

Reduced T_H1/T_H17 CD4 T cell numbers are associated with impaired purified protein derivative-specific cytokine responses in patients with HIV-1 infection

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Background: In HIV-1-infected patients impaired IFN- γ responses to purified protein derivative (PPD) are associated with an increased risk of active tuberculosis. Tuberculosis antigen-specific cells are found in the T_H1/T_H17 subset of CD4 T cells, which support HIV-1 replication. Selective loss of T_H1/T_H17 cells in patients with HIV-1 infection might contribute to reduced tuberculosis-induced immune responses and an increased susceptibility to active tuberculosis.

Objectives: We sought to investigate the association between T_H1/T_H17 cells and PPD-specific cytokine responses in HIV-1-infected patients.

Methods: A cross-sectional study was performed on healthy control subjects, HIV-1-infected patients receiving successful antiretroviral therapy (ART⁺), and ART-naïve HIV-1-infected patients (ART⁻). All patients studied had evidence of BCG vaccination. Four discrete CD4 T-cell subsets were assessed by flow cytometry: T_H1/T_H17 cells (CXCR3⁺CCR6⁺CCR4⁻), T_H1 cells (CXCR3⁺CCR6⁻CCR4⁻), T_H17 cells (CXCR3⁻CCR6⁺CCR4⁺), and T_H2 cells (CXCR3⁻CCR6⁻CCR4⁺). IFN- γ and IL-2 PPD-specific cytokine responses were assessed in PBMCs by using the enzyme-linked immunospot assay.

Results: Twenty-nine healthy control subjects, 34 ART⁺ patients, and 26 ART⁻ patients were recruited. The number and frequency of T_H1/T_H17 and T_H1/T_H17 CCR5⁺ CD4 T cells were significantly reduced in HIV-1-infected patients. IFN- γ and IL-2 PPD responses were significantly lower in ART⁻ patients and were partially reconstituted with successful ART. Loss of T_H1/T_H17 CCR5⁺ cells was associated with reduced IFN- γ and IL-2 PPD responses.

Conclusions: Selective loss of T_H1/T_H17 cells may be a risk factor for the development of active tuberculosis in patients with HIV-1 infection and might be a useful biomarker in the development of tuberculosis vaccines. (J Allergy Clin Immunol 2011;■■■■:■■■■-■■■■.)

Key words: HIV, *Mycobacterium tuberculosis*, tuberculosis, CD4, T_H1/T_H17 cells, IFN- γ , IL-2

Tuberculosis is the leading cause of death in HIV-1-seropositive patients in Africa and is also the most common presenting illness in patients receiving antiretroviral therapy (ART).¹ The risk of active tuberculosis is significantly increased in patients with progressive HIV-1 infection.² Unlike other opportunistic infections, active tuberculosis can occur early in the natural history of HIV-1 infection, and the clinical manifestations of tuberculosis are influenced by the CD4 T-cell count.^{3,4} Mechanisms of immunity against *Mycobacterium tuberculosis* are not fully understood; however, the development of a functional CD4 helper (T_H1) immune response characterized by the secretion of IFN- γ , IL-2, and TNF- α and consequent activation of tissue macrophages is believed to be important in host defence.^{5,6} In patients with HIV-1 infection, the loss of functional T_H1 immune responses to mycobacterial proteins can occur early in the natural history of infection^{7,8} and can persist even in patients who are receiving long-term successful ART.⁹ By using the differential expression of CCR4, CCR6, and CXCR3 (see Table E1 in this article's Online Repository at www.jacionline.org) on CD4 T cells, a number of investigators have identified 4 subsets of memory CD4 T cells that express lineage-specific transcription factors for T_H1, T_H17, T_H2, and T_H1/T_H17 CD4 T cells and exhibit characteristic cytokine profiles.¹⁰⁻¹² *M tuberculosis*-specific memory T cells are found in the CCR4⁻CXCR3⁺CCR6⁺ (T_H1/T_H17) subset of CD4 T cells, which secrete IL-17 and IFN- γ , whereas *Candida albicans* antigen-specific immune responses are detected in the CCR4⁺CXCR3⁻CCR6⁺ (T_H17) subset of CD4 T cells, which express the cytokine IL-17.¹¹ Cytomegalovirus-induced memory responses are found in the CXCR3⁺CCR4⁻CCR6⁻ (T_H1) subset of CD4 T cells, which are characterized by secretion of IFN- γ .¹⁰ A recent study demonstrated that the T_H1/T_H17 and T_H17 subsets of CD4 T cells express the HIV-1 coreceptors CCR5 and CXCR4 and are highly permissive to infection with HIV-1.¹³ It has been suggested that depletion of the T_H1/T_H17 subset of CD4 T cells might account, in part, for the reduced systemic *M tuberculosis*-specific CD4 T-cell immune responses seen in patients with HIV-1 infection and increased susceptibility to active

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Abbreviations used

APC:	Allophycocyanin
ART:	Antiretroviral therapy
BAL:	Bronchoalveolar lavage
CV:	Coefficient of variation
ELISPOT:	Enzyme-linked immunospot
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
PPD:	Purified protein derivative

tuberculosis.¹³ The aim of this study was to determine whether there is an association between the number of CD4⁺CCR4[−]CCR6⁺CXCR3⁺CCR5⁺ T cells and purified protein derivative (PPD)-specific cytokine responses in healthy control subjects and HIV-1-infected patients with a history of BCG vaccination. We found that the frequency and number of CD4 T_H1/T_H17 T cells are reduced in HIV-1-infected patients who are ART naive or receiving ART compared with healthy control subjects. There is a significant association between the number of T_H1/T_H17 CCR5⁺ T cells and the capacity of PBMCs to secrete IFN- γ and IL-2 in response to PPD in healthy control subjects and HIV-1-infected patients. The loss of T_H1/T_H17 cells might be a risk factor for the development of active tuberculosis in patients with HIV-1 infection, and quantitation of this lymphocyte subset might be a useful biomarker in tuberculosis vaccine development.

METHODS**Study population**

We performed a cross-sectional study on healthy control subjects, HIV-1-infected patients receiving ART (ART⁺) with suppressed viral load (<50 copies/mL), and ART-naive HIV-1-infected patients (ART[−]). The characteristics of these patient groups are outlined in Table I. All control subjects (laboratory staff and health care workers) had a self-reported history of BCG vaccination, whereas only patients with visual evidence of BCG scars, as confirmed by the study investigators, were enrolled in this study. Exclusion criteria for the study were a past or current history of tuberculosis or the presence of a positive tuberculosis enzyme-linked immunospot (ELISPOT) test result. Ethical approval and informed consent was provided by all study participants.

Plasma HIV-1 viral load and immunophenotyping of T-cell subsets

The plasma HIV viral load was measured by means of PCR by using branched DNA amplification technology (Bayer Healthcare, Tarrytown, NY) with a lower limit of detection of 50 copies/mL. Quantification of lymphocyte subsets was determined by using 4-color flow cytometry (see the Methods section in this article's Online Repository at www.jacionline.org). The phenotypic characterization of T-cell subsets was performed on whole blood with 6-color flow cytometry. Monoclonal antibodies used for staining included CD3 allophycocyanin (APC)-Cy7, CD4 peridinin-chlorophyll-protein complex (PerCP), CD8 APC, CCR4 phycoerythrin (PE)-Cy7, CCR5 PE, CXCR3 APC (all from BD Biosciences, Mississauga, Ontario, Canada), and CCR6 fluorescein isothiocyanate (FITC; R&D Systems, Minneapolis, Minn) (see Table E2 in this article's Online Repository at www.jacionline.org). The expression of chemokine receptors allowed for the definition of 4 discrete CD4 T-cell subsets (Fig 1): CD4⁺CCR4[−]CXCR3⁺CCR6⁺ (T_H1/T_H17 cells), CD4⁺CCR4[−]CXCR3⁺CCR6[−] (T_H1 cells), CD4⁺CCR4⁺CXCR3[−]CCR6⁺ (T_H17 cells), and CD4⁺CCR4⁺CXCR3[−]CCR6[−] (T_H2 cells). For each CD4 subset, the proportion of T cells expressing CCR5 was then defined. Acquisition was performed on an LSRII flow cytometer (BD

Biosciences), and at least 30,000 CD3⁺CD4⁺ lymphocytes were acquired. Compensation beads and fluorescence minus one controls were used to control for spectral overlap in whole-blood samples. FACS Diva software (version 6.1.2, BD Biosciences) was used for subsequent data analysis. Matched isotype controls were used to gate chemokine receptors on CD3⁺CD4⁺ T cells. Boolean gates from single-positive chemokine events on histograms were then used to determine the frequency of CD4 T-cell memory subsets.

Isolation of PBMCs and stimulation conditions

PBMCs were isolated from heparinized blood by means of density gradient centrifugation and resuspended in AIM-V medium (Gibco, Carlsbad, Calif). Cells were stimulated with PPD (at 0.5 μ g/mL estimated and derived from *Mycobacterium avium*). Unstimulated cells served as a negative control, and PHA (1 μ g/mL; Sigma, St Louis, Mo) or staphylococcal enterotoxin B (200 ng/mL, Sigma) was used as a positive control.

Detection of cytokine responses by means of ELISPOT

IFN- γ and IL-2 cytokine responses were assessed in PBMCs by means of ELISPOT, as per the manufacturer's instructions (Diaclone, Stamford, Conn). Briefly, 96-well polyvinylidene difluoride-backed plates (Millipore, Temecula, Calif) were coated with capture antibodies at 4°C overnight. Nonspecific binding sites were blocked with 20% FBS (Sigma) in AIM-V medium for 1 hour at 37°C. The PBMCs (2.5×10^6 PBMCs per well) were incubated with stimulus in the plates for 18 to 20 hours at 37°C in a 5% CO₂ atmosphere. ELISPOT plates were developed with biotinylated detection antibody, followed by streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium chloride substrate. Washes were performed with 0.1% PBS-Tween. After drying, spots were counted with a Zeiss ELISPOT Reader System with KS ELISPOT v4.4 Software (Carl Zeiss Vision, GmbH, Aalen, Germany). The intra-assay and interassay coefficient of variation (CV) for PPD antigen-specific cytokine responses ranged from 11% to 28% (see the Methods section in this article's Online Repository).

Detection of intracellular cytokine responses by means of flow cytometry

Intracellular cytokine responses to PPD and staphylococcal enterotoxin B were measured in PBMCs by means of flow cytometry. PBMCs (2×10^6 cells/mL) and stimulus were incubated for 18 to 20 hours at 37°C in a 5% CO₂ atmosphere. Brefeldin A (10 μ g/mL, Sigma) was added for the final 4 hours of stimulation. Stimulated PBMCs were stained with surface markers (CD3 APC-Cy7, CD4 PerCP, and CD8 PE; BD Biosciences) and then permeabilized with Fix/Perm solution (BD Biosciences). Permeabilized PBMCs were stained with antibodies to accumulated intracellular cytokines (IFN- γ PE-Cy7 and IL-2 APC) after blocking with 20% FBS. Acquisition was performed on an LSRII (BD Biosciences). Because the lower limit of cytokine detection was set at 0.01%, we estimated that it was necessary to acquire to 100,000 CD4 or CD8 parent events to acquire 100 cytokine events of interest to obtain an acceptable CV of 10% for each cytokine-positive event being detected within a sample. Cytometric data were analyzed by using FACS Diva software (BD Biosciences) and exported to Microsoft Excel (Microsoft, Redmond, Wash). A positive T-cell cytokine response was empirically defined as one that fulfilled the following criteria: (1) it was at least 3-fold the magnitude of the unstimulated control, (2) the net cytokine response (total cytokine response minus unstimulated control cytokine response) was greater than 0.01%, and (3) negative values that arose as a result of the background subtraction or cytokine responses of less than 0.01% were assigned a value of 0.001%.

Statistical analysis

Distribution of results were summarized by using medians and interquartile ranges. Relationships between variables were assessed by using Spearman rank correlation tests, and partial correlations were performed to control for a third variable. Differences between groups were tested for statistical

TABLE I. Characteristics of healthy control subjects and HIV-1–infected patients receiving ART (ART⁺) and treatment-naïve HIV-1–infected patients (ART[−])

	HC (n = 29)	ART ⁺ (n = 34)	ART [−] (n = 26)
Age (y), median (IQR)	32 (27-37)	45 (40-50)	38 (30-46)
Male sex, no. (%)	16 (55)	32 (94)	25 (96)
CD3 (%), median (IQR)	78.1 (74.0-81.1)	79.7 (76.8-83.9)	82.1 (78.5-87.9)†,
CD3 cells/μL, median (IQR)	1,248 (1,084-1,403)	1,689 (1,261-2,023)*	1,116 (879-1,430)
CD4 (%), median (IQR)	48.3 (44.7-52.9)	30.8 (28.8-41.1)*	21.6 (17.7-25.8)#,
CD4 cells/μL, median (IQR)	793 (621-888)	647 (556-824)	267 (243-352)†,
CD8 (%), median (IQR)	26.7 (22.4-31.0)	44.1 (36.5-48.8)*	59.5 (51.2-65.2)#,
CD8 cells/μL, median (IQR)	440 (361-502)	860 (637-1,224)*	792 (565-994)‡
Nadir CD4 cells/μL, median (IQR)	NA	187 (77-290)	264 (233-312)§, **
Viral load, median (IQR)	NA	<50††	16,650 (10,237-53,295)¶
Years since HIV-1 diagnosis, median (IQR)	NA	16 (8-22)‡‡	5 (2-12) ,§§
Years on HAART, median (IQR)	NA	10 (4-13)‡‡	NA
Years after diagnosis before HAART, median (IQR)	NA	5 (1-12)‡‡	NA

Statistically significant differences between the medians for each group as determined by using the Mann-Whitney *U* test are indicated as follows: HC versus ART⁺.

HAART, Highly active antiretroviral therapy; HC, healthy control subjects; IQR, interquartile range; NA, not applicable.

**P* ≤ .001; HC versus ART[−].

†*P* < .01.

‡*P* ≤ .001; ART⁺ versus ART[−].

§*P* < .05.

||*P* ≤ .001.

¶*P* < .000.

#*P* < .001.

**Lowest known CD4 count; data not available for 1 patient.

††Viral load of less than 50 copies/mL for all but 1 patient, who had a blip with a viral load of 3,975 at the time of the study.

‡‡Data not available for 3 patients.

§§Data not available for 4 patients.

|||Data not available for 7 patients.

significance by using the Mann-Whitney *U* test (for unrelated samples) and the Wilcoxon signed-rank test (for related samples) as indicated. The χ^2 test was also used to test for differences between patient groups. The threshold for statistical significance was set at a *P* value of less than .05. All statistical calculations were performed with SPSS version 14 (SPSS, Inc, Chicago, Ill).

RESULTS

Study population

Twenty-nine healthy control subjects and 60 HIV-1–seropositive patients were enrolled in this study; details of T-cell counts and HIV-1 viral loads are presented in Table I. The median nadir CD4 T-cell counts (187 cells/μL) of the patients receiving ART (ART⁺) was significantly lower than the median of the CD4 count (264 cells/μL) of the ART-naïve group (ART[−]; Table I). The median number of years since the initial diagnosis of HIV-1 was significantly greater in the ART⁺ group (16 years) than in the ART[−] group (5 years). There was no significant difference in time since BCG vaccination between control subjects and the HIV-1–infected patient groups.

There was a significant reduction in both the frequency and number of T_H1/T_H17 cells in both HIV-1–infected patient groups compared with the healthy control subjects (Table II). The number of T_H1/T_H17 cells were significantly increased in the ART⁺ group compared with those in the ART[−] group (Table II). There were no significant differences in the frequencies of the T_H1, T_H17, and T_H2 cell subsets between healthy control subjects and the HIV-1–infected patient groups (Table II). Numbers of T_H1, T_H17, and T_H2 cells were significantly lower in the ART[−] group compared with those seen in healthy control subjects and the ART⁺ group (Table II). The expression of the

HIV-1 coreceptor CCR5 was assessed on each of the CD4 T-cell subsets in healthy control subjects and HIV-1–infected patients (Table II and Fig 2). In the healthy control group the T_H1/T_H17 subset had the highest proportion of cells expressing CCR5, followed by T_H1 and T_H17 subsets; CCR5 expression was lowest on T_H2 cells (Table II and Fig 2, A). In HIV-1–infected patients the differential expression of CCR5 on T_H1/T_H17, T_H1, T_H17, and T_H2 cells was similar to that seen in healthy control subjects (Table II and Fig 2, A). The median fluorescent intensity of CCR5 on T_H1/T_H17 and T_H1 cells was similar in healthy control subjects and was significantly higher than that of T_H17 and T_H2 cells (Table II and Fig 2, B). Both HIV-1–infected patient groups had a significant reduction in the frequency and number of T_H1/T_H17 CCR5⁺ cells compared with those seen in healthy control subjects (Table II and Fig 3). The number of T_H1/T_H17 CCR5⁺ cells was higher in the ART⁺ group compared with that in the ART[−] group (Table II and Fig 3). Differences observed in the number and frequency of T_H1 CCR5⁺ and T_H17 CCR5⁺ cells mirrored those found in the T_H1 and T_H17 subsets for the ART⁺ and ART[−] groups. In contrast, the expression of CCR5 on the T_H2 subset was significantly higher in the ART⁺ and ART[−] groups compared with that seen in the healthy control subjects.

PPD-specific IFN- γ and IL-2 responses were significantly lower in the ART[−] group and were partially reconstituted on suppressive ART

There were no significant differences in the magnitude of PHA-induced IFN- γ responses between healthy control subjects and the HIV-1–infected patients (Table III). However, the

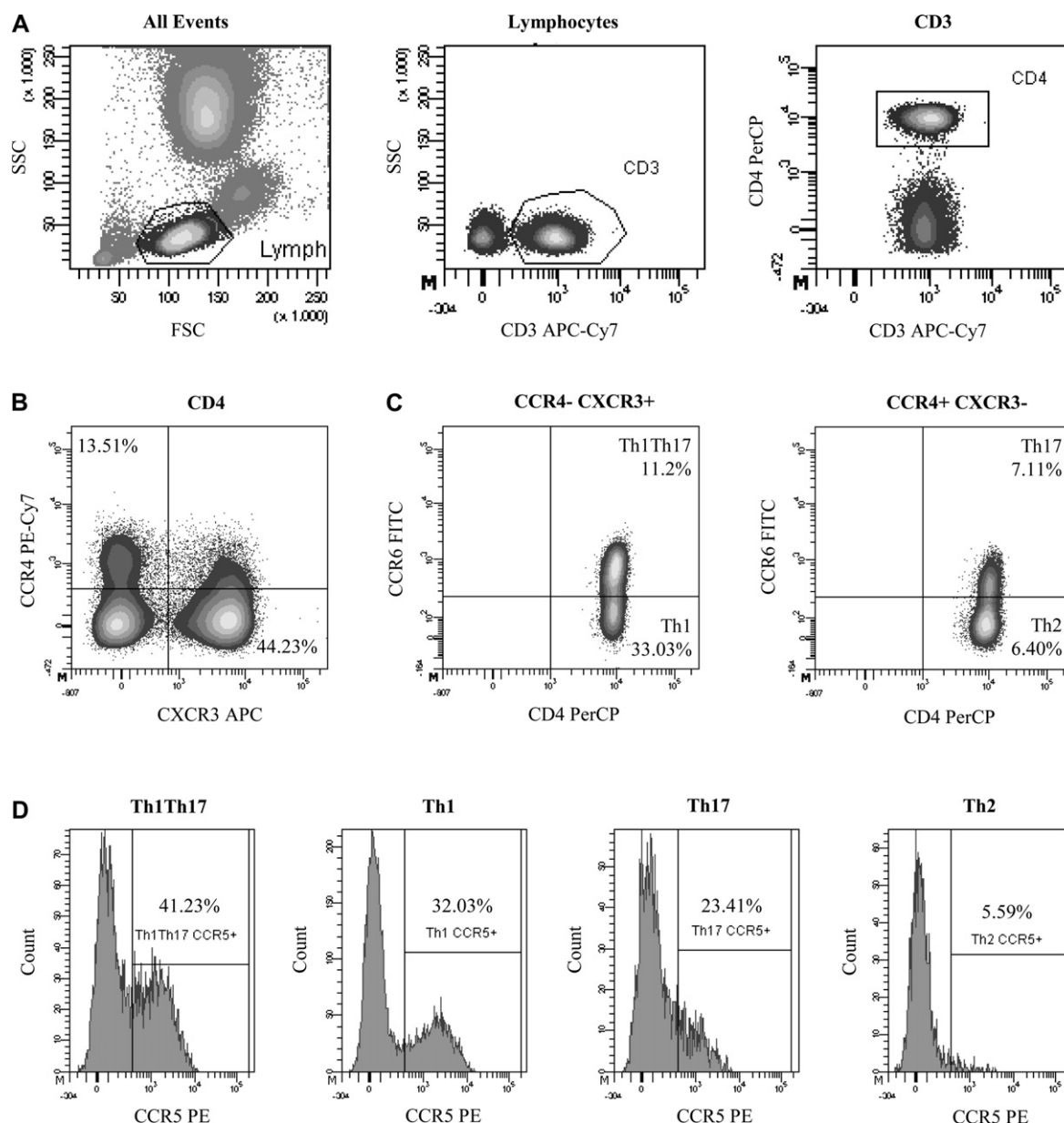


FIG 1. A, CD3⁺CD4⁺ lymphocytes were identified. B, CCR4⁺CXCR3⁺ and CCR4⁺CXCR3⁻ CD4 T-cell subsets were identified. C, CCR4⁻CXCR3⁺ and CCR4⁺CXCR3⁻ subsets were further subdivided into Th1/Th17, Th1, Th17, and Th2 subsets based on their CCR6 expression as follows: CD4⁺CCR4⁻CXCR3⁺CCR6⁺ (Th1/Th17 cells), CD4⁺CCR4⁻CXCR3⁺CCR6⁻ (Th1 cells), CD4⁺CCR4⁺CXCR3⁻CCR6⁺ (Th17 cells), and CD4⁺CCR4⁺CXCR3⁻CCR6⁻ (Th2 cells). D, Expression of CCR5 on Th1/Th17, Th1, Th17, and Th2 subsets. The frequency of positive events within each gating region is shown. FSC, Forward scatter; SSC, side scatter.

magnitude of IL-2 responses after mitogenic stimulation was significantly lower in ART⁻ patients compared with that seen in ART⁺ patients and healthy control subjects. As previously reported,⁷⁻⁹ PPD-specific IFN- γ and IL-2 responses were significantly reduced in ART⁻ patients compared with those seen in healthy control subjects. ART resulted in a partial recovery of PPD-specific IFN- γ responses; however, they were still significantly lower than those obtained in healthy control subjects. PPD-specific IL-2 responses in the ART⁺ group did not differ significantly from those in healthy control subjects.

Intracellular flow cytometry was performed to identify the potential cellular source of IFN- γ and IL-2 (see Fig E1 in this article's Online Repository at www.jacionline.org). PPD-specific CD4 IFN- γ immune responses were detected more often in healthy control subjects compared with those seen in both types of HIV-1-infected patients (see Table E3 in this article's Online Repository at www.jacionline.org). PPD-specific IL-2 immune responses were exclusively found in CD4 T cells. There was no significant difference in the frequency of PPD-specific IL-2 CD4 T-cell immune responses (see Table E3).

TABLE II. The frequency and number of T_H1/T_H17 (CCR4⁺CXCR3⁺CCR6⁺), T_H1 (CCR4⁺CXCR3⁺CCR6⁻), T_H17 (CCR4⁺CXCR3⁻CCR6⁺), and T_H2 (CCR4⁺CXCR3⁻CCR6⁻) CD4 T-cell subsets in healthy control subjects, HIV-1-infected patients receiving HAART (ART⁺), and treatment-naïve HIV-1-infected patients (ART⁻)

	HCs (n = 22)	ART ⁺ (n = 24)	ART ⁻ (n = 18)
T _H 1/T _H 17 (%)	4.14 (2.66-6.00)	1.48 (0.88-2.42) [‡]	1.54 (0.54-2.82)
T _H 1/T _H 17 cells/μL	30 (20-38)	11 (6-18) [‡]	4 (2-9) ,#
T _H 1/T _H 17 CCR5 ⁺ (%)	1.90 (1.35-3.52)	0.55 (0.36-0.89) [‡]	0.63 (0.28-1.35)§
T _H 1/T _H 17 CCR5 ⁺ cells/μL	16 (10-23)	4 (3-6) [‡]	2 (1-4) ,¶
T _H 1 (%)	23.23 (17.90-27.01)	25.83 (21.02-30.07)	27.30 (19.75-31.76)
T _H 1 cells/μL	185 (138-218)	169 (148-206)	75 (49-85) ,**
T _H 1 CCR5 ⁺ (%)	8.34 (3.78-10.92)	7.60 (5.23-10.86)	10.23 (7.74-12.62)
T _H 1 CCR5 ⁺ cells/μL	64 (35-85)	48 (36-71)	28 (23-39) ,**
T _H 17 (%)	4.71 (3.38-5.60)	3.95 (2.17-6.21)	2.73 (1.88-6.74)
T _H 17 cells/μL	33 (27-39)	25 (15-44)	8 (5-16) ,**
T _H 17 CCR5 ⁺ (%)	1.20 (0.93-1.79)	1.45 (0.88-2.46)	1.25 (0.94-2.82)
T _H 17 CCR5 ⁺ cells/μL	9 (8-16)	10 (6-16)	4 (2-6) ,**
T _H 2 (%)	8.02 (6.40-9.93)	6.46 (5.62-9.10)	6.35 (4.23-10.50)
T _H 2 cells/μL	61 (45-72)	47 (34-56)*	18 (13-25) ,**
T _H 2 CCR5 ⁺ (%)	0.46 (0.34-0.85)	1.18 (0.62-2.28) [‡]	1.59 (0.52-2.75)§
T _H 2 CCR5 ⁺ cells/μL	4 (3-6)	8 (4-14) [‡]	4 (2-9)#

The number of subjects in each group for which data were obtained is indicated. The median and interquartile range of each subset in each group are reported.

Statistically significant differences between the medians for each group as determined by using the Mann-Whitney *U* test are indicated as follows: HC versus ART⁺.

HAART, Highly active antiretroviral therapy; HC, healthy control subjects.

**P* < .05.

†*P* < .005.

‡*P* ≤ .001; HC versus ART⁻.

§*P* < .005.

||*P* ≤ .001; ART⁺ versus ART⁻.

¶*P* < .05.

#*P* < .01.

***P* ≤ .001.

Loss of CCR4⁺CXCR3⁺CCR6⁺CCR5⁺ CD4 T_H1/T_H17 cells is associated with reduced capacity to secrete IFN-γ and IL-2 after PPD stimulation in HIV-1-infected patients

There was a significant correlation between the number of T_H1/T_H17 cells and the magnitude of PPD-specific IFN-γ responses for healthy control subjects and HIV-1-infected patients (Spearman rho [*r*] = 0.69, *P* < .001). This relationship still remained valid after statistical adjustment for total CD4 T-cell counts (*r* = 0.37, *P* < .03, Fig 4). There was no association between the number of T_H1, T_H17, and T_H2 cells and the magnitude of PPD-specific IFN-γ responses after statistical correction for total CD4 T-cell counts (data not shown). Given the increased expression of the HIV-1 coreceptor by T_H1/T_H17 cells, we looked for an association between the number of T_H1/T_H17 CCR5⁺ T cells and PPD-specific IFN-γ responses. The number of T_H1/T_H17 CCR5⁺ T-cell numbers and PPD-specific IFN-γ responses were significantly correlated (*r* = 0.69, *P* < .001), and this association persisted after controlling for total CD4 T-cell counts (*r* = 0.51, *P* = .001, Fig 4). PPD-specific IL-2 responses were also significantly associated with the number of T_H1/T_H17 T cells (*r* = 0.56, *P* = .001); however, controlling for CD4 count resulted in loss of statistical significance (*r* = 0.25, *P* = .16, Fig 4). T_H1/T_H17 CCR5⁺ T-cell counts and PPD-specific IL-2 responses were significantly correlated (*r* = 0.60, *P* < .001), and this association was still significant after correction for total CD4 T-cell counts (*r* = 0.36, *P* < .04, Fig 4). There was no association between the number of T_H1, T_H17, and T_H2 cells and the magnitude of PPD-specific IL-2 responses after statistical correction for total

CD4 T-cell counts (data not shown). No significant associations were found between nadir CD4 T-cell counts, the duration of known HIV-1 infection viral load, and T_H1/T_H17 counts (see the Results section in this article's Online Repository at www.jacionline.org). Analysis of ART⁻ patients stratified by CD4 T-cell counts into greater or less than 300 cells/μL showed that T_H1/T_H17 counts (median, 6.50 cell/μL; interquartile range, 2.500-9.50; *P* < .001) and percentages (median, 1.93%; interquartile range, 0.68% to 2.66%; *P* < .005) were still significantly lower than in healthy control subjects.

DISCUSSION

The results of this study show that there is a selective loss of T_H1/T_H17 cells and T_H1/T_H17 CCR5⁺ cells in ART-naïve patients with HIV-1 infection. ART led to a significant increase in the number and frequency of T_H1/T_H17 and T_H1/T_H17 CCR5⁺ cells; however, values obtained were still significantly less than those seen in healthy control subjects. There was a significant correlation between the number of T_H1/T_H17 and T_H1/T_H17 CCR5⁺ cells and the capacity of PBMCs in ELISPOT cultures to secrete IFN-γ and IL-2 in response to stimulation with PPD, even after adjustment for total CD4 T-cell counts. In contrast, no correlation between T_H1 and T_H17 cell counts and PPD antigen-specific cytokine responses was observed after statistical adjustment for overall CD4 T-cell numbers.

There are several reasons why the loss of T_H1/T_H17 cells might be relevant to the increased risk of active tuberculosis

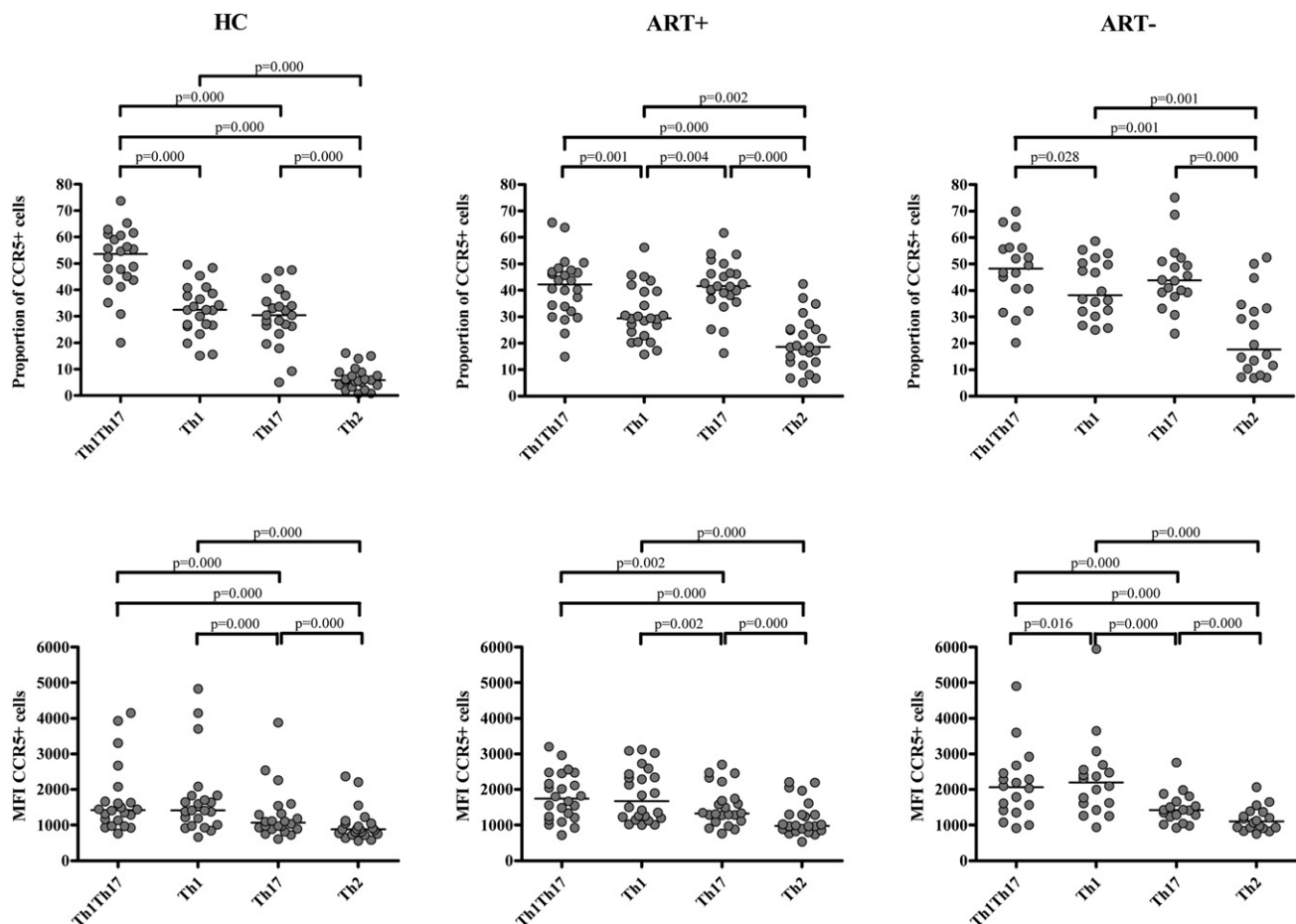


FIG 2. The proportion (**A**) and mean fluorescent intensity (**MFI**; **B**) of CCR5 expression on T_H1/T_H17 , T_H1 , T_H17 , and T_H2 cells from healthy control subjects (**HC**) and **ART**⁺ and **ART**⁻ patients is shown. The horizontal line denotes the median value for each subset. Statistically significant differences between each subset were determined by using the Wilcoxon signed-rank test, and *P* values were reported. Analysis of the data includes all outlier points; however, exclusion of outlier points did not alter the significance of the results.

in patients with HIV-1 infection. T_H1/T_H17 cells express significantly higher levels of CCR5 (HIV-1 coreceptor) compared with other CD4 T-cell subsets and are preferential targets for HIV-1 replication.¹³ Loss of T_H1/T_H17 cells has been described in patients with HIV-1 infection,¹³ and data showing preservation of T_H1 CXCR3⁺CCR6⁻ proportions agree with a recent study showing preferential depletion of *M tuberculosis* antigen-specific but not cytomegalovirus antigen-specific CD4 T cells in blood samples obtained from patients with HIV-1.¹⁴ Although there is no specific chemokine receptor marker that is unique to the lungs, it is known that CD4 T cells isolated from bronchoalveolar lavage (BAL) samples of patients with inflammatory lung disease exhibit enriched expression of CCR6, CXCR3, and CCR5¹⁵⁻¹⁷ and that T_H1/T_H17 cells express the mucosal homing receptor $\alpha 4\beta 7$ integrin.¹³ Most studies have shown that the proportion of CD4 T cells found in the blood mirrors that seen in BAL samples in HIV-1-seropositive patients with no symptoms and signs of lung disease.^{18,19} This indicates that redistribution of T_H1/T_H17 cells to mucosal tissues, such as the lungs, is unlikely to account for loss of this T-cell subset in blood. There is increasing evidence that both the magnitude and quality of PPD

antigen-specific immune responses are significantly reduced in BAL samples obtained from patients with HIV-1 infection, suggesting that mucosal immune responses are also affected by HIV-1 infection.¹⁹⁻²¹

Failure of ART to fully reconstitute PPD antigen-specific immune responses and T_H1/T_H17 cells might be a reflection of the advanced disease stage of our cohort or the limited efficacy of current antiviral therapy. Neither nadir CD4 T-cell count nor length of HIV-1 infection influenced T_H1/T_H17 numbers and the finding of significant depletion in T_H1/T_H17 cell numbers in ART-naïve patients with modest immunodeficiency, suggesting that failure of ART to restore mucosal CD4⁺CCR5⁺ cell subsets might account for the limited immune reconstitution observed. It is recognized that immune reconstitution after ART is limited in gastrointestinal tissues compared with that seen in peripheral blood, and one study has shown that ART might not restore polyfunctional antigen-specific mucosal BAL immune responses.^{21,22} The immunologic findings recapitulate some of the clinical and experimental features of HIV-1/tuberculosis coinfection, such as the increased risk of active tuberculosis early in infection^{3,23} and the effect of ART in reducing the risk of active disease,²⁴ and provide further

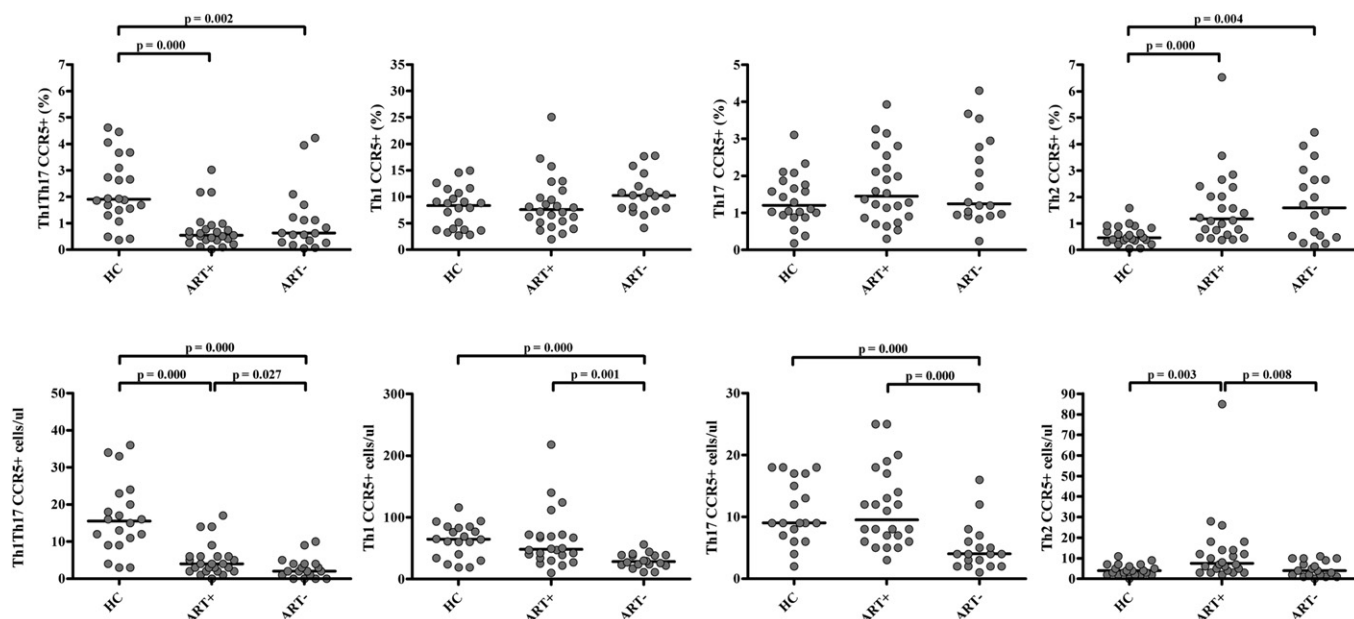


FIG 3. The frequency (*top panel*) and number (*bottom panel*) of T_H1/T_H17 CCR5⁺, T_H1 CCR5⁺, T_H17 CCR5⁺, and T_H2 CCR5⁺ CD4 T-cell subsets in healthy control subjects (HC) and ART⁺ and ART⁻ patients. The horizontal line denotes the median value for each subset. Statistically significant differences in CCR5⁺ cells of each subset between each group were determined by using the Mann-Whitney *U* test, and *P* values were reported. Analysis of the data includes all outlier points; however, exclusion of outlier points did not alter the significance of the results.

TABLE III. PHA- and PPD-specific IFN- γ and IL-2 responses in healthy control subjects, HIV-1-infected patients receiving ART (ART⁺), and treatment-naive HIV-1-infected patients (ART⁻)

Cytokine	Stimulus	HC	ART ⁺	ART ⁻
No.		26	19	13
IFN- γ	PPD	172 (133-221)	68 (48-194)*	4 (1-23)†,§
	PHA	5650 (4511-7128)	6652 (4865-7237)	7630 (4928-7901)
No.		24	21	14
IL-2	PPD	105 (48-153)	42 (23-99)	7 (0-30)†,§
	PHA	4317 (3521-6466)	4088 (2388-5845)	2905 (1333-3605)†,‡

For each group, the median and interquartile range of spot-forming cells per million PBMCs, as determined by means of ELISPOT, are reported for each cytokine. The number of patients for which data were obtained is indicated.

Statistically significant differences between groups for each subset as determined by using the Mann-Whitney *U* test are indicated as follows: HC versus ART⁺.

HC, Healthy control subjects.

**P* < .01; HC versus ART⁺.

†*P* ≤ .001; ART⁺ versus ART⁻.

‡*P* < .05.

§*P* ≤ .001.

evidence for use of ART early in the natural history of HIV-1 infection.²⁵

Longitudinal studies with paired blood and BAL fluid samples should aim to confirm that there is a selective depletion in T_H1/T_H17 cells in the respiratory mucosa of patients with HIV-1 infection and to determine the effect, if any, of ART on immune reconstitution of this lymphocyte subset. Analysis of the capacity of T_H1/T_H17 cells to secrete individual or polyfunctional (≥2 simultaneously) cytokines after PPD and region of difference-1 peptide stimulation is needed to assess the potential importance of this lymphocyte subset in control of tuberculosis infection in patients with HIV-1. Finally, the relationship, if any, between T_H1/T_H17 cells and other components of the immune system believed to be important in the control of tuberculosis, such as CD8 T cells,

CD4 regulatory T cells, $\gamma\delta$ cells, and CD1 restricted T cells, needs to be analyzed.^{5,26}

In conclusion, we show that reduced T_H1/T_H17 cell counts are associated with impaired *M tuberculosis* PPD-specific IFN- γ and IL-2 immune responses. The loss of the T_H1/T_H17 subset of CD4 T cells might be a risk factor for the development of active tuberculosis in patients with HIV-1 infection. Analysis of T_H1/T_H17 cell counts can be potentially used as a biomarker for tuberculosis diagnostics, vaccine development, or both.

We acknowledge the performance of plasma HIV-1 viral load measurement and the quantification of lymphocyte subsets by the Department of Immunology, Chelsea & Westminster Hospital, Imperial College Healthcare NHS Trust.

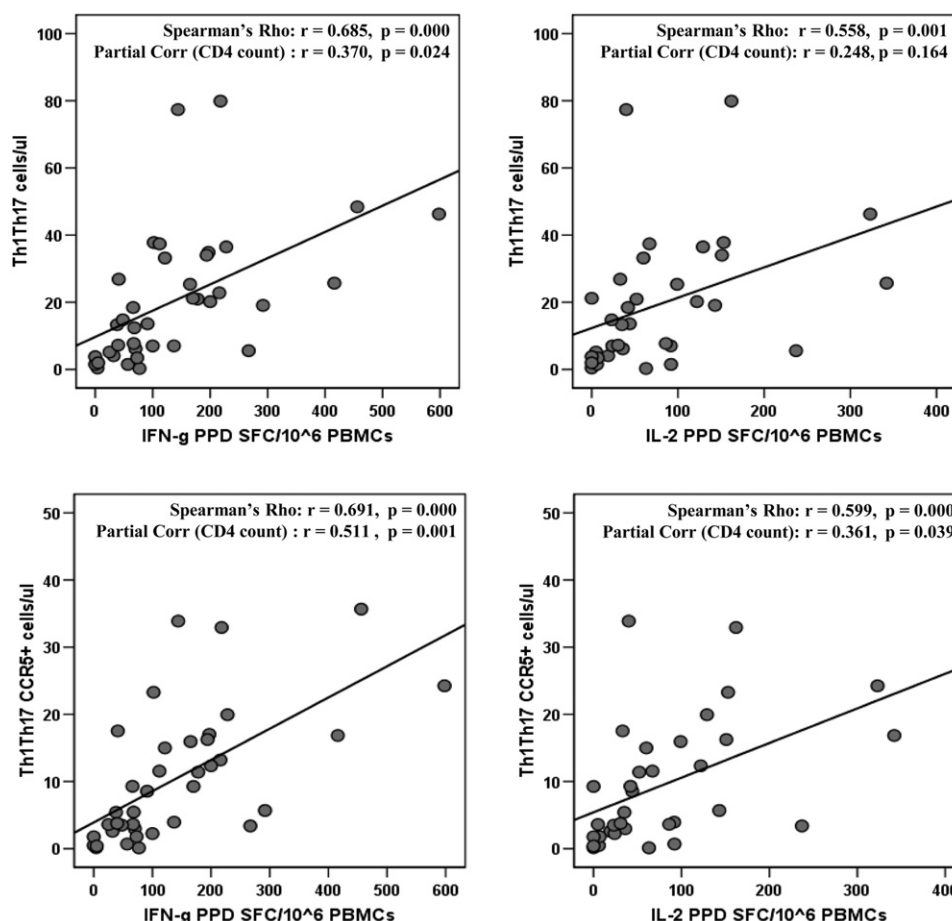


FIG 4. The relationship between the magnitude of IFN- γ and IL-2 PPD responses and the number of T_H1/T_H17 and T_H1/T_H17 CCR5⁺ cells in healthy control subjects and HIV-1-infected patients. The best-fit line for the relationship is shown. The Spearman rho correlation coefficient, partial correlation coefficient (when controlling for CD4 count), and significant *P* values are reported. *SFC*, Spot-forming cells.

Clinical implications: Preferential loss of CD4 T_H1/T_H17 memory cells may explain the increased risk of active tuberculosis in patients with HIV-1 infection and might be a useful biomarker for tuberculosis vaccine development.

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METHODS

Plasma HIV-1 viral load and immunophenotyping of T-cell subsets

Briefly, whole blood (EDTA) samples were stained with a panel of mAbs (CD3, CD4, CD8, and CD45 or CD3, CD56, CD19, and CD45) for 30 minutes in the dark and was evaluated with a Cytomics FC500 flow cytometer (Beckman Coulter, Inc, High Wycombe, United Kingdom). Whole-blood samples were lysed and fixed with Beckman Coulter TQ Prep (IMMUNOPREP Reagent System, ref: 7546999). A 100- μ L sample of Flow Count Fluorospheres (ref: 7547053) was then added to each sample. T-cell counts and percentages were then evaluated by using a Cytomics FC500 flow cytometer.

The phenotypic characterization of T-cell subsets was performed on whole blood with 6-color flow cytometry. Monoclonal antibodies used for staining were CD3 APC-Cy7, CD4 PerCP, CD8 APC, CCR4 PE-Cy7, CCR5 PE, CXCR3 APC (all from BD Biosciences), and CCR6 FITC (R&D Systems). Isotype controls included FITC IgG_{2b} (R&D Systems, CCR6 isotype), PE IgG_{2b} (BD Biosciences, isotype for CCR5), IgG₁ PE-Cy7 (BD Biosciences, isotype for CCR4), and IgG₁ APC (BD Biosciences, isotype CXCR3). Antibodies were added to 150 μ L of whole blood by using the panels outlined in Table E2. After a 30-minute incubation, whole-blood samples were lysed and fixed with the Beckman Coulter TQ Prep IMMUNOPREP Reagent System (ref: 7546999). Cells were washed twice with 2 mL of PBS (7 minutes at 400g) and resuspended in 400 μ L of PBS. The expression of chemokine receptors allowed for the definition of 4 discrete CD4 T-cell subsets (Fig 1): CD4⁺CCR4⁻CXCR3⁺CCR6⁺ (T_H1/T_H17 cells), CD4⁺CCR4⁻CXCR3⁺CCR6⁻ (T_H1 cells), CD4⁺CCR4⁺CXCR3⁻CCR6⁺ (T_H17 cells), and CD4⁺CCR4⁺CXCR3⁻CCR6⁻ (T_H2 cells). For each CD4 subset, the proportion of T cells expressing CCR5 was then defined. Acquisition was performed on an LSRII flow cytometer (BD Biosciences), and at least 30,000 CD3⁺CD4⁺ lymphocytes were acquired. Compensation beads and fluorescence minus one controls were used to control for spectral overlap in whole-blood samples. FACS Diva software (version 6.1.2, BD Biosciences) was used for subsequent data analysis. Lymphocytes were selected on the basis of forward-scatter and side-scatter properties, and a CD3 gate was used to identify T cells. CD4 T cells were identified within the CD3 lymphocytes. Matched isotype controls were used to gate chemokine receptor-positive CD4 T cells. Boolean gating from positive chemokine events on histograms were used to determine CD4 T-cell memory subsets. The absolute counts (cells per microliter of whole blood) of CD4 memory cell subsets were calculated from the frequency at which the subset occurred and the absolute count of the CD4 parent population measured with the Beckman Coulter flow cytometer.

Detection of cytokine responses by means of ELISPOT

The intra-assay CV for PPD-specific IFN- γ spot-forming cell counts is 11%. The intra-assay CV for PPD-specific IL-2 spot-forming cell counts

is 25%. The interassay CV for PPD-specific IFN- γ spot-forming cell counts is 28%. The interassay CV for PPD-specific IL-2 spot-forming cell counts is 18%.

Detection of intracellular cytokine responses by means of flow cytometry

The interassay CV for IFN- γ intracellular cytokine responses is 14%. The interassay CV for IL-2 intracellular cytokine responses is 17%.

RESULTS

PPD-specific IFN- γ and IL-2 responses were significantly lower in the ART⁻ group and were partially reconstituted on suppressive ART

Table E3 shows that the proportion of patients with positive CD4 PPD-specific IFN- γ immune responses is significantly reduced in HIV ART⁻ patients compared with that seen in control subjects ($P < .05$, χ^2 test). There is no difference in the proportion of positive CD8 PPD-specific IFN- γ responses between study group participants. CD4 PPD-specific IL-2 immune responses are detectable in a minority of study participants and do not differ among study participants. CD8 PPD-specific IL-2 immune responses were not observed in this study.

Loss of CCR4⁻CXCR3⁺CCR6⁺CCR5⁺ CD4 T_H1/T_H17 cells is associated with reduced capacity to secrete IFN- γ and IL-2 after PPD stimulation in HIV-1-infected patients

Analysis of nadir CD4 T-cell counts for ART⁻ and ART⁺ patients showed no association between nadir CD4 T-cell counts and CD4 T_H1/T_H17 ($r = 0.05$ and $P > 0.05$ for ART⁻ patients and $r = 0.03$ and $P > 0.05$ for ART⁺ patients). We found no association between the length of HIV-1 infection for both HIV-infected patient groups ($r = -0.27$ and $P > .05$ for the ART⁺ group and $r = -0.08$ and $P > .05$ for the ART⁻ group). There was no relationship between viral load and CD4 T_H1/T_H17 counts for the ART⁻ group ($r = 0.12$, $P > .05$). The combination of HIV-1-infected patient groups showed no significant association between the duration of HIV-1 infection ($r = 0.23$, $P > .05$) or nadir CD4 T-cell counts ($r = -0.12$, $P > .05$).

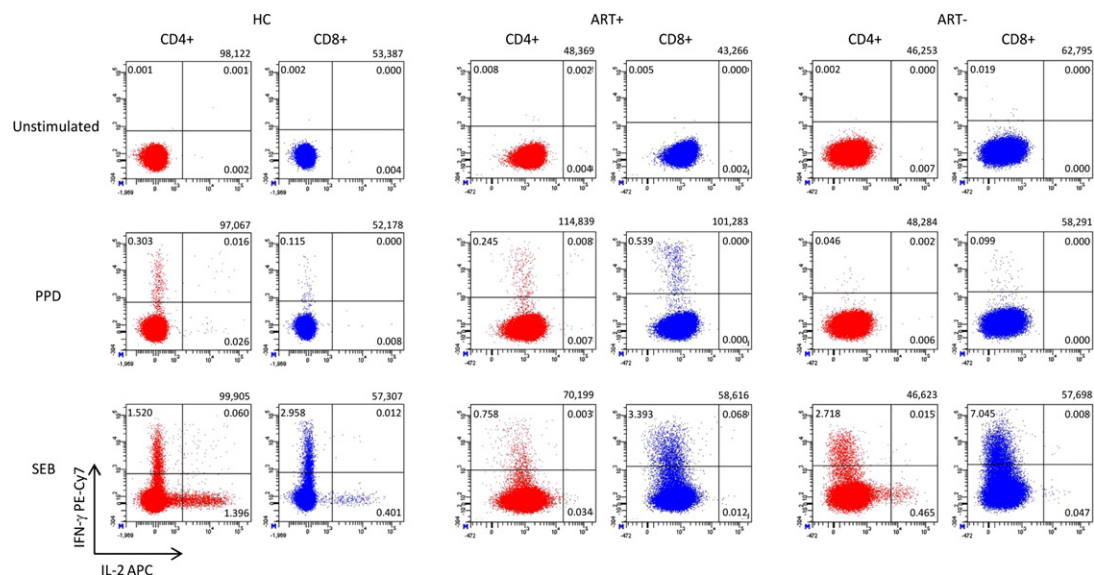


FIG E1. Unstimulated cells and SEB stimulated controls serve as the negative and positive controls for intracellular cytokine detection. Within each quadrant the percentage of IFN- γ ⁺, IL-2⁺ and IFN- γ ⁺/IL-2⁺ cells are indicated. The number of CD4⁺ or CD8⁺ T cells viewed within each dot plot is indicated on the top, right of each dot plot. The gating strategy incorporated a time gate to check for integrity of sample acquisition, followed by a singlet gate to exclude doublets. Lymphocytes were selected on the basis of forward and side scatter properties and T cell were selected on the basis of CD3 expression. CD4 and CD8 T cells were then identified on the CD3 gate. Gates that for the discrimination of positive and negative cytokine events were set on unstimulated PBMC stained for cytokine. The gate was placed half a log above the negative population to control for nonspecific binding of cytokine by dead, dying cells and an overestimation of true antigen specific cytokine responses.

TABLE E1. Principal immunologic role of chemokine receptors and their ligands used to define CD4 T-cell memory subsets in this study

Chemokine receptor	Chemokine ligand		Function
	Original name	Systematic name	
CXCR3	MIG	CXCL-9	Recruitment of T cells to chronic inflammatory sites
	IP-10	CXCL-10	
	I-TAC	CXCL-11	
CCR4	TARC	CCL-17	Recruitment of T cells and basophils to inflammatory sites
	MDC	CCL-22	
CCR5	MIP-1 α	CCL-3	Recruitment of lymphocytes, monocytes, and dendritic cells to inflammatory sites
	MIP-1 β	CCL-4	
	RANTES	CCL-5	
CCR6	MIP-3 α	CCL-20	Regulation of lymphocyte and dendritic cell migration

IP-10, IFN-inducible protein 10; *I-TAC*, INF-inducible T-cell alpha chemoattractant; *MDC*, macrophage-derived chemokine; *MIG*, monokine induced by INF- γ ; *MIP*, macrophage inflammatory protein; *TARC*, thymus and activation-regulated chemokine.

TABLE E2. Monoclonal antibody panels for T-cell immunophenotyping

Tube	FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7
Chemokine receptor expression on CD4 T cells	CCR6 20 μ L	CCR5 10 μ L	CD4 5 μ L	CCR4 5 μ L	CXCR3 10 μ L	CD3 5 μ L
Cytokine tube		CD8 10 μ L	CD4 5 μ L	IFN- γ 2 μ L	IL-2 2 μ L	CD3 5 μ L
FMO tube for FITC		CCR5 10 μ L	CD4 5 μ L	CCR4 5 μ L	CXCR3 10 μ L	CD3 5 μ L
FMO tube for PE	CCR6 10 μ L		CD4 5 μ L	CCR4 5 μ L	CXCR3 10 μ L	CD3 5 μ L
FMO tube for PerCP	CCR6 10 μ L	CCR5 10 μ L		CCR4 5 μ L	CXCR3 10 μ L	CD3 5 μ L
FMO tube for PE-Cy7	CCR6 10 μ L	CCR5 10 μ L	CD4 5 μ L		CXCR3 10 μ L	CD3 5 μ L
FMO tube for APC	CCR6 10 μ L	CCR5 10 μ L	CD4 5 μ L	CCR4 5 μ L		CD3 5 μ L
FMO tube for APC-Cy7	CCR6 10 μ L	CCR5 10 μ L	CD4 5 μ L	CCR4 5 μ L	CXCR3 10 μ L	
Chemokine receptor expression on CD4 T cells, isotype control	MsIgG _{2b} 10 μ L	MsIgG _{2a} 10 μ L	CD4 5 μ L	MsIgG ₁ 5 μ L	MsIgG ₁ 10 μ L	CD3 5 μ L
Intracellular cytokine SEB and unstimulated		CD8 5 μ L	CD4 5 μ L	IFN- γ 2 μ L	IL-2 2 μ L	CD3 5 μ L

FMO, Fluorescence minus one; *Ms*, murine; *SEB*, staphylococcal enterotoxin B.

TABLE E3. Proportion of positive CD4 and CD8 cytokine responses to PPD in study participants

	Healthy control subjects	HIV-infected ART ⁺ patients	HIV-infected ART ⁻ patients
CD4 PPD-specific IFN- γ	84%	57%	33%
CD4 PPD-specific IL-2	32%	14%	17%
CD8 PPD-specific IFN- γ	68%	60%	50%
CD8 PPD-specific IL-2	0%	0%	0%