$[a]					
1	−	+	259	149	<50	Undetected[c]	AZT, 3TC, NVP
2	−	+	1842	230	50	900[d]	ddI, d4T, IDV
3	−	+	152	130	15,500	28,500[d]	3TC, d4T, NVP
4	−	+	178	2	<50	>100,000[d]	3TC, d4T, IDV
5	−	+	426	90	600	>100,000[d]	ABC, AZT, 3TC, NFV
6	−	+	615	448	1,000	>100,000[d]	3TC, d4T, NFV
7	+	−	177	150	7,000	15,100[d]	3TC, d4T, NFV
8	−	+	253	50	100	89,500[d]	ABC, AZT, 3TC, EFV, RTV
9	+	−	641	263	1,100	11,700	3TC, d4T, NFV
10	−	+	193	41	100	>100,000	ABC, 3TC, TDF, IDV, RTV
11	−	−	510	510	<50	>100,000	AZT, 3TC, TDF
12	−	+	424	314	15,600	40,940	ABC, 3TC, NVP
13	−	+	757	213	2,800	>100,000	AZT, 3TC, NVP
14	−	+	434	30	<50	59,630	ABC, AZT, 3TC, NVP
15	−	+	458	140	<50	>100,000	AZT, 3TC, NFV
16	+	+	201	122	45,400	>100,000	3TC, d4T, EFV
17	+	+	520	10	300	>100,000	AZT, ddI, NFV, RTV

[a] $CD14^{high}/CD16^{-}$ and $CD16^{+}$ monocyte subsets were isolated from 9 ml of blood by either Dynabead sorting (patients 1–5), flow cytometric sorting (patients 6–9), or MACS bead sorting (patients 10–17).

[b] ABC, abacavir; AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; ddI, didanosine; TDF, tenofovir; EFV, efavirenz; NVP, nevirapine; IDV, indinavir; NFV, nelfinavir; RTV, ritonavir.

[c] Viral load has been undetectable since November 1996 in 33 assays. No prior viral load measurement was obtained.

[d] Highest recorded viral load (pretherapy viral load data were unavailable for these patients).

recombinant luciferase reporter viruses (4 RTU/cell) in IH10 by spinoculation 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 luciferase 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-$1_{Ba\text{-}L}$ 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 copy per cell in a HIV-1-negative PBMC background.

Semiquantitative 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 A2/B2 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 8E5 cells (one proviral copy per cell). Densitometry (Fuji Image Gauge version 3.3) of PCR products was used to estimate HIV-$1_{Ba\text{-}L}$ entry levels into 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-$1_{Ba\text{-}L}$ stocks in some experiments. In these cases, the level of background viral DNA in control samples was subtracted from the matched sample cultured at 37°C.

HIV-1 receptor expression

Peripheral blood was collected in K_3EDTA 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-CD16-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 fluorescence. $CD14^{high}CD16^{-}$, $CD14^{high}CD16^{+}$, and $CD14^{low}CD16^{+}$ 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 specific staining of cells from each donor.

APOBEC3G analysis

Monocytes were enriched from PBMC by countercurrent elutriation. Contaminating 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 chromatography and Western blotting as previously described (24).

Statistical analysis

Flow cytometric data of HIV-1 receptors on monocyte subsets were analyzed 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 conventional 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 $CD14^{high}CD16^{-}$, $CD16^{+}$, 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.

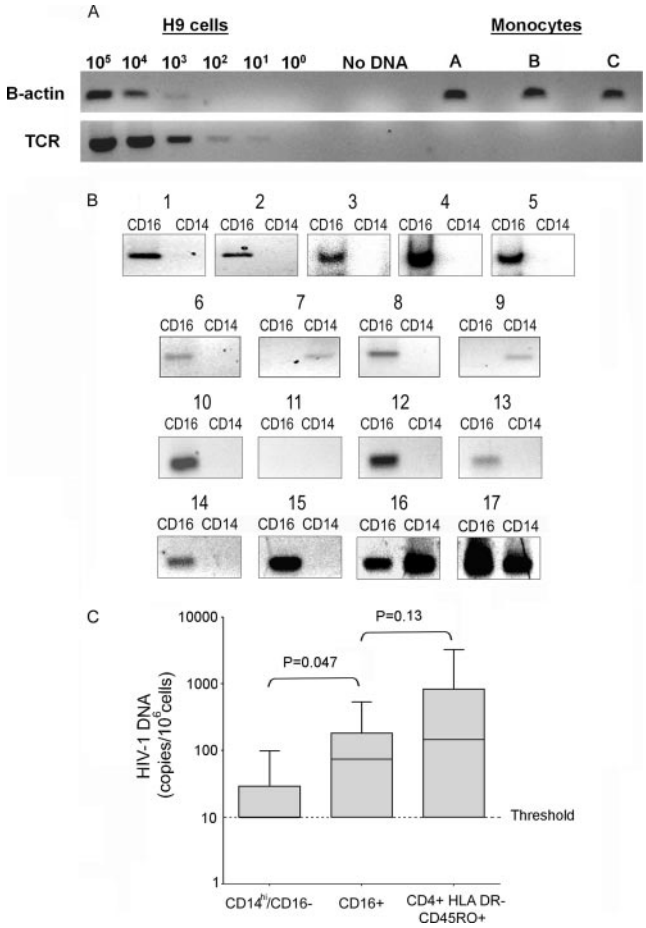

FIGURE 1. CD16$^+$ monocytes preferentially harbor HIV-1 in patients receiving HAART. *A*, Three monocyte isolation preparations (*lanes* marked A, B, and C) were tested for T cell contamination by an in-house TCR RT-PCR. *B*, Detection of HIV-1 DNA in 1×10^4 CD16$^+$ and CD14^{high}CD16$^-$ (labeled CD14) monocytes of patients ($n = 17$) by PCR against *gag* (limit of detection ≤10 copies). *C*, Median and interquartile range of HIV-1 copy numbers in CD14^{high}CD16$^-$ monocytes (0–99.0 copies) and CD16$^+$ monocytes (0–535 copies) compared with resting memory T cells (0–3280 copies) per 1×10^6 cells ($n = 11$; limit of detection 10 copies per 1×10^6 cells).

We compared the detection of HIV-1 DNA in highly purified CD14^{high}CD16$^-$ and CD16$^+$ monocyte subsets from these 17 patients. Purified monocyte subsets contained <0.01% total T cell contamination (Fig. 1*A*) 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*. This assay consistently detected HIV-1 DNA from ≤10 8E5 cells, which harbor a single integrated copy of HIV-1 DNA per cell and no unintegrated viral DNA (55). Using three different methods of isolation involving both magnetic bead and flow cytometric techniques, HIV-1 DNA was detected in CD16$^+$ monocytes only (12 of 17 patients), in CD14^{high}CD16$^-$ monocytes only (two of 17 patients), in both monocyte subsets (two of 17 patients), and not detected in either subset (one of 17 patients) (Table I and Fig. 1*B*). The preferential detection of HIV-1 DNA in CD16$^+$ compared with CD14^{high}CD16$^-$ monocytes was statistically significant ($p < 0.01$). No correlation was found between the detection of HIV-1 in the CD16$^+$ monocyte subsets and the patient's current or nadir CD4 count, plasma viral load, or antiretroviral drug regimen. The HIV-1 DNA copy number contained within the monocyte subsets was also determined by real-time PCR and compared with

Table II. *Quantification of HIV-1 copy number in monocyte subsets and comparison to resting memory CD4$^+$ T cells*

Patient No.	HIV-1 Copies Per 10^6 Cells: CD14^{high}CD16$^{-a,b}$	CD16$^{+a,b}$	CD4$^+$HLA DR$^-$CD45RO^{+a}	Percentage of CD16$^+$ Monocytes
18	29	121	147	11.5
19	37	75	627	10.4
20	<10	<10[c]	<10	8.2
21	17	536	<10[c]	7.9
22	<10	433	84	12.2
23	<10[c]	<10	3278	36.4
24	<10	182	1991	11.2
25	<10	<10	60	12.1
26	<10[c]	143	<10[c]	8.0
27	<10[c]	<10	832	21.5
28	99	17	663	19.5

[a] CD14^{high}CD16$^-$ monocytes, CD16$^+$ monocytes, and CD4$^+$HLA DR$^-$CD45RO$^+$ memory T cells from patients' blood were sorted by flow cytometry and the HIV-1 copy number for each population was determined by real time *gag*-PCR.

[b] Sorted monocyte populations were assayed for CD4 T cell contamination. The CD14^{high}CD16$^-$ monocyte preparation had a median range of 0.3% (0.1–1.1%) CD4$^+$ T cell contamination and the CD16$^+$ monocyte had a median range CD4$^+$ T cell contamination of 0.6% (0.2–1.4%).

[c] HIV-1 DNA was detectable but below a quantifiable threshold of 10 copies per 1×10^6 cells.

that in CD4$^+$HLA DR-CD45RO$^+$ memory T cells for another group of 11 patients on HAART (Table II and Fig. 1*C*). The resting memory T cells contained approximately twice the DNA copy number found

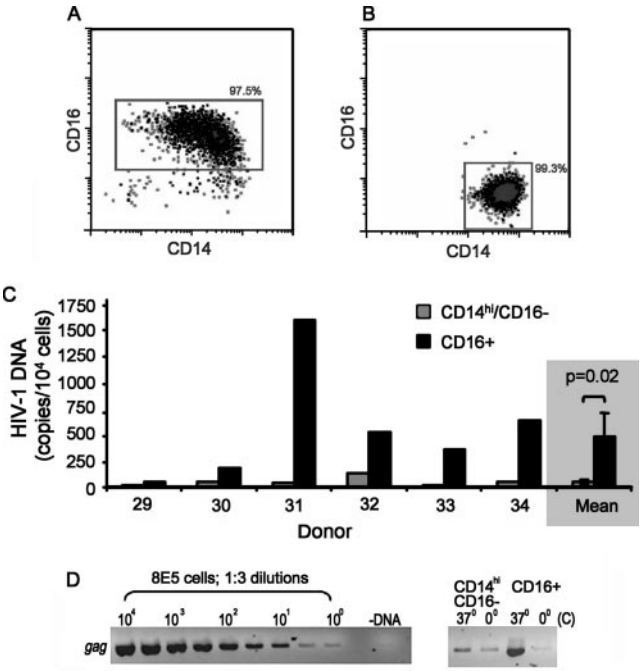

FIGURE 2. CD16$^+$ monocytes were more permissive to infection with HIV-$1_{Ba\text{-}L}$ in vitro. *A* and *B*, CD14^{high}CD16$^-$ and CD16$^+$ monocyte subsets were isolated by flow cytometric sorting from six HIV-seronegative buffy coats on the day of venepuncture. Equal numbers of monocyte subsets were immediately exposed to DNase-treated HIV-$1_{Ba\text{-}L}$ 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°) by conventional PCR is also shown (*D*, *right panel*).

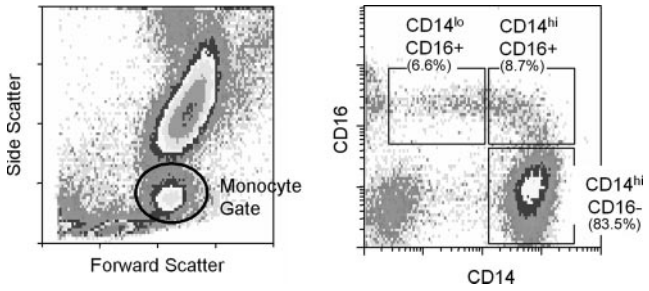

FIGURE 3. Gating of monocyte subsets for analysis of HIV-1 receptor expression. Monocyte subsets present in whole blood were identified using light scatter characteristics (*left panel,* Monocyte Gate) and CD14/CD16 expression patterns (*right panel*). Three subsets of monocytes, $CD14^{high}CD16^{-}$,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

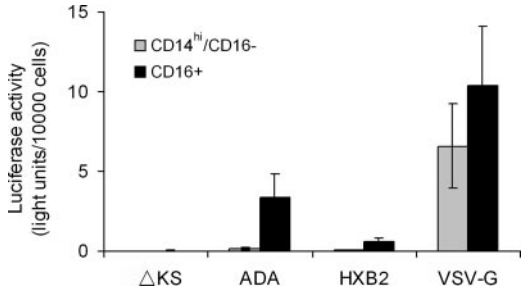

FIGURE 4. $CD16^{+}$ monocytes were more susceptible to infection by CCR5 using reporter strains and are inherently more permissive for HIV-1 replication than $CD14^{high}CD16^{-}$ monocytes. $CD14^{high}CD16^{-}$-sorted (gray bars) and $CD16^{+}$-sorted (filled bars) monocyte populations were exposed to reporter strains of HIV-1 carrying CCR5-using ($HIV\text{-}1_{ADA}$) or CXCR4-using ($HIV\text{-}1_{HXB2}$) 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 ($HIV\text{-}1_{\Delta KS}$) was used as a negative control to determine the background levels of luciferase activity.

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. $CD14^{high}CD16^{-}$ 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 $HIV\text{-}1_{Ba\text{-}L}$ (4 RTU/cell; multiplicity of infection of ~0.06 as assessed by a 50% tissue culture infectious dose ($TCID_{50}$) performed in monocyte-derived macrophages) for 2 h. Viral entry was determined by quantifying HIV-1 DNA following culture for 36 h postinfection by either real-time PCR ($n = 2$) or densitometric analysis of semi-quantitative PCR ($n = 4$; Fig. 2*C*). Although susceptibility to infection varied between donors, more HIV-1 DNA was detected in the $CD16^{+}$ compared with $CD14^{high}CD16^{-}$ monocytes in all cases, on average ~10-fold higher ($p = 0.02$) (Fig. 2*D*).

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. 3*A*). Three monocyte subsets were defined using CD14CD16 expression patterns, namely $CD14^{high}CD16^{-}$ monocytes, $CD14^{high}CD16^{+}$ monocytes, and $CD14^{low}CD16^{+}$ monocytes (Fig. 3*B*). Compared with $CD14^{high}CD16^{-}$ and $CD14^{low}CD16^{+}$ monocyte subsets, $CD14^{high}CD16^{+}$ 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, $CD14^{high}CD16^{-}$ monocytes expressed 4-fold higher CXCR4 compared with $CD14^{low}CD16^{+}$ monocytes and ~2-fold higher CXCR4 than $CD14^{high}CD16^{+}$ 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 $CD14^{high}CD16^{-}$ monocytes ($p = 0.07$), $CD14^{high}CD16^{+}$ monocytes ($p = 0.09$), and $CD14^{low}$

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

Receptor	Donor Type[a]	*n*	Data Type	$CD14^{high}CD16^{-}$[b]	$CD14^{high}CD16^{+}$[b]	$CD14^{low}CD16^{+}$[b]
CD4	HIV^{-}	5	Percentage	92.6 ± 1.2	91.1 ± 1.9	89.7 ± 1.8
			MFI	21.3 ± 2.8	27.5 ± 3.6	17.0 ± 3.8
	HIV^{+}	7	Percentage	89.8 ± 1.1	89.1 ± 1.3	91.7 ± 0.9
			MFI	22.8 ± 0.6	27.8 ± 0.8	17.8 ± 1.3
CCR5	HIV^{-}	14	Percentage	13.5 ± 5.4	42.9 ± 7.9	18.8 ± 5.7
			MFI	1.2 ± 0.5	5.1 ± 1.2	1.9 ± 0.6
	HIV^{+}	37	Percentage	8.8 ± 1.5	41.4 ± 3.7	17.6 ± 2.4
			MFI	1.3 ± 0.3	5.5 ± 0.5	2.3 ± 0.4
CXCR4	HIV^{-}	6	Percentage	52.3 ± 13.8	35.6 ± 13.8	20.2 ± 8.7*
			MFI	12.3 ± 4.8	6.7 ± 4.2	2.6 ± 1.6
	HIV^{+}	10	Percentage	86.8 ± 4.6	67.6 ± 7.1	49.8 ± 8.7*
			MFI	20.7 ± 3.1	13.9 ± 3.6	7.2 ± 2.3

[a] Whole blood from non-infected and HIV-infected patients was stained for HIV-1 coreceptor percent expression and mean fluorescent intensity (MFI).

[b] Monocytes were gated according to CD14 and CD16 expression.

*, Significant difference ($p = 0.04$) was found between the percentage of $CD14^{low}CD16^{+}$ monocytes that express CXCR4 from HIV-1-infected donors compared with uninfected donors.

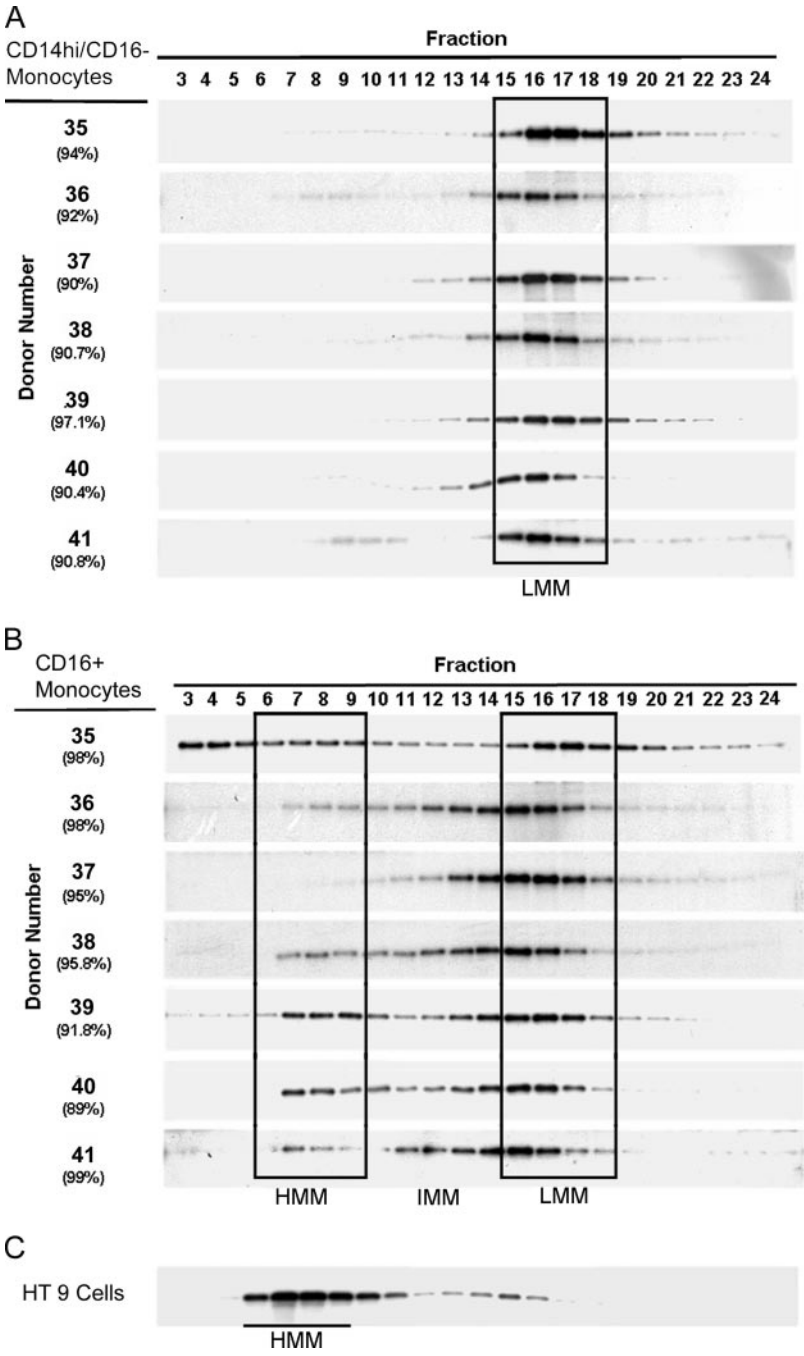

FIGURE 5. APOBEC3G is present in an active low molecular mass form in $CD14^{high}CD16^{-}$ monocytes and high molecular mass forms in $CD16^{+}$ monocytes. *A* and *B*, Lysates of $CD14^{high}CD16^{-}$ (*A*) and $CD16^{+}$ (*B*) monocytes isolated from PBMC from HIV-negative buffy coats ($n = 7$) were fractionated using gel permeation chromatography. *C*, Fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-APOBEC3G Abs. As a control, lysates from H9 cells (a T cell line that is readily susceptible to HIV-1) were also analyzed and show HMM and intermediate molecular mass (IMM) forms of APOBEC3G. The percentage under each donor number represents the purity of the isolated monocytes.

$CD16^{+}$ 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 $HIV\text{-}1_{Ba\text{-}L}$ 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 $HIV\text{-}1_{ADA}$ 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 $HIV\text{-}1_{Ba\text{-}L}$. The CXCR4-using $HIV\text{-}1_{HXB2}$ 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-$1_{Ba\text{-}L}$ and HIV-1_{ADA}) preferentially infect $CD16^+$ monocytes and that the infection of $CD14^{high}CD16^-$ monocytes may be restricted at viral entry.

To determine whether the infection of $CD14^{high}CD16^-$ 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_{ADA} and HIV-1_{HXB2}) virions, infection by a VSV-G-pseudotyped virus was evident in $CD14^{high}CD16^-$ monocytes as well as the $CD16^+$ monocyte subset, confirming the restriction at the level of HIV-1 entry. However, the luciferase activity of $CD16^+$ monocytes following infection with a VSV-G-pseudotyped virus was almost double that seen in $CD14^{high}CD16^-$ monocytes ($n = 6$; $p = 0.036$; Fig. 4), suggesting that other intracellular restrictions to HIV-1 permissiveness also exist in $CD14^{high}CD16^-$ monocytes. These data suggest that HIV-1 infection of $CD14^{high}CD16^-$ monocytes is restricted at multiple levels, including both early (either at entry or reverse transcription) and later stages of viral replication; these blocks to HIV-1 replication were not observed in $CD16^+$ monocytes. In all experiments the envelope defective virus ΔKS failed to initiate infection.

Another factor that could restrict HIV-1 replication following entry within monocyte subsets is the deoxycytidine deaminase APOBEC3G (24). We therefore analyzed APOBEC3G ribonucleoprotein complexes in $CD14^{high}CD16^-$ and $CD16^+$ monocytes by size exclusion chromatography. In $CD14^{high}CD16^-$ monocytes APOBEC3G was found in the LMM form (Fig. 5*A*), which has been previously shown to have inhibitory activity (24). However, the $CD16^+$ monocytes additionally expressed high and intermediate molecular mass complexes (Fig. 5*B*). The H9 T cell line, which is highly permissive to HIV-1 infection, also expressed predominantly high (and some intermediate) molecular mass complexes (Fig. 5*C*) 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 $CD16^+$ monocytes to HIV-1 in vitro and in vivo.

Discussion

In this study, we have shown that the minor $CD16^+$ subset of monocytes from HIV-1-infected individuals receiving HAART preferentially harbors HIV-1 compared with the major $CD14^{high}CD16^-$ monocyte subset. Of note, HIV-1 was identified within the $CD16^+$ 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 $CD16^+$ monocytes are more permissive to HIV-1 infection than $CD14^{high}CD16^-$ 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 $CD16^+$ 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 $CD4^+CD45RO^+$ HLA DR^- resting memory T cell is recognized as a major HIV-1 reservoir (1–4, 61–63). We found that $CD16^+$ 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 $CD16^+$ 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 $CD14^{high}CD16^-$ monocytes expressed more CXCR4 than $CD14^{high}CD16^+$ or $CD14^{low}CD16^+$ monocytes, both $CD14^{high}CD16^-$ and $CD16^+$ monocytes were relatively refractory to a virus using the CXCR4-tropic envelope of HIV-1_{HXB2}. Low levels of CXCR4-mediated infection were detected in $CD16^+$ monocytes even though they expressed less CXCR4 than $CD14^{high}CD16^-$ monocytes. This suggests that coreceptor expression is not the only limiting factor to infection of the major $CD14^{high}CD16^-$ monocyte subset. We show here that monocytes that coexpress CD16 and CD14 at high levels also express higher levels of CCR5 than $CD14^{high}CD16^-$ 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-$1_{Ba\text{-}L}$ and a reporter virus expressing the HIV-1_{ADA} envelope) showed that $CD14^{high}CD16^-$ monocytes are resistant to HIV-1 infection at the level of viral entry when compared with $CD16^+$ 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 $CD16^+$ monocytes, providing evidence for the resistance to infection of $CD14^{high}CD16^-$ 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 freshly isolated monocytes but that early and late viral reverse transcripts were inefficiently produced (67). 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 deoxycytidine deaminase APOBEC3G restricts HIV-1 replication and blocks late reverse transcription in

quiescent CD4$^+$ T cells and likely in monocytes (24). APOBEC3G resides as an inactive ribonucleoprotein complex of HMM in permissive cells such as activated CD4$^+$ T cells and monocyte-derived macrophages (24). APOBEC3G-specific siRNA treatment restored HIV-1 reverse transcription in unactivated CD4$^+$ T cells (24) and immature monocyte-derived dendritic cells (68), which strengthens the hypothesis that APOBEC3G also regulates reverse transcription in monocytes. Our findings that CD16$^+$ monocytes contain the inactive HMM form as well as in an intermediary form of APOBEC3G provides a mechanism by which these cells are more permissive to HIV-1 infection after entry. Because the CD14^{high}CD16$^-$ monocyte subset is considered to be a less mature subset than CD16$^+$ monocytes, the high, intermediate, and low molecular mass form of APOBEC3G in the latter may reflect alterations in APOBEC3G complexes associated with cellular maturation or monocyte heterogeneity within this population (31, 36). As the HMM form of APOBEC3G is comprised of many different components, the intermediate molecular mass APOBEC3G is most likely due to the transition from the LMM form to the HMM form and to the recruitment of the various components of the HMM complex (25). Similar results have recently been published showing that noninfected stimulated CD4 T cells from a HIV-GFP cultures predominantly expressed LMM APOBEC3G, whereas the T cells positive for HIV-GFP expressed significantly greater levels of HMM APOBEC3G (69). Because of the large volume of blood required to isolate sufficient CD16$^+$ monocytes for APOBEC3G analysis, we were unable to correlate APOBEC3G molecular mass with susceptibility to HIV-1 infection or to investigate the activation state of APOBEC3G in these HIV-1-infected patients.

Given the recent reports that CD16$^+$ monocytes render resting CD4$^+$ T cells more permissive for HIV-1 replication (37, 70) and that this cell population has a migratory capacity (36), CD16$^+$ monocytes are being increasingly shown to be important in viral dissemination and may carry infection to tissues to establish viral reservoirs in sanctuary sites such as the brain. An analysis of brain tissue from patients with HIV-related dementia shows perivascular infiltration of HIV-1-infected CD16$^+$ monocytes (71). Shiramizu et al. reported isolating CD14^{high}CD16$^+$ monocytes from the peripheral blood of five HIV-1 infected individuals, three of whom were clinically defined as having HIV-related dementia. These cells harbored HIV-1 DNA in all five donors, although the infection of CD14^{high}CD16$^-$ monocytes was not examined (72).

The persistence of HIV-1 in the CD16$^+$ peripheral blood monocyte subset despite HAART remains a significant hurdle to eliminating the virus from infected persons. Our laboratory and others have shown that, even in individuals with viral loads suppressed below detection for prolonged periods of time, HIV-1 continues to replicate at very low levels in monocytes and that infected monocytes can transmit HIV-1 to other susceptible cells (12, 13, 17). These data suggest that these cells are potentially important for the rebound of viral replication upon the interruption or cessation of antiretroviral therapy and in the dissemination of the virus to other tissues. We propose that the CD16$^+$ monocyte subset is more permissive to HIV-1 infection than the majority of monocytes, is clinically and functionally important in the dissemination of HIV-1 into tissues, and is a source of viral persistence in patients receiving HAART.

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Disclosures

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