Circulating CXCR5-Expressing CD8+ T-Cells Are Major Producers of IL-21 and Associate With Limited HIV Replication

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Background: Despite advances made with the highly active antiretroviral therapy (HAART) in the control of the HIV 1 infection, a cure has not been achieved because of the persistence of viral reservoirs. The major HIV reservoirs remain in the lymphoid follicles because of, among other factors, the partial absence of CD8+ T-cells in these structures. Recently, lymphoid follicle–confined and circulating CD8+ T-cells expressing the C-X-C chemokine receptor type 5 (CXCR5) were described, possessing antiviral mechanisms that could help to control HIV replication.

Setting and Methods: By flow cytometry, we characterized the phenotype and function of circulating CXCR5-expressing CD8+ T-cells in HIV-infected patients with natural or HAART-induced control of HIV replication.

Results: Circulating CXCR5-expressing CD8+ T-cells exhibited low or null expression of the C–C chemokine receptor type 7 (CCR7) and had a transitional memory phenotype. Particular redistributions of CXCR5-expressing CD8+ T-cells were found in HIV-infected patients, and they were partially restored by HAART. The frequency of $CXCR5^{hi}CCR7^{-/lo}$ CD8+ T-cells was higher in spontaneous HIV controllers and negatively correlated with plasma HIV RNA levels. Total and HIV-specific CXCR5+ CD8+ T-cells were major producers of interleukin-21, and this function was positively associated with their interferon-γ production.

Conclusions: Circulating CXCR5-expressing CD8+ T-cells are associated with low-level HIV replication; these cells could be novel correlates of protection, and potentially useful in the eradication of HIV reservoirs.

Key Words: HIV, follicular, circulating, CXCR5, CD8, IL-21

(*J Acquir Immune Defic Syndr* 2018;78:473–482)

Received for publication December 28, 2017; accepted April 2, 2018.

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The authors have no funding or conflicts of interest to disclose.

Supported by Universidad de Antioquia, UdeA, COLCIENCIAS (Code: 111571249724) and Corporación Universitaria Remington—Uniremington (Code: 4000000063-16).

F.P.-C., N.A.T., and M.T.R. conceived the study. F.P.-C. performed the experiments. F.P.-C., N.A.T., and M.T.R. analyzed the results and wrote the manuscript.

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INTRODUCTION

Although the highly active antiretroviral therapy (HAART) has prevented around 7.6 million of AIDS-related deaths worldwide, the HIV-1 infection is still a public health concern worldwide considering the lack of a vaccine or a cure.[1] In addition, these drugs can only clear replicating virus, so that HIV reservoirs are not eliminated, and treatment interruptions lead to viral rebound.[2,3]

The major HIV reservoirs are follicular CD4+ T-cells, which are infected by virions attached to membrane receptors in follicular dendritic cells. Their infection is enhanced by the low levels of antiretroviral drugs within the lymphoid follicles and a partial absence of CD8+ T-cells in these sites,[4–6] among other factors.[7] However, a population of CD8+ T-cells within lymphoid follicles was described (follicular CD8+ T-cells),[8] characterized by the expression of the C-X-C chemokine receptor type 5 (CXCR5) and low or null expression of the C–C chemokine receptor type 7 (CCR7). CD8+ T-cells also infiltrate lymphoid follicles during HIV infection.[9–11] This follicle-confined CD8+ T-cell population exhibits lytic and nonlytic mechanisms and has been associated with lentiviral infections control.[12,13] Interestingly, although the main localization of follicular T-cells are the secondary lymphoid organs, CXCR5-expressing T-cells are also found in peripheral blood.[14,15] However, the dynamics and role of circulating CXCR5-expressing CD8+ T-cells during HIV infection is unknown. The study of these cells might provide new correlates of protection, disease progression, or treatment response, pointing toward potential therapeutic strategies.

Here, we characterized circulating CXCR5-expressing CD8+ T-cells in healthy and HIV-infected individuals and analyzed their association with the spontaneous or HAART-induced viral control. A potential role of $CXCR5^{hi}CCR7^{-/lo}$ CD8+ T-cells in the setting of low-level HIV replication was suggested. In addition, the production of interleukin (IL)-21 by HIV-specific CXCR5+ CD8+ T-cells was associated with their interferon (IFN)-γ response.

METHODS

Patients and Samples

This study and the informed consent were approved by the Ethical Committee of the Universidad de Antioquia

(certificates 15-08-634 and 11-08–352). All experiments followed the principles expressed in the Declaration of Helsinki. Three groups of HIV-infected individuals were included (Table 1): (1) HIV noncontrollers (n = 15): patients who exhibited $CD4^+$ T-cell counts >250 cells per milliliter and plasma HIV RNA levels between 2000–1,000,000 copies per milliliter and naive for antiretroviral therapy. (2) Spontaneous HIV controllers (n = 14): patients with viral load <2000 copies per milliliter for at least 1 year and naïve for antiretroviral therapy, including 5 elite controllers (with undetectable viral load). (3) Patients on suppressive HAART (n = 21): with viral load <50 HIV RNA copies per milliliter for more than 1 year and receiving only one therapeutic scheme throughout this time (62% receiving abacavir/lamivudine/efavirenz; 19% on efavirenz/emtricitabine/tenofovir; and 19% receiving raltegravir/tenofovir/emtricitabine). A group of HIV-seronegative volunteers were included as controls (n = 20). From each individual, 5 mL of venous blood was collected in ethylenediaminetetraacetic acid–containing tubes; phenotyping of circulating T-cells was performed immediately. Plasma was used for determining viral load with the approved clinical diagnostic test RT-PCR Ampliprep-Cobas (Roche, Indianapolis, IN), after the manufacturer's protocol, with a detection limit of 50 copies per milliliter. The cellular fraction was used for the isolation of peripheral blood mononuclear cells (PBMCs).

Detection of Circulating CXCR5-Expressing T-Cells

One hundred μL of whole blood was incubated for 30 minutes at room temperature with optimized doses and combinations of the following antibodies from Becton Dickinson (BD; San Jose, CA) fluorescein isothiocyanate (FITC)–labeled mouse antihuman CD3 (clone HIT3a); allophycocianin-labeled mouse antihuman CD4 (clone RPA T4); peridinin–chlorophyll–protein complex (PerCP)-cyanine (cy) 5.5-labeled rat antihuman CXCR5 (clone RF8B2); and from eBioscience (San Diego, CA), Alexa Fluor 700–labeled mouse antihuman CD8 (clone OKT8) and phycoerythrin (PE)-labeled rat antihuman CCR7 (clone 3D12). In addition, for some analysis, we included the following mouse antihuman antibodies: FITC-labeled anti-CD45RA (clone HI100), PE-labeled anti-CD45RO (clone UCHL1), PE-labeled anti-CD28 (clone L293), FITC-labeled anti-CD95 (clone DX2), and FITC-labeled anti-CD62L (clone DREG.56) (all from BD). Next, red blood cells were eliminated with 1X FACS Lysing Solution (BD) for 20 minutes at room temperature. Finally, leukocytes were washed once with phosphate-buffered saline (Lonza, Basel, Switzerland) and resuspended in 1% paraformaldehyde. Cells were acquired on the LSR Fortessa cytometer (BD), using the FACS Diva software v.6.0, within an hour of completing the staining; at least 30,000 $CD3^+$ events were acquired. Data were analyzed with the FlowJo Software version 10.4 (Tree Star, Inc., Ashland, OR). Fluorescence minus one controls were included to define positive thresholds. T-cells absolute numbers were calculated according to the absolute white blood cell count.

Evaluation of the Functionality of CXCR5-Expressing T-Cells

The PBMCs were isolated using a Ficoll density gradient (Ficoll Histopaque-1077; Sigma-Aldrich, St. Louis, MO) and cryopreserved in 10% (vol/vol) dimethyl sulfoxide in fetal bovine serum. At the time of analysis, PBMCs were thawed and rested for 12 hours in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM of L-glutamine (complete medium) (all from Gibco, Carlsbad, CA). Cell viability was

TABLE 1. Characteristics of the Cohort of Study

Parameter	Healthy, n = 20	Controllers, n = 14	Noncontrollers, n = 15	HAART, n = 21	*P* Healthy vs. HIV*	*P* all Groups†
Age, yr; median (range)	29 (21–55)	29 (22–43)	27 (22–54)	30 (18–54)	0.9	0.7
Sex, male, n (%)	10 (50)	6 (43)	12 (80)	13 (72.2)	0.1	0.001‡
Time since diagnosis, mo; median (range)	N/A	37 (20–65)	16 (14–60)	25 (13–132)	N/A	≤0.03§
Treatment time, mo; median (range)	N/A	0 (0)	0 (0)	23 (15–84)	N/A	<0.0001‖
$CD4^+$ T-cell count, cells/μL; median (range)	756 (580–1468)	977 (348–2732)	360 (271–1239)	535 (356–1156)	0.4	0.1
$CD8^+$ T-cell count, cells/μL; median (range)	291 (176–373)	638 (128–3011)	642 (254–1398)	620 (306–1246)	0.001	≤0.04¶
CD4/CD8 ratio, median (range)	2.7 (1.7–3.6)	1.2 (0.3–2.2)	0.5 (0.2–1.5)	0.8 (0.3–1.5)	<0.0001	≤0.003¶
Viral load, RNA copies/mL; median (range)	N/A	230 (25–1900)	11,990 (2785–478,735)	25 (25)	N/A	≤0.007‖#

*Mann–Whitney or Fisher test.
†Kruskal–Wallis and Dunn post hoc test or χ^2 test.
‡Controllers vs. healthy, noncontrollers, and HAART.
§Noncontrollers vs. controllers and HAART.
‖HAART vs. controllers and noncontrollers.
¶Healthy vs. controllers, noncontrollers, and HAART.
#Controllers vs. noncontrollers.
N/A, does not apply.

higher than 90% (assessed by trypan blue exclusion staining). Then, 2×10^6 cells per milliliter were separately stimulated in 96-well V-bottom plates (Costar, Corning, NY) with mouse antihuman CD28 and CD49d functional grade–purified antibodies alone (both at 1 μg/mL; clones CD28.2 and 9F10, respectively, both from eBioscience; used as negative control), anti-CD28 and anti-CD49d plus a pool of HIV 1 consensus B Gag peptides (at 5 μg/mL) (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; Cat: 8117, Lot: 140303), or with phorbol 12-myristate 13-acetate (PMA) and ionomycin (at 50 and 500 ng/mL, respectively; both from Sigma-Aldrich; used as positive control) and incubated for 12 hours at 37°C in 5% CO_2, all in the presence of 10 μg/mL of Brefeldin A and monensin (both from eBioscience), as well as an optimized dose of allophycocianin-labeled mouse antihuman CD107a (clone H4A3, BD FastImmune). After incubation, PBMCs were harvested and washed with 2 mL of 1X phosphate-buffered saline. Afterward, lineage antibody cocktail for cell surface staining was added and incubated for 30 minutes at 4°C, light-protected, followed by a wash, cell fixation, and permeabilization with Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Subsequently, 10 μL of Fc Receptor Binding Inhibitor Polyclonal Antibody (eBioscience) was added and incubated 10 minutes at room temperature. Then, proper doses of the following mouse antihuman antibodies were added and incubated for 30 minutes at 4°C, light-protected: brilliant violet 421-labeled anti-IL-21 (clone 3A3-N2.1, BD), PE-Cy7–labeled anti–IFN-γ (clone 4S.B3, eBioscience), and PE-labeled antiperforin (clone B-D48; Biolegend, San Diego, CA). Finally, the cells were washed twice with 1X permeabilization solution (eBioscience) and acquired on an LSR Fortessa cytometer (BD). At least 30,000 $CD3^+$ events were acquired. Fluorescence minus one controls were also included. Because of limited sample volume, in some analysis, it was not possible to include all the individuals.

Statistical Analysis

GraphPad Prism software v. 7.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Data are presented as medians and ranges, and nonparametric analyses were performed because of the lack of normality and homoscedasticity (Shapiro–Wilk and Levene tests). The Mann–Whitney and Wilcoxon tests were used for comparison of 2 independent and paired data, respectively, and the Kruskal–Wallis test for more than 2 independent groups. If the Kruskal–Wallis P value was <0.05, the Dunn multiple comparison test was performed. The degree of correlation between variables was determined with the Spearman test. In all the analyses, a value equal to the half of the limit of detection of the assay (50 copies/mL) was assigned to samples with undetectable viral load. Fisher and χ^2 tests were used for frequency analysis. In all cases, a P value <0.05 was considered significant.

RESULTS

Circulating CXCR5-Expressing $CD8^+$ T-Cells Exhibit Low Levels of CCR7 and a Transitional Memory Phenotype

The phenotype and frequency of circulating CXCR5-expressing $CD8^+$ T-cells were evaluated in peripheral blood from healthy individuals and compared with $CD4^+$ T-cells, the classically described CXCR5-expressing T-cell population.[14] To indirectly assess the potential ability of migration to lymphoid follicles or extrafollicular zones, we determined the expression of CXCR5 and CCR7 in circulating T-cells. As shown in Figure 1A, and according to previous reports,[16] 2 populations of T-cells ($CXCR5^+$ and $CXCR5^{hi}$) were identified, based on a 2-fold difference in the median fluorescence intensities (MeFIs) of CXCR5 between subsets. As expected, the frequency of $CXCR5^+$ and $CXCR5^{hi}$ cells was higher in $CD4^+$ compared with $CD8^+$T populations (Fig. 1B). When compared with $CXCR5^-CCR7^+$ cells, both $CXCR5^+$ and $CXCR5^{hi}$ $CD4^+$ and $CD8^+$ T-cells had low expression of CCR7 (Fig. 1C), suggesting a potential capability of migration to lymphoid follicles.

Similarly, when the coexpression of CXCR5 and CCR7 in circulating T-cells was evaluated (Fig. 1D), four populations of $CD4^+$ T-cells were identified, which in descending order of frequency were $CXCR5^-CCR7^+$, $CXCR5^-CCR7^-$, $CXCR5^+CCR7^{lo}$, and $CXCR5^+$ $CCR7^-$ cells. On the other hand, a different distribution of these populations was observed in $CD8^+$ T-cells, which in descending order of frequency were $CXCR5^-CCR7^-$, $CXCR5^-CCR7^+$, $CXCR5^+$ $CCR7^-$, and $CXCR5^+CCR7^{lo}$ $CD8^+$ T-cells (Fig. 1D). The proportion of these populations was significantly different between $CD4^+$ and $CD8^+$ T-cells (Fig. 1E).

CCR7 is a homing receptor, allowing for T-cell migration to secondary lymphoid tissues, and particularly recruiting them into T-cell zones.[17] Because circulating CXCR5-expressing T-cells did not express CCR7, we evaluated CD62L (L-selectin), an adhesion molecule also involved in migration to secondary lymphoid tissues through binding to sialyl lewis X ligands on high endothelial venules.[18] Similar to previous reports,[15] $CXCR5^+$ $CCR7^-$ $CD4^+$ and $CD8^+$ T-cells expressed high levels of CD62L, similar to $CXCR5^-CCR7^+$ cells (Fig. 1F). Thus, CD62L could be driving these cells into secondary lymphoid tissues, and, in turn, the lack of CCR7 is allowing them to migrate into lymphoid follicles.

In addition, we characterized the differentiation state of circulating CXCR5-expressing T-cells by the expression of CD45RA, CD45RO, CD28, and CD95. As shown in Figure 2A, a low frequency of $CXCR5^+$ $CD4^+$ and $CD8^+$ T-cells expressed CD45RA. On the other hand, $CXCR5^+$T-cells expressed CD45RO, CD95, and CD28 (Fig. 2B), coinciding with a transitional memory phenotype.[19]

The Circulating $CXCR5^+$ Subset Is the Major Source of IL-21 Within Total $CD8^+$ T-Cells

To explore the functionality of CXCR5-expressing $CD8^+$ T-cells, we stimulated PBMCs from healthy individuals with PMA and ionomycin and evaluated the production of IFN-γ, perforin, and the expression of CD107a.[20] In

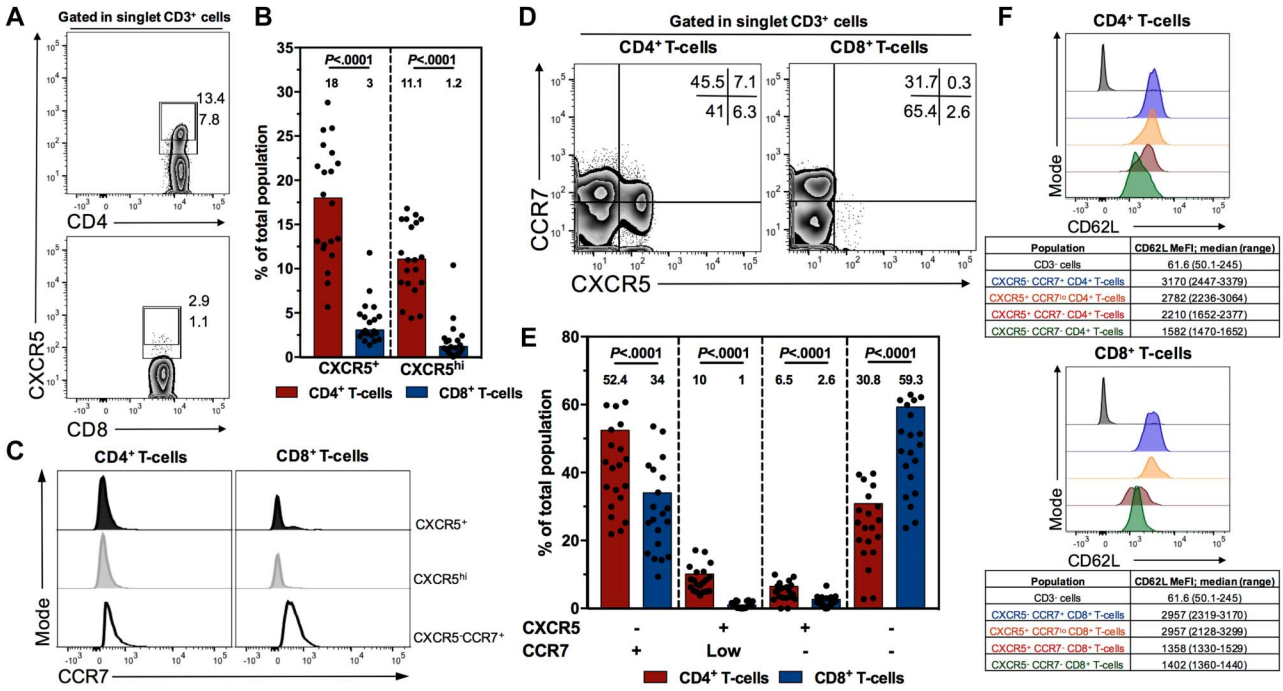

FIGURE 1. CXCR5-expressing T-cells have low expression of CCR7. A, Gating strategy for the detection of circulating CXCR5$^+$ and CXCR5^{hi} CD4$^+$ and CD8$^+$ T-cells. Representative zebra plots from a healthy individual are shown. Peripheral blood lymphocytes were first gated and duplicates were excluded (FSC-A/FSC-H). T-cells were identified as CD3$^+$. The numbers next to the gates indicate the percentage of the respective population. B, Frequency of circulating CXCR5$^+$ and CXCR5^{hi} CD4$^+$ and CD8$^+$ T-cells in healthy individuals (n = 20). C, Expression of CCR7 in CXCR5$^+$, CXCR5^{hi}, and CXCR5$^-$CCR7$^+$CD4$^+$ and CD8$^+$ T-cells from a representative healthy individual, of a total of 20. D, Gating strategy for the detection of circulating CXCR5/CCR7-expressing CD4$^+$ or CD8$^+$ T-cells. Representative zebra plots from a healthy individual are shown. Peripheral blood lymphocytes were first gated and duplicates were excluded (FSC-A/FSC-H). T-cells were identified as CD3$^+$. The numbers in the upper right quadrants indicate the percentage of each population. E, Frequency of circulating CXCR5$^-$CCR7$^+$, CXCR5$^+$CCR7^{lo}, CXCR5$^+$ CCR7$^-$, and CXCR5$^-$CCR7$^-$ CD4$^+$ and CD8$^+$ T-cells in healthy individuals (n = 20). F, Expression of CD62L in CD3$^-$ cells (as controls), CXCR5$^-$CCR7$^+$, CXCR5$^+$CCR7^{lo}, CXCR5$^+$ CCR7$^-$, and CXCR5$^-$CCR7$^-$ CD4$^+$ and CD8$^+$ T-cells from a representative healthy individual, of a total of 5. The summary of the CD62L MeFI in each subset is shown in the lower panel. In (B and E) the median (shown at the top of each bar), ranges, and the *P* value of the Wilcoxon test are shown.

addition, we analyzed the expression of IL-21, a cytokine that characterizes follicular CD4$^+$ T-cells.[21] Of note, when stimulated with the polyclonal stimuli, CD8$^+$ T-cells increased the expression of CXCR5, similar to previous reports, where CXCR5 has been shown to be an activation marker,[22] but maintained low expression of CCR7 (data not shown). CXCR5$^+$ CD8$^+$ T-cells had higher production of IL-21 than the CXCR5$^-$ counterparts (Fig. 2C), with comparable expression of IFN-γ and de novo perforin (coexpression of perforin and CD107a)[23] between both populations (Fig. 2D and E). Similar results were found for CD4$^+$ T-cells, where the expression of CXCR5 was enriched in IL-21–producing but not within IFN-γ–producing cells (data not shown), as previously reported.[24]

The Redistribution of Circulating CXCR5-Expressing CD8$^+$ T-Cells in HIV-Infected Patients Is Partially Restored by HAART

Subsequently, the frequencies of circulating CXCR5-expressing CD8$^+$ T-cells in HIV-infected individuals were explored. In comparison with healthy controls, total HIV-infected patients had similar percentages of CXCR5$^+$ CCR7$^{-/lo}$CD8$^+$ T-cells (Fig. 3A), but lower frequencies of circulating CXCR5^{hi}CCR7$^{-/lo}$ (Fig. 3B), CXCR5$^-$CCR7$^+$, and CXCR5$^+$CCR7^{lo} CD8$^+$ T-cells (Fig. 3C), and a higher frequency of CXCR5$^-$CCR7$^-$ CD8$^+$ T-cells (Fig. 3C). These alterations were apparently CD8 specific because we did not find any significant differences within CD4$^+$ T-cell populations ($P \geq 0.5$; data not shown). Thus, despite the high CD8$^+$ T-cell counts observed in HIV-infected patients (Table 1), the proportions of CXCR5^{hi}CCR7$^{-/lo}$, CXCR5$^-$CCR7$^+$, and CXCR5$^+$CCR7^{lo} CD8$^+$ T-cells are decreased at expenses of the increase of CXCR5$^-$CCR7$^-$ CD8$^+$ T-cells.

Partial redistribution of CD8$^+$ T-cell subsets has been demonstrated in HIV-infected patients receiving HAART.[25] To explore whether HAART induces a redistribution of the frequencies of circulating CXCR5-expressing CD8$^+$ T-cells, we compared the percentages of these populations in HIV-infected patients receiving HAART and healthy controls. As shown in Figure 3D, the frequencies of CXCR5$^+$ CCR7$^{-/lo}$ and CXCR5^{hi}CCR7$^{-/lo}$ CD8$^+$ T-cells were comparable between healthy controls and patients on suppressive HAART ($P \geq 0.3$), suggesting a redistribution of these

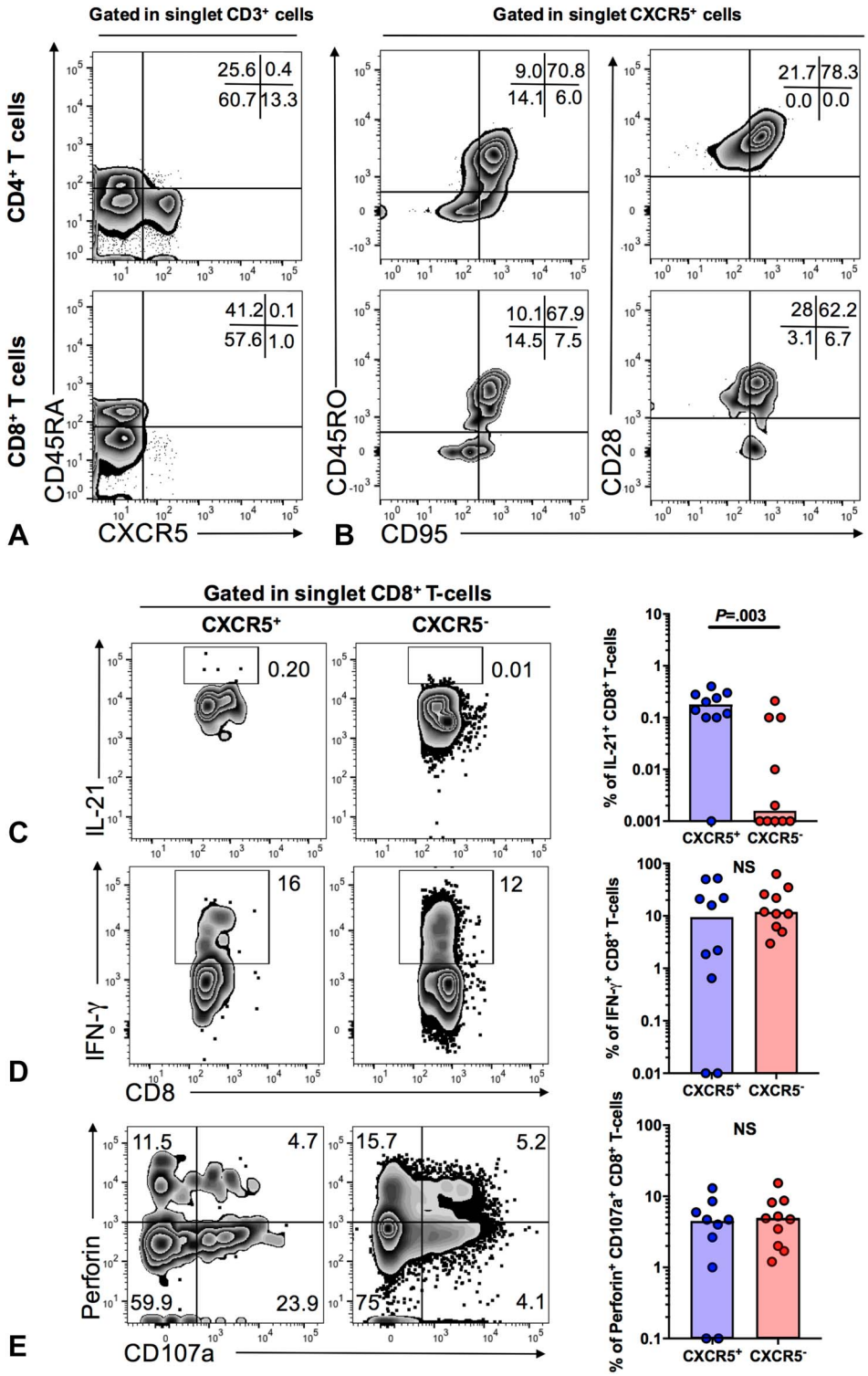

FIGURE 2. Circulating CXCR5-expressing CD8+ T-cells have a transitional memory phenotype and are major producers of IL-21. A, Expression of CD45RA in CXCR5+ CCR7−, CXCR5+CCR7lo, CXCR5−CCR7+, and CXCR5−CCR7− CD4+ and CD8+ T-cells from a representative healthy individual. B, Expression of CD45RO, CD95, and CD28 in CXCR5+ CD4+ and CD8+ T-cells from a representative healthy individual. The numbers in the upper right quadrants indicate the percentage of each population. n = 5. C–E, Representative zebra plots and frequencies of IL-21+ (C), IFN-γ+ (D), and perforin+ CD107a+ (E) CXCR5+ and CXCR5−CD8+ T-cells after stimulation of PBMC from healthy individuals (n = 10) with PMA and ionomycin. The numbers in the zebra plots indicate the percentage of each population. The median of each group and the *P* value of the Mann–Whitney test are shown.

subsets. However, in patients on HAART, the proportions of CXCR5+CCR7lo and CXCR5−CCR7− CD8+ T-cells remain decreased or increased, respectively (Fig. 3E). Of note, the redistribution of these populations was not associated with the treatment delay, treatment length, or therapeutic scheme ($P > 0.1$; data not shown).

Circulating CXCR5hiCCR7−/lo CD8+ T-Cells Are Associated With Limited HIV Replication

To explore the association of circulating CXCR5-expressing T-cells with the natural control of HIV replication, we compared the respective frequencies in spontaneous HIV controllers and noncontrollers, both HAART-naive. As

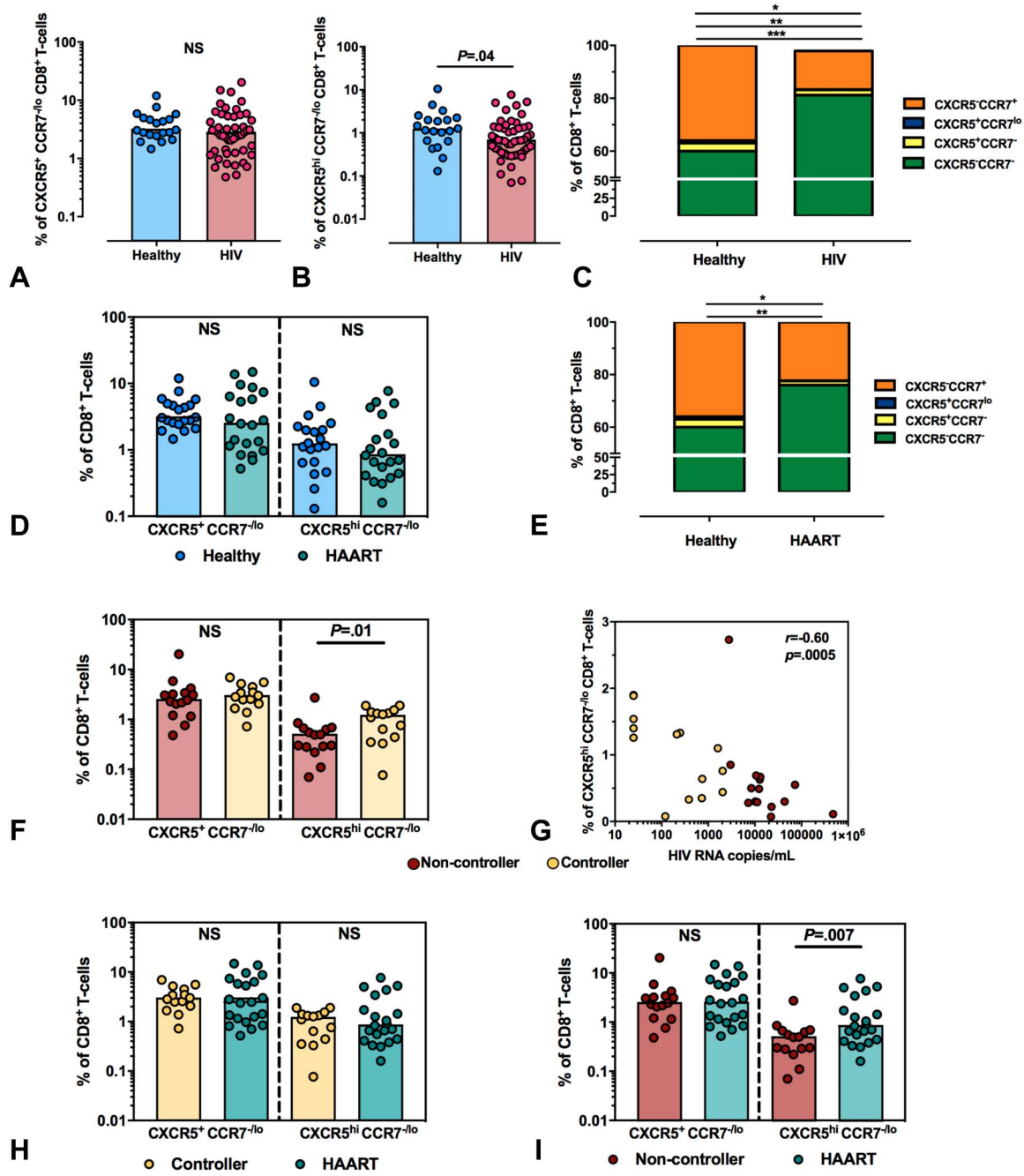

FIGURE 3. CXCR5-expressing CD8⁺ T-cells during HIV infection. Percentages of CXCR5⁺ CCR7$^{-/lo}$ (A), CXCR5^{hi}CCR7$^{-/lo}$ (B), and CXCR5^{-}CCR7⁺, CXCR5⁺CCR7^{lo}, CXCR5⁺ CCR7^{-}, and CXCR5^{-}CCR7^{-} (C) CD8⁺ T-cells in HIV-infected patients (noncontrollers, controllers, and patients on suppressive HAART) and healthy controls. The median of each group and the *P* value of the Mann–Whitney test are shown. In (C): $*P = 0.001$ in CXCR5^{-}CCR7⁺CD8⁺ T-cells; $**P < 0.0001$ in CXCR5⁺CCR7^{lo} CD8⁺ T-cells; $***P < 0.0001$ in CXCR5^{-}CCR7^{-} CD8⁺ T-cells. D–E, Percentages of CXCR5⁺ CCR7$^{-/lo}$ and CXCR5^{hi}CCR7$^{-/lo}$ (D) and CXCR5^{-}CCR7⁺, CXCR5⁺CCR7^{lo}, CXCR5⁺ CCR7^{-} and CXCR5^{-}CCR7^{-} (E) CD8⁺ T-cells in healthy controls and HIV-infected patients on suppressive HAART. The median of each group and the *P* value of the Mann–Whitney test are shown. In (E): $*P = 0.0006$ in CXCR5⁺CCR7^{lo} CD8⁺ T-cells; $**P = 0.002$ in CXCR5^{-}CCR7^{-} CD8⁺ T-cells. F, Percentages of CXCR5⁺ CCR7$^{-/lo}$ and CXCR5^{hi}CCR7$^{-/lo}$ CD8⁺ T-cells in HIV noncontrollers and controllers. The median of each group and the *P* value of the Mann–Whitney test are shown. G, Correlation between the percentages of CXCR5^{hi}CCR7$^{-/lo}$ CD8⁺ T-cells and the viral load in HIV noncontrollers (red dots) and spontaneous controllers (yellow dots). The rho and *P* values of the Spearman test are shown. H–I, Percentages of CXCR5⁺ CCR7$^{-/lo}$ and CXCR5^{hi}CCR7$^{-/lo}$ CD8⁺ T-cells in patients on suppressive HAART and HIV controllers (H) and noncontrollers (I). The median of each group and the *P* value of the Mann–Whitney test are shown.

shown in Figure 3F, both groups of patients had similar frequencies of $CXCR5^+$ $CCR7^{-/lo}CD8^+$ T-cells ($P = 0.4$), but HIV controllers had a higher frequency of $CXCR5^{hi}CCR7^{-/lo}$ $CD8^+$ T-cells than noncontrollers. Accordingly, a significant negative correlation was found between the frequency of $CXCR5^{hi}CCR7^{-/lo}$ $CD8^+$ T-cells with viral load in these individuals (Fig. 3G).

One possibility is that the lower levels of viral antigens within secondary lymphoid tissues in HIV controllers result in the recirculation of $CXCR5^{hi}CCR7^{-/lo}$ $CD8^+$ T-cells, increasing their frequency in blood. This redistribution has been observed in patients with HAART-induced HIV suppression.[26] To evaluate this hypothesis, we compared the frequencies of $CXCR5^+$ and $CXCR5^{hi}CCR7^{-/lo}$ $CD8^+$ T-cells between patients on suppressive HAART versus HIV controllers and noncontrollers. As shown in Figure 3H, patients on suppressive HAART and HIV controllers had comparable frequencies of both cell populations. However, patients on suppressive HAART had higher frequencies of $CXCR5^{hi}CCR7^{-/lo}$ $CD8^+$ T-cells compared with HIV noncontrollers (Fig. 3I). These results suggest a redistribution of this subset after the spontaneous or HAART-induced control of viral replication.

Antigen-Specific Circulating $CXCR5^+$ $CD8^+$ T-Cells Produce IL-21, and This Function Is Associated With the Production of IFN-γ

The production of IL-21, IFN-γ, perforin, and the expression of CD107a by antigen-specific $CXCR5^+$ $CD8^+$ T-cells was evaluated through the stimulation of PBMC from HIV-infected patients with a pool of Gag peptides. As shown in Figure 4, HIV-specific IL-21–producing $CD8^+$ T-cells were detected, and the frequency was higher in $CXCR5^+$ $CD8^+$ T-cells in comparison with $CXCR5^-CD8^+$ T-cells (Fig. 4A). Similar frequencies of IFN-γ– and de novo perforin–producing $CXCR5^+$ and $CXCR5^-CD8^+$ T-cells were detected (Fig. 4B and 4C). On the other hand, in comparison with $CXCR5^-CD8^+$ T-cells, antigen-specific $CXCR5^+$ $CD8^+$ T-cells had similar MeFIs for IL-21 (Fig. 4A) but higher and lower MeFIs for IFN-γ and perforin, respectively (Fig. 4B and C).

To determine whether the functionality of antigen-specific $CXCR5^+$ $CD8^+$ T-cells is associated with the spontaneous or HAART-induced control of HIV replication, we compared the production of IL-21, IFN-γ, and de novo perforin in the 3 groups of HIV-infected individuals. However, we did not find any statistically significant difference in the production of these molecules between the groups of patients, both in their percentage and MeFIs ($P \geq 0.4$, Fig. 4D and data not shown). Similarly, we did not find a significant correlation between the production of these molecules by $CXCR5^+$ $CD8^+$ T-cells and the viral load, $CD4^+$ T-cell count, or CD4:CD8 ratio ($P \geq 0.6$; data not shown). Interestingly, the frequency of IL-21–producing $CXCR5^+$ $CD8^+$ T-cells was positively correlated with the total $CD8^+$ T-cell count (Fig. 4E) and with the frequency of IFN-γ–producing $CXCR5^+$ $CD8^+$ T-cells (Fig. 4F).

DISCUSSION

Although their main localization is the lymphoid follicle, circulating CXCR5-expressing $CD4^+$ and $CD8^+$ T-cells have also been described.[15,27] Although their role in blood is unclear, for $CD4^+$ T-cells, it has been proposed that they might represent mature follicular cells, differentiated after antigen encounter, traveling from one lymphoid organ to another.[27] Indeed, these peripheral CXCR5-expressing $CD4^+$ T-cells have been proposed as counterparts of follicle-confined cells.[14,28] In the case of circulating CXCR5-expressing $CD8^+$ T-cells, they could contribute to the elimination of HIV-infected cells through the production of antiviral cytokines and cytotoxic molecules.[8,12,16] In addition, CXCR5-expressing $CD8^+$ T-cells with a regulatory profile could reduce HIV replication through the control of inflammation and cellular activation.[29] Certainly, the study of circulating CXCR5-expressing T-cells might be useful for determining novel correlates of protection or disease progression in HIV infection, and some insights of their dynamics in the lymphoid follicle could be extrapolated, specially taking into account the difficulty in obtaining lymphoid tissues from infected, or even healthy individuals.

In our study, we identified circulating $CXCR5^+$ and $CXCR5^{hi}$ $CD4^+$ and $CD8^+$ T-cells, which exhibited low or null expression of CCR7, suggesting their potential ability to migrate into lymphoid follicles. The level of expression of CXCR5 is accompanied by a transcriptional and phenotypic profile, and a differential ability to follicle homing, particularly in $CD4^+$ T-cells.[30] Thus, $CXCR5^{hi}CD8^+$ and $CD4^+$ T-cells are characterized by high expression of the transcription factors T-cell factor-1 and B-cell lymphoma-6 (BCL-6), both associated with a follicular profile and the absence of the B lymphocyte-induced maturation protein (BLIMP)-1.[31–34] $CXCR5^{hi}$ $CD4^+$ T-cells also express higher levels of the programmed death (PD) protein-1 than $CXCR5^+$ cells, a molecule associated with the follicular subset.[35] Finally, $CXCR5^{hi}$ $CD4^+$ T-cells have a higher chemotaxis response to CXCL13 than $CXCR5^{lo}CD4^+$ T-cells, as well as a higher ability to migrate into follicles.[35,36] Together, these data indicate that the density of expression of CXCR5 distinguishes those T-cells with a follicular profile, influencing their ability to migrate into lymphoid follicles. However, studies evaluating the migratory ability of $CD8^+$ T-cells according to the expression of CXCR5 are required.

In addition, similar to previous reports,[14,15,28,37] circulating CXCR5-expressing T-cells did not express CD45RA, whereas expressed CD45RO, CD95, and CD28 coinciding with a transitional memory profile.[19] This differentiation state could confer them a higher and sustained proliferation, self-renewal potential, and recall responses in comparison with central memory or terminal effector T-cells.[38,39] Together, our data suggest that the pattern of expression of CXCR5 and CCR7 correlates with the step-wise differentiation pathway of circulating T-cells; the reported distributions of the circulating memory and naive T-cell populations in healthy individuals (predominant naive and central memory $CD4^+$ T-cells and naive and effector $CD8^+$ T-cells, respectively)[40] may explain the different pattern and distribution of the CXCR5/CCR7-expressing $CD4^+$ and $CD8^+$ T-cells that we observed.

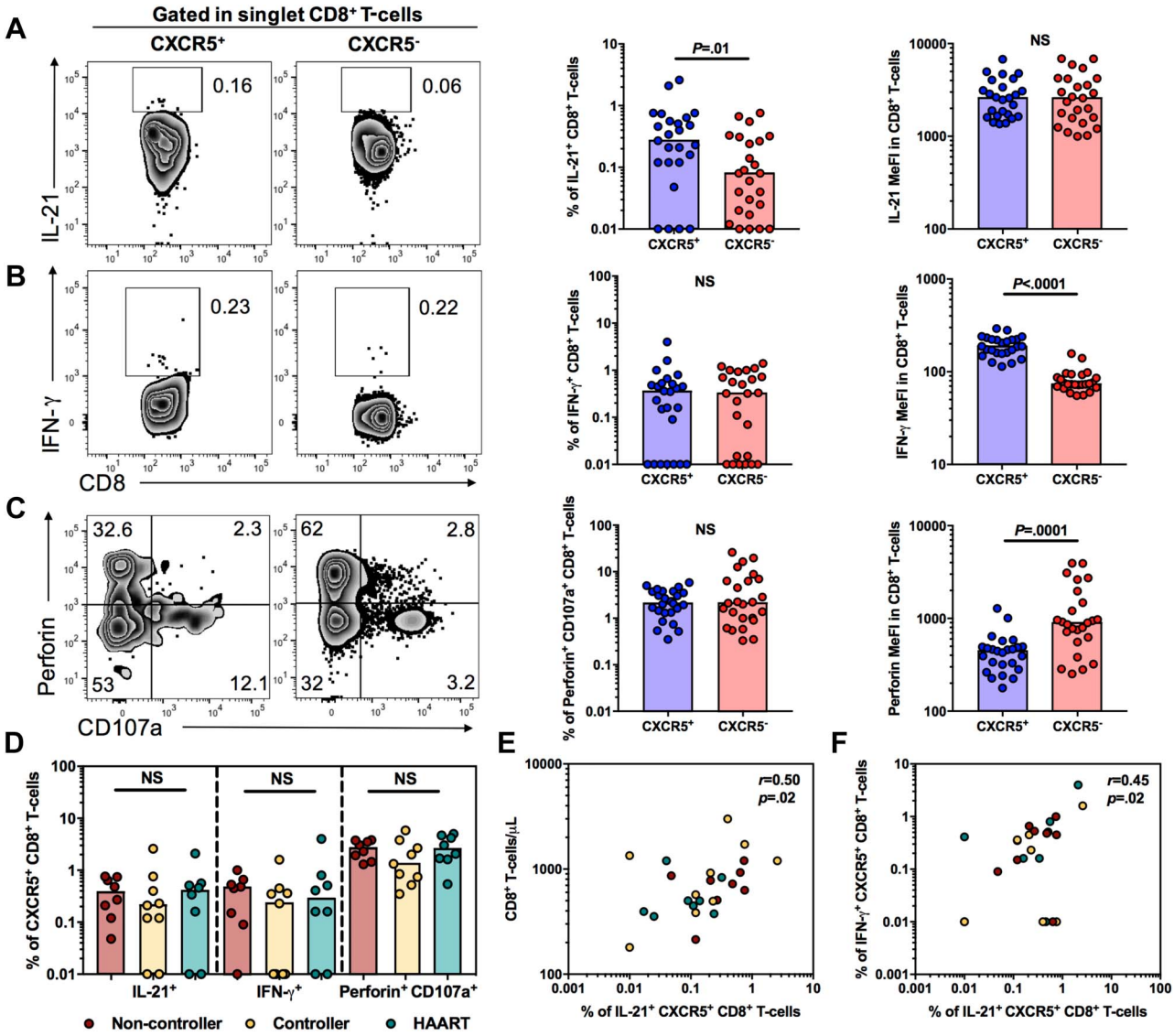

FIGURE 4. Antigen-specific CXCR5$^+$ CD8$^+$ T-cells are detectable and major producers of IL-21. Representative zebra plots, frequencies, and MeFIs of IL-21$^+$ (A), IFN-γ$^+$ (B), and perforin$^+$ CD107a$^+$ (C) CXCR5$^+$ and CXCR5$^-$CD8$^+$ T-cells after stimulation of PBMC from HIV-infected individuals [noncontrollers (n = 8), controllers (n = 9), and patients on suppressive HAART (n = 8)] with a pool of Gag peptides. The numbers in the zebra plots indicate the percentage of each population. D, Frequencies of HIV-specific IL-21$^+$, IFN-γ$^+$, and perforin$^+$ CD107a$^+$ CXCR5$^+$ CD8$^+$ T-cells in the 3 groups of HIV-infected patients. E–F, Correlation between the frequency of IL-21$^+$CXCR5$^+$ CD8$^+$ T-cells and the total CD8$^+$ T-cells count (E) and the frequency of IFN-γ$^+$CXCR5$^+$ CD8$^+$ T-cells (F) in HIV noncontrollers (red dots), spontaneous controllers (yellow dots), and patients on suppressive HAART (green dots). The rho and *P* values of the Spearman test are shown. In (A–D), the median of each group and the *P* value of the Mann–Whitney test (A–C) and Kruskal–Wallis and Dunn post hoc test (D) are shown.

Interestingly, despite the high numbers of total circulating CD8$^+$ T-cell characteristic in HIV infection,[41] a lower frequency of CXCR5^{hi}CCR7$^{-/lo}$ CD8$^+$ T-cells was found in HIV-infected patients, suggesting a redistribution of these cells, most likely to lymphoid tissues.[42] Indeed, during HIV infection, there is an increase of CXCR5^{hi}CD8$^+$ T-cells within lymphoid follicles.[16] Of note, although we did not evaluate whether cell death is responsible for the lower frequencies of some circulating CXCR5-expressing CD8$^+$ T-cell subsets in HIV-infected patients, the absolute counts of these populations were not decreased in these patients, in comparison with healthy controls, suggesting that a redistribution rather than elimination of these subsets is the associated mechanism. In addition, the increased frequency of effector and terminal effector memory circulating CD8$^+$ T-cells in HIV-infected patients at expenses of decreased naive and central memory CD8$^+$ T-cells[43] explains, for instance, the increased frequency of CXCR5$^-$CCR7$^-$ at expenses of decreased CXCR5$^-$CCR7$^+$CD8$^+$ T-cells.

Despite the lack of treatment, spontaneous HIV controllers partially regulated the disturbances in the CXCR5-expressing CD8$^+$ T-cell populations, with a higher frequency

of CXCR5^{hi}CCR7$^{-/lo}$ CD8$^+$ T-cells in comparison with noncontrollers, which could account for their natural control of viral replication, as previously reported for activated CD8$^+$ T-cells.[44] A negative correlation was also obtained between the frequency of these cells with viral load, similar to a previous report.[12] However, the redistribution of this subset in the setting of low-level viral replication might as well account for the observed results, as patients on suppressive HAART also had higher frequencies of CXCR5^{hi}CCR7$^{-/lo}$ CD8$^+$ T-cells than HIV noncontrollers. Thus, although CXCR5-expressing CD8$^+$ T-cells might be involved in viral control, the recirculation dynamics should be taken into account. In addition, simian immunodeficiency virus–infected elite-controller macaques have persistent viral replication in lymphoid follicles,[45] whereas follicle-confined CD8$^+$ T-cells only partially suppress simian immunodeficiency virus–infected cells.[46] Thus, CXCR5-expressing CD8$^+$ T-cells may fail to completely prevent HIV replication within lymphoid follicles. Importantly, taking into account that HAART-induced suppression of viral replication did not completely restore the frequencies of circulating CXCR5-expressing CD8$^+$ T-cells, other factors, in addition to viral replication, such as the inflammatory environment, could be responsible for these alterations[16]; a longer treatment could be required for their normalization, as found for other immunological markers.[47]

As previously reported,[12] HIV-specific circulating CXCR5$^+$ CD8$^+$ T-cells were detectable and had a higher production of IL-21 than CXCR5$^-$ cells, with comparable frequencies of IFN-γ– and de novo perforin–producing cells between both subsets. Contrary to a previous report, where a higher frequency of IL-21–producing HIV-specific CD45RO$^+$CD8$^+$ T-cells was observed in HIV elite controllers,[48] we did not find an association between the production of IL-21, IFN-γ, or de novo perforin by CXCR5$^+$ CD8$^+$ T-cells and the natural or HAART-induced control. Nonetheless, the higher frequency of these cells in spontaneous HIV controllers or other still undefined mechanisms are possibly exerting the viral control.

The finding related to IL-21 is remarkable because it not only promotes the differentiation and function of follicular CD4$^+$T and B-cells,[49] but also promotes the survival, expansion, and cytotoxic responses of antigen-specific CD8$^+$ T-cells.[50–52] Taking into account the dysfunction of antigen-specific CD8$^+$ T-cells during HIV infection,[53] the production of IL-21 by CXCR5$^+$ CD8$^+$ T-cells could compensate, by stimulating their effector functions. Strikingly, we found a positive correlation between the frequency of HIV-specific IL-21–producing CXCR5$^+$ CD8$^+$ T-cells and the total CD8$^+$ T-cell count, as well as with the frequency of HIV-specific IFN-γ–producing CXCR5$^+$ CD8$^+$ T-cells, supporting this hypothesis. Indeed, IL-21 along with IL-15 induces the expression of IFN-γ in T-cells using the signal transducer and activator of transcription (STAT)1 and STAT4,[54] and through this mechanism, IL-21 promotes virus-specific CD8$^+$ T-cell responses.[55] Conversely, there was no association between IL-21 and perforin production by CXCR5$^+$ CD8$^+$ T-cells (data not shown). Although the IL-21 stimulation of perforin expression in total CD8$^+$ T-cells has been demonstrated,[52] the case of circulating CXCR5-expressing or follicular CD8$^+$ T-cells is particular because their transcriptional profile (upregulation of BCL-6 protein and downregulation of BLIMP-1) modulates their cytotoxic mechanisms.[56] Indeed, BLIMP-1 is responsible of the CD8$^+$ T-cells response to IL-21,[57] so that in the case of the IL-21–induced expression of cytotoxic molecules in circulating CXCR5$^+$ CD8$^+$ T-cells, the effect is not as evident as in CXCR5$^-$CD8$^+$ T-cells.

Our study explores the phenotype and functionality of circulating CXCR5-expressing CD8$^+$ T-cells during HIV infection, pointing to their potential role in the natural and/or HAART-induced control of viral replication. Studies addressing the antiviral mechanisms of circulating CXCR5-expressing CD8$^+$ T-cells and evaluating their potential in the elimination of latently infected cells could define the relevance of the use of these cells as novel therapeutic strategies.

ACKNOWLEDGMENTS

The authors thank the Laboratorio de Ciencias de la Vida, Corporación Universitaria Remington—Uniremington and to Gustavo Castro, Jorge Luján, Kevin León and SIES Salud for their help in patient's recruitment.

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