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Memory CCR6+CD4+ T Cells Are Preferential Targets for Productive HIV Type 1 Infection Regardless of Their Expression of Integrin β7

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HIV type 1 infection is associated with a rapid depletion of Th17 cells from the GALT. The chemokine receptor CCR6 is a marker for Th17 lineage polarization and HIV permissiveness in memory CD4+ T cells. CCR6+ T cells have the potential to migrate into the GALT via the gut-homing integrin αβ7, a newly identified HIV-gp120 binding receptor. In this study, we investigated whether memory T cells coexpressing CCR6 and integrin β7 are selective HIV targets and whether retinoic acid (RA)-induced imprinting for gut-homing selectively increases CCR6+ T cell permissiveness to infection. We demonstrated that β7−R6− and β7−R6+ compared with β7−R6− and β7−R6+ T cells were highly permissive to HIV, produced Th17 cytokines, and their frequency was decreased in the peripheral blood of HIV-infected subjects. RA upregulated integrin α7 and β7 coexpression in both CCR6+ and CCR6− T cells, but increased HIV permissiveness selectively in CCR6+ T cells via entry (CCR5 upregulation) and postentry mechanisms. In conclusion, these results demonstrate that CCR6, but not the integrin β7, is a discriminative marker for memory T cells imprinted with a transcriptional program favorable to HIV replication. Nevertheless, given the ability of integrin β7 to regulate cell migration into the GALT and bind HIV-gp120, CCR6+ T cells coexpressing integrin β7 and CCR5 might have an extraordinary ability to disseminate HIV from the portal sites of entry. Understanding the molecular mechanisms of memory CCR6+ T cell differentiation is critical for the design of new therapeutic strategies that should interfere with viral permissiveness but not Th17 lineage commitment and gut-homing potential in CCR6+ T cells. The Journal of Immunology, 2011, 186: 4618–4630.
to cross intestinal vascular walls [e.g., integrin αβ7 (15, 16)], and then by chemokine receptors that further direct cell localization into lymphoid or nonlymphoid structures underneath the endothe-
lium [e.g., CCR6 (17), CCR9 (18)]. The expression of the gut-
homing marker integrin αβ2 is controlled in part by the retinoic acid 
(RA) activation pathway, an active derivative of vitamin A 
(retinol) generated mainly by mucosal dendritic cells (18). The integrin αβ2 binds to the mucosal addressin cell adhesion 
molecule-1 (MadCAM-1) expressed on gut endothelial cells (15).

Recent studies identified the integrin αβ2 as a new receptor for 
HIV-gp120 binding that facilitates viral replication by 
triggering lymphocyte function-associated Ag-1 (LFA-1) activa-
tion and increasing HIV cell-to-cell transfer (19). The integrin 
αβ2 is expressed on peripheral blood T cells at low levels and 
under an inactive state, with the transition toward an activated 
form being induced by RA (20). The HIV-gp120 binds only the 
activated form of the αβ2 integrin, which forms a complex with 
CD4 and is coexpressed with CCR5 on a small subset of RA-
treated CD4+ T cells (21). Of interest, integrin αβ2 identifies 
a subset of memory CD4+ T cells producing IL-17 that is pref-
erentially infected and depleted during acute SIV infection (17, 
22). Peripheral blood CD4+ T cells expressing integrin αβ2 are 
also depleted during primary HIV infection (23). Thus, HIV 
binding onto integrin αβ2 may be a very important mechanism 
during HIV transmission and might explain the depletion of Th17 
cells from the GALT in HIV-infected subjects.

Among gut-homing chemokine receptors, CCR6 is critical for 
cell migration into Peyer’s patches of the distal small intestine 
(ileum) (17, 24, 25), whereas CCR9 mediates cell infiltration into 
lamina propria of the proximal small intestine (20, 26, 27). In 
addition to being a homing receptor, CCR6 is a well-established 
marker for human Th17 cells (28, 29). Recent studies by us and 
others identified CCR6 as a marker for discrete CD4+ T cell 
subsets that coexpress the HIV coreceptor CCR5 and are highly 
permissive to R5 and X4 HIV replication (30, 31). Superior HIV-
DNA integration in memory CCR6+ T cells (30) is associated with 
a significant decrease in the frequency of these cells in the pe-
ripheral blood of HIV-infected individuals (30, 32), and this de-
crease remained persistent despite the normalization of CD4 
counts under viral suppressive therapy (30). The disease pro-
gression of SIV/HIV is associated with depletion of Th17 cells 
from the GALT, a process that subsequently leads to impaired 
mucosal permeability and microbial translocation from the gut 
(33–37). Antiretroviral therapy (ART) is associated with a partial 
restoration of the CD4+ T cell and Th17 into the GALT (38–40).

Thus, it appears that HIV causes dramatic alterations in mucosal immunity by efficiently infecting CD4+ T cells that are present at the portal sites of entry.

Considering the fact that the integrin αβ2 mediates recruitment of 
CCR6+ T cells across the intestinal endothelium, we sought to 
determine whether there was a link between HIV permissiveness 
and imprinting for gut-homing in CCR6+ T cells. With this in mind, 
we measured HIV replication in memory CD4+ T cells expressing 
CCR6 and/or integrin β2, and investigated the consequences of RA 
expression on HIV permissiveness in CCR6+ and CCR6+ T cells. 
We found that CCR6, but not integrin β2, is a marker for T cell 
subsets that are highly permissive to HIV replication and produced 
both Th17 and Th1 cytokines, whereas expression of integrin β2 
alone identified T cells resistant to infection that produced Th1 
cytokines only. The RA upregulated integrin αβ2 expression on 
both CCR6+ and CCR6+ T cells, but upregulation of CCR5 ex-
pression and enhancement of HIV replication were observed preferentially in RA-treated CCR6+ T cells. In conclusion, these 
findings point to the fact that CCR6 expression coincides with 
a transcriptional program compatible with HIV replication in 
memory CD4+ T cells expressing or not the gut-homing adhesion 
molecule integrin αβ2; therefore, this indicates the need for future 
research into the molecular mechanisms of epigenetic mod-
fications (41) associated with CCR6+ T cell differentiation that 
might be exploited by HIV for efficient replication.

Materials and Methods

Subjects

Subjects infected with HIV-1 (n = 14) and uninfected donors (n = 24) were 
recruited at the Montreal Chest Institute, McGill University Health Centre 
and Saint-Luc Hospital (Montreal, QC, Canada), through the Fonds de la 
Recherche en Santé Québec/AIDS and Infectious Diseases Network 
(Montreal, QC, Canada). Informed consent and Internal Review Board 
approval were obtained for all participants. Table I summarizes immuno-
ological, virological, and clinical data of HIV-infected patients. Among 
HIV-infected patients, three subjects received ART, which included a pro-
tease inhibitor, a nonnucleoside reverse transcriptase inhibitor, and nu-
cleoside reverse transcriptase inhibitors. Plasma viral load was measured 
using the Amplicor HIV-1 monitor ultrasensitive method (Roche). PBMCs 
(10–1010 cells) were collected from HIV-infected and uninfected indi-
viduals by leukapheresis as previously reported (42). For specific experi-
ments, 10 ml fresh blood was collected from uninfected donors.

Abs and polychromatic flow cytometry analysis

Fluorochrome-conjugated Abs used for polychromatic flow cytometry 
analysis were anti–CD3-Pacific blue (UCHT1), anti–CD4-Alexa700 (RPA-
T4), anti–CD45RA-allophycocyanin-Cy7 (H1001), anti–CCR5-FITC (2D7), 
anti–CCR6-PE (11A9), anti–CXCR4-PE (12G5), anti–LFA-1–FITC (G43-
25B), anti–CD38-FITC (HIT2), anti–HLA-D-related (HLA-DR)–FITC 
(G46-6), anti–CXCR3-PE-Cy5, and anti–B2–FITC (6CC8) (BD Phar-
mingen), anti–CCL3–FITC (93342) and anti–CCR7–FITC (150503) (R&D 
Systems), integrin anti–α4-PE (4H4), anti–integrin β2–FITC (FB504), 
anti–integrin β7–PE (FB504), anti–integrin β7–Cy5 (FB504), anti– 
CD56–FITC (MEM188), anti–IL-17–PE (64DEC17), anti–CD27–PE-Cy7 
(G0323), anti–CD127–FITC (eBioRDR5) (eBioscience), anti–CD14–FITC 
(My4) and anti–HIV-p24–FITC (HFI900-1) (Beckman Coulter), anti– 
CD25–FITC (BC96) and anti–PD-1–FITC (EH12-2H7) (BioLegend), 
anti–CD8–FITC (BW135/80), anti–CD19–FITC (LTL9), and anti–IFN-γ– 
PE (45-15) Abs (Miltenyi). Cell phenotype was analyzed by flow cytom-
etry using the BD LSRII cytometer and BD Diva software. A viability staining 
Vivid (Invitrogen) was included in each staining mixture to ex-
clude dead cells from our data. FACs analysis was performed using the 
BD LSRII cytometer and BD Diva software. For multicolor analysis, all 
Abs were titrated for an optimal noise/signal ratio, all Ab cocktails were 
validated by comparing single to multiple staining, and gates were 
established using fluorescence minus one and/or isotype controls, as 
previously described (30).

MACS and FACS

PBMCs were isolated from fresh blood or leukapheresis by Ficoll-
Paque (Amersham Biosciences) gradient density centrifugation (43). The 
CD4+ T cells were sorted from PBMCs by negative selection using magnetic beads (MACS; Miltenyi) with a purity >95%, as determined 
by triple staining with CD3, CD4, and CD8 Abs and FACS analysis. For 
experiments in Figs. 1 and 2, memory β7+ R6–, β7+ R6+, β2+ R6–, and 
β2+ R6+ CD4+ T cell subsets were sorted on staining with anti–CD45RA-
allophycocyanin-Cy7, anti–integrin β2–FITC, and anti–CCR6–PE Abs. The 
sorting gate was set on CD45RA+ T cells as depicted in Supplemental 
Fig. 1 using the BD Aria II magnetic beads (MACS; Miltenyi) with a purity 
98% pure and 99% viable and B cells (CD19). The sorting gate was set on 
CD45RA+ T cells that lack FITC staining as depicted in Supplemental 
Fig. 3 using the BD Aria II magnetic beads (MACS; Miltenyi) with a purity 
98% pure and 99% viable and B cells (CD19). The sorting gate was set on 
CD45RA+ T cells that lack FITC staining as depicted in Supplemental 
Fig. 3 using the BD Aria II magnetic beads (MACS; Miltenyi) with a purity 
98% pure and 99% viable and B cells (CD19). The sorting gate was set on 
CD45RA+ T cells that lack FITC staining as depicted in Supplemental 
Fig. 3 using the BD Aria II magnetic beads (MACS; Miltenyi) with a purity 
98% pure and 99% viable and B cells (CD19). The sorting gate was set on

Intracellular staining for cytokines

PBMCs or CD4+ T cell subsets were stimulated with PMA (50 ng/ml) and 
imonomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml) for 18 h. The production of IL-17A was measured by intracellular staining with

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appropriate Abs using the BD cytofix/cytoperm fixation/permeabilization solution kit (BD Biosciences) according to the manufacturer’s protocols.

**HIV infection and quantification of viral replication**

The following HIV-1 molecular clones were used in this study: 1) replication-competent CCR5-using (R5) NL4.3-BaL and CCRX4-using (X4) NL4.3 HIV expressing gfp in place of nef; and 2) replication-defective VSVG-GFP-pseudotyped HIV (VSVG-GFP-HIV, an envelope-deficient NL4.3 provirus pseudotyped with the VSVG envelope and expressing gfp in place of nef). The HIV stocks were produced by transfection of 293T cells with the appropriate plasmids using Fugene6 (Roche) (44). Viral stocks were quantified by a HIV-p24 ELISA assay (45) and titrated for infectiousness on CD4+CCR5/CXCR4+ MAGI cells and primary CD5/CD28-activated CD4+ T cells. Sorted CD4+ T cell subsets were exposed to HIV (50 ng HIV-p24/10^6 cells corresponding to a multiplicity of infection 0.01/10^6 cells, as previously used) (30, 44) for 3 h at 37°C. Unbound HIV was removed by extensive washing, and cells were cultured at 10^6/ml in RPMI 1640 10% FBS and IL-2 (5 ng/ml; R&D Systems). For NL4.3 BaL and NL4.3 HIV infection, cell culture supernatants were harvested every 3 d and HIV replication was measured by HIV-p24 ELISA. In parallel, cells were harvested at day 3 postinfection, and cell lysates were used for real-time PCR quantification of integrated HIV-DNA. For VSVG-GFP-HIV infection, cells were harvested 3 d postinfection, and the frequency of GFP-expressing cells (indicative of HIV-DNA integration and transcription) was analyzed by flow cytometry.

**Real-time PCR quantification of HIV-DNA**

The quantification of integrated HIV-DNA was performed as previously described (45, 46). In brief, T cells were digested in a proteinase K buffer (Invitrogen), and 10^5 cells/15 µl lysisate were used per amplification. Integrated HIV-DNA was amplified first (12 cycles) using two outward-facing Alu primers and one HIV long terminal repeat (LTR) primer tagged with a λ sequence; the CD3 gene was amplified in the same reaction (30, 45, 46). The HIV and CD3 amplicons were then amplified in separate reactions (Light Cycler; Roche Diagnostics). The HIV-DNA was amplified with a fluorescent probe specific for HIV LTR (46). The CD3 DNA was amplified using a λ-specific primer and an inner LTR primer in the presence of two fluorescent probes specific for HIV LTR (46). The CD3 DNA was amplified using inner primers and two fluorescent probes specific for CD3 (45). Amplification reactions were carried out with Jumpstart Taq Ready Mix (Sigma) and Taq Polymerase (Invitrogen). The ACH2 cells carrying one copy of integrated HIV-DNA per cell (National Institute of Allergy and Infectious Diseases reagent program) were used as standard curve (45).

**ELISA quantification of cytokine production**

Cytokine levels in cell culture supernatants were quantified by ELISA assays specific for CCL20 (R&D Systems), IL-17, and IFN-γ (eBioscience) according to the manufacturers’ protocols.

**Statistics**

Statistical significance between groups (p < 0.05 was considered significant) was calculated using Mann–Whitney U test (comparison between unpaired samples), paired t test, and Wilcoxon signed rank test or paired t test (comparison between paired samples), as specified in the figure legends. All statistical analyses were performed using the GraphPad Prism 5 software.

**Results**

**CCR6, but not integrin β7, is a marker for memory CD4+ T cell subsets highly permissive to HIV replication**

CCR6 is a marker for memory CD4+ T cells highly permissive to HIV replication (17, 24, 30, 31), and the gut-homing integrin α4β7, identified as a new binding receptor for HIV-gp120 (15, 16). We hypothesized that CCR6+ T cells with gut-homing potential are major targets for HIV replication. Flow cytometry analysis of integrin β7 and CCR6 expression identified the following four memory (CD45RA2) CD5/CD28+ T cell subsets in the peripheral blood of HIV-uninfected individuals: β7−CCR6+, β7−CCR6+, β7+CCR6+, and β7+CCR6+ (Fig. 1A). These four T cell subsets were sorted by flow cytometry (Supplemental Fig. 1), stimulated via CD3/CD28, and assessed for the expression of the major HIV coreceptors CCR5 and CXCR4 before HIV exposure. The highest expression of CCR5 was detected on β7−CCR6+ and β7+CCR6+ T cells, with β7−CCR6− and β7−CCR6− expressing low CCR5 levels (Fig. 1B). The CXC4R4 expression was similarly high on the four memory T cell subsets (Fig. 1B). The integrin β7 and CCR6 phenotype was stable on TCR stimulation of sorted T cell subsets (data not shown). Subsequently, T cells were tested for their ability to support R5 HIV replication in vitro. Levels of HIV-p24 detected by ELISA in cell supernatants, indicative of productive HIV infection, were strikingly greater in β7−CCR6+ and β7−CCR6+ compared with β7−CCR6− and β7−CCR6+ T cells at days 3, 7, and 10 postinfection (Fig. 1C). Similarly, HIV-DNA integration, as quantified by real-time PCR, was significantly higher in β7+CCR6+ and β7+CCR6+ compared with β7−CCR6− and β7−CCR6− T cells, respectively, at day 3 postinfection (Fig. 1D).

Together, these results demonstrate that memory T cell subsets expressing CCR6 in the presence or absence of integrin β7 are highly permissive to R5 HIV replication, whereas cells expressing the integrin β7 only, or lacking both gut-homing markers, are relatively resistant to R5 HIV replication. Increased permissiveness to R5 HIV replication in β7−CCR6+ and β7+CCR6+ T cell subsets is most likely related to a highly efficient CCR5-mediated viral entry.

**CCR6, but not integrin β7, is a marker of Th17 specification in memory CD4+ T cells**

The CCR6 is a well-established marker for CD4+ T cells producing Th17 cytokines (28, 29). The integrin β7 was also reported to be a marker for Th17 cells (22). In this study, we investigated the link between Th17 lineage specification and HIV permissiveness in CD4+ T cell subsets expressing CCR6 and/or integrin β7. Memory β7−CCR6+, β7−CCR6+, β7+CCR6+, and β7+CCR6+ T cell subsets were sorted by flow cytometry, and their ability to produce Th1 (IL-17, CCL20) and Th1 (IFN-γ) cytokines on TCR triggering was quantified by ELISA. Memory β7−CCR6+ and β7−CCR6+ T cells were the only cells able to produce IL-17, and CCL20 levels produced by these cells were significantly greater compared with those produced by β7+CCR6+ and β7−CCR6+ T cells (Fig. 2, left and middle panels). Similarly, single-cell analysis of intracellular IL-17 expression demonstrated that 23 ± 8% and 31 ± 12% (mean ± SD; n = 5) of β7−CCR6+ and β7−CCR6+ T cells produced IL-17, respectively, whereas <8% of β7−CCR6− and β7+CCR6+ T cells were IL-17+ (Supplemental Fig. 2). CCR6+ compared with CCR6− T cell subsets expressed significantly higher frequencies of IL-17+ cells (paired t test, p < 0.05), whereas differences between β7−CCR6+ and β7+CCR6+ T cells were only marginally significant (p = 0.07) (Supplemental Fig. 2). In contrast, IFN-γ was produced at high levels by all four T cell subsets with some minor, although statistically significant, differences (Fig. 2, right panel). Together, these results demonstrate that CCR6, but not integrin β7, is a marker for Th17 polarized cells, and that β7−CCR6+ compared with β7−CCR6+ T cell subsets tend to be enriched in IL-17+ cells. Thus, the Th17 lineage specification of β7−CCR6+ and β7+CCR6+ T cells coincides with their superior permissiveness to HIV replication.

**CCR6 and integrin β7 identify four memory CD4+ T cell subsets that are highly heterogeneous in terms of differentiation status**

Previous studies demonstrated that different stages of CD4+ T cell differentiation exhibit distinct HIV permissiveness (4, 45). To get insights into the developmental relation between the four memory T cell subsets, β7−CCR6+, β7−CCR6+, β7+CCR6+, and β7+CCR6+ from uninfected individuals were analyzed for the expression of the following differentiation markers: CCR7 (a chemokine receptor mediating cell migration into lymph nodes) (47),...
CD27 (a member of the TNFR superfamily) (48), CD127 (the receptor for IL-7, a cytokine critical for homeostatic T cell proliferation) (49), and CD11a (the α-chain of LFA-1, an integrin critical for HIV infection of memory T cells) (50). Differential expression of CCR7 and CD27 distinguishes central (CM; CCR7+CD27+) and transitional (TM; CCR7−CD27+) CD4+ T cells (51), with their distinct ability to harbor integrated HIV-DNA in infected subjects receiving ART (45). The four memory T cell subsets were heterogeneous in terms of CM/EM/TM differentiation stage; β7+CCR6− and β7+CCR6+ subsets included the highest frequencies of TM cells and high frequencies of EM cells, whereas the β7+CCR6+ subset was enriched in CM cells with very low frequencies of EM and TM cells (Fig. 3A, 3B). The expression of CD127 was significantly higher on β7+CCR6− and β7+CCR6+ compared with β7+CCR6− and β7+CCR6− subsets, respectively.
were quantified in cell culture supernatants by ELISA (mean HIV permissiveness in Fig. 1b).

uninfected individuals, but the frequency of CD57+CD11a. The relative resistance to HIV observed in the subsets included the highest frequencies of Ki-67+ cells (data not shown), indicative of increased homeostatic cycling ability. The CD11a expression was significantly higher on CD57+, similar among the four subsets, with the highest MFI observed on terminal differentiation (CD57) (56, 57), and survival (Bcl2, an antiapoptotic protein) (58).

The frequency of circulating T cell subsets expressing CCR6 and/or integrin β7 is reduced in HIV-infected subjects

Infection with HIV in CD4+ T cells leads to increased susceptibility to apoptosis and depletion in HIV-infected individuals (52–54). To determine whether superior HIV permissiveness in β7+CCR6+ and β7+CCR6− subsets in vitro is associated with selective depletion in vivo, the frequency of peripheral blood memory CD4+ T cell subsets expressing the integrin β7 and/or CCR6 was analyzed in a cohort of HIV-infected subjects (HIV+: median time since infection, 5.5 mo; median CD4 counts, 506 cells/µl; and median plasma viral load, 6500.5 HIV RNA copies/ml; n = 14) (Table I) and uninfected control subjects (HIV−: n = 13). The frequency of β7+CCR6+, β7+CCR6−, and β7+CCR6−, but not β7−CCR6− T cell subsets, was significantly decreased in HIV-infected compared with uninfected individuals (Fig. 4A, 4B).

To further explain these alterations, we analyzed cells from four different HIV-infected individuals for the expression of several activation (CD25 and HLA-DR) (54), exhaustion (PD-1) (55), terminal differentiation (CD57) (56, 57), and survival (Bcl2, an antiapoptotic protein) (58) markers by flow cytometry. The frequency of CD25+ cells was significantly higher on β7−CCR6+ and β7−CCR6+ compared with β7+CCR6− and β7+CCR6− subsets, respectively, whereas the greatest levels of HLA-DR expression (mean fluorescence intensity [MFI]) were detected on the β7−CCR6− subsets (Fig. 4C). The frequency of PD-1+ cells was similar among the four subsets, with the highest MFI observed on β7−CCR6− cells (Fig. 4C). The highest frequency of CD57+ T cells was observed within the β7−CCR6− subset (Fig. 4C). For CD57, similar results were obtained with cells from HIV-uninfected individuals, but the frequency of CD57+β7−CCR6− cells was significantly higher in HIV-infected compared with uninfected individuals (46 ± 12 versus 10 ± 3%; Mann–Whitney U test, p = 0.01; n = 4) (Fig. 4C and data not shown). Finally, Bel-2 levels (% and MFI) were significantly greater in β7−CCR6− and β7−CCR6+ compared with β7+CCR6− and β7+CCR6− subsets, respectively (Fig. 4D). Together, these results reveal decreased frequencies of memory T cell subsets expressing CCR6 and/or integrin β7 during the first year of HIV infection. This suggests a potential link between these alterations and the expression of activation and/or exhaustion, but not with terminal differentiation or survival markers.

RA-mediated upregulation of integrin αβ7 is associated with increased permissiveness to R5 HIV replication in CCR6+ but not CCR6− T cells

All-trans-RA (ATRA) imprints T cells with gut-homing potential via induction of integrin αβ7 (20) and enhances permissiveness to R5 HIV replication in CD4+ T cells by a mechanism involving HIV-gp120 binding on activated integrin αβ7 (19, 21). To further explore the role of CCR6 as a marker for T cells selectively programmed to support HIV replication (30, 31), we investigated the effects of ATRA on CCR6+ and CCR6− T cell permissiveness to HIV. Memory CCR6+ and CCR6− T cells from three different HIV-uninfected individuals were sorted by flow cytometry (Supplemental Fig. 3) and stimulated via CD3/CD28 in the presence or absence of physiological doses of ATRA (10 nM) (19, 21). No significant changes in CCR6 expression were induced by ATRA (data not shown). Both CCR6+ and CCR6− T cells expressed similar levels of the RA receptor α (RARα) mRNA (data not shown), a prerequisite for ATRA responsiveness in CD4+ T cells (18). Consistently, TCR triggering in the presence of ATRA resulted in enhanced frequencies of αβ7+ cells within both CCR6+ and CCR6− T cell subsets, whereas the frequencies of αβ6+β7+ remained relatively low (<5%) (Fig. 5A, 5B). The activation marker CD25 was expressed at similarly high levels on CCR6+ and CCR6− T cells cultured in the presence or absence of ATRA (data not shown), indicative of a similar state of activation in these cells. Of particular interest, CCR6− compared with CCR6+ T cells expressed higher levels of CCR5, and ATRA treatment significantly increased CCR5 expression on CCR6+ but not on CCR6− T cells (Fig. 5C, 5D). In addition, treatment with ATRA did not interfere with total CD4+ T cell ability to produce the CCR5 binding chemokine CCL3 (data not shown). This result suggests the possibility that ATRA increases permissiveness to R5 HIV replication in CCR6+ but not in CCR6− T cells. Consistent with this hypothesis, exposure to R5 NL4.3BaL HIV strain demonstrated that ATRA preferentially increased viral replication in CCR6+, but not CCR6− T cells, at days 3 and 7 postinfection (Fig. 6A). Superior levels of R5 HIV replication coincided with significantly greater levels of HIV-DNA integration in ATRA-treated CCR6+ compared with CCR6− T cells at day 3 post-infection (Fig. 6B). Similarly, ATRA increased the MFI of
CXCR4 expression on CCR6+ but not CCR6− T cells (data not shown) and resulted in significantly greater levels of X4 NL4.3 HIV replication in ATRA-treated compared with untreated total CD4+ T cells (Supplemental Fig. 4) or CCR6+ T cells (data not shown). Thus, ATRA effects on T cell permissiveness to HIV are not restricted to R5 strains.

ATRA might increase CCR6+ T cell permissiveness to HIV replication by augmenting viral entry efficacy via integrin β7, CCR5, and CXCR4 upregulation, as well as by inducing a transcriptional program compatible with a more efficient HIV reverse transcription, integration, and/or transcription in CCR6+ but not CCR6− T cells. To distinguish between these two possibilities, we exposed cells to a VSVG-GFP pseudotyped HIV, which enters cells independently of the HIV receptor and coreceptors. The frequency of GFP+ T cells at day 3 postinfection, indicative of viral integration and transcription (as the GFP expression is under the control of the HIV LTR), was significantly higher in ATRA-treated compared with untreated CCR6+ T cells; ATRA had no significant effects on GFP expression in CCR6− T cells (Fig. 6C).

Consistent with differences observed in GFP expression, the

**FIGURE 3.** The integrin β7 and CCR6 identify four distinct stages of memory CD4+ T cell differentiation. The PBMCs from uninfected individuals were stained with a mixture of fluorochrome-conjugated Abs including anti–CD3-Pacific blue, anti–CD4-Alexa700, anti–CD45RA-allophycocyanin-Cy7, anti–CCR6-PE, anti–integrin β7-PE-Cy5, anti–CD27-PE-Cy7, and anti–CCR7-FITC, anti–CD127-FITC, or anti–CD11a-FITC Abs. The four memory CD3+ CD4+CD45RA− T cells expressing integrin β7 and/or CCR6 were analyzed for the expression of CCR7, CD27, CD127, and CD11a by polychromatic flow cytometry. A and B, The coexpression of CCR7 and CD27 identified CCR7+CD27−, CM (CCR7+CD27−), EM (CCR7−CD27−), and TM (CCR7−CD27+) T cells with distinct frequencies within the four memory T cell subsets. Shown are representative dot plots (%) (A) and statistical analysis (B) of the frequencies of CM, EM, and TM cells within the four memory T cell subsets (mean ± SEM; n = 5). Shown are representative histograms (% and MFI) (C, E) and statistical analysis (D, F) of CD127 and CD11a expression on the four memory T cell subsets (mean ± SEM; n = 5). Paired t test p values are indicated on the graphs.
quantification of integrated HIV-DNA showed significantly greater levels of proviral DNA in CCR6+ compared with CCR6− T cells treated with ATRA (Fig. 6D). Together, these results demonstrated that ATRA differentially acts on CCR6+ and CCR6− T cells, and preferentially increases HIV permissiveness in CCR6+ T cells by interfering with viral replication efficacy at entry (integrin β7 and CCR5) and postentry (integration and/or transcription) levels.

Discussion

In this study, we demonstrated that memory CD4+ T cells expressing CCR6 in the presence or absence of the gut-homing integrin α4β7 are highly permissive to HIV and exhibit a Th17 lineage specification profile. In addition, we demonstrated that ATRA, which triggers a signaling pathway critical for the imprinting for gut-homing (18), differentially acts on CCR6+ and CCR6− T cells and selectively increases HIV permissiveness in CCR6+ T cells by acting at entry (integrin β7 and CCR5 expression) and yet unidentified postentry levels. These findings reveal that expression of CCR6 coincides with a transcriptional program favorable for HIV replication and suggest that the integrin α4β7 might have the potential to enhance HIV infection in cis only in CCR6+ T cells. CCR6+ T cells are in majority HIV-specific (32), and their permissiveness to productive viral infection may contribute to altered immunity against HIV. In addition, by their ability to migrate into the GALT via integrin β7 and CCR6 (17, 24, 25), and also into the brain via CCR6 (59), CCR6+ T cells may significantly contribute to HIV dissemination from the portal site of entry and the establishment of HIV reservoirs in immunoprivileged tissues.

HIV infection is associated with a dramatic and persistent decrease in CD4+ T cell counts in the peripheral blood and mucosal levels (60, 61). However, productive HIV infection is observed in only a small fraction of memory CD4+ T cells (62, 63), and the phenotypic and functional characteristics of those cells started to emerge (4, 45, 64). Recent studies in fundamental immunology identified the chemokine receptor CCR6, in combination with CCR4 and CXCR3, as a marker for Th17 (CCR4+CCR6+) and Th1-Th17 (CXCR3+CCR6+) T cells that are specific for Candida albicans and Mycobacterium tuberculosis, respectively (28, 29).

We and others extrapolated this knowledge to HIV pathogenesis and reported that CCR6 identifies Th17 polarized memory T cells highly permissive to HIV replication in vitro and HIV-DNA integration in vivo (30–32) with decreased frequencies during HIV disease progression (30, 32). This indicated that CCR6 expression and Th17 polarization are linked to HIV permissiveness (30, 31).

Table I. Clinical parameters of primary and chronically HIV-infected subjects (HIV*)

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<th>Patient ID</th>
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<th>CD8 (Cells/μl)</th>
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<th>Time Since Infection (mo)</th>
<th>ART</th>
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Recent studies identified the integrin α4β7 as a new receptor for HIV-gp120 binding, critical for viral cell-to-cell transmission (19, 21), and reported that the integrin α4β7 memory T cells include the majority of IL-17-producing T cells and are preferentially infected during acute SIV infection (22). In this context, we now characterize the contribution of four subsets of memory T cells with distinct expression of CCR6 and integrin β7 to HIV-1 pathogenesis. The results in our study revealed that memory β7−CCR6+ and β7−CCR6− T cell subsets exhibited Th17 polarization profiles (IL-17, CCL20) in addition to Th1 (IFN-γ) and were highly permissive to R5 HIV replication. The CCR6 ligand CCL20 was recently reported to contribute to viral dissemination from the portal site of entry (65). However, at very high concentration, CCL20 appears to increase APOBEC3G expression and render T cells resistant to HIV infection (66). CCR6+ T cell subsets are selectively permissive to HIV, although they produce CCL20; thus, the role of autocrine CCL20 production in regulating HIV replication in Th17 cells remains unexplored. In contrast with Th17-polarized β7−CCR6+ and β7−CCR6− T cells, we demonstrated that β7−CCR6− and β7−CCR6− T cells produced Th1 but not Th17 cytokines and were resistant to R5 HIV infection. Permissiveness to R5 HIV replication correlated with the intensity of CCR5 expression, which was superior on T cell subsets expressing CCR6 regardless of their expression of integrin β7. In line with these results, our previous work demonstrated that integrin β7 is expressed on T cell subsets permissive or resistant to HIV replication, with the highest levels being detected on CXCX3CXCR6+ T cells (30). Differences observed in HIV permissiveness could not be explained by only superior CCR5 expression because similar results were obtained with X4 viruses. Therefore, other unidentified postentry mechanisms may explain these differences. Indeed, the cell cycle marker Ki67 was expressed at the highest levels on β7−CCR6+ and β7−CCR6− T cells, thus suggesting that these subsets are more prone to support HIV replication as they are in a metabolically active state.

Permissiveness to HIV was previously linked to the stage of CD4+ T cell differentiation. Studies by Chomont et al. (45) used CD45RA, CCR7, and CD27 as markers to identify EM (CCR7−CD27−), TM (CCR7−CD27+), and CM (CCR7+CD27+) CD4+ T cells, and demonstrated the preferential ability of CM and TM cells to harbor integrated HIV-DNA in infected subjects receiving ART with high and low CD4 counts, respectively. By using the same markers, we demonstrated that β7−CCR6+ and β7−CCR6− T cell subsets, which are permissive to HIV replication, were heterogeneous in terms of EM, TM, and CM phenotype, with
significantly higher frequencies of EM and TM and lower frequencies of CM compared with \( b_7^{+}\text{CCR6}^2 \) T cells. These findings are in line with a scenario in which EM and TM are selectively infected by HIV, and in which persistence of HIV reservoirs in CM cells of ART-treated subjects occurs as a result of the linear differentiation of EM and TM into CM cells (67). Our results further suggest that the long-term survival of \( b_7^+\text{CCR6}^+ \) and \( b_7^{+}\text{CCR6}^2 \) T cell subsets might be controlled by IL-7 because...
FIGURE 5. ATRA-induced integrin $\alpha_4\beta_7$ expression is associated with CCR5 upregulation on memory CCR6$^+$ but not CCR6$^-$ T cells. Total CD4$^+$ T cells were isolated from PBMCs of uninfected individuals by MACS; then memory CCR6$^+$ and CCR6$^-$ T cells were sorted by FACS. Cells were stimulated via CD3/CD28 in the presence or absence of ATRA (10 nM) for 4 d. The expression of the integrins $\beta_7$, $\alpha_4$, and $\alpha_E$ (A, B) and that of the HIV coreceptor CCR5 (C, D) were analyzed by polychromatic flow cytometry. A, Dot plots of integrins $\beta_7$, $\alpha_4$, and $\alpha_E$ coexpression on T cell subsets from one experiment representative of experiments performed with cells from three different donors; (B) statistical analysis of the frequency of $\alpha_4^+\beta_7^+$ and $\alpha_E^+\beta_7^+$ cells within CCR6$^+$ and CCR6$^-$ T cells cultured in the presence or absence of ATRA (mean $\pm$ SD; $n = 3$). C, Histograms of CCR5 expression from experiments performed with cells from three different donors, and (D) statistical analysis of relative CCR5 expression (% and MFI relative to CCR6$^-$/media) on CCR6$^+$ and CCR6$^-$ T cells cultured in the presence or absence of ATRA (mean $\pm$ SD; $n = 3$). Paired $t$ test $p$ values are indicated on the graphs.
these cells expressed greater levels of the IL-7R, CD127, compared with CCR6+ T cells. This is consistent with a recent study demonstrating the critical role of IL-7 in the homeostatic proliferation of murine Th17 cells (68). Whether the administration of IL-7 to HIV-infected subjects may selectively enhance survival of Th17 cells remains to be investigated. Finally, we revealed that among the four memory T cell subsets, β7+CCR6+ cells were resistant to infection and expressed the lowest levels of CD11a, which together with CD18 forms the LFA-1. A critical role is played by LFA-1 in HIV transmission in trans by facilitating cell–cell contacts on integrin β7-mediated activation (19). As well, LFA-1 contributes to HIV infection in cis as its ligand, ICAM-1, is incorporated into the virions that could subsequently bind onto LFA-1+ cells (50). Consequently, the low expression of CD11a, together with the low expression of CCR5, on β7+CCR6+ T cells might explain, in part, their relative resistance to HIV replication in vitro.

Depletion of memory CD4+ T cells is more pronounced in GALT than in the peripheral blood and persists throughout the course of HIV/SIV infection (10, 60). Because GALTs include ~80% of the total lymphocyte pool, depletion of this compartment within weeks after HIV infection is of paramount importance. The depletion of circulating β7+CD4+ T cells in SIV-infected
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macaques coincided with a parallel loss of intestinal CD4+ T cells during SIV infection (69), suggesting that the two compartments are closely connected. Thus, studies on peripheral blood T cells expressing gut-homing markers should be highly indicative of events taking place into the GALT. These findings in monkeys were consistent with earlier studies in humans demonstrating that peripheral blood CD4+ T cells expressing the integrin α4β7 are depleted during primary HIV infection (23). Recent studies in humans further defended the idea that alterations in T cell homoeostasis occur concomitantly in the blood and the GALT (40). Results included in our study demonstrated that the frequency of cells expressing CCR6 and/or integrin β7 was reduced, whereas the frequency of β7−CCR6− T cell subsets was increased in recently HIV-infected patients compared with uninfected control subjects. Although β7+CCR6+ and β7−CCR6+ T cells might be depleted from the peripheral blood as a result of their increased permissiveness to HIV infection, the alteration of β7−CCR6− T cell frequency during the first year of HIV infection might be caused by a global alteration in the differentiation, distribution, and/or survival of T cells imprinted with gut-homing potential. By analyzing the expression of different markers of activation (CD25 and HLA-DR) (54), exhaustion (PD-1) (55), terminal differentiation (CD57) (56, 57), and survival (Bcl-2, an antiapoptotic protein) (58), our study suggests a potential link between T cell frequency alterations and the expression of activation and/or exhaustion, but not terminal differentiation or survival markers. In contrast with our prediction, β7−CCR6+ and β7−CCR6+ T cells had the highest levels of Bcl-2 expression; this is consistent with their increased expression of CD127 (Fig. 3) and the ability of IL-7 to induce Bcl-2 expression (70). Of note, the β7−CCR6+ T cell subset expressed the highest levels of CD57, a marker for terminally differentiated cells (56, 57). The fact that β7−CCR6− T cells expressing CD57 are resistant to HIV infection is consistent with previous studies demonstrating that CD57+CD4+ T cells harbor relatively low levels of HIV-DNA in HIV-infected subjects (4) and produce antiviral factors (MIP-1β, granzyme A/B, perforin) similar to CD8+ T cells (57). Further studies are needed to determine whether alterations in the frequency of T cells expressing CCR6 and integrin β7 observed in the blood parallel changes in the GALT during HIV disease progression, and to identify molecular mechanisms responsible for these alterations.

Imprinting for gut-homing is mediated by ATRA (18). There are two different isomeric forms of RA, ATRA and 9-cis-RA, the most abundant form being ATRA. The effects of ATRA on HIV gene expression, transcription, and replication have been studied by several groups, but depending on the cell type and concentration being used, they remain controversial. Nevertheless, recent studies by Arthos et al. (19) and Cicala et al. (21) demonstrated that ATRA, at physiological doses (10–20 nM in human plasma) (71), increased CD4+ T cell permissiveness to HIV replication, and this by a mechanism involving the expression of the integrin α4β7 under an active form able to bind HIV-gp120 and colocalize with CD4 and CCR5. In this study, we used ATRA stimulation as a tool to determine whether CCR6+ T cells were selectively committed to support HIV replication. We report in this article for the first time, to our knowledge, that ATRA dramatically increased R5 HIV replication and HIV-DNA integration preferentially in CCR6+ compared with CCR6− T cells, despite similar upregulation of integrin α4 and β2 coexpression on these two cell subsets. This difference could be explained by a most efficient HIV entry, as suggested by the selective upregulation of CCR5 expression on CCR6+ T cells on ATRA treatment. The effect of ATRA was not limited to R5 HIV strains as demonstrated by increased X4 HIV replication in ATRA-treated memory T cells. In this study, we did not investigate the role of the integrin α4β7 in HIV binding. Based on previous studies (19, 21), it is likely that integrin α4β7 selectively contributes to HIV binding and subsequent replication in CCR6+ T cells. However, CD4+ T cells in the rectal mucosa, a primary site of HIV transmission, express the integrin β7 but not CCR6 (P. Ancuta, unpublished observations). Thus, whether CCR6+ T cells are able to capture HIV from this site via integrin α4β7 and promote the trans infection of CCR6+ T cells and other cell types within the mesenteric lymph nodes remains to be investigated.

As well, ATRA enhanced HIV replication in CCR6+ T cells at postr sty levels, as demonstrated by increased GFP expression and HIV-DNA integration on exposure to VSVG-GFP pseudotyped HIV. Multiple signaling pathways and cellular functions are regulated by ATRA (18, 72). The ATRA binds to nuclear receptors such asRAR and retinoid X receptor (73). There are three different isoforms of RAR (α, β, and γ), with RARα playing an important role in human T cell activation (74). It is known that RARα forms heterodimers with retinoid X receptor and binds to RA-responsive elements or retinoid X-responsive elements in the promoters of their target genes (18, 72, 73). Although the effects of ATRA on HIV replication remains a subject of debate, the presence of RA-responsive elements was identified in the HIV promoter (75). Levels of RARα mRNA were similar in CCR6+ and CCR6− T cells, and both cell subsets were able to respond to ATRA by upregulating the integrin α4 and β7 coexpression. The fact that GFP expression on cell exposure to VSVG-GFP pseudotyped HIV was selectively upregulated by ATRA in CCR6+ T cells suggests the possibility that ATRA directly enhances HIV-DNA integration and/or transcription. However, because HIV-DNA integration was also higher in CCR6+ compared with CCR6− T cells exposed to VSVG-GFP pseudotyped HIV, another possibility is that ATRA acts selectively on CCR6+ T cells, thus resulting in highly efficient HIV reverse transcription and integration. Finally, similar to results obtained with β7−CCR6− T cells, ATRA-treated CCR6+ T cells upregulated integrin α4β7, however, the increase in HIV replication and viral DNA integration was insignificant. Of note, ATRA did not interfere with the production of CCL3, a CCR5-binding chemokine demonstrated to play a role in protecting cells from infection in an autocrine manner (8). Thus, increased HIV replication in ATRA-treated CCR6+ T cells cannot be linked to a decreased protection against CCR5-mediated HIV entry. This is consistent with the finding that ATRA similarly enhanced permissiveness to X4 HIV strains.

In conclusion, we demonstrated that: 1) memory β7−CCR6+ and β7−CCR6+CD4+ T cell subsets were highly permissive to HIV and exhibited a Th17 lineage commitment profile; 2) the frequency of peripheral blood T cells expressing CCR6 and/or the integrin β7 was altered during the first year of HIV infection; and 3) ATRA selectively enhanced HIV replication in CCR6+ compared with CCR6− T cells by acting at entry and postentry levels. Given the ability of integrin β7 to control cell migration into the GALT (15) and to bind HIV-gp120 (15, 16), CCR6+ T cells coexpressing integrin β7 and CCR5 might have an extraordinary ability to disseminate HIV from the portal sites of entry. Our findings strongly suggest that CCR6 is a molecular signature on the surface of memory T cells imprinted with a transcriptional program favorable to productive HIV infection. Epigenetic modifications are known to control the differentiation of memory CD4+ T cells (41). Thus, it is likely that mechanisms governing memory CCR6+ T cell differentiation are exploited by HIV to ensure its successful replication. Future investigations will be critical for the design of new pharmaceutical strategies to interfere with HIV replication in
CCCR6+ T cell subsets, whereas preserving their Th17 polarization and role in mucosal immunity.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

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**Supplemental online material**

**Supplemental Figure 1: Purity of flow cytometry sorted CD4+ T-cell subsets expressing integrin $\beta_7$ and/or CCR6.** Total CD4$^+$ T-cells were isolated from PBMC of uninfected individuals by negative selection using magnetic beads (MACS, Miltenyi). Highly pure CD4$^+$ T-cells were stained with anti-CD45RA-APC-Cy7, CCR6-PE, and integrin $\beta_7$-FITC Abs. Four memory (CD45RA$^-$) expressing integrin $\beta_7$ and/or CCR6 were sorted by FACS (BD Aria II) as follows: $\beta_7^{+}$CCR6$^+$, $\beta_7^{+}$CCR6$^-$, $\beta_7^{-}$CCR6$^+$, and $\beta_7^{-}$CCR6$^-$. Shown is CD45RA, integrin $\beta_7$, and CCR6 expression before (A) and after cell sorting (B). The percentage (%) of each subset is indicated on the figure. Results are representative of experiments performed with cells from >3 different donors.

**Supplemental Figure 2: CCR6$^+$ T-cells expressing or not the integrin $\beta_7$ produce IL-17.** The PBMC from uninfected individuals were stimulated with PMA (50 ng/ml) and Ionomycin (1 μg/ml), in the presence of Brefeldin A (10 μg/ml), for 18 hours. Cells were then stained with a cocktail of fluorochromes conjugated Abs including anti-CD3-Pacific Blue, CD4-Alexa700, CD45RA-APC-Cy7, integrin $\beta_7$-PE-Cy5, CCR6-PE (surface staining) and IL-17-FITC Abs (intracellular staining) then analyzed by polychromatic flow cytometry. The frequency of IL-17-expressing cells was analyzed in the following four subsets of memory CD4$^+$ T-cells subsets: $\beta_7^{-}$R6$^-$, $\beta_7^{+}$R6$^+$, $\beta_7^{+}$R6$^-$, and $\beta_7^{+}$R6$. Shown is statistical analyses of the percentages (%) and mean fluorescence intensity (MFI) of IL-17 expression in the four T-cell subsets (mean±SEM; n=5). Paired t-test p-values are indicated in the figures.

**Supplemental Figure 3: Purity of flow cytometry sorted memory CCR6$^+$ and CCR6$^-$ T-cell subsets.** Total CD4$^+$ T-cells were isolated from PBMC of uninfected individuals by negative selection using magnetic beads (MACS, Miltenyi). The CD4$^+$ T-cells were stained with anti-
CD45RA-APC-Cy7 and CCR6-PE Abs and a cocktail of FITC conjugated Abs against monocytes (CD14), CD8⁺ T-cells (CD8), B cells (CD19), and NK cells (CD56). Memory (CD45RA⁻) CCR6⁺ and CCR6⁻ T-cells lacking FITC staining were sorted by FACS (BD Aria II). Shown are FITC, CD45RA, and CCR6 expression before (A) and after cell sorting (B). The percentage (%) of each subset is indicated on the figure. Results are representative of experiments performed with cells from >5 different donors.

Supplemental Figure 4: ATRA enhances memory CD4⁺ T-cells permissiveness to X4 HIV strain replication. Memory CD4⁺ T-cells were isolated by MACS and stimulated via CD3/CD28 for four days in the presence or absence of ATRA (10 nM). Cells were then exposed to X4 NL4.3 HIV strains (50 ng HIV-p24/10⁶ cells) for 3h at 37 °C. Unbound virus was removed by extensive washing and cells were cultured at 10⁶ cells/ml in RPMI 10% FBS and IL-2 (5 ng/ml) in the presence or absence of ATRA (10 nM). Supernatants were harvested at day 10 post-infection, and levels of HIV-p24 were quantified by ELISA. (A) Shown are independent results obtained with cells from three different donors (mean±SD of triplicate wells) and (B) statistical analysis of relative HIV-p24 expression in supernatants from CD4⁺ T-cells cultured in the presence or absence of ATRA (Median, n=3). Paired t-test p-values are indicated in the figures.
A  Phenotype before FACS sort

B  Phenotype after FACS sorting

Monteiro et al., Supplemental Figure 1
Monteiro et al., Supplemental Figure 4