JVI Journals.ASM.org

High Levels of CD2 Expression Identify HIV-1 Latently Infected Resting Memory CD4$^+$ T Cells in Virally Suppressed Subjects

Maria Iglesias-Ussel,[a] Claire Vandergeeten,[b] Luigi Marchionni,[c] Nicolas Chomont,[b] Fabio Romerio[a]

Institute of Human Virology, University of Maryland School of Medicine, Baltimore, Maryland, USA[a]; VGTI Florida, Port St. Lucie, Florida, USA[b]; Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA[c]

Resting memory CD4$^+$ T cells are the largest reservoir of persistent infection in HIV-1-positive subjects. They harbor dormant, stably integrated virus despite suppressive antiretroviral therapy, posing an obstacle to a cure. Surface markers that identify latently infected cells remain unknown. Microarray analyses comparing resting latently infected and uninfected CD4$^+$ T cells generated *in vitro* showed profound differences in the expression of gene programs related to transcriptional and posttranscriptional regulation, cell proliferation, survival, cycle progression, and basic metabolism, suggesting that multiple biochemical and metabolic blocks contribute to preventing viral production in latently infected cells. We identified 33 transcripts encoding cell surface markers that are differentially expressed between latently infected and uninfected cells. Quantitative reverse transcriptase PCR (RT-QPCR) and flow cytometry analyses confirmed that the surface marker CD2 was expressed at higher levels on latently infected cells. To validate this result *in vivo*, we sorted resting memory CD4$^+$ T cells expressing high and low surface levels of CD2 from six HIV-1-infected subjects successfully treated with antiretroviral drugs for at least 3 years. Resting memory CD4$^+$ CD2^{high} T cells from all subjects harbored higher HIV-1 DNA copy numbers than all other CD4$^+$ T cell subsets. Moreover, after *ex vivo* viral reactivation, robust viral RNA production was detected only from resting memory CD4$^+$ CD2^{high} T cells but not from other cell subsets. Altogether, these results show that a high CD2 expression level is a hallmark of latently infected resting memory CD4$^+$ T cells *in vivo*.

Current antiretroviral therapy (ART) is ineffective at eradicating human immunodeficiency virus type 1 (HIV-1) infection. Low-level viremia (<50 copies/ml) is often detected with ultrasensitive assays in patients on suppressive ART (1); occasional bursts of viral replication ("blips") occur (2, 3), and eventually, viremia rebounds after ART interruption in all but exceptional cases (4).

HIV-1 establishes persistent infection in a number of cell types at different anatomical compartments via different mechanisms (reviewed in references 5, 6, 7, 8, and 9). Latent reservoirs harbor stably integrated HIV-1 DNA in the absence of viral particle production, are refractory to antiretroviral drugs, and are invisible to immune surveillance. The largest latent reservoir consists of resting memory CD4$^+$ T cells, with a major contribution from central and transitional memory CD4$^+$ T cells (10).

Numerous factors, such as the quiescent nature of resting memory CD4$^+$ T cells, the site of viral integration, and many others, contribute to the establishment and maintenance of HIV-1 latency. Some of these mechanisms are currently being exploited to flush the latent provirus from its reservoirs in the absence of T cell activation (5, 11–13) by combining latency-reversing agents (LRAs) that reactivate latent proviruses, causing the demise of the infected cell, with highly active ART (HAART) intensification, which prevents infection of new target cells. While this approach presents potential pitfalls (the fraction of replication-competent latent proviruses that can be reactivated with LRAs [A. Cillo, M. Sobolewski, J. Coffin, and J. Mellors, presented at the 20th Conference on Retroviruses and Opportunistic Infections, Atlanta, GA, 2013; Y. C. Ho, L. Shan, J. Wang, N. Hosmane, J. Blankson, and R. Siliciano, presented at the 20th Conference on Retroviruses and Opportunistic Infections, Atlanta, GA, 2013], the fraction of latently infected CD4$^+$ T cells that survives after viral reactivation [14], incomplete efficacy of HAART in sanctuary sites [15–17], and the impact of LRAs on reservoirs other than CD4$^+$ T cells and on other latent viruses [18, 19]), reactivation of latent reservoirs merits further investigation. Nevertheless, there is also a need to develop alternative therapeutic approaches to eliminate or reduce the number of latently infected cells. The identification of cell surface biomarkers would provide an impetus in that direction. Indeed, uniquely or differentially expressed surface molecules could be used to isolate a partially purified subpopulation of latently infected cells for further characterization. Moreover, they could provide new therapeutic targets for the elimination of latently infected cells even in the absence of viral reactivation. To gain insight into the cellular mechanisms contributing to viral latency, and with the goal of identifying distinctive markers that distinguish latently infected CD4$^+$ T cells, we have used an *in vitro* model developed in our laboratory (20) to study the expression profile of latently infected CD4$^+$ T cells by microarray analysis. The results that we report in this study point to new mechanisms for the establishment and maintenance of latency in CD4$^+$ T cells that could be exploited for exploring novel therapies aimed at targeting this reservoir. In addition, this survey identified a panel of genes encoding cell surface molecules that were differentially expressed in latently infected versus uninfected cells, which may have diagnostic as well as therapeutic implications. Among the

Received 14 May 2013 Accepted 5 June 2013
Published ahead of print 12 June 2013
Address correspondence to Fabio Romerio, fromerio@ihv.umaryland.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.01297-13.

doi:10.1128/JVI.01297-13

markers identified in our study, CD2 was particularly interesting because of its know therapeutic applications (21–31). Sorting of resting memory CD4$^+$ T cells expressing high levels of the CD2 receptor from HIV-1-infected subjects on suppressive ART allowed a significant enrichment of latently infected cells able to produce robust levels of viral particles following *ex vivo* reactivation. Therefore, the studies presented below demonstrate that high levels of CD2 expression identify latently infected resting memory CD4$^+$ T cells in virally suppressed HIV-1-infected subjects.

MATERIALS AND METHODS

Ethics statement. All of the subjects provided their informed written consent to participate in the study. Peripheral blood mononuclear cells (PBMCs) of 4 HIV-1-negative donors (donors 3, 111, 112, and 113) were obtained with signed informed consent, after approval of the Institutional Review Board of the University of Maryland, Baltimore. PBMCs of 6 HIV-1-seropositive subjects (subjects ST045, ST101, ST102, ST104, ST109, and ST113) with undetectable viremia on suppressive ART for at least 3 years were obtained with signed informed consent and approved by the Institutional Review Board at Martin Memorial Health Systems (Stuart, FL).

Generation of latently infected CD4$^+$ T cells *in vitro*. The model for generation of latently infected CD4$^+$ T cells *in vitro* was described previously (20), except for the modifications described in the supplemental material.

Sorting of *in vitro*-generated latently infected cells, total RNA isolation, and microarray analysis. Procedures for sorting of *in vitro*-generated latently infected cells, total RNA isolation, and microarray analysis are described in the supplemental material and were described previously (32).

***In vitro* validation of mRNA expression by QPCR.** Total RNA was isolated as described above, and cDNA was generated by using the high-capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative real-time PCRs (QPCRs) were performed in triplicate on a Bio-Rad IQ5 instrument by using TaqMan gene expression assays (Applied Biosystems) (see Table S4 in the supplemental material), according to the manufacturer's instructions. Expression levels were compared to the levels of MED19, since it did not show differential expression in the microarray.

***In vitro* validation of surface protein expression by flow cytometry.** Surface expression of CD2, CD6, and CD130 was analyzed on CD4$^+$ T cell cultures latently infected with HIV-1 carrying a green fluorescent protein (GFP) reporter gene in place of Nef (pNL4-3-GFP). Further details are available in the supplemental material.

***Ex vivo* validation of CD2 expression in HIV-1-infected patients on ART.** We obtained PBMCs from 5 HIV-1-seropositive subjects with undetectable viremia who were on suppressive ART for at least 3 years (see Table S3 in the supplemental material). CD4$^+$ T cells were enriched from PBMCs by negative selection. Quantification of total HIV-1 DNA was performed with 300,000 cells of each subset by using ultrasensitive HIV-1 DNA PCR assays (sensitivity, 1 copy), as previously described (10). For viral reactivation, naive CD4$^+$ T cells and resting memory CD4$^+$ T cells expressing high or low levels of CD2 (5×10^5 cells per subset) were stimulated with anti-CD3/CD28 Dynabeads for 6 days in the presence of antiretrovirals (180 nM zidovudine [AZT], 100 nM efavirenz [EFV], and 200 nM raltegravir [RALT]) to prevent new rounds of infection. We then used ultrasensitive reverse transcriptase PCR (RT-PCR) (detection limit, 1 HIV-1 RNA copy) to quantify HIV-1 RNA copies in supernatants. Finally, the percentages of naive, central memory, transitional memory, effector memory, and terminally differentiated cells were determined by flow cytometry by gating on total CD4$^+$ T cells and on the subset of CD4$^+$ T cells expressing high and low levels of CD2. Further details for all these procedures are provided in the supplemental material.

Microarray data accession number. Complete results from this comparative host gene expression profiling analysis can be found at the public Gene Expression Omnibus (GEO) database under accession number GSE40550.

RESULTS

Latently infected CD4$^+$ T cells have gene expression profiles distinct from those of uninfected cells from the same culture. Latently infected cells were generated *in vitro* from CD4$^+$ T cells of 4 HIV-1-negative donors (donors 3, 111, 112, and 113) according to our previously described model (20), with the modifications shown in Fig. S1A in the supplemental material and described in Materials and Methods. After infection and expansion, cells were allowed to rest for 1 week, which allowed them to achieve cell quiescence, as shown by the lack of the activation markers HLA-DR, CD69, and Ki67 (see Fig. S1B in the supplemental material).

We have previously shown that the HIV-1 p24^{gag} antigen synthesized during productive infection persists in the cytoplasm of infected cells for several days and slowly declines during the latency phase (20). Detection of p24^{gag} in the cytoplasm of latently infected cells does not reflect new rounds of viral infection or *de novo* synthesis, in that the addition of AZT or cycloheximide does not affect the slope of p24^{gag} decay (20). Moreover, RT activity was detectable in culture supernatants of cells from two different donors at the peak of infection, but it was undetectable after 1 week of rest, indicating that by that time, active viral production had ceased (see Fig. S1C in the supplemental material). Therefore, we exploited cytoplasmic p24^{gag} to sort latently infected from uninfected cells from the same initial cell culture by flow cytometry, as we described recently (32). As cytoplasmic p24^{gag} declines over time, the frequency of p24^{gag}-negative latently infected cells sorting with, and thus contaminating, the truly HIV-1-negative population grows as a function of the length of the resting phase. We circumvented this potential pitfall by employing a resting phase of 1 week, since this was shown to be sufficient to obtain quiescent, latently infected cells (see above).

Total RNA was isolated from sorted latently infected and uninfected cells generated from CD4$^+$ T cells of four different donors. Paired RNA samples from infected and uninfected cells were labeled with Cy3 and Cy5 to allow dual-color competitive hybridization (20). Moreover, to control for the dye bias in our experiments, we implemented a dye swap protocol (reciprocal labeling) for paired RNA samples from 2 donors, as described previously (20), in Materials and Methods, and in Fig. 1A. RNA quality diagnostics did not reveal any artifacts (20).

Complete results from this comparative host gene expression profiling analysis can be found in Table S1 in the supplemental material and at the public GEO database (accession number GSE40550). A total of 533 genes were upregulated in latently infected cells, while 342 genes were downregulated (false discovery rate [FDR], <5%). Of particular interest are a panel of genes downmodulated in latently infected cells that could contribute to halt HIV-1 transcription due to their involvement in cellular metabolism, chromosome organization, gene expression, cell activation, proliferation, survival, and cell cycle progression (Fig. 1B).

Some of the factors known to have a role in HIV-1 infection and latency were also deregulated in latently infected cells. Thus, the mRNA of the chromatin-remodeling enzyme responsible for transcription repression, histone deacetylase 1 (*HDAC1*) (33), and the natural antiviral factor apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (*APOBEC3G*) (34) were up- and downregulated, respectively, in latently infected com-

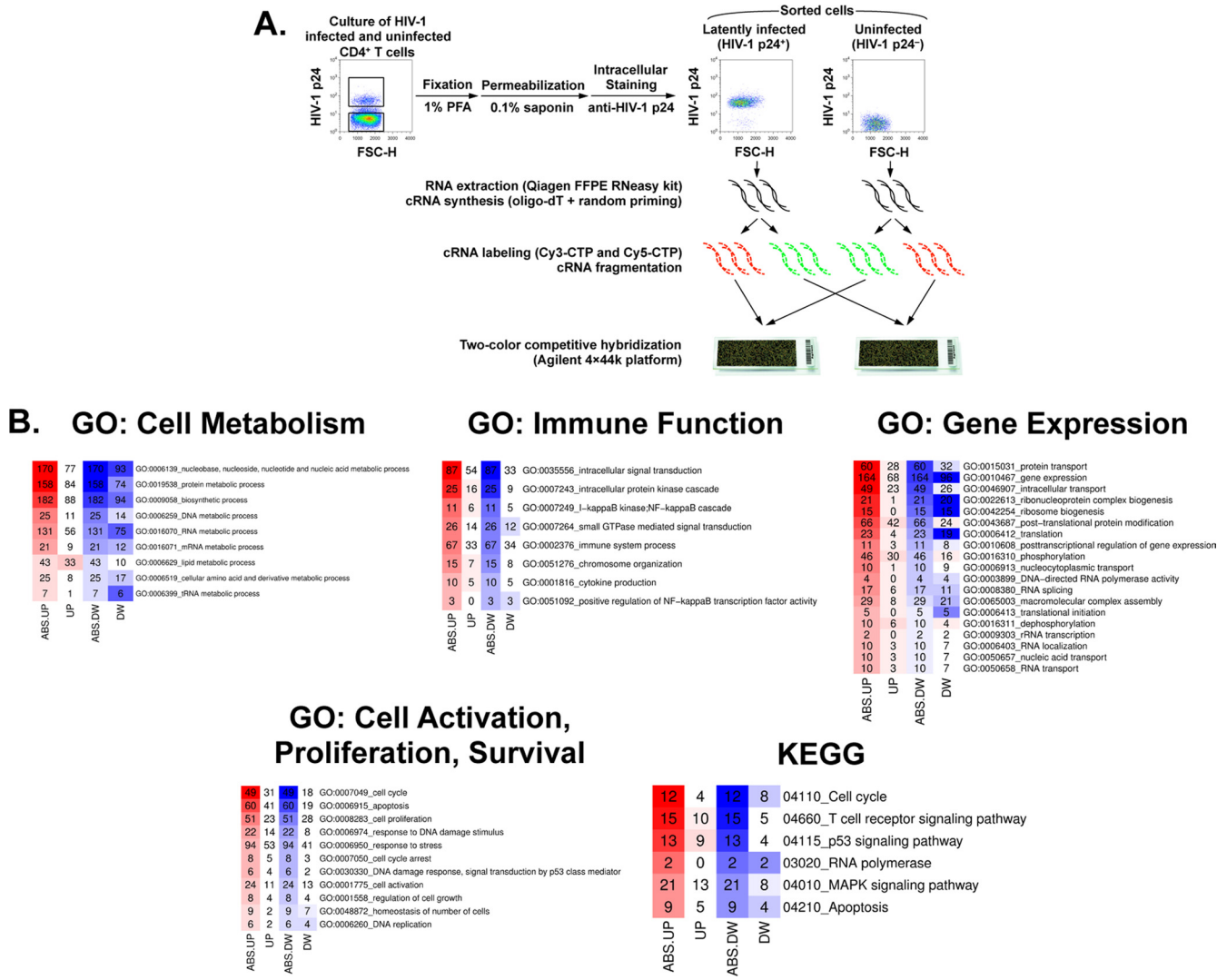

FIG 1 Sorting of latently infected and uninfected cells from the same culture for microarray studies. (A) Experimental sorting procedure and microarray labeling. Latently infected and uninfected cells generated *in vitro* were sorted based on the presence or absence of intracellular HIV-1 $p24^{gag}$ expression using fluorescein isothiocyanate-labeled KC57 antibody. Paired RNA samples from $p24^+$ and $p24^-$ cells from the same donor were labeled by using a two-color design with dye swap control. PFA, paraformaldehyde; FSC, forward scatter; FFPE, formalin-fixed paraffin-embedded. (B) Gene categories identifying pathways significantly deregulated (FDR, <5%) in HIV-1 latently infected $CD4^+$ T cells with a possible role in latency. Gene set enrichment was evaluated by a Wilcoxon rank-sum test for upregulation (UP) (shown in red), downregulation (DW) (shown in blue), and differential expression irrespective of the direction of the change in expression (columns labeled with the prefix "ABS," duplicated and shown with both color schemes). The number of significant differentially expressed genes (FDR, <5%) is reported for each category.

pared to uninfected cells. We also found reduced expression levels of a number of transcripts that might potentially impact entry into or maintenance of HIV-1 latency: mRNAs for several eukaryotic initiation-of-translation factors (EIFs), *1AX*, *2A*, *4A1*, *4B*, *4G2*, and *5A* (required for Rev function [35]); *CCND2* and *CCND3* (involved in G_1 progression and G_1/S transition in human T cells [36]); and *MYST4* (a histone methyltransferase involved in chromatin condensation [37]) were expressed at lower levels in latently infected than in uninfected cells.

Finally, a number of transcripts involved in various steps of the virus life cycle were also found to be differentially modulated when latently infected cells were compared to uninfected cells from the same culture. For instance, we found that the transcripts of the HIV-1-suppressive factors *CCL4/MIP-1*β and *CCL5/RANTES* were downregulated in latently infected cells. However, mRNAs of the HIV-1 receptor *CD4* and coreceptor *CXCR4* genes were upregulated in these cells. Transcripts of factors involved in RNA splicing (*PRPF4B*, *SFRS16*, and *SFRS17A*) were upregulated in latently infected cells, whereas factors mediating nuclear import of Rev (*IPO5/RANBP5* [38] and *IPO7* [39]) and Tat (*NUP93* and *NUP153* [40]) were downmodulated in these cells.

The results of our microarray analysis also revealed deregulated expression of many genes in Gene Ontology (GO) categories such as nucleic acid, protein, and lipid metabolic processes; RNA processing; posttranscriptional regulation; posttranslational protein modifications; protein localization; and nucleocytoplasmic transport. Moreover, genes involved in vesicle transport, endocy-

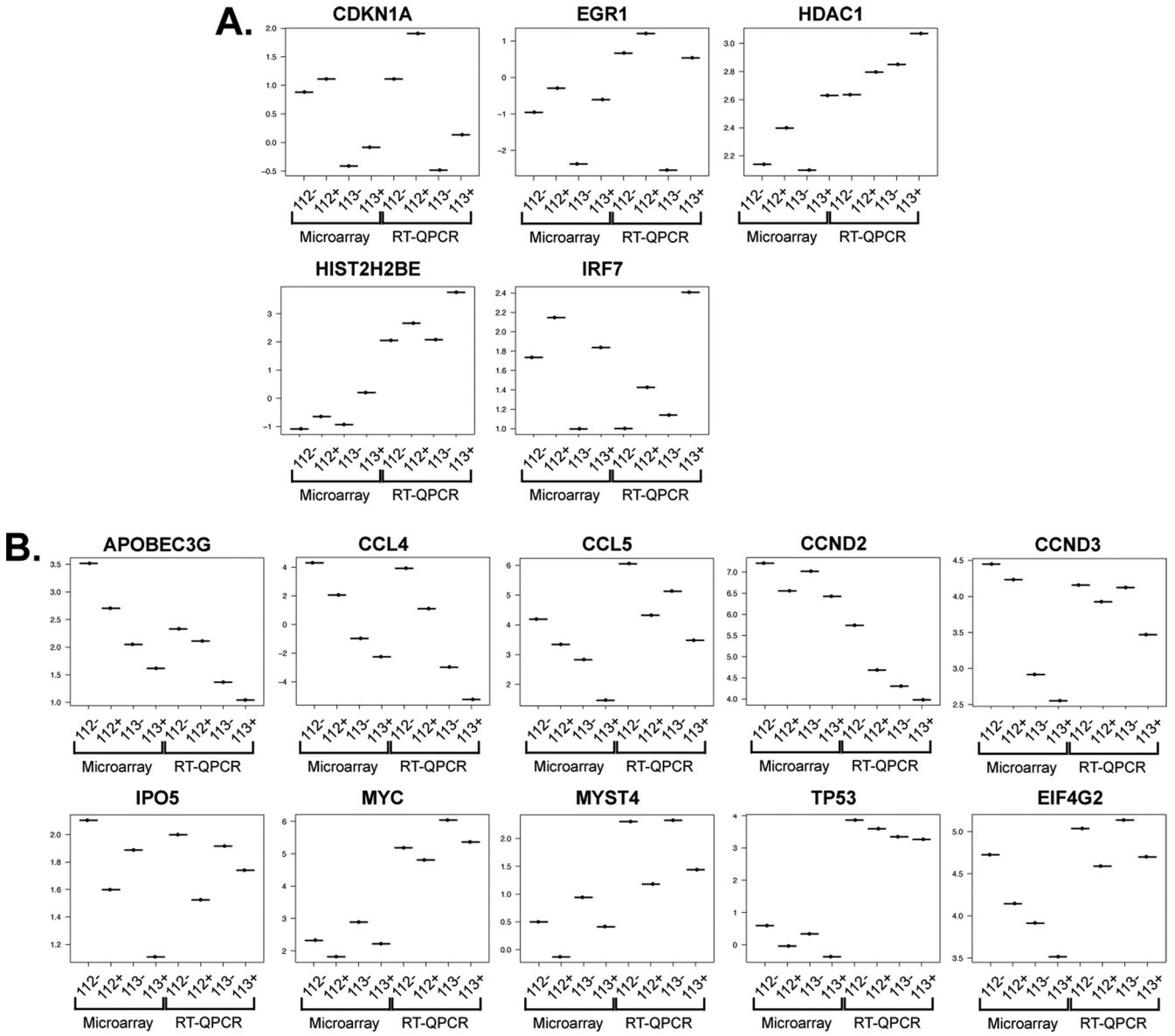

FIG 2 *In vitro* validation of the microarray by RT-QPCR. Total RNA was extracted from latently infected and uninfected cells generated *in vitro* from two donors, sorted based on intracellular p24 expression. Expression levels of transcripts encoding several intracellular markers were determined by RT-QPCR and compared to the results obtained by microarray analysis using the same RNA samples. (A) Genes upregulated in latently infected compared to uninfected cells; (B) genes downregulated in latently infected compared to uninfected cells. The figure shows the $\log_2$ fold change relative to levels of the housekeeping gene MED19. For the microarray analysis, a single data point was obtained, while for the QPCR analysis, we show the weighted geometric means across three replicate samples. 112−, uninfected cells from donor 112; 112+, infected cells from donor 112; 113−, uninfected cells from donor 113; 113+, infected cells from donor 113.

tosis, and membrane invagination were also differently regulated in latently infected cells. Data for additional enrichment analyses, including the updated analysis of functional annotation (AFA), can be found at the GEO database (accession number GSE40550).

In relation to proliferation or survival, both pro- and antiapoptotic factors were found to be up- and downmodulated in latently infected cells. Thus, based on these results, it is difficult to ascertain whether latent infection confers a cell survival benefit.

To validate our data set, we performed real-time RT-PCR with total RNA from latently infected and uninfected cells from 2 donors for 15 genes identified in the microarray analysis. The results obtained by quantitative RT-PCR (RT-QPCR) for all the genes were in accordance with the differential expression found by microarray analyses: 5 out of 5 genes were upregulated (Fig. 2A), and 10 out of 10 were downregulated (Fig. 2B).

Latently infected CD4$^+$ T cells differentially express a panel of cell surface receptors. We found that 89 transcripts for membrane-associated proteins were differentially expressed (56 up- and 33 downregulated) in latently infected compared to uninfected CD4$^+$ T cells generated *in vitro* (see Table S2 in the supplemental material). Among these 89 transcripts, 33 encode cell surface markers belonging to the cluster-of-differentiation (CD) protocol, including 20 genes upregulated and 13 downregulated in latently infected CD4$^+$ T cells (Table 1). We used RT-QPCR to

TABLE 1 Transcripts of CD surface markers differentially expressed in latently infected versus uninfected $CD4^+$ T cells generated *in vitro*[a]

Function	Gene	Log FC
Upregulated transcripts		
TCR associated	CD2	0.42
	CD3δ	0.30
	CD4	0.45
	CD6	0.49
	CD38	1.11
	CD278/ICOS	0.57
TNFR family	CD262	0.72
	CD270	0.37
	APO-3	0.57
TNF family	CD40L	0.54
	CD70	0.52
Cytokine receptors	CD124/IL4RA	0.36
	CD130/IL6RB	0.67
	CD218A/IL18RA	0.62
	CD218B/IL18RB	0.52
	IFNGR2	0.41
Chemokine receptors	CD197/CCR7	0.57
	CD184/CXCR4	0.43
Other	CD32	0.36
	CD69	1.17
Downregulated transcripts		
Integrins	CD49/α1	−0.54
	CD11b/αM	−0.42
	CD11c/αX	−0.73
	CD29/β1	−0.47
Chemokine receptors	CD183/CXCR3	−0.59
	CD186/CXCR6	−0.54
Costimulatory receptors	CD8b	−1.02
	CD48	−0.49
	CD80	−0.44
Other	CD35	−0.65
	CD73	−1.01
	CD164	−0.35
	CD117/c-kit	−0.71

[a] Log FC, $\log_2$ fold change in infected versus uninfected cells; TNFR, TNF receptor.

validate differential expression of 10 upregulated and 3 downregulated transcripts with total RNA from cells generated with our *in vitro* latency model. As shown in Fig. 3A and B, all 13 genes (except CD6 with RNA from donor 112) were validated in samples from two donors. Next, we decided to confirm whether some of these receptors were also deregulated at the protein level by flow cytometry. We focused on surface molecules that were upregulated, since they may have more therapeutic and diagnostic potential, and we selected three receptors expressed at high (CD2), intermediate (CD6), and low (CD130/IL6RB) levels on $CD4^+$ T cells. We used our *in vitro* model to generate cultures latently infected with full-length, replication-competent HIV-1 expressing GFP, which facilitates flow cytometry analysis. As GFP, like HIV-1 $p24^{gag}$, persists in the cytoplasm of infected cells for several days during the latency phase (see Fig. S2 in the supplemental material), we measured the expression levels of these three markers on latently infected and uninfected cells by gating on the GFP-positive (GFP^+) and GFP-negative (GFP^-) fractions, respectively. The results were validated by using latently infected cultures generated using our *in vitro* model of HIV-1 latency. As shown in Fig. 3C, surface expression levels of all three receptors were higher on latently infected than on uninfected cells.

High levels of expression of the CD2 receptor identify infected $CD4^+$ T cells in virally suppressed subjects. Next, we sought to determine whether the cell surface markers identified through the microarray analysis described above could be used to identify latently infected cells *in vivo*. As the differential expression level of the CD2 receptor between latently infected and uninfected cells *in vitro* was higher than those of CD6 and CD130 (Fig. 3C), we chose to perform our studies with clinical samples using CD2 first. Thus, we obtained a large number of PBMCs (5×10^9 to 15×10^9 cells) through leukapheresis from six HIV-1-seropositive subjects with undetectable viremia and who were on suppressive ART for at least 3 years (see Table S3 in the supplemental material). We enriched $CD4^+$ T cells by negative selection, and we stained these cells with fluorochrome-conjugated antibodies to CD3, CD4, HLA-DR, CD45RA, and CD2, as described in Materials and Methods. After gating on the live-cell population, we sorted naive $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD45RA^+$), activated memory $CD4^+$ T cells ($CD3^+$ $CD4^+$ HLA-DR^+ $CD45RA^-$), and resting memory $CD4^+$ T cells ($CD3^+$ $CD4^+$ HLA-DR^- $CD45RA^-$) that fell within the 20th highest and lowest percentiles of CD2 fluorescence ($CD2^{high}$ and $CD2^{low}$, respectively) (Fig. 4A). First, we measured the copies of HIV-1 DNA in the 4 sorted cell subsets, using $CD4^+$ T cells as controls. We found that resting memory $CD4^+$ $CD2^{high}$ T cells from all six subjects harbored higher HIV-1 DNA copy numbers than the other cell subsets (Fig. 4B). The median of the enrichment of HIV-1 DNA copies in the $CD4^+$ $CD2^{high}$ subset compared to total $CD4^+$ T cells was 5.7-fold (range, 3- to 10.8-fold; $P = 0.013$) (Fig. 4B).

Next, we measured viral RNA production following *in vitro* reactivation of resting memory $CD4^+$ $CD2^{high}$, resting memory $CD4^+$ $CD2^{low}$, and naive $CD3^+$ $CD4^+$ $CD45RA^+$ T cells. Sorted cells from 3 of the 6 subjects analyzed as described above (subjects ST101, ST104, and ST113) were stimulated with anti-CD3/CD28 Dynabeads for 6 days in the presence of antiretrovirals to prevent new rounds of infection. We then used ultrasensitive RT-PCR to quantify HIV-1 RNA copies in culture supernatants after pelleting of viral particles by ultracentrifugation. We detected robust levels of viral RNA production in the culture medium of resting memory $CD4^+$ $CD2^{high}$ T cells, whereas little or no viral particles were detected in the supernatants of resting memory $CD4^+$ $CD2^{low}$ and naive $CD4^+$ T cells (Fig. 4C). Thus, these results indicate that a high expression level of the surface receptor CD2 identifies resting memory $CD4^+$ T cells harboring replication-competent HIV-1 in peripheral blood of virally suppressed patients.

Finally, we used flow cytometry to determine the frequencies of all major cell differentiation subsets (naive, central memory, transitional memory, effector memory, and terminally differentiated) within the total $CD4^+$ T cell, $CD4^+$ $CD2^{low}$, and $CD4^+$ $CD2^{high}$ populations of 5 out of 6 subjects as described above. As shown in Fig. 5, the $CD4^+$ $CD2^{high}$ population was markedly enriched in cells displaying a memory phenotype. Thus, these results are in line with a previous study showing that integrated HIV-1 is found primarily within central ($CD45RA^-$ $CCR7^+$ $CD27^+$), transitional ($CD45RA^-$ $CCR7^-$ $CD27^+$), and effector ($CD45RA^-$ $CCR7^-$ $CD27^-$) memory $CD4^+$ T cells (10).

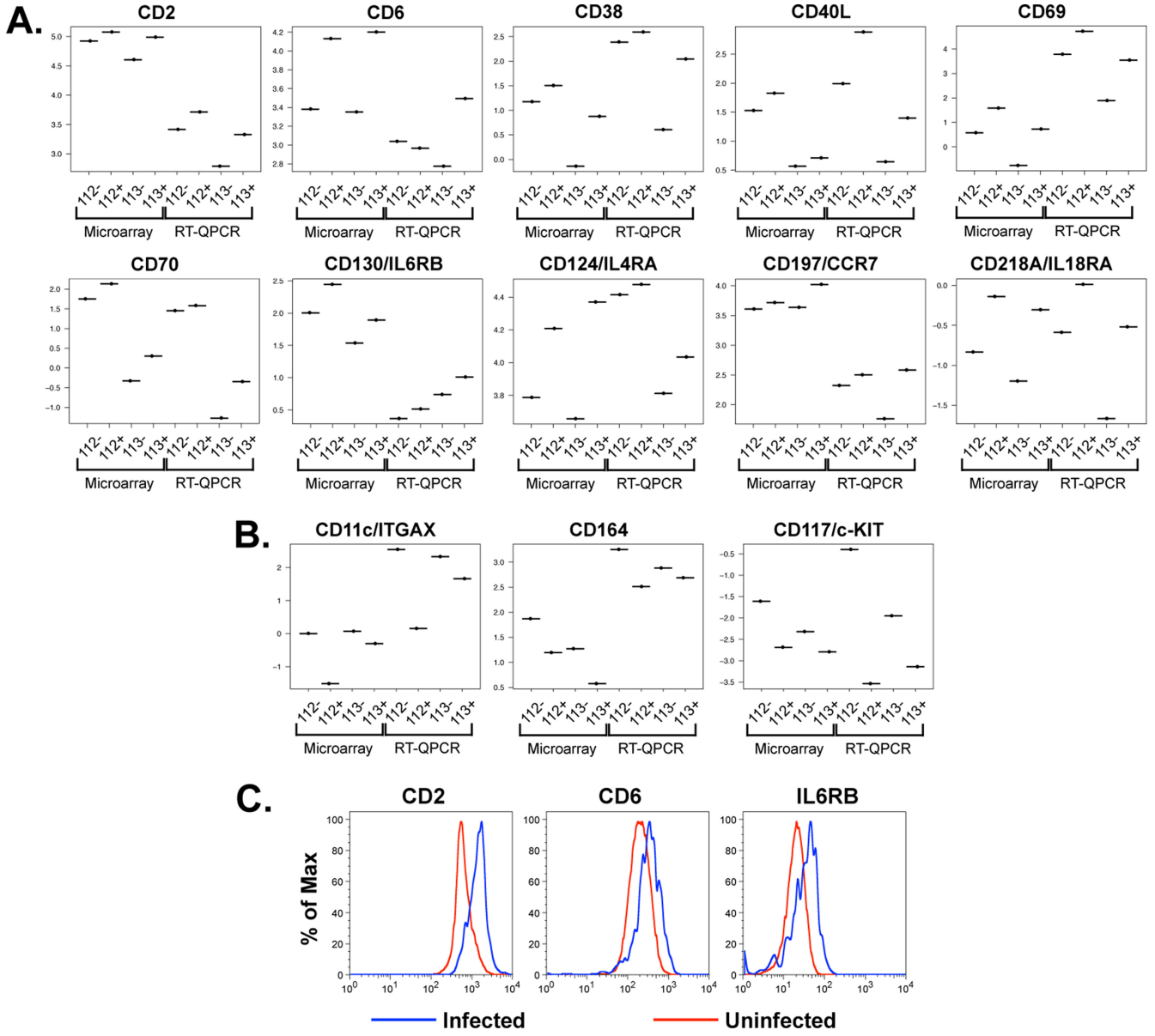

FIG 3 *In vitro* validation of cell surface receptors shown to be deregulated by microarray in HIV-1 latently infected cells. (A) Validation of upregulated transcripts by real-time RT-PCR. Total RNA was extracted from *in vitro*-generated latently infected and uninfected cells sorted based on intracellular p24 expression. Expression levels of transcripts encoding several intracellular markers obtained by RT-PCR were compared to the results obtained by microarray analysis using the same RNA samples. The figure shows the $\log_2$ fold change relative to levels of the housekeeping gene MED19. For the microarray analysis, a single data point was obtained, while for the RT-PCR analysis, we show the weighted averages of four replicate samples. (B) Validation of downregulated transcripts by real-time RT-PCR. See above (A) for details. (C) Validation of differential cell surface expressions of CD2, CD6, and CD130/IL6RB on *in vitro*-generated latently infected and uninfected CD4$^+$ T cells by flow cytometry. CD4$^+$ T cells were infected with full-length, replication-competent HIV-1 expressing GFP and allowed to return to quiescence for 7 days. Cells were then harvested; stained with antibodies to CD2, CD6, and CD130/IL6RB; and then analyzed by flow cytometry gating on the GFP$^+$ and GFP$^-$ populations. The plots shown are representative of three independent experiments carried out with cells from three different donors.

DISCUSSION

Several limitations hinder the study of HIV-1 latency with clinical samples, including the lack of distinctive surface markers for latently infected cells, the low frequency of latently infected resting memory CD4$^+$ T cells in ART patients, and the fact that clinical samples allow studying only reactivation from latency but not the mechanisms underlying its establishment. These reasons have compelled several groups to develop *in vitro* models to generate and study proviral latency in resting CD4$^+$ T cells (20, 41–50; reviewed in reference 9).

The model developed in our laboratory (20) recapitulates the activation of primary CD4$^+$ T cells via T cell receptor (TCR) engagement, infection with HIV-1, and return back to quiescence through a resting phase in the presence of low levels of interleukin-7 (IL-7). Our model is amenable to providing new insights into the nature of latency and identifying new biomarkers and

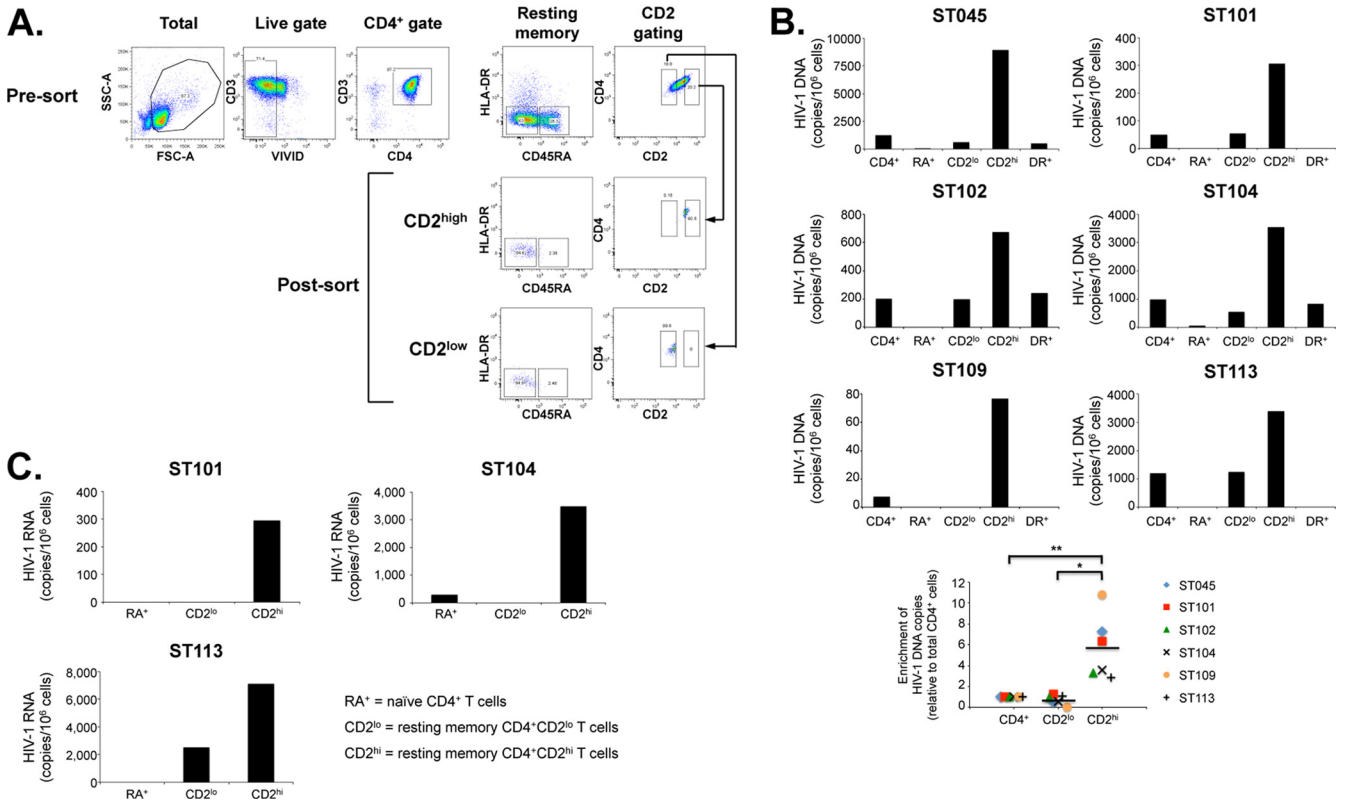

FIG 4 High levels of CD2 expression can be used to enrich latently infected resting memory $CD4^+$ T cells *ex vivo*. (A) Cell sorting strategy to obtain resting memory $CD4^+$ T cells expressing low or high levels of the CD2 receptor from $CD4^+$ T cells of aviremic HIV-1-infected patients on suppressive ART. Memory resting $CD4^+$ T cells were defined as $CD3^+$ $CD4^+$ $CD45RA^-$ $HLA\text{-}DR^-$. Unsorted $CD4^+$ T cells were used as the control. After cell sorting, highly pure populations were obtained. (B) Copies of integrated HIV-1 DNA in total $CD4^+$ T cells ($CD4^+$) and in sorted naive (RA^+), activated memory (DR^+), and resting memory $CD4^+$ T cells expressing low ($CD2^{lo}$) or high ($CD2^{hi}$) levels of CD2. HIV-1 DNA copy numbers were measured by ultrasensitive PCR. For the enrichment of HIV-1 DNA copies, values obtained with $CD2^{lo}$ and $CD2^{hi}$ cells from each patient were normalized to the value obtained with the corresponding total $CD4^+$ T cells. Black horizontal lines indicate the medians. Statistical significance was determined with the two-tailed paired *t* test (*, $P = 0.015$; **, $P = 0.013$). (C) Viral production upon stimulation with anti-CD3/CD28 Dynabeads of sorted naive $CD4^+$ T cells (RA^+) and resting memory $CD4^+$ T cells expressing low ($CD2^{lo}$) or high ($CD2^{hi}$) levels of CD2. Viral production was measured in culture supernatants after 6 days of culture by ultrasensitive RT-PCR.

therapeutic targets. The modifications of our original model that we adopted in the current study for the purpose of isolating latently infected cells—most notably a shortened resting phase—did not affect our ability to generate truly quiescent latently infected cells, as shown by the lack of surface and intracellular activation markers (HLA-DR, CD69, and Ki67) and by the absence of RT activity in the culture supernatants. The choice of using RT activity as a measure of viral latency was dictated by the evidence that cell-associated viral RNA can be detected in latently infected resting memory $CD4^+$ T cells of HIV-1-infected subjects under effective antiretroviral therapy (11, 51), and therefore, this parameter might not provide a stringent measure of latency. This result underscores the role of low doses of IL-7 in the establishment of immunological memory in $CD4^+$ T cells (52–54). We have previously shown that the HIV-1 antigen $p24^{gag}$ produced during active viral replication persists in the cytoplasm of infected cells for several days, even during latent infection, which allows us to distinguish latently infected $CD4^+$ T cells from their uninfected counterparts (20). Thus, we took advantage of this marker to perform, for the first time, comparative genomic profiling of primary latently infected resting memory $CD4^+$ T cells versus their uninfected counterparts sorted from the same culture. Previous studies were carried out with latently infected cell lines (55–57), but no studies using primary $CD4^+$ T cells have been reported. Krishnan et al. showed altered expression levels of genes encoding components of the proteasome, histone deacetylases, and transcription factors in the chronically infected cell lines ACH-2, J1.1, and U1 compared to the parental uninfected cell lines A3.01, Jurkat, and U937 (56). The same data set was used later on to identify several networks at the latent stage, including an enrichment of proteins associated with HIV-1 replication, apoptosis, and cell growth regulation (58). Since latently infected cells cannot be distinguished from uninfected cells *in vivo*, only a comparative study of resting memory $CD4^+$ T cells from viremic and aviremic subjects has been done so far (59).

Our microarray analysis indicates that latently infected resting memory $CD4^+$ T cells have a very different gene expression profile than uninfected cells. It should be noted that because of the temporal course of our experimental design, it is unclear whether the genes identified in our screen are related to entry into latency, maintenance of latency, or both. Resolving this issue will require a more accurate follow-up study. The affected gene ontology not only covers pathways related to transcriptional and posttranscriptional regulation, as described previously (13, 60), but also illustrates more profound changes affecting cell proliferation, cell cycle progression, as well as basic cellular functions such as nucleic

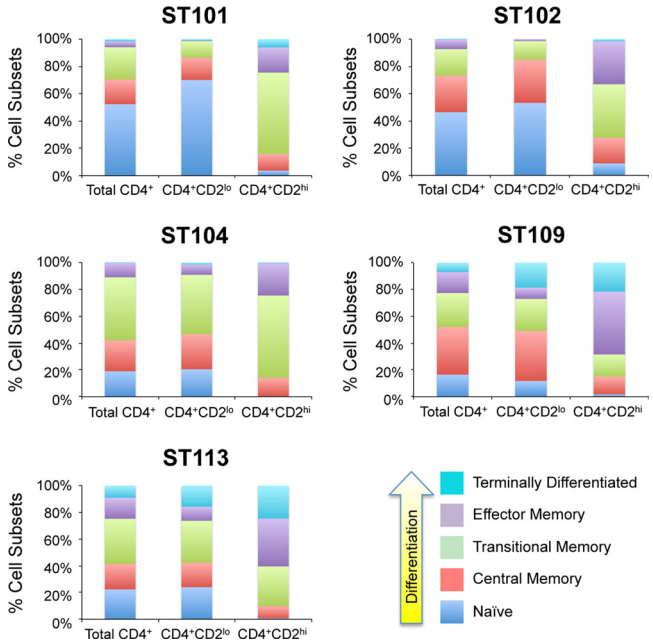

FIG 5 Phenotyping of CD4$^+$ T cells based upon expression levels of CD2. Percentages of naive (N), effector memory (Em), transition memory (Tm), central memory (Cm), and terminally differentiated (Td) cells in CD4$^+$ T cells expressing different levels of CD2 are shown.

acid, lipid, carbohydrate, and amino acid metabolic processes. Altogether, these changes may contribute to the establishment of a cellular environment that is conducive to cell quiescence and viral latency. Recent *ex vivo* studies suggested that a single treatment with current LRAs induces viral reactivation in only a small percentage of latently infected cells (Cillo et al., presented at the 20th Conference on Retroviruses and Opportunistic Infections; Ho et al., presented at the 20th Conference on Retroviruses and Opportunistic Infections). Furthermore, recent *in vivo* studies showed that administration of a single dose and multiple doses of the HDAC inhibitor vorinostat led to transient increases in acetylation of histone H3 and in HIV-1 *gag* cell-associated RNA copy numbers. Therefore, treatment with vorinostat also affects the transcription activity of the HIV-1 long terminal repeat (LTR) *in vivo*. However, this effect was not paralleled by a spike in viremia (11; J. Elliott, A. Solomon, F. Wightman, M. Smith, S. Palmer, M. Prince, J. Watson, J. Hoy, J. McMahon, and S. R. Lewin, presented at the 20th Conference on Retroviruses and Opportunistic Infections, Atlanta, GA, 2013). A possible interpretation of these results may be that an increase in the level of cell-associated viral RNA following exposure to LRAs did not lead to viral production by latently infected cells. The results of our microarray analysis may suggest that latently infected cells lack the proper biochemical machinery required to support viral production despite higher intracellular levels of viral RNA. The use of concomitant LRAs targeting different transcription blocks was clearly shown to be more effective than single-agent approaches *in vitro* (43, 61). Moreover, it is conceivable that targeting of more downstream steps, including some of the ones identified in this study, may lead to more sustained viral production, especially in more refractory latent reservoirs.

The establishment of HIV-1 latency has been portrayed largely as a passive process. In other words, HIV-1 expression is shut down—and consequently, latency is achieved—as part of the process by which the host cell returns to quiescence. However, it is plausible that viral proteins expressed during productive infection of activated CD4$^+$ T cells could play an active role by deregulating gene programs within the host cell so as to generate a cellular environment conducive to cell quiescence and viral latency. Indeed, our microarray study indicated that in latently infected cells, gene programs were modulated in a way that is consistent with cell quiescence, cell survival, transcription silencing, and evasion of the immune system. Since HIV-1 proteins have a profound effect on many host cell functions that are relevant to its life cycle (55, 58, 62–69), it is conceivable that they might also affect cell functions relevant to latency. On the other hand, it is possible to speculate that a subset of CD4$^+$ T cells with a gene expression profile similar to the one emerging from our microarray analysis already preexists in peripheral blood of healthy individuals, so that upon HIV-1 infection, it provides an ideal environment for the establishment of latency.

Our microarray analysis found that the expressions of transcripts encoding a number of cell surface markers were deregulated in latently infected CD4$^+$ T cells. This finding is of particular relevance since specific surface biomarkers to distinguish these cells have not yet been identified. Of these, CD2 was selected for validation in clinical samples, using peripheral blood of aviremic HIV-1-infected subjects under suppressive antiretroviral therapy for at least 3 years. Selection based on high expression levels of this CD2 receptor allowed strong *ex vivo* enrichment of a bona fide latent reservoir of resting memory CD4$^+$ T cells. In all subjects analyzed, this CD2^{high} population was shown to contain higher copy numbers of HIV-1 DNA and to give rise to more robust viral production upon reactivation than total resting memory CD4$^+$ T cells. On the other hand, our data also show that the majority of CD2^{high} cells are not infected. Moreover, we detected HIV-1 sequences in the CD2^{low} subset of 5/6 subjects as well as HIV-1 RNA in the culture supernatant of CD2^{low} cells of 1/3 subjects following *ex vivo* reactivation. Therefore, expression of high CD2 levels does not fully capture the latent reservoir, and while it represents a useful tool for future research, it is a limited one. The identification of new markers remains a high priority, and the list of transcripts identified in our microarray study might represent a valid starting point.

Two lines of evidence provide a possible basis for the identification of CD2^{high} as a biomarker of bona fide latently infected CD4$^+$ T cells. Indeed, binding of CD2 to its ligand, CD58/LFA-3, facilitates T cell interactions with antigen-presenting cells and perhaps enhances virus transmission. Additionally, LFA-3 is found on the membrane of HIV-1 virions and has been proposed to mediate viral targeting to particular tissues and specific cell types (70). Thus, cells overexpressing CD2 might bind HIV-1 and become infected more readily.

CD2 might represent a potential target for the elimination of latently infected CD4$^+$ T cells. Whereas some anti-CD2 antibodies induce T cell proliferation (71) and viral transcription in PBMCs and resting CD4$^+$ T cells (72, 73), binding of certain epitopes on CD2 induces T cell death via antibody-dependent cellular cytotoxicity (ADCC) induced by NK cells and activated monocytes. For instance, alefacept, a fusion protein composed of the first extracellular domain of human LFA-3 fused to the hinge, CH2, and CH3 domains of human IgG1, as well as rat IgG2b

monoclonal antibody LO-CD2a/BTI-322 inhibit T cell proliferation and induce NK cell-mediated death of T cell memory subsets (74, 75). LO-CD2a/BTI-322 and its humanized version (siplizumab; MEDI-507) have been used for the treatment of acute kidney transplant rejection (22, 26, 28, 30), acute graft-versus-host disease (GVHD) (21, 24, 29), and psoriasis (23) and is being tested for the treatment of T cell malignancies (25, 27, 31). In addition, alefacept is FDA approved to kill memory T cells expressing higher levels of CD2 for the treatment of chronic psoriasis (76, 77). These studies suggest the possibility of targeting CD2 for the depletion of latently infected $CD4^+$ T cells in HIV-1 infection. Although CD2 is also expressed on uninfected T and NK cells, the FDA has approved targeting of receptors such as CD20 for cell depletion in autoimmune diseases and cancer, despite the fact that CD20 is also expressed on normal B and T cells (78, 79).

Besides CD2, our microarray study found other interesting receptors that, on the basis of their known function and signaling pathways, could be explored to target or deplete this latent reservoir (Table 1). For example, engagement of the tumor necrosis factor (TNF) receptors CD262, CD270, and APO-3, found upregulated in our microarray in latently infected resting $CD4^+$ T cells, triggers caspase activation and apoptosis (80, 81). In addition, two receptors listed in Table 1 are known to trigger cell death through the p38 mitogen-activated protein kinase (MAPK) pathway. Thus, antibodies to CD70, a TNF family member (80, 82), trigger death of malignant cells (83). Also, it is well documented that CXCR4 mediates p38 MAPK-dependent cell death in HIV-1 infection (84, 85). In addition, we found differential expressions of transcripts encoding cell surface activation markers such as CD38, CD69, and CD154/CD40L. This result is intriguing because our flow cytometry analysis showed that CD69 is not expressed on the cell surface after 1 week of a resting phase, and it further underscores the need to validate microarray results at the protein level.

In summary, microarray analysis using HIV-1 latently infected resting memory $CD4^+$ T cells generated with our *in vitro* model points toward possible new pathways of latency beyond transcription that could be explored as new therapeutic targets aimed at eradicating this reservoir. In addition, we identified a panel of surface makers differentially expressed in latently infected cells, which seem worth investigating for their potential use as biomarkers. Indeed, they might allow the enrichment of this latent reservoir for in-depth molecular studies, for monitoring the size of the latent reservoir in the clinical setting, as well as for the development of novel therapeutic strategies aimed at eradicating HIV-1. Among them, CD2 has proven to allow efficient enrichment of latently infected resting memory $CD4^+$ T cells *ex vivo*.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI084711 (F.R.). L.M. was also supported by NIH-NCI grant P30CA006973.

REFERENCES

1. **Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, Polis M, Dewar RL, Planta A, Liu S, Metcalf JA, Mellors JW, Coffin JM.** 2003. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. **41:**4531–4536.
2. **Cohen Stuart JW, Wensing AM, Kovacs C, Righart M, de Jong D, Kaye S, Schuurman R, Visser CJ, Boucher CA.** 2001. Transient relapses ("blips") of plasma HIV RNA levels during HAART are associated with drug resistance. J. Acquir. Immune Defic. Syndr. **28:**105–113.
3. **Havlir DV, Bassett R, Levitan D, Gilbert P, Tebas P, Collier AC, Hirsch MS, Ignacio C, Condra J, Gunthard HF, Richman DD, Wong JK.** 2001. Prevalence and predictive value of intermittent viremia with combination HIV therapy. JAMA **286:**171–179.
4. **Ortiz GM, Wellons M, Brancato J, Vo HT, Zinn RL, Clarkson DE, Van Loon K, Bonhoeffer S, Miralles GD, Montefiori D, Bartlett JA, Nixon DF.** 2001. Structured antiretroviral treatment interruptions in chronically HIV-1-infected subjects. Proc. Natl. Acad. Sci. U. S. A. **98:**13288–13293.
5. **Choudhary SK, Margolis DM.** 2011. Curing HIV: pharmacologic approaches to target HIV-1 latency. Annu. Rev. Pharmacol. Toxicol. **51:**397–418.
6. **Coiras M, Lopez-Huertas MR, Perez-Olmeda M, Alcami J.** 2009. Understanding HIV-1 latency provides clues for the eradication of long-term reservoirs. Nat. Rev. Microbiol. **7:**798–812.
7. **Dahl V, Josefsson L, Palmer S.** 2010. HIV reservoirs, latency, and reactivation: prospects for eradication. Antiviral Res. **85:**286–294.
8. **Iglesias-Ussel MD, Romerio F.** 2011. HIV reservoirs: the new frontier. AIDS Rev. **13:**13–29.
9. **Tyagi M, Romerio F.** 2011. Models of HIV-1 persistence in the CD4+ T cell compartment: past, present and future. Curr. HIV Res. **9:**579–587.
10. **Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel MR, Ghattas G, Brenchley JM, Schacker TW, Hill BJ, Douek DC, Routy JP, Haddad EK, Sekaly RP.** 2009. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat. Med. **15:**893–900.
11. **Archin NM, Liberty AL, Kashuba AD, Choudhary SK, Kuruc JD, Crooks AM, Parker DC, Anderson EM, Kearney MF, Strain MC, Richman DD, Hudgens MG, Bosch RJ, Coffin JM, Eron JJ, Hazuda DJ, Margolis DM.** 2012. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature **487:**482–485.
12. **Lehrman G, Hogue IB, Palmer S, Jennings C, Spina CA, Wiegand A, Landay AL, Coombs RW, Richman DD, Mellors JW, Coffin JM, Bosch RJ, Margolis DM.** 2005. Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. Lancet **366:**549–555.
13. **Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ.** 2009. The challenge of finding a cure for HIV infection. Science **323:**1304–1307.
14. **Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang HC, Zhang H, Margolick JB, Blankson JN, Siliciano RF.** 2012. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. Immunity **36:**491–501.
15. **Antinori A, Perno CF, Giancola ML, Forbici F, Ippolito G, Hoetelmans RM, Piscitelli SC.** 2005. Efficacy of cerebrospinal fluid (CSF)-penetrating antiretroviral drugs against HIV in the neurological compartment: different patterns of phenotypic resistance in CSF and plasma. Clin. Infect. Dis. **41:**1787–1793.
16. **Solas C, Lafeuillade A, Halfon P, Chadapaud S, Hittinger G, Lacarelle B.** 2003. Discrepancies between protease inhibitor concentrations and viral load in reservoirs and sanctuary sites in human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. **47:**238–243.
17. **Valentin A, Morrow M, Poirier RH, Aleman K, Little R, Yarchoan R, Pavlakis GN.** 2010. Identification of a potential pharmacological sanctuary for HIV type 1 in a fraction of CD4(+) primary cells. AIDS Res. Hum. Retroviruses **26:**79–88.
18. **Contreras-Galindo R, Almodovar-Camacho S, Gonzalez-Ramirez S, Lorenzo E, Yamamura Y.** 2007. Comparative longitudinal studies of HERV-K and HIV-1 RNA titers in HIV-1-infected patients receiving successful versus unsuccessful highly active antiretroviral therapy. AIDS Res. Hum. Retroviruses **23:**1083–1086.
19. **Contreras-Galindo R, Kaplan MH, Contreras-Galindo AC, Gonzalez-Hernandez MJ, Ferlenghi I, Giusti F, Lorenzo E, Gitlin SD, Dosik MH, Yamamura Y, Markovitz DM.** 2012. Characterization of human endogenous retroviral elements in the blood of HIV-1-infected individuals. J. Virol. **86:**262–276.
20. **Marini A, Harper JM, Romerio F.** 2008. An in vitro system to model the establishment and reactivation of HIV-1 latency. J. Immunol. **181:**7713–7720.
21. **Adkins D, Ratanatharathorn V, Yang H, White B.** 2009. Safety profile and clinical outcomes in a phase I, placebo-controlled study of siplizumab in acute graft-versus-host disease. Transplantation **88:**198–202.
22. **Besse T, Malaise J, Mourad M, Pirson Y, Hope J, Awwad M, White-Scharf M, Squifflet JP.** 1997. Prevention of rejection with BTI-322 after

renal transplantation (results at 9 months). Transplant. Proc. **29:**2425–2426.
23. **Bissonnette R, Langley RG, Papp K, Matheson R, Toth D, Hultquist M, Geba GP, White B.** 2009. Humanized anti-CD2 monoclonal antibody treatment of plaque psoriasis: efficacy and pharmacodynamic results of two randomized, double-blind, placebo-controlled studies of intravenous and subcutaneous siplizumab. Arch. Dermatol. Res. **301:**429–442.
24. **Brochstein JA, Grupp S, Yang H, Pillemer SR, Geba GP.** 2010. Phase-1 study of siplizumab in the treatment of pediatric patients with at least grade II newly diagnosed acute graft-versus-host disease. Pediatr. Transplant. **14:**233–241.
25. **Fanale MA, Younes A.** 2007. Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma. Drugs **67:**333–350.
26. **Mourad M, Besse T, Malaise J, Baldi A, Latinne D, Bazin H, Pirson Y, Hope J, Squifflet JP.** 1997. BTI-322 for acute rejection after renal transplantation. Transplant. Proc. **29:**2353. doi:10.1016/S0041-1345(97)00398-9.
27. **O'Mahony D, Morris JC, Stetler-Stevenson M, Matthews H, Brown MR, Fleisher T, Pittaluga S, Raffeld M, Albert PS, Reitsma D, Kaucic K, Hammershaimb L, Waldmann TA, Janik JE.** 2009. EBV-related lymphoproliferative disease complicating therapy with the anti-CD2 monoclonal antibody, siplizumab, in patients with T-cell malignancies. Clin. Cancer Res. **15:**2514–2522.
28. **Pruett TL, McGory RW, Wright FH, Pescovitz MD, Yang H, McClain JB.** 2009. Safety profile, pharmacokinetics, and pharmacodynamics of siplizumab, a humanized anti-CD2 monoclonal antibody, in renal allograft recipients. Transplant. Proc. **41:**3655–3661.
29. **Przepiorka D, Phillips GL, Ratanatharathorn V, Cottler-Fox M, Sehn LH, Antin JH, LeBherz D, Awwad M, Hope J, McClain JB.** 1998. A phase II study of BTI-322, a monoclonal anti-CD2 antibody, for treatment of steroid-resistant acute graft-versus-host disease. Blood **92:**4066–4071.
30. **Squifflet JP, Besse T, Malaise J, Mourad M, Delcorde C, Hope JA, Pirson Y.** 1997. BTI-322 for induction therapy after renal transplantation: a randomized study. Transplant. Proc. **29:**317–319.
31. **Zhang Z, Zhang M, Ravetch JV, Goldman C, Waldmann TA.** 2003. Effective therapy for a murine model of adult T-cell leukemia with the humanized anti-CD2 monoclonal antibody, MEDI-507. Blood **102:**284–288.
32. **Iglesias-Ussel MD, Marchionni L, Romerio F.** 20 February 2013. Isolation of microarray-quality RNA from primary human cells after intracellular immunostaining and fluorescence-activated cell sorting. J. Immunol. Methods doi:10.1016/j.jim.2013.02.003.
33. **Coull JJ, Romerio F, Sun JM, Volker JL, Galvin KM, Davie JR, Shi Y, Hansen U, Margolis DM.** 2000. The human factors YY1 and LSF repress the human immunodeficiency virus type 1 long terminal repeat via recruitment of histone deacetylase 1. J. Virol. **74:**6790–6799.
34. **Sheehy AM, Gaddis NC, Choi JD, Malim MH.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646–650.
35. **Ruhl M, Himmelspach M, Bahr GM, Hammerschmid F, Jaksche H, Wolff B, Aschauer H, Farrington GK, Probst H, Bevec D, Hauber J.** 1993. Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating transactivation. J. Cell Biol. **123:**1309–1320.
36. **Modiano JF, Domenico J, Szepesi A, Lucas JJ, Gelfand EW.** 1994. Differential requirements for interleukin-2 distinguish the expression and activity of the cyclin-dependent kinases Cdk4 and Cdk2 in human T cells. J. Biol. Chem. **269:**32972–32978.
37. **Utley RT, Cote J.** 2003. The MYST family of histone acetyltransferases. Curr. Top. Microbiol. Immunol. **274:**203–236.
38. **Henderson BR, Percipalle P.** 1997. Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. J. Mol. Biol. **274:**693–707.
39. **Arnold M, Nath A, Hauber J, Kehlenbach RH.** 2006. Multiple importins function as nuclear transport receptors for the Rev protein of human immunodeficiency virus type 1. J. Biol. Chem. **281:**20883–20890.
40. **Gautier VW, Gu L, O'Donoghue N, Pennington S, Sheehy N, Hall WW.** 2009. In vitro nuclear interactome of the HIV-1 Tat protein. Retrovirology **6:**47. doi:10.1186/1742-4690-6-47.
41. **Bosque A, Planelles V.** 2009. Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. Blood **113:**58–65.
42. **Burke B, Brown HJ, Marsden MD, Bristol G, Vatakis DN, Zack JA.** 2007. Primary cell model for activation-inducible human immunodeficiency virus. J. Virol. **81:**7424–7434.
43. **Burnett JC, Lim KI, Calafi A, Rossi JJ, Schaffer DV, Arkin AP.** 2010. Combinatorial latency reactivation for HIV-1 subtypes and variants. J. Virol. **84:**5958–5974.
44. **Gondois-Rey F, Biancotto A, Pion M, Chenine AL, Gluschankof P, Horejsi V, Tamalet C, Vigne R, Hirsch I.** 2001. Production of HIV-1 by resting memory T lymphocytes. AIDS **15:**1931–1940.
45. **Lassen KG, Hebbeler AM, Bhattacharyya D, Lobritz MA, Greene WC.** 2012. A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs. PLoS One **7:**e30176. doi:10.1371/journal.pone.0030176.
46. **Sahu GK, Lee K, Ji J, Braciale V, Baron S, Cloyd MW.** 2006. A novel in vitro system to generate and study latently HIV-infected long-lived normal CD4+ T-lymphocytes. Virology **355:**127–137.
47. **Saleh S, Solomon A, Wightman F, Xhilaga M, Cameron PU, Lewin SR.** 2007. CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency. Blood **110:**4161–4164.
48. **Swiggard WJ, Baytop C, Yu JJ, Dai J, Li C, Schretzenmair R, Theodosopoulos T, O'Doherty U.** 2005. Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. J. Virol. **79:**14179–14188.
49. **Tyagi M, Pearson RJ, Karn J.** 2010. Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. J. Virol. **84:**6425–6437.
50. **Yang HC, Xing S, Shan L, O'Connell K, Dinoso J, Shen A, Zhou Y, Shrum CK, Han Y, Liu JO, Zhang H, Margolick JB, Siliciano RF.** 2009. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. J. Clin. Invest. **119:**3473–3486.
51. **Pasternak AO, Jurriaans S, Bakker M, Prins JM, Berkhout B, Lukashov VV.** 2009. Cellular levels of HIV unspliced RNA from patients on combination antiretroviral therapy with undetectable plasma viremia predict the therapy outcome. PLoS One **4:**e8490. doi:10.1371/journal.pone.0008490.
52. **Bradley LM, Haynes L, Swain SL.** 2005. IL-7: maintaining T-cell memory and achieving homeostasis. Trends Immunol. **26:**172–176.
53. **Khaled AR, Durum SK.** 2003. Death and Baxes: mechanisms of lymphotrophic cytokines. Immunol. Rev. **193:**48–57.
54. **Swain SL, Agrewala JN, Brown DM, Jelley-Gibbs DM, Golech S, Huston G, Jones SC, Kamperschroer C, Lee WH, McKinstry KK, Roman E, Strutt T, Weng NP.** 2006. CD4+ T-cell memory: generation and multifaceted roles for CD4+ T cells in protective immunity to influenza. Immunol. Rev. **211:**8–22.
55. **de la Fuente C, Santiago F, Deng L, Eadie C, Zilberman I, Kehn K, Maddukuri A, Baylor S, Wu K, Lee CG, Pumfery A, Kashanchi F.** 2002. Gene expression profile of HIV-1 Tat expressing cells: a close interplay between proliferative and differentiation signals. BMC Biochem. **3:**14. doi:10.1186/1471-2091-3-14.
56. **Krishnan V, Zeichner SL.** 2004. Host cell gene expression during human immunodeficiency virus type 1 latency and reactivation and effects of targeting genes that are differentially expressed in viral latency. J. Virol. **78:**9458–9473.
57. **Munier S, Delcroix-Genete D, Carthagena L, Gumez A, Hazan U.** 2005. Characterization of two candidate genes, NCoA3 and IRF8, potentially involved in the control of HIV-1 latency. Retrovirology **2:**73. doi:10.1186/1742-4690-2-73.
58. **Bandyopadhyay S, Kelley R, Ideker T.** 2006. Discovering regulated networks during HIV-1 latency and reactivation. Pac. Symp. Biocomput. **2006:**354–366.
59. **Chun TW, Justement JS, Lempicki RA, Yang J, Dennis G, Jr, Hallahan CW, Sanford C, Pandya P, Liu S, McLaughlin M, Ehler LA, Moir S, Fauci AS.** 2003. Gene expression and viral production in latently infected, resting CD4+ T cells in viremic versus aviremic HIV-infected individuals. Proc. Natl. Acad. Sci. U. S. A. **100:**1908–1913.
60. **Mbonye U, Karn J.** 2011. Control of HIV latency by epigenetic and non-epigenetic mechanisms. Curr. HIV Res. **9:**554–567.
61. **Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E.** 2009. Epigenetic regulation of HIV-1 latency by cytosine methylation. PLoS Pathog. **5:**e1000495. doi:10.1371/journal.ppat.1000495.
62. **Brooks DG, Zack JA.** 2002. Effect of latent human immunodeficiency virus infection on cell surface phenotype. J. Virol. **76:**1673–1681.
63. **Chen BK, Gandhi RT, Baltimore D.** 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1

involves independent activities of vpu, env, and nef. J. Virol. **70:**6044–6053.
64. **Chugh P, Bradel-Tretheway B, Monteiro-Filho CM, Planelles V, Maggirwar SB, Dewhurst S, Kim B.** 2008. Akt inhibitors as an HIV-1 infected macrophage-specific anti-viral therapy. Retrovirology **5:**11. doi:10.1186/1742-4690-5-11.
65. **Chugh P, Fan S, Planelles V, Maggirwar SB, Dewhurst S, Kim B.** 2007. Infection of human immunodeficiency virus and intracellular viral Tat protein exert a pro-survival effect in a human microglial cell line. J. Mol. Biol. **366:**67–81.
66. **Emerman M, Malim MH.** 1998. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. Science **280:**1880–1884.
67. **Poon B, Grovit-Ferbas K, Stewart SA, Chen IS.** 1998. Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents. Science **281:**266–269.
68. **Selliah N, Finkel TH.** 2001. Biochemical mechanisms of HIV induced T cell apoptosis. Cell Death Differ. **8:**127–136.
69. **Tanaka M, Ueno T, Nakahara T, Sasaki K, Ishimoto A, Sakai H.** 2003. Downregulation of CD4 is required for maintenance of viral infectivity of HIV-1. Virology **311:**316–325.
70. **Bastiani L, Laal S, Kim M, Zolla-Pazner S.** 1997. Host cell-dependent alterations in envelope components of human immunodeficiency virus type 1 virions. J. Virol. **71:**3444–3450.
71. **Meuer SC, Hussey RE, Fabbi M, Fox D, Acuto O, Fitzgerald KA, Hodgdon JC, Protentis JP, Schlossman SF, Reinherz EL.** 1984. An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. Cell **36:**897–906.
72. **Bressler P, Pantaleo G, Demaria A, Fauci AS.** 1991. Anti-CD2 receptor antibodies activate the HIV long terminal repeat in T lymphocytes. J. Immunol. **147:**2290–2294.
73. **Shen A, Yang HC, Zhou Y, Chase AJ, Boyer JD, Zhang H, Margolick JB, Zink MC, Clements JE, Siliciano RF.** 2007. Novel pathway for induction of latent virus from resting CD4(+) T cells in the simian immunodeficiency virus/macaque model of human immunodeficiency virus type 1 latency. J. Virol. **81:**1660–1670.
74. **Cooper JC, Morgan G, Harding S, Subramanyam M, Majeau GR, Moulder K, Alexander DR.** 2003. Alefacept selectively promotes NK cell-mediated deletion of CD45R0+ human T cells. Eur. J. Immunol. **33:**666–675.
75. **Nizet Y, Chentoufi AA, de la Parra B, Lewalle P, Rouas R, Cornet A, Besse T, Mourad M, Malaise J, Squifflet JP, Bazin H, Latinne D.** 2000. The experimental (in vitro) and clinical (in vivo) immunosuppressive effects of a rat IgG2b anti-human CD2 mAb, LO-CD2a/BTI-322. Transplantation **69:**1420–1428.
76. **da Silva AJ, Brickelmaier M, Majeau GR, Li Z, Su L, Hsu YM, Hochman PS.** 2002. Alefacept, an immunomodulatory recombinant LFA-3/IgG1 fusion protein, induces CD16 signaling and CD2/CD16-dependent apoptosis of CD2(+) cells. J. Immunol. **168:**4462–4471.
77. **Majeau GR, Meier W, Jimmo B, Kioussis D, Hochman PS.** 1994. Mechanism of lymphocyte function-associated molecule 3-Ig fusion proteins inhibition of T cell responses. Structure/function analysis in vitro and in human CD2 transgenic mice. J. Immunol. **152:**2753–2767.
78. **Algino KM, Thomason RW, King DE, Montiel MM, Craig FE.** 1996. CD20 (pan-B cell antigen) expression on bone marrow-derived T cells. Am. J. Clin. Pathol. **106:**78–81.
79. **Withers DR, Fiorini C, Fischer RT, Ettinger R, Lipsky PE, Grammer AC.** 2007. T cell-dependent survival of CD20+ and CD20− plasma cells in human secondary lymphoid tissue. Blood **109:**4856–4864.
80. **Aggarwal BB, Gupta SC, Kim JH.** 2012. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood **119:**651–665.
81. **Herbein G, O'Brien WA.** 2000. Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. Proc. Soc. Exp. Biol. Med. **223:**241–257.
82. **Denoeud J, Moser M.** 2011. Role of CD27/CD70 pathway of activation in immunity and tolerance. J. Leukoc. Biol. **89:**195–203.
83. **Park GB, Kim YS, Lee HK, Song H, Cho DH, Lee WJ, Hur DY.** 2010. Endoplasmic reticulum stress-mediated apoptosis of EBV-transformed B cells by cross-linking of CD70 is dependent upon generation of reactive oxygen species and activation of p38 MAPK and JNK pathway. J. Immunol. **185:**7274–7284.
84. **Berndt C, Mopps B, Angermuller S, Gierschik P, Krammer PH.** 1998. CXCR4 and CD4 mediate a rapid CD95-independent cell death in CD4(+) T cells. Proc. Natl. Acad. Sci. U. S. A. **95:**12556–12561.
85. **Trushin SA, Algeciras-Schimnich A, Vlahakis SR, Bren GD, Warren S, Schnepple DJ, Badley AD.** 2007. Glycoprotein 120 binding to CXCR4 causes p38-dependent primary T cell death that is facilitated by, but does not require cell-associated CD4. J. Immunol. **178:**4846–4853.