Susceptibility of Human Th17 Cells to Human Immunodeficiency Virus and Their Perturbation during Infection

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Background. Identification of the Th17 T cell subset as important mediators of host defense and pathology prompted us to determine their susceptibility to human immunodeficiency virus (HIV) infection.

Methods and results. We found that a sizeable portion of Th17 cells express HIV coreceptor CCR5 and produce very low levels of CCR5 ligands macrophage inflammatory protein (MIP)–1α and MIP-1β. Accordingly, CCR5^{+} Th17 cells were efficiently infected with CCR5-tropic HIV and were depleted during viral replication in vitro. Remarkably, HIV-infected individuals receiving treatment had significantly reduced Th17 cell counts, compared with HIV-uninfected subjects, regardless of viral load or CD4 cell count, whereas treatment-naive subjects had normal levels. However, there was a preferential reduction in CCR5^{+} T cells that were also CCR6 positive, which is expressed on all Th17 cells, compared with CCR6^{−}CCR5^{+} cells, in both treated and untreated HIV-infected subjects. This observation suggests preferential targeting of CCR6^{+}CCR5^{+} Th17 cells by CCR5-tropic viruses in vivo. Th17 cell levels also inversely correlated with activated CD4^{+} T cells in HIV-infected individuals who are receiving treatment.

Conclusions. Our findings suggest a complex perturbation of Th17 subsets during the course of HIV disease potentially through both direct viral infection and virus indirect mechanisms, such as immune activation.

Th17 cells, a subset of helper T cells, mediate inflammation [1] and development of autoimmunity [2–7]. Th17 cells are also important in protective immune responses to a variety of pathogens in animal models [8–13]. In humans, it was recently reported that patients with hyper immunoglobulin E syndrome, char-

acterized by recurrent *Staphylococcus* and *Candida* infections [14, 15], have impaired Th17 development [16, 17], further highlighting the importance of Th17 responses in normal host defense against these pathogens.

Human immunodeficiency virus (HIV) infection perturbs T cell subsets [18], and the systemic depletion of CD4^{+} T cells leads to immunosuppression and development of opportunistic infections, such as *Pneumocystis jiroveci* and *Candida* infection [19]. Several groups have also reported the depletion of mucosa-associated memory CD4^{+}CCR5^{+} T cells and linked this to HIV disease progression [20–22]. HIV-infected patients also have higher levels of chronic immune activation markers, which correlate with disease progression and CD4^{+} cell depletion [23, 24]. Th17 cells could play a role in host defense mechanisms against HIV-associated opportunistic infections [11, 13]. Th17 cells are also enriched in the lamina propria of the gastrointestinal tract [25, 26] and may play an important role in the defense against microbes, particularly at mucosal surfaces [27]. Thus, perturbation of Th17 cells during

HIV infection could compromise mucosal defenses against resident and pathogenic microbes, which, in turn, could result in immune activation [28].

Little is known about the role of Th17 cells in HIV pathogenesis. Two recent studies of Th17 cells in HIV infection showed that HIV-infected children with detectable virus loads had lower levels of interleukin (IL)–17-secreting cells than did HIV-uninfected children [29], and in adults, Th17 cells were lost in the gastrointestinal tract of HIV-infected individuals [30]. However, another cross-sectional study suggested significantly higher levels of IL-17 in HIV-infected individuals, compared with HIV-uninfected volunteers [31]. It is also not clear whether Th17 cells are directly infected and depleted by HIV or whether their numbers are perturbed due to generalized immune activation.

Here we sought to determine the susceptibility of Th17 subsets to CCR5-tropic (R5-tropic) HIV infection and the relative proportion of these effector cells in HIV-infected individuals. We found that a sizeable portion of Th17 cells expressed CCR5 and low levels of CCR5 ligands macrophage inflammatory protein (MIP)–1α and MIP-1β and were highly susceptible to infection with R5-tropic viruses. Th17 cells were reduced in the blood of HIV-infected individuals who were receiving antiretroviral therapy (ART) but not in untreated (ie, treatment-naive) subjects, compared with HIV-uninfected subjects. Remarkably, reduction in the number of Th17 cells in HIV-infected individuals receiving ART with undetectable viral load was highly correlated with increased immune activation parameters, suggesting this may be a potential reason for perturbation of Th17 cells in this group of patients.

MATERIALS AND METHODS

Subjects. Thirty-seven ART-receiving and 11 treatment-naive HIV-infected individuals were recruited in accordance with an institutional review board–approved protocol and consent. The HIV loads were determined by HIV RNA polymerase chain reaction (PCR), with the results reported as the number of copies per milliliter. Clinical details for each subject are shown in Table 1. HIV-infected individuals who were receiving ART had a median CD4 count of 336 cells/mm³. Twenty-six patients who were receiving ART had an HIV load <50 copies/mL, and the remaining had a median HIV load of 997 copies/mL. Treatment-naive HIV-infected individuals had a median HIV load of 23,300 copies/mL and a median CD4 cell count of 418 cells/mm³, which was not statistically different from the CD4 cell counts of HIV-infected individuals who were receiving ART. For HIV-uninfected controls, 33 random blood samples were obtained from the blood bank.

Staining and fluorescence-activated cell sorting analysis. Cells were stained with corresponding antibodies, as previously described [32]. For intracellular staining, fixation and permea-

Table 1. Clinical Data on Human Immunodeficiency Virus (HIV)–Infected Subjects

Subject	CD4+ cell count, cells/mm³	HIV load, copies/mL	Duration of ART, months
1	23	<50	1
2	296	100,000	1
3	266	904	1
4	223	<50	2
5	600	3320	3
6	62	<50	4
7	176	<50	8
8	322	<50	9
9	344	<50	16
10	297	<50	16
11	375	<50	21
12	356	<50	24
13	525	<50	24
14	184	<50	24
15	111	<50	43
16	242	144	46
17	749	<50	54
18	205	158	≥60
19	517	<50	≥60
20	496	<50	≥60
21	484	<50	≥60
22	1083	<50	≥60
23	137	<50	≥60
24	953	<50	≥60
25	476	<50	≥60
26	200	600	≥60
27	25	13,100	≥60
28	414	<50	≥60
29	419	5480	≥60
30	167	<50	≥60
31	1075	131	≥60
32	408	6670	≥60
33	651	<50	≥60
34	148	433	≥60
35	681	<50	≥60
36	472	<50	≥60
37	239	1090	≥60
38	686	4990	Naive
39	336	21,900	Naive
40	332	100,000	Naive
41	418	24,300	Naive
42	476	100,000	Naive
43	567	33,000	Naive
44	104	70,600	Naive
45	617	721	Naive
46	652	23,300	Naive
47	322	10,800	Naive
48	188	15,700	Naive

bilization were performed using a kit (BD Biosciences) in accordance with the manufacturer's instructions. Analyses were performed using LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star). The following anti-human antibodies were used for staining: CD3, CD25, CD38, CD45RO,

CCR5, CCR6, MIP-1α, MIP-1β, (BD Biosciences), CD4, CD8, interferon (IFN)-γ, and IL-17 (eBioscience). Intracellular HIV p24 staining was done using phycoerythrin-conjugated p24 antibody (Coulter), as described above. Chemokine secretion was measured from T cells activated with plate-bound anti-CD3 and soluble anti-CD28 for 16 h using a cytometric bead array (BD Biosciences).

HIV production. HIV pseudotyped with vesicular stomatitis virus (VSV)–G envelope (VSV-G.HIV) was generated as described elsewhere [33]. Viral supernatants from replication competent CCR5-tropic HIV were prepared by transfecting HEK293T cells with HIV that encoded R5-tropic (BaL) envelope. These viruses expressed either murine CD24 (mCD24) or green fluorescent protein (GFP) in place of the *nef* gene as marker genes, as described elsewhere [32–34]. In some experiments R5-tropic viruses that also encode *nef* were used and the infections were monitored by intracellular p24 staining as described elsewhere [32–34]. Viral titers were measured as described elsewhere [32, 33] and ranged from 10×10^6 to 5×10^6 infectious units/mL for replication-competent viruses and $10–30 \times 10^6$ infectious units/mL for VSV-G.HIV.

T cell purifications. Peripheral blood mononuclear cells from the blood of HIV-uninfected and HIV-infected individuals were prepared using Ficoll-paque plus (GE Health care). CD4$^+$ T cells were isolated using Dynal CD4 Positive Isolation Kit (Invitrogen) and were >99% pure. CD4$^+$ cells from healthy donors were further sorted by flow cytometry (FACSAria; BD Biosciences) on the basis of expression of CD45RO and CCR6 or CCR5 and were used for the in vitro experiments. Sorted subsets were >99% pure and were kept at 37°C and 5% CO$_2$ in Roswell Park Memorial Institute 1640 medium with 10% fetal calf serum [32].

T cell activation and HIV infection. Sorted CD4$^+$ T cell subsets were stimulated and maintained in IL-15 (10 ng/mL; R&D Systems) during the course of infection experiments. Five days later, cells were infected with VSV-G.HIV, or R5.HIV at different multiplicities of infection. Alternatively, cells were activated with anti-CD3/CD28 conjugated beads. For intracellular cytokine or chemokine staining, cells were reactivated for 5 h with Phorbol ester (20 ng/mL; Sigma), ionomycin (500 ng/mL; Sigma), and GolgiStop (Brefeldin A; BD Biosciences) to prevent protein secretion.

Statistical analysis. All statistical analyses were performed with GraphPad Prism software, version 5.0 (GraphPad Software). The significance in in vitro studies was determined using a 2-tailed 2-sample Student *t* test. Comparisons of HIV-infected and HIV-uninfected individuals were analyzed using the Mann-Whitney *U* test, and the results were considered statistically significant if the *P* value was <.05. Correlation between IL-17, IFN-γ, and T cell activation markers was calculated by the Spearman rank test.

RESULTS

Expression of HIV coreceptor CCR5 on Th17 cells. Human Th17 cells are part of the CD4$^+$ memory subset and have been reported to express the chemokine receptor CCR6 [8, 35, 36]. Infection with R5- and X4-tropic HIV requires expression of CCR5 or CXCR4, respectively, in addition to CD4. We found that a sizeable and significantly higher proportion of CCR6$^+$ T cells than the CCR6$^-$ subset, expressed CCR5, which is in contrast to another recent study [37]. The expression of CXCR4 was high and similar in both subsets (Figure 1*A* and 1*B*). CD45RO$^+$ memory cells were then sorted into 4 subsets on the basis of these chemokine receptor profiles shown in Figure 1*C*: (1) CCR6$^+$CCR5$^-$, (2) CCR6$^+$CCR5$^+$, (3) CCR6$^-$CCR5$^+$, and (4) CCR6$^-$CCR5$^-$. The sorted cells were activated, and the percentage of IL-17– and IFN-γ–secreting cells within each subset was determined using intracellular staining. In accordance with previous publications [8, 35, 36], all IL-17-secreting T cells were CCR6$^+$ (Figure 1*D*). CCR6$^+$CCR5$^+$ T cells also included ~2-fold more IL-17–secreting T cells, compared with the CCR6$^+$CCR5$^-$ subset (Figure 1*E*). Interestingly, the CCR6$^+$CCR5$^+$ subset contained the majority of Th17 cells that also produced IFN-γ (IFN-γ^+IL-17$^+$ cells).

Expression of MIP-1α and MIP-1β in Th17 cells. The finding that Th17 cells expressed high levels of CCR5 prompted us to determine the percentage of Th17 cells that secrete CCR5 ligands MIP-1α and MIP-1β, which can act as entry inhibitors of HIV infection [38]. For this experiment, memory CCR6$^+$ and CCR6$^-$ T cells from healthy individuals were sorted and activated as described in the methods section. These cells were then stained for intracellular IL-17 and IFN-γ in conjunction with MIP-1α and MIP-1β (Figure 2*A*). We found that Th17 cells produced significantly less of either chemokine than did IFN-γ^+ T cells, both in CCR6$^+$ and CCR6$^-$ subsets (Figure 2*B*). This finding suggests that the Th17 T cell subset would be predicted to have less capability to suppress CCR5-mediated HIV entry through chemokine expression during T cell activation.

HIV infection of Th17 cells. On the basis of the findings above, we then determined the susceptibility of Th17 cells to HIV infection. T cells require activation either through the T cell receptor (TCR) or via cytokines to become susceptible to HIV [33]. For this purpose, we stimulated sorted memory CCR6$^+$ and CCR6$^-$ cells either through the TCR or via IL-15 alone, which renders resting T cells susceptible to infection [33] and enhances the production of IL-17 [39]. Cells stimulated with IL-15 displayed 3 effector subsets (ie, IL-17$^+$, IFN-γ^+, or IFN-γ^+IL-17$^+$ subsets) after 6-day culture in vitro (Figure 3*A*) and maintained this for up to 3 weeks in culture (data not shown). Th17 cells were not induced in the CCR6$^-$ subset with either stimulus (Figure 3*A*).

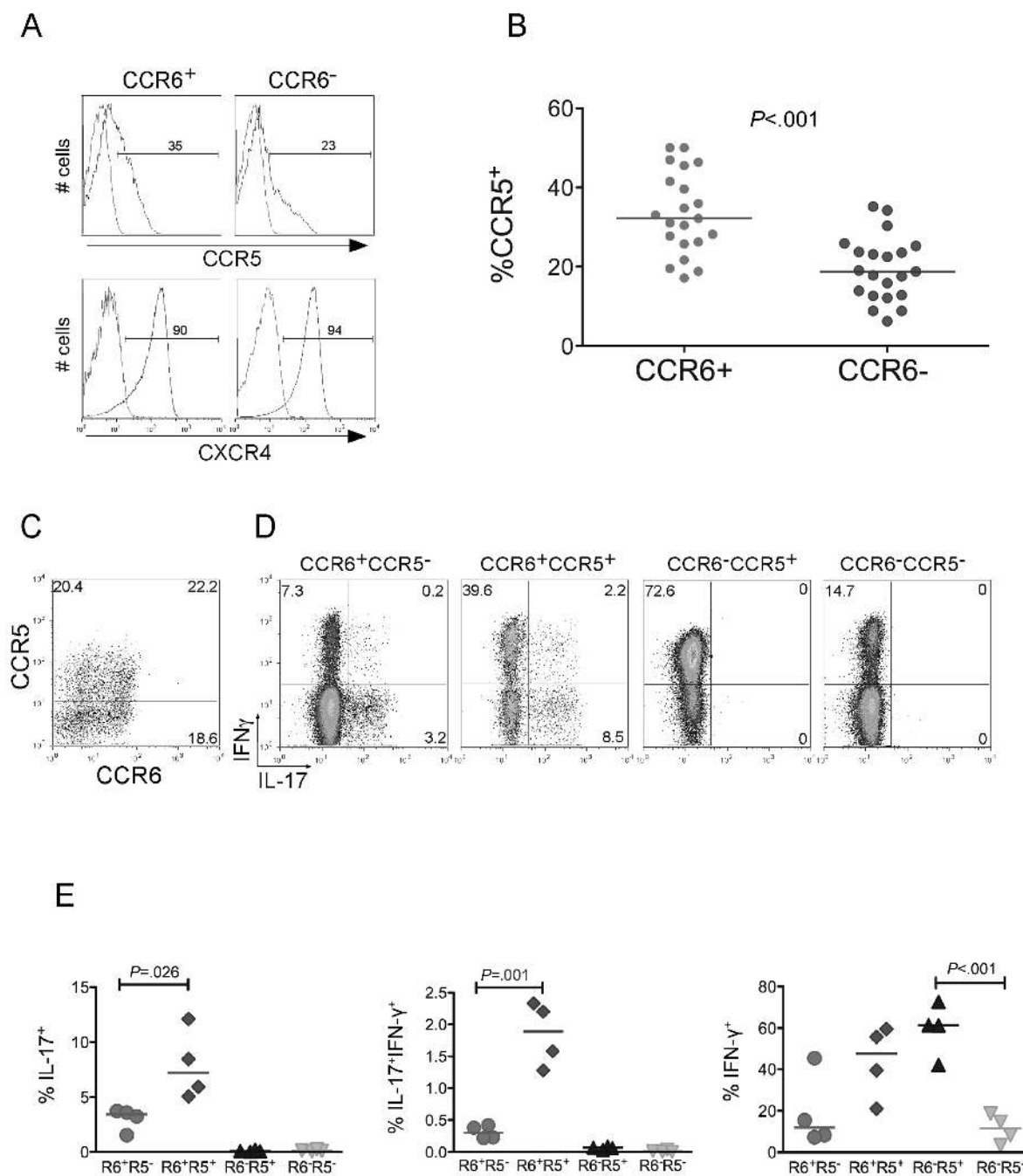In subsequent experiments, we focused on using IL-15 stim-

Figure 1. CCR5 segregation of Th17 cells into interferon (IFN)–γ⁻IL-17⁺ and IFN-γ⁺interleukin (IL)–17⁺. *A,* Resting CD4⁺, CD45RO⁺, CCR6⁺, or CCR6⁻ T cells purified as described in Methods and stained with CCR5-phycoerythrin or CXCR4-PerCP-cy5.5 antibodies or isotype-matched controls *(far left).* *B,* Percentage of memory CCR5⁺ cells in CD4⁺ cells stained for CCR5 and CCR6. Each circle is representative of 1 adult healthy donor. *C,* Resting memory CD4⁺ T cells stained with CCR6-biotin and CCR5-phycoerythrin. *D,* Stained cells in *panel C* sorted into 4 memory (CD45RO⁺) subsets: CCR6⁺CCR5⁻, CCR6⁺CCR5⁺, CCR6⁻CCR5⁺, and CCR6⁻CCR5⁻. To detect IL-17– and IFN-γ–producing cells, we activated sorted subsets using Phorbol ester, ionomycin and Golgi stop. Cells were fixed, permeabilized, and stained with IFN-γ-phycoerythrin-cy7 and IL-17-FITC antibodies. An isotype-matched control did not show any background staining (data not shown). *E,* Percentage of IL-17⁺, IFN-γ⁺, and IFN-γ⁺IL-17⁺ in memory CCR6⁺CCR5⁻, CCR6⁺CCR5⁺, CCR6⁻CCR5⁺, and CCR6⁻CCR5⁻ T cell subsets. Each symbol represents 1 adult healthy donor. *P* values were calculated using the 2-sample Student *t* test.
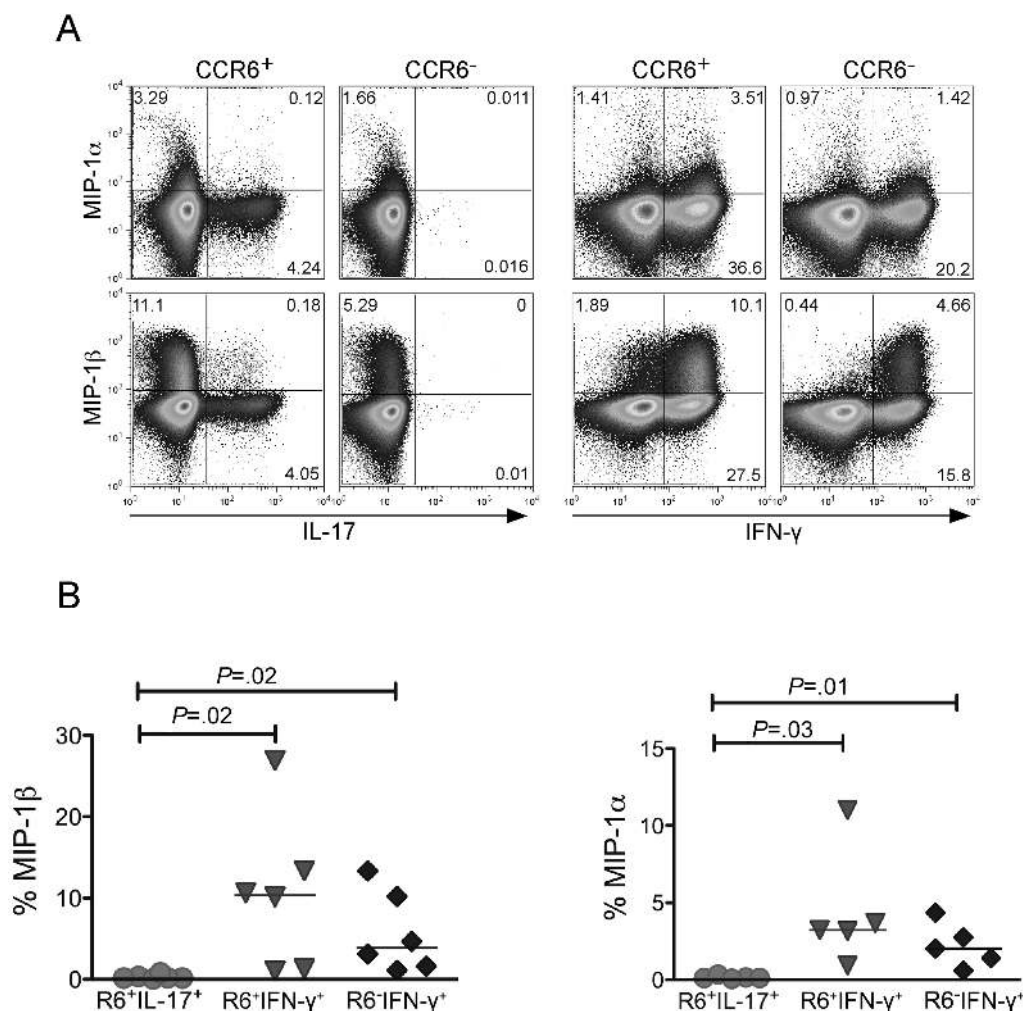
Figure 2. Th17 cells expression of lower levels of macrophage inflammatory protein (MIP)–1α and MIP-1β. Resting CD4$^+$ T cells from peripheral blood were further purified based on CD45RO and CCR6 markers into CD45RO$^+$CCR6$^+$ and CD45RO$^+$CCR6$^-$. Purified subsets were then stimulated with Phorbol ester and ionomycin in the presence of Golgi Stop. *A,* Cells costained with anti–interleukin (IL)–17-FITC, interferon (IFN)–γ-pecy7, and either MIP-1α or MIP-1β -phycoerythrin. *B,* The percent MIP-1α and MIP-1β producing cells determined in CCR6$^+$IL-17$^+$, CCR6$^+$IFN-γ^+, and CCR6$^-$IFN-γ^+ subsets in different healthy donors. *P* values were calculated using the 2-sample Student *t* test.

ulation to determine susceptibility of Th17 subsets to HIV infection, because we found that activation of T cells through the TCR, as opposed to IL-15, causes extensive proliferation of T cells, which skews IL-17-secreting effector populations in vitro from experiment to experiment. Furthermore, the TCR activation–induced differentiation and proliferation also occasionally selected for cells that could have differential susceptibility to HIV, possibly in association with changes in receptor expression or chemokine secretion, whereas IL-15 activation of resting T cells has minimal effect on T cell proliferation while still rendering them susceptible to HIV. Therefore, we challenged CCR6$^+$ memory T cells cultured in the presence of IL-15 with either replication-incompetent VSV-G-pseudotyped HIV (VSV-G.HIV), which bypasses a receptor requirement, or with replication-competent R5-tropic HIV (R5.HIV). We found

that IL-15 stimulation rendered Th17 cells susceptible to infection with both viruses (Figure 3*B*).

The IL-15–stimulated and HIV-infected T cells were then harvested every 3 days and evaluated for GFP expression as a marker for infected T cells and stained for IL-17 and IFN-γ to monitor the changes of effector cells in the cultures. We found that, although IFN-γ^+ and IL-17$^+$ subsets were similarly infected with R5.HIV or VSV-G.HIV (Figure 3*C*), the IFN-γ^+IL-17$^+$ T cells were relatively more susceptible with R5.HIV and VSV-G.HIV, compared with the other 2 subsets of T cells (Figure 3*C*). Of interest, CCR6$^-$ T cells that were IFN-γ^+ were significantly less susceptible to HIV infection both with R5.HIV as well as VSVG.HIV compared to CCR6$^+$ IFN-γ^+ T cells (Figure 3*D*). On the basis of this result, we hypothesized that T cell subsets infected with replicating HIV would also be preferen-
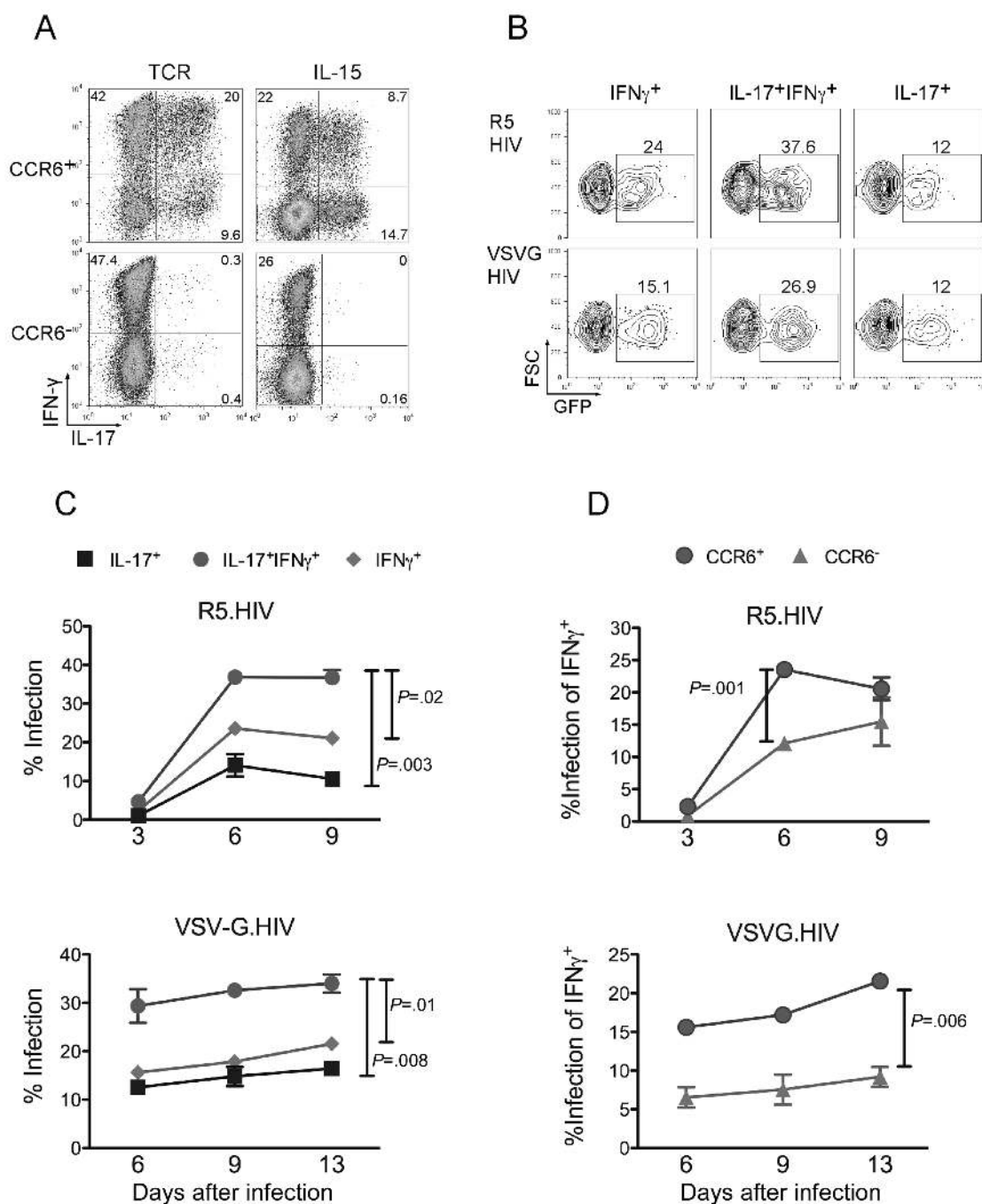
Figure 3. Human immunodeficiency virus (HIV) infection of interleukin (IL)–15–stimulated Th17 cells. *A,* Resting CD4+ T cells stained with CD45RO and CCR6 antibodies and sorted into CD45RO+CCR6+ and CD45RO+CCR6−. Purified cells were cultured in IL-15 for 5 days or were activated using anti-CD3/CD28 conjugated beads. Intracellular staining for interferon (IFN)–γ and IL-17 was performed at day 6 post activation as described in Figure 1. *B,* IL-15–stimulated cells infected with either CCR5-tropic HIV (R5.HIV), or vesicular stomatitis virus (VSV)–G pseudotyped HIV (VSVG.HIV) having a green fluorescent protein (GFP) marker. They were activated using Phorbol ester and ionomycin and stained for IFN-γ and IL-17, as above. GFP expression was determined in IFN-γ+, IL-17+IFN-γ+, and IL-17+ cell subsets. *C,* T cells stimulated with IL-15 and infected with R5.HIV or VSVG.HIV having a GFP marker. At different time points after infection, cells were restimulated with Phorbol ester and ionomycin and stained for IL-17 and IFN-γ expression. The percentage of infected cells within each cytokine-secreting group was determined by GFP expression after gating on IL-17+, IL-17+IFN-γ+ and IFN-γ+ cells following intracellular staining. *D,* The percentage of infected IFN-γ+ cells determined at different time points after infection with R5.HIV or VSVG.HIV in CCR6+ and CCR6− cells. *P* values represent 1 time point after infection and were calculated using the 2-sample Student *t* test.
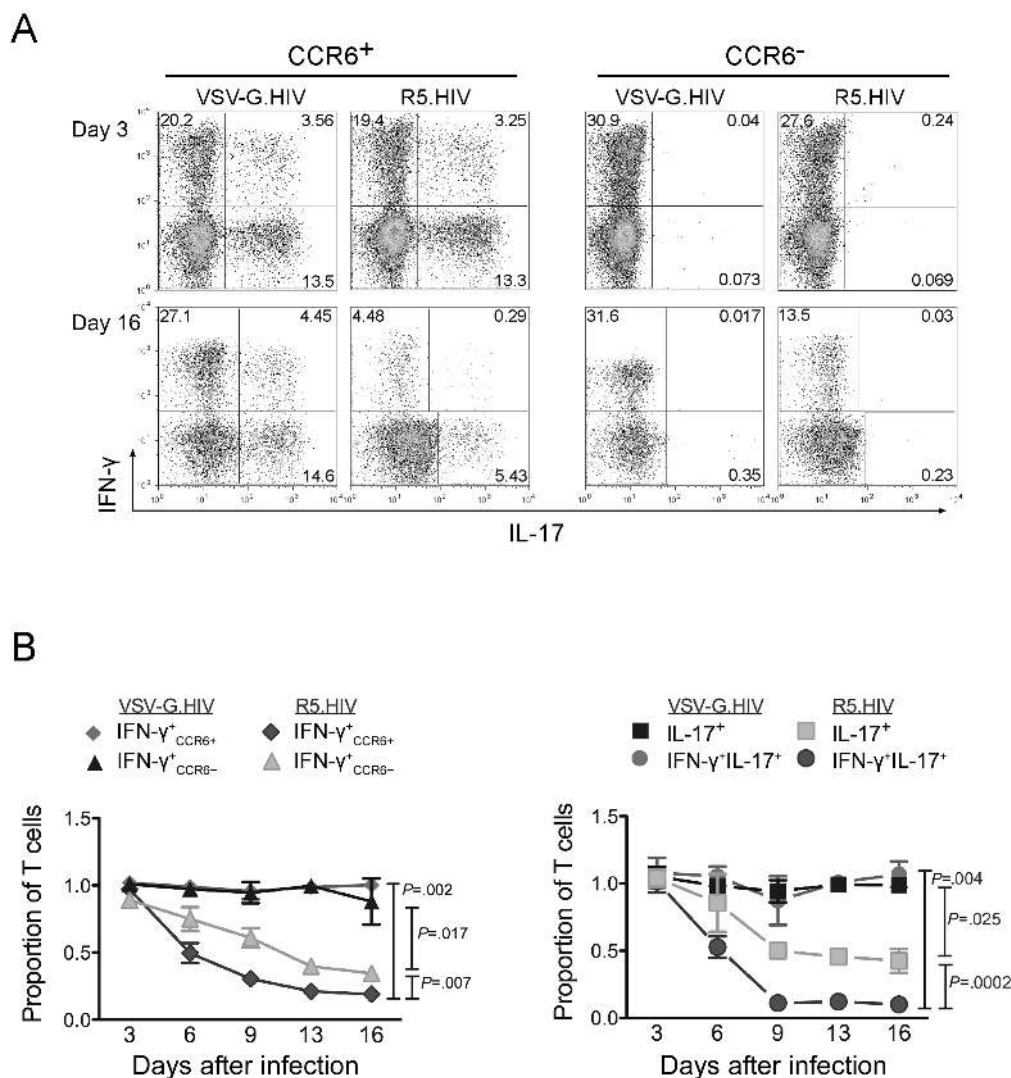
Figure 4. Th17 cells depleted after infection with R5-tropic HIV. *A,* Fluorescence-activated cell sorting plot of CD4+ CD45RO+CCR6+ and CD45RO+CCR6− cells cultured in IL-15 and stained with IL-17 and IFN-γ at 3 and 16 days post infection with vesicular stomatitis virus (VSV)–G.HIV or R5.HIV. *B,* Relative proportion of IFN-γ+ in CCR6+ and CCR6− T subsets and that of IL-17+ and IFN-γ+IL-17+ cells in CCR6+ cultures at different time points post infection with VSV-G. HIV or R5. HIV. *P* values were calculated for the entire data set using the paired 2-sample Student *t* test.

tially depleted during in vitro culture. We therefore determined the proportion of IFN-γ+–, IFN-γ+IL-17+–, and IL-17+–producing cells in culture at each indicated time point after infection. We found that cells that were infected with VSV-G.HIV persisted stably at similar percentages during the period of culture, whereas R5-tropic replication–competent viruses profoundly depleted IFN-γ– or IL-17–secreting T cells in these cultures over time (Figure 4*A* and 4*B*). In these cytokine-producing populations infected with R5.HIV, IFN-γ+IL-17+ cells were depleted faster and more drastically than were IFN-γ+ and IL-17+ cells. Consistent with infection results, CCR6− IFN-γ+ persisted longer in cultures infected with R5.HIV, compared with CCR6+IFN-γ+ (Figure 4*B*). Similar results were obtained from different donors at different time points after infection

and using wild-type viruses that also contain the *nef* gene (data not shown).

Analysis of the Th17 cell subset in peripheral blood of HIV-infected individuals. To complement in vitro HIV infection studies, we determined levels of IL-17– and IFN-γ–producing cells in the peripheral blood of HIV-infected and HIV-uninfected individuals. Because Th17 cells are found in the memory cell population, and because most HIV-infected individuals have a predominance of memory over naive cells, we report the percentage of IL-17– or IFN-γ–producing cells in the memory population gated on CD45RO+ cells. We divided HIV-infected individuals by CD4+ cell counts of <350 cells/mm³ or ⩾350 cells/mm³. HIV-infected individuals with CD4 cell counts of <350 cells/mm³ had a marked decrease in IL-17+ and IFN-
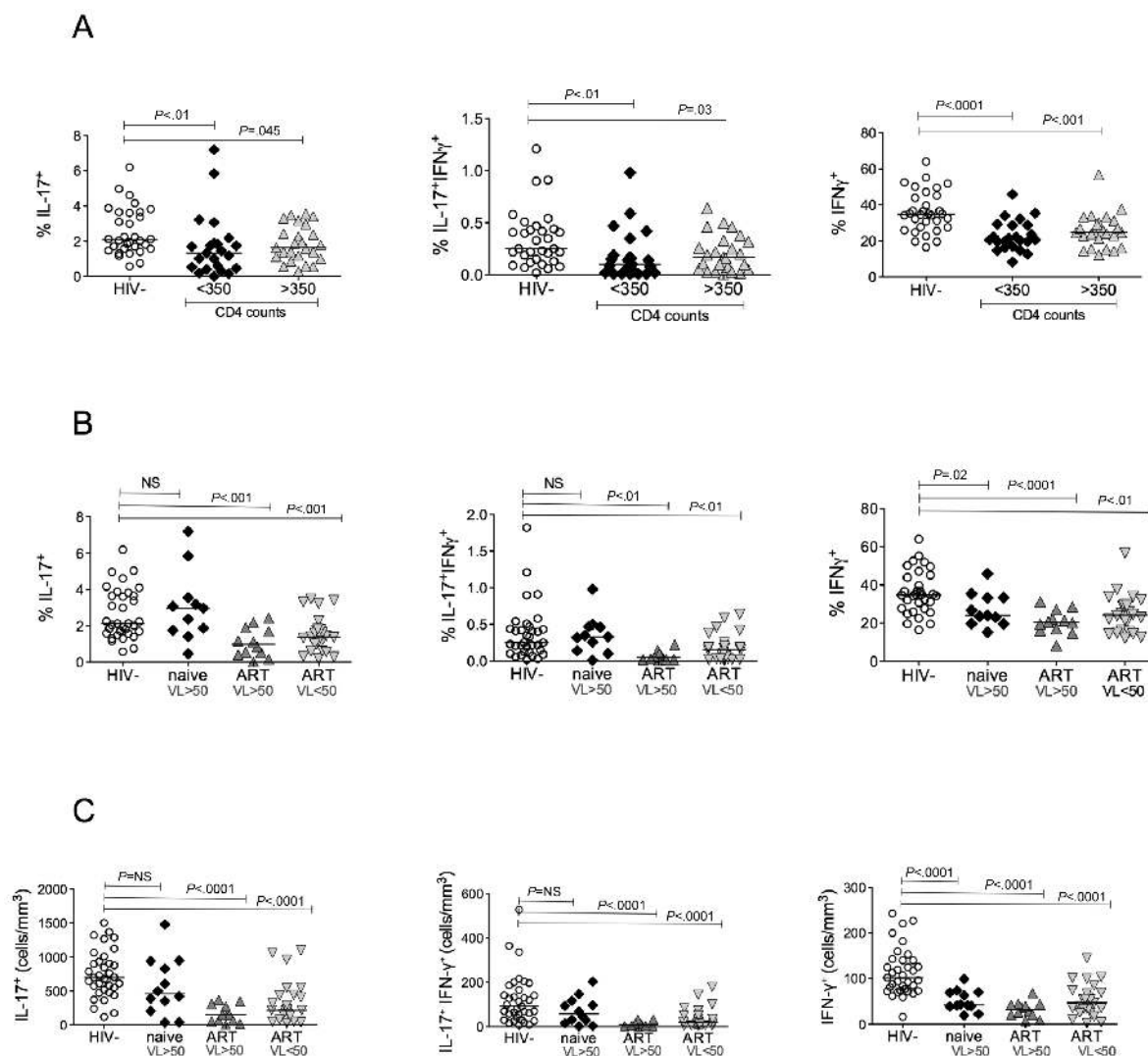
Figure 5. Th17 cells depleted in the peripheral blood of human immunodeficiency virus (HIV)–infected individuals who were receiving antiretroviral therapy (ART) but not in treatment-naive subjects. Purified CD4$^+$ cells were stimulated with Phorbol ester and ionomycin in the presence of Golgi stop. They were then stained for interleukin (IL)–17-FITC and interferon (IFN)–γ-APC and analyzed by LSR-II. IFN-γ$^+$, IL-17$^+$, and IFN-γ$^+$IL-17$^+$ subsets are shown in HIV-infected versus HIV-uninfected individuals gated on memory CD4$^+$ cells. The memory population was determined by gating on CD3$^+$CD4$^+$CD45RO$^+$ cells. *A*, IFN-γ$^+$, IL-17$^+$, and IFN-γ$^+$IL-17$^+$ cell subsets shown in healthy individuals versus HIV-infected individuals with CD4$^+$ cell counts of <350 or ≥350 cells/mm^3. IFN-γ$^+$, IL-17$^+$, and IFN-γ$^+$IL-17$^+$ cell subsets presented as percentages *(B)* or as absolute cell counts *(C)* in healthy individuals, compared with HIV-infected individuals who are either antiretroviral therapy (ART) naive or who were receiving ART and had an HIV RNA level <50 or ≥50 copies/mL, as determined by polymerase chain reaction.

γ$^+$IL-17$^+$ cell counts (Figure 5*A*). Those with CD4 cell counts ≥350 cells/mm^3 also had lower IL-17$^+$ and IFN-γ$^+$IL-17$^+$ cell counts, but of less statistical significance (Figure 5*A*). Similarly IFN-γ–secreting cells were also significantly lower in HIV-infected groups regardless of the CD4$^+$ cell numbers (Figure 5*A*). There was no correlation between total CD4 cell counts and either IL-17$^+$ or IFN-γ$^+$ T cell counts (data not shown).

We next compared the HIV-infected population on the basis of antiretroviral treatment and HIV load. All treatment-naive subjects had HIV loads >50 copies/mL, by determined by HIV RNA PCR. The subjects who were receiving ART were further divided by HIV load <50 copies/mL or ≥50 copies/mL. Remarkably, the portion of IL-17$^+$ and IFN-γ$^+$IL-17$^+$ T cells in treatment-naive subjects was not significantly different from that in the HIV-uninfected subjects (Figure 5*B*). This observation was confirmed even when we calculated the absolute cell numbers of each cytokine-producing subset in which we did not find significant difference between absolute cell counts of IL-17$^+$ and IFN-γ$^+$IL-17$^+$ T cells in naive subjects, compared with HIV-uninfected individuals (Figure 5*C*). In contrast, subjects who were receiving ART, with or without detectable HIV loads, had significantly lower IL-17$^+$ and IFN-γ$^+$IL-17$^+$ T cell

values—both in percentages (Figure 5B) and, more significantly, in absolute numbers (Figure 5C). All HIV-infected individuals had significantly fewer IFN-γ⁺–producing cells, regardless of ART therapy and HIV load (Figure 5B).

Since one would predict that R5-tropic viruses would preferentially deplete CCR5⁺ T cells, we hypothesized that there would be fewer CCR5⁺ Th17 cells in HIV infected individuals. Because it was difficult to measure CCR5 expression in conjunction with IL-17 staining due to transient downregulation of CCR5 after activation and lower sensitivity during intracellular staining (data not shown), we determined the percent of memory CCR5⁺ T cells as well as the percent of CCR5⁺ cells within the CCR6⁺ subset, since all Th17 cells express the latter marker. HIV-infected subjects overall had lower CCR5⁺ memory cells both in naive and ART HIV-infected subjects (Figure 6A). Interestingly, the total proportion of CCR6⁺ cells was significantly increased in both naive and ART groups compared to HIV-uninfected subjects (Figure 6A). In contrast, CCR5⁺ within CCR6⁺ T cells were greatly reduced in both HIV-infected groups, (Figure 6B), whereas the proportion of CCR5⁺ within CCR6⁻ T cells was not different compared to healthy donors (Figure 6B). Taken together, these findings demonstrate a preferential reduction in the proportion of CCR5⁺CCR6⁺ memory T cells *in vivo* in HIV-infected individuals, which may be a consequence of direct infection and depletion with R5-tropic HIV due to high susceptibility of these cells to infection.

Despite T cell depletion, it is well known that HIV-infected individuals demonstrate evidence of chronic immune activation. Consistent with prior studies, HIV-infected individuals had significantly higher expression of immune activation marker CD38 within CD4⁺ T cells, (Figure 6C and 6D), an independent predictor of disease progression [40, 41]. Remarkably, in the group of HIV-infected individuals who were receiving ART and who had undetectable HIV RNA levels, the reduction in IL-17 correlated positively with the percentage of CD4⁺CD38⁺ cells (Figure 6E). A similar correlation was seen between CD4⁺CD38⁺ cells and IFN-γ–secreting cells. There was no statistical correlation between CD4⁺CD38⁺ T cells and IL-17– or IFN-γ–secreting cells both in HIV-negative individuals (Figure 6E) and in HIV-infected individuals who were treatment naive or who were receiving ART and who had HIV RNA levels ⩾50 copies/mL (data not shown).

DISCUSSION

Our findings that a portion of Th17 cells express high levels of CCR5 and lower levels of CCR5 ligands MIP-1α and MIP-1β, correlated with a high infection rate of this subset with R5-tropic HIV. This finding suggests that CCR5⁺ Th17 cells are conceivably infected with HIV in vivo and provide an explanation for their preferential depletion in the gut of HIV-infected patients [30]. Interestingly, we found that infection and deple-

tion of IFN-γ⁺IL-17⁺ cells were more pronounced than were IFN-γ⁻IL-17⁺ T cells. This finding may be partly due to the level of CCR5 expression, which was consistently higher on IFN-γ⁺IL-17⁺ cells. In addition, we found that the IFN-γ⁺IL-17⁺ cells were also more susceptible to VSV-G-pseudotyped HIV, suggesting post-entry intrinsic host factors that modulate HIV infection of this subset compared to other effector T cell subsets. More detailed characterization of this subset including their phenotypic profile and effector functions might give us clues as to their origin and function in normal immune responses as well as their role in HIV pathogenesis.

Variations in *MIP-1α* gene dose and *CCR5* genotypes were shown to act as host factors that affect HIV transmission, viral load, disease progression and immune recovery during antiretroviral therapy. In fact, the gene encoding MIP-1α encompasses many segmental duplications and people with a copy number of the *MIP-1α* gene lower than the population average have markedly enhanced HIV/AIDS susceptibility [42, 43]. It will be interesting in the future to determine the level of Th17 cells in these individuals with genetic differences in their CCR5 ligand levels or expression.

We showed here that HIV targets Th17 cells in in vitro experiments, which predicted that they would be depleted preferentially in subjects with higher viral loads. However, we found that Th17 subsets, along with IFN-γ⁺ T cells, were greatly reduced in HIV-infected subjects who were receiving treatment and even with undetectable viremia. It was also puzzling that in treatment-naive HIV-infected individuals there was no decrease in IL-17⁺ T cells. How then can our results showing in vitro HIV-susceptibility of Th17 cells be reconciled with these perplexing ex vivo findings? We think during early stages of infection, Th17 cells are indeed targeted by HIV in vivo, but one would expect only the CCR5⁺ Th17 subset to be susceptible to infection with R5-tropic viruses. Indeed we found a dramatic decrease in the CCR5⁺ portion of CCR6⁺ T cells both in treatment-naive subjects and in ART recipients (Figure 6B). This finding is also consistent with a recent report describing preferential and rapid depletion of Th17 cells in the gut mucosa of HIV-infected individuals, which would be expected to be mostly CCR5 positive [30]. We postulate that perhaps the relative decrease in CCR5⁺CCR6⁺ T cells, which contain a substantial portion of the Th17 subset, causes a perturbation that results in a compensatory increase in CCR5⁻ Th17 cells in naive HIV-infected subjects. In addition, although naive subjects in our study may not have reached their nadir CD4 cell counts, some of the ART subjects could have started with a lower nadir CD4 cell count prior to initiation of ART. It is conceivable that as CD4⁺ T cells progressively decline during the course of HIV infection, Th17 cells are depleted, and ART treatment may fail to restore their levels once lost to preinfection levels, perhaps because of a compromise in Th17 cell differentiation or sur-
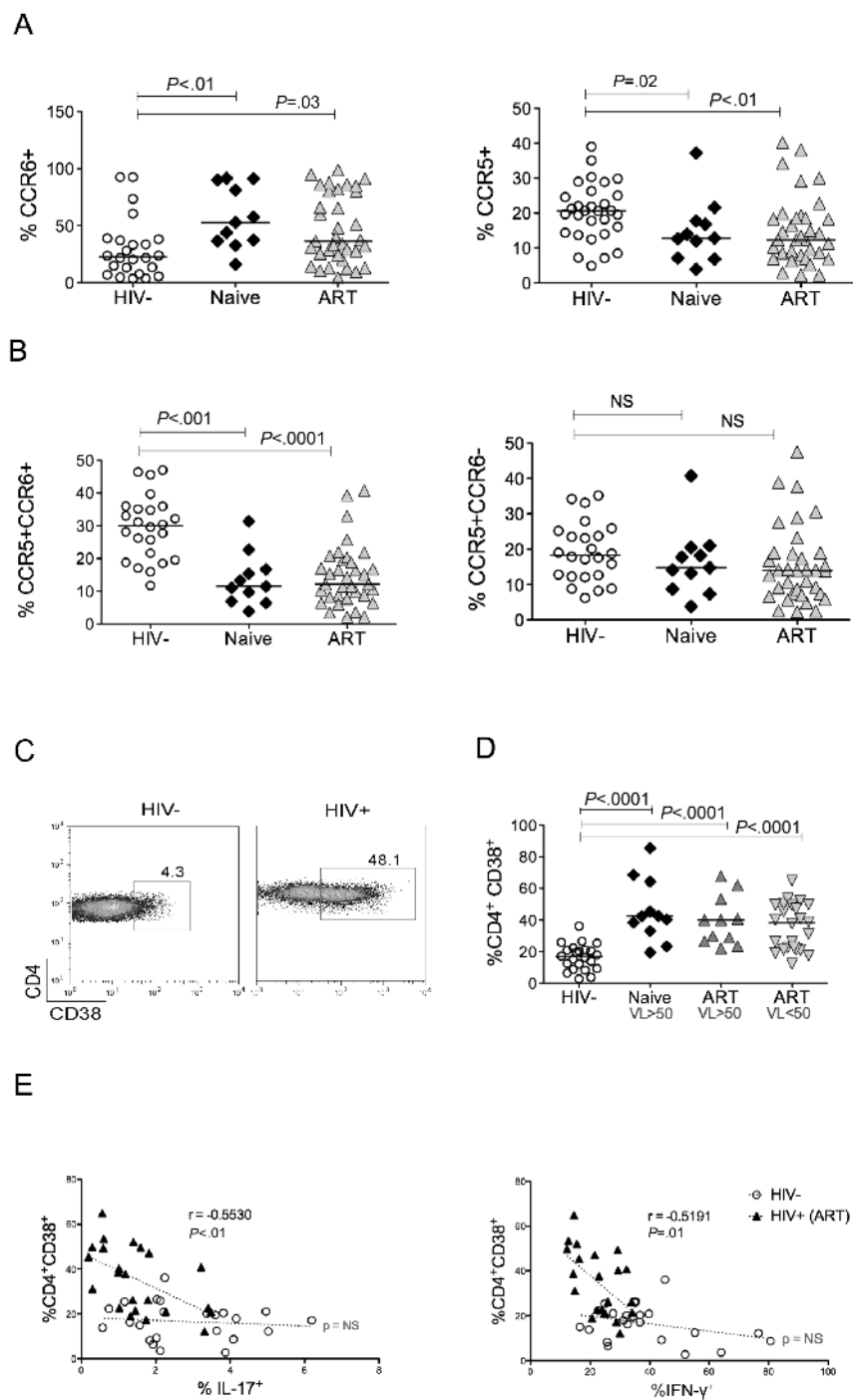
Figure 6. Changes in CCR6+ and CCR5+ T cells and correlation of T cell activation with Th17 cells during human immunodeficiency virus (HIV) infection. Peripheral blood mononuclear cells were stained for CD45RO-FITC, CCR5-phycoerythrin, and CCR6-biotin. Cells were gated first on CD45RO. *A,* Total CCR6+ and CCR5+ T cells within the memory subset are shown for both HIV-infected and HIV-uninfected donors. *B,* The percentage of memory CCR5+ cells within CCR6+ and CCR6− memory T cells, as determined in HIV-uninfected and in HIV-infected subjects who were either treatment naive or who were receiving antiretroviral therapy (ART). *C,* Peripheral blood mononuclear cells from HIV-uninfected and HIV-infected individuals were stained for CD3-PerCP-cy5.5, CD4-Alexa 750, and activation marker CD38-phycoerythrin as described in Methods, then gated first on CD3+CD4+ T cells and then for CD38+ populations. A representative FACS data plot comparing the percent of CD4+CD38+ cells in 1 HIV-uninfected and 1 HIV-infected subject is shown. *D,* CD4+CD38+ cells are shown in HIV-uninfected and HIV-infected individuals. *E,* interleukin (IL)–17– or interferon (IFN)–γ-producing cells were identified in memory CD4+ T cells and the percentage of IL-17+ or IFN-γ+ cells was plotted against the percent of CD38+ cells within CD4+ T cells in HIV- and HIV-infected individuals who were receiving ART with an HIV load <50 copies/mL. Statistical correlations were determined by the Spearman rank test.

vival. However, because our study was not longitudinal, it is difficult to predict when during the course of HIV infection this decline occurs and whether antiretroviral therapy restores Th17 cells. An alternative possibility is that, IL-17 as a marker underestimates the presence of lineage committed Th17 cells. It is conceivable that constant immune activation or immune-exhaustion suppresses Th17 or Th1 cells from displaying their effector functions such as secretion of IL-17 and IFN-γ. In support of this notion of immune-exhaustion, Nixon and colleagues recently showed that a molecule called Tim-3 is up-regulated in chronically activated T cells in HIV-infected subjects and that these cells did not secrete IFN-γ unless the signals from Tim-3 were blocked by neutralizing its interaction with its ligand [44]. It will be interesting to determine in future studies whether secretion of IL-17 can also be rescued by suppressing Tim-3 or other negative signaling molecules such as PD-1 on CCR6+ T cells.

How could reduced levels of IL-17–secreting cells impact HIV pathogenesis? A potential scenario can be envisaged, where the loss of Th17 cells may compromise the mucosal immune system during HIV infection. Th17 cells assist *in vivo* in mucosal host defense against many pathogens in the gut, lungs and skin through regulating antimicrobial peptide production by epithelial cells and keratinocytes [27, 45]. The loss of Th17 cells during HIV infection might also contribute to microbial translocation of bacterial products from mucosal tissues to the circulation resulting in immune activation [28, 46]. Hence, the loss of Th17 cells during HIV infection could also potentially be a biomarker for disease progression. More studies will be needed to understand the impact of Th17 depletion in HIV pathogenesis and its relationship with other T cell subsets, such as regulatory T cells. Future prospective studies will also be important to determine if Th17 cells decline immediately post-treatment of naive subjects and whether Th17 cells are reconstituted when immune activation is subdued.

In conclusion, we have shown that Th17 cells represent ideal targets for HIV by virtue of high expression of CCR5 and lower secretion of CCR5 ligands MIP-1α and MIP-1β. The high susceptibility of Th17 cells to HIV in vitro was reflected by their in vivo depletion in the peripheral blood of HIV-infected individuals. Association of this preferential depletion with immune activation was particularly striking and highlights the potential significance of Th17 cell depletion during HIV immunopathogenesis.

Acknowledgments

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