

# Association between Tuberculin Skin Test Reactivity, the Memory CD4 Cell Subset, and Circulating FoxP3-Expressing Cells in HIV-Infected Persons

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**Background.** Lack of reactivity to the tuberculin skin test (TST) is widely observed in individuals with advanced human immunodeficiency virus type 1 (HIV-1) infection.

**Methods.** Biopsy specimens from the TST reaction site and from skin not infiltrated with purified protein derivative were obtained from 15 HIV-1-infected and 23 uninfected persons who did not have active tuberculosis and who were from a community in which the incidence of tuberculosis was very high. Histologic sections (size, 8  $\mu$ m) were immunohistochemically stained for CD4, CD8, CD28, CD45RA, CD45RO, CD62L, CD1a, human leukocyte antigen (HLA)-DR, granulysin, interferon- $\gamma$ , and FoxP3 and were analyzed by single-cell in situ digital imaging. Peripheral blood mononuclear cells were analyzed using a fluorescence-activated cell sorter.

**Results.** Biopsy specimens obtained from TST-reactive skin of HIV-1-infected persons demonstrated fewer CD4<sup>+</sup> T cells at the TST site ( $P = .036$ ) but more HLA-DR<sup>+</sup> T cells ( $P = .037$ ) than did such biopsy specimens obtained from HIV-1-uninfected persons. Among HIV-1-infected persons, the total number of cells ( $P = .008$ ) and numbers of CD45RO<sup>+</sup> memory T cells ( $P = .003$ ) were significantly higher in TST-reactive persons than in TST-unreactive persons. For HIV-1-infected persons, TST induration was inversely correlated with the numbers of FoxP3<sup>+</sup> T cells in the blood ( $P = .026$ ) but was unrelated to the number of circulating CD4<sup>+</sup> T cells.

**Conclusions.** For HIV-1 infected persons, the TST depends on memory T cells and is more strongly associated with the numbers of circulating FoxP3<sup>+</sup>CD4<sup>+</sup> T cells than with the total number of CD4<sup>+</sup> T cells.

HIV infection and tuberculosis (TB) are the most pressing public health problems in Africa [1]. The epidemiologic

profile and clinical characteristics of HIV-associated TB have been well researched, but few studies have analyzed in detail the immunophenotype of T cell populations in HIV-1/TB coinfection.

HIV infects immune cells that bear CD4, and the most characteristic and clinically useful index of immune depletion in HIV infection is the CD4<sup>+</sup> lymphocyte count. Therefore, as the CD4<sup>+</sup> lymphocyte count decreases, susceptibility to TB and the likelihood of exhibiting a false-negative response to the protein purified derivative (PPD) of tuberculin increase [2, 3]. In advanced HIV-1 infection, the ability to mount an immune response to a protein challenge decreases, which is one of the reasons why vaccination of HIV-1-infected individuals is, at best, only partially effective [4, 5]. Thus, administration of the tuberculin skin test (TST) provides an opportunity to better understand the response to protein subunits in HIV-1-infected people. It is a safe and relatively standardized test, and its route of administration (the

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intradermal route) facilitates antigen presentation to *Mycobacterium tuberculosis*-reactive T cells via dendritic cells. These cells expand after restimulation in the skin and give rise to the characteristic type IV delayed-type hypersensitivity reaction, although positive TST reactions can also be observed in individuals infected with nontuberculous mycobacteria, including the vaccine strain bacille Calmette-Guérin.

In addition to expanding T cells that assist intracellular containment of *M. tuberculosis*, PPD may also stimulate regulatory or anti-inflammatory T cells that can be characterized by the expression of the phenotypic marker FoxP3 [6]. Expansion of peripheral blood CD4<sup>+</sup>FoxP3<sup>+</sup> T cells has been described in cases of HIV-associated TB [7, 8]. Detailed immunologic analysis of TST reactions in HIV-infected and -uninfected people could therefore provide an invaluable window to study, at a site of inflammation, the immune dysregulation that better characterizes the increased susceptibility of HIV-infected people to TB. In the present study, we compared, in HIV-1-infected and -uninfected persons from a South African community with a very high incidence of TB, the immunophenotypes of cells obtained from peripheral blood and from skin at the site of tuberculin injection.

## SUBJECTS AND METHODS

**Subject recruitment and skin biopsy.** The study was approved by the research ethics committees of the University of Cape Town (Cape Town, South Africa) (REC 175/2005) and the University of Lübeck (Lübeck Germany) (REC 04/176). All persons who participated in the study provided written informed consent. The clinical setting of the study in Khayelitsha Township, South Africa, has been described elsewhere [9, 10]. Khayelitsha has a TB incidence of 1612 cases/100,000 persons, and previous studies of the prevalence of latent TB in young adults, as determined by both in vitro and skin test methods, indicated that ~80% of people are infected with *M. tuberculosis* [9–11].

Adults who were residents of Khayelitsha and who attended voluntary counseling and underwent testing for HIV-1 infection were invited to participate in the study either after their second clinic visit after receiving voluntary counseling and testing, on the day that they received a negative test result, or at the time of their first visit to the HIV/AIDS clinic. Exclusion criteria included previous TB, receipt of isoniazid as preventive therapy, receipt of steroid therapy, pregnancy, and, in HIV-1-infected persons, either a Karnofsky score of  $\leq 60$  or a current opportunistic infection. Assessment of eligibility included a symptom screening questionnaire and physical examination for active TB that was based on validated approaches [12, 13]. The presence of any one of a list of symptoms (cough, chest pain, recent weight loss, night sweats, fever, loss of appetite, swelling of the lymph

nodes, or generalized tiredness) formed an exclusion criterion and triggered referral.

At the first study visit, blood samples were obtained, a 2-tuberculin unit TST (RT23; Statens Serum Institute) was placed on the volar aspect of the forearm, and the site was marked with a circle with the use of a felt-tip pen. At 48 h, the transverse diameter of TST induration was determined using the ballpoint pen and ruler method [14]. Administration of the TST and its assessment were performed by 2 investigators (R.J.W. and M.X.R.) with 23 years of combined experience in TB research.

A locally sterile field was established, the surrounding skin was infiltrated with 1% lidocaine anesthetic, and a 4-mm punch biopsy specimen of the TST reaction (irrespective of whether it was reactive or unreactive) was obtained [15]. Some of the biopsy specimens showed incipient central necrosis that was avoided. Otherwise, biopsy specimens were of clearly indurated, raised skin. Wounds were closed using steristrips. A second 2-mm biopsy specimen of healthy skin was also obtained from the volar aspect of the other forearm, for the purposes of making a baseline comparison. Biopsy specimens were placed directly into a U-bottom, 1-mL, screw-topped, sterile Eppendorf tube and snap-frozen in liquid nitrogen at the clinic. Participants were reviewed at 1 week, to ensure healing, and HIV-1-infected persons with Mantoux reactions of  $\geq 5$  mm of induration were offered isoniazid as preventive therapy, according to the national guidelines of South Africa. The skin specimens were transported on liquid nitrogen to the Center of Infectious Medicine, Karolinska Institute (Stockholm, Sweden), where further analyses were conducted in the laboratory of J.A.

**Peripheral blood mononuclear cell (PBMC) separation and 4-channel flow cytometric analysis.** After 20 mL of venous blood was obtained, PBMCs were separated using standard protocols, and the cells were left resting overnight in 96-well U-bottom plates at  $10^6$  cells/mL of cell culture medium (RPMI 1640)/10% fetal calf serum (FCS) plated at 200  $\mu$ L/well. In the morning, cells were stained for 20 min on ice, by use of the following fluorescent antibodies (all of which were obtained from BD Biosciences): CD4-PerCP, CD62L-FITC, CD45RA-PE, CD45RO-FITC, HLA-DR-PE, CD28-FITC, and CD8-PerCP. After washing, stained cells were fixed in PBS/2% FCS containing 1.6% paraformaldehyde and analyzed on a BD FACSCalibur flow cytometer. Intracellular staining for FoxP3 was performed using the phycoerythrin (PE) anti-human FoxP3 staining set from eBioscience. In brief, cells were first surface stained, as described above, and then incubated for 45 min on ice in eBioscience Fix/Perm buffer. Cells were washed in eBioscience permeabilization buffer and incubated for 15 min on ice in 2% serum from healthy rats; they then were incubated for 30 min on ice in 10  $\mu$ L of anti-human FoxP3 (PCH101) antibody. Cells were washed again, fixed in PBS/2% FCS containing 1.6% paraformaldehyde, and analyzed on a BD FACSCalibur flow cytometer.

**Interferon (IFN)- $\gamma$  enzyme-linked immunospot (ELISPOT) analysis.** Blood was drawn for overnight IFN- $\gamma$  ELISPOT analysis by use of the recombinant *M. tuberculosis* region of difference (RD1)-encoded antigens culture filtrate protein-10 and early secretory antigenic target-6 exactly as described elsewhere [10]. A positive response was defined by the presence of  $\geq 20$  spot-forming cells/million PBMCs. Positive control wells containing phytohemagglutinin (5  $\mu$ g/mL) were included. The absence of reactivity to this stimulus in the absence of reactivity to any antigen was scored as an indeterminate result.

**Immunohistochemical staining of biopsy specimens.** Sections of cryopreserved biopsy specimens (8  $\mu$ m) were cut and then were fixed on glass slides in 2% formaldehyde and stored at  $-20^{\circ}\text{C}$ . Immunohistochemical staining was performed using the ABC (avidin-biotin-enzyme complex) method, as described elsewhere [16]. After blocking endogenous peroxidase and biotin in the tissue, primary antibodies were incubated overnight with the monoclonal antibodies against CD4, CD8, HLA-DR (BD Biosciences), CD1a, CD28, CD45RA, CD45RO, CD62L (Dako Cytomation), and anti-IFN- $\gamma$  (Mabtech). Antigranulysin (Santa Cruz Biotechnology) and anti-FoxP3 (Abcam) were both polyclonal goat anti-human antibodies. Negative controls used to observe for potential nonspecific binding were mouse IgG1 and IgG2a (Dako) and goat IgG (R&D Systems). The secondary antibodies (polyclonal anti-mouse antibodies and polyclonal anti-goat antibodies; Dako Cytomation) were applied and labeled using ABC complex containing horseradish peroxidase for the enzyme (Vectastain Elite ABC Kit; Vector Laboratories). As enzyme substrate, we used 3,3'-diaminobenzidine (Vector Laboratories). Counterstaining was performed in hematoxylin. Acquired computerized image analysis was performed on the immunohistochemically stained sections, as described elsewhere [17], by use of the Leica DMR-X microscope (Leica) and the computerized image analysis system Leica Qwin 550IW (Leica Imaging System), which allowed for quantification of the positive immunoreactivity at the single-cell level relative to the total area of the section.

**Statistical analysis.** The normality of the data was assessed using the Shapiro-Wilk test. Normally distributed variables are shown, and the mean value ( $\pm$ SD) and between-group comparisons were assessed using Student's unpaired *t* test. Nonparametric variables are expressed as the median value (interquartile range [IQR]), and between-group comparisons were conducted using the Mann-Whitney *U* test. When normality was incongruent between clinical groups, nonparametric distributions were assumed and were analyzed appropriately.

## RESULTS

**Characteristics at baseline.** Thirty-eight persons (15 anti-retroviral-naïve HIV-1-infected persons and 23 HIV-1-uninfected persons) were recruited for the study; their demographic and clinical characteristics are shown in table 1. The sex distribution, age, and TB exposure history of these individuals did not differ between

**Table 1. Characteristics at baseline of the subjects enrolled in the study.**

Characteristic	HIV-1 status		<i>P</i>
	Infected ( <i>n</i> = 15)	Uninfected ( <i>n</i> = 23)	
Sex, no. (%) female	10 (67)	14 (61)	1.0
Age, mean $\pm$ SD, years	31.6 $\pm$ 5.2	31.4 $\pm$ 8.6	.94
CD4 cell count, median (range)	325 (125–972)	NR	NA
Induration, <sup>a</sup> mm			
Mean $\pm$ SD	10.6 $\pm$ 10.0	17.1 $\pm$ 6.6	.022
>15	6 (40)	18 (78)	.037
>10	9 (60)	20 (87)	.12
>5	9 (60)	22 (96)	.01
0–5	6 (40)	1 (4)	.01
RD1-based ELISPOT finding			
Positive	8/11	11/13	.63
Negative	2/11	1/13	.58
Indeterminate	1/11	1/13	1.0

**NOTE.** ELISPOT, enzyme-linked immunospot; NA, not available; NR, not reported; RD1, region of difference.

<sup>a</sup> As determined by the tuberculin skin test.

groups. The mean diameter ( $\pm$ SD) of the TST reaction was greater in the HIV-1-uninfected group than in the HIV-1-infected group (17.1  $\pm$  6.7 mm vs. 10.6  $\pm$  10 mm; *P* = .022). This difference was reflected in the greater proportion of HIV-1-uninfected persons who exhibited a positive TST reaction at each cutoff level, significantly so for the cutoff levels of 5 and 15 mm (*P*  $\leq$  .037). For further analysis, we used a cutoff of 5 mm of induration to define positive TST reactions. In the HIV-1-infected group, the CD4 cell count and TST induration were not significantly correlated, although the mean CD4 cell count in persons who were unreactive to tuberculin was lower (243  $\pm$  100 vs. 442  $\pm$  260 cells/mm<sup>3</sup>; *P* = .75). For 24 patients (11 HIV-1-infected patients and 13 HIV-1-uninfected patients) for whom sufficient numbers of mononuclear cells were available, we also performed RD1-based IFN- $\gamma$  ELISPOT analysis. For one assay in each group, an indeterminate result was noted; 11 of 13 patients in the HIV-1-uninfected group had a positive result, as did 8 of 11 patients in the HIV-1-infected group (including positive results for 3 of the 6 patients who did not have a TST reaction).

**Phenotype of PBMCs.** The mean total percentage of CD4<sup>+</sup> cells in the blood of HIV-1-infected persons was lower than that in HIV-1-uninfected persons (16.0%  $\pm$  2.1% vs. 27.4%  $\pm$  1.9%; *P* = .0004) (table 2). The median frequency (IQR) of CD4<sup>+</sup> T cells bearing the costimulatory marker CD28 was also significantly reduced in HIV-1-infected persons (20.8% [13.2%–23.5%] vs. 34.7% [30.1%–42.9%]; *P* = .0001) (table 2). In TST-unreactive HIV-1-infected persons, there was a decrease in the percentage of CD4<sup>+</sup>CD45RA<sup>−</sup>CD45RO<sup>+</sup> cells, compared with that noted in TST-reactive HIV-1-infected persons (median [IQR], 19.2%

**Table 2. Analysis of the phenotype of cells in the peripheral blood of subjects, by HIV-1 status and tuberculin skin test (TST) reaction.**

Marker	HIV-1 status				
	Uninfected		Infected		
	All (n = 22)	Reactive (n = 21)	All (n = 15)	Reactive (n = 9)	Unreactive (n = 6)
Total CD4 <sup>+</sup>	27 ± 1.9	28 ± 2.2	16 ± 2.1 <sup>a</sup>	17 ± 3.3 <sup>b</sup>	15 ± 2.0
CD4 <sup>+</sup> CD28 <sup>+</sup>	35 ± 1.9	37 ± 2.0	21 ± 2.5 <sup>c</sup>	18 ± 2.8 <sup>d</sup>	22 ± 4.9
CD4 <sup>+</sup> HLA-DR <sup>+</sup>	2.7 (2.3–3.9)	2.7 (2.2–4.2)	2.4 (2.0–4.5)	3.4 (2.2–5.6)	2.2 (1.7–3.6)
CD4 <sup>+</sup> CD45RA <sup>+</sup> CD45RO <sup>+</sup>	39 (30–45)	38 (29–44)	26 (20–40)	32 (26–55)	19 (17–26) <sup>e</sup>
CD4 <sup>+</sup> CD45RA <sup>+</sup> CD62L <sup>+</sup>	44 (34–50)	44 (35–49)	56 (31–63)	53 (26–62)	59 (41–67)
CD4 <sup>+</sup> FoxP3 <sup>+</sup>	8.1 (6.7–9.1)	7.8 (6.5–9.4)	8.5 (7.1–13.0)	7.7 (6.4–10.0)	14 (7.8–17.0) <sup>f</sup>
CD8 <sup>+</sup> CD28 <sup>+</sup>	14 (13–19)	14 (13–17)	14 (10–19)	14 (7–20)	15 (10–18)
CD8 <sup>+</sup> HLA-DR <sup>+</sup>	11 ± 1.0	10 ± 1.1	19 ± 3.2 <sup>g</sup>	25 ± 4.8 <sup>h</sup>	20 ± 3.4
CD8 <sup>+</sup> CD45RA <sup>+</sup> CD45RO <sup>+</sup>	8.2 (3.3–12.0)	8.3 (3.4–11.0)	11 (7.1–17.0)	15 (8.9–22.0) <sup>i</sup>	8 (4.9–14.0)
CD8 <sup>+</sup> CD45RA <sup>+</sup> CD62L <sup>+</sup>	45.0 ± 2.3	45.0 ± 2.2	34.0 ± 2.7 <sup>j</sup>	35.0 ± 4.5 <sup>k</sup>	33.0 ± 2.2

**NOTE.** Data are the mean % of cells positive for the marker (±SD) or the median % of cells positive for the marker (interquartile range).

<sup>a</sup> *P* = .0004, compared with HIV-1-uninfected persons.

<sup>b</sup> *P* = .009, compared with TST-reactive, HIV-1-uninfected persons.

<sup>c</sup> *P* < .001, compared with HIV-1-uninfected persons.

<sup>d</sup> *P* < .0001, compared with TST-reactive, HIV-1-uninfected persons.

<sup>e</sup> *P* = .005, compared with TST-reactive, HIV-1-infected persons.

<sup>f</sup> *P* = .036, compared with TST-reactive, HIV-1-infected persons.

<sup>g</sup> *P* < .0001, compared with HIV-1-uninfected persons.

<sup>h</sup> *P* = .0002, compared with TST-reactive HIV-1-uninfected persons.

<sup>i</sup> *P* = .040, compared with TST-unreactive HIV-1-infected persons.

<sup>j</sup> *P* = .004, compared with HIV-1-uninfected persons.

<sup>k</sup> *P* = .036, compared with TST-reactive HIV-1-uninfected persons.

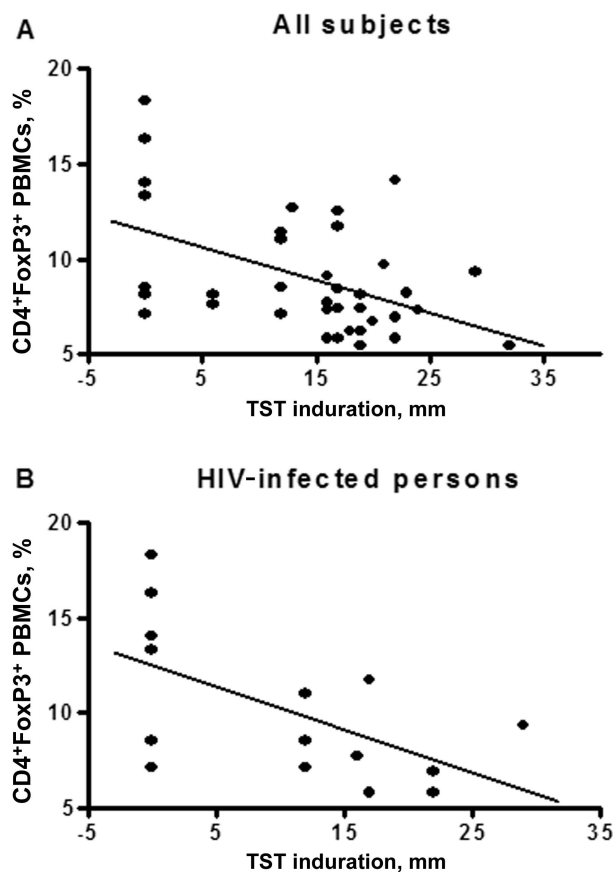
[16.6%–26.1%] vs. 31.6% [26.1%–54.7%]; *P* = .005). Conversely, the median percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> cells was 1.78 times higher in TST-unreactive HIV-1-infected persons than in TST-reactive HIV-1-infected persons (13.7% [7.8%–17.3%] vs. 7.7% [6.4%–10.2%]; *P* = .036). There was a greater percentage of activated (HLA-DR<sup>+</sup>) and memory (CD45RA<sup>+</sup>CD45RO<sup>+</sup>) CD8<sup>+</sup> cells in the PBMCs of HIV-1-infected persons than in the PBMCs of HIV-1-uninfected persons (mean [±SD] for CD8<sup>+</sup>HLA-DR<sup>+</sup> cells, 18.9% ± 3.2% vs. 10.8% ± 1.0% [*P* < .0001]; median [IQR] for CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup> cells, 11.3% [7.1%–16.6%] vs. 8.2% [3.3%–11.9%]; *P* = .006). There was a trend toward a lower percentage of CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup> cells in the PBMCs of TST-unreactive HIV-1-infected persons, but this finding was not statistically significant (*P* = .088).

We next explored whether there existed a correlation between the diameter of the TST reaction and any peripheral T cell subset. The only significant correlations were found to be negative: between the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> PBMCs in the entire patient group (Spearman's correlation coefficient, *r* = −0.39 [95% confidence interval {CI}, −0.07 to −0.64]; *P* = .015) and, more strongly, in the HIV-1-infected group (Spearman's correlation coefficient, *r* = −0.57 [95% CI, −0.06 to −0.84]; *P* = .026) (figure 1). An absolute CD4 cell count was available for the HIV-1-infected patients and also allowed an alternative

exploration of the correlation with absolute numbers. When this type of analysis was done, a weak but significant positive association was noted between the total number of CD4 HLA-DR<sup>+</sup> cells and the diameter of induration observed after the TST was performed (Spearman's correlation coefficient, *r* = 0.53 [95% CI, 0.01–0.82]; *P* = .04).

**Skin biopsy specimens.** One biopsy specimen obtained from an HIV-1-uninfected person was excluded from analysis because it was inadequate in size and quality, such that appropriate analysis was impossible. We first wished to establish the percentage of cells that stained positive for CD4, CD8, and CD1a in a section of healthy skin in relation to the total area of the section. In control biopsy specimens of healthy skin obtained from a subset of 13 HIV-1-infected and 19 HIV-1-uninfected persons, the median percentage of CD4-positive cells was 4.39% (IQR, 1.39%–5.79%) in HIV-1-infected persons and 3.32% (IQR, 0.54%–9.16%) in HIV-1-uninfected persons (*P* = .69). Similarly, there was no significant difference between the frequencies of CD8-positive cells, although the frequency tended to be lower in HIV-1-infected persons (median 0.93% [IQR, 0.56%–3.84%] vs. 2.41% [IQR, 0.78%–3.96%]; *P* = .41). Neither were there significant differences between the numbers of antigen-presenting CD1a-positive cells in HIV-1-infected persons (median, 0.42 cells [IQR, 0.26–0.83 cells] and HIV-1-uninfected persons (median, 0.67 cells [IQR, 0.48–0.8 cells]; *P* = .42).



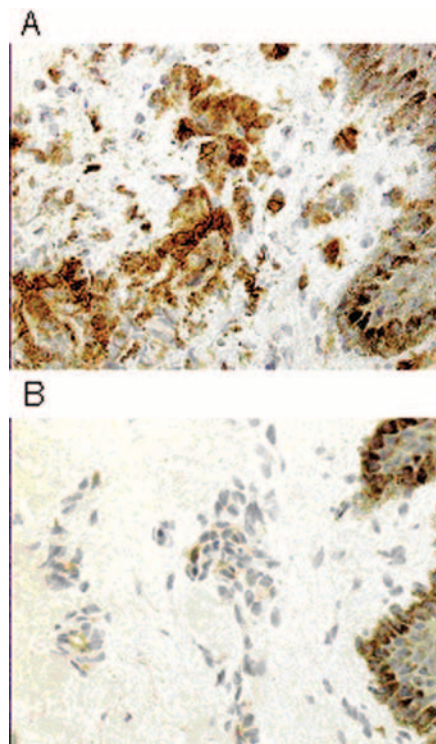


**Figure 1.** Association of the induration of the tuberculin skin test (TST) reaction with the no. of circulating CD4<sup>+</sup>FoxP3<sup>+</sup> peripheral blood mononuclear cells (PBMCs) per microliter of blood in all subjects (A) and in the HIV-1-infected subset (B). In HIV-1-infected persons, there was a significant negative correlation ( $r = 0.571$ ;  $P = .026$ ) between the TST induration diameter on the skin and the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T cells in the blood. There was no such correlation among HIV-1-uninfected persons.

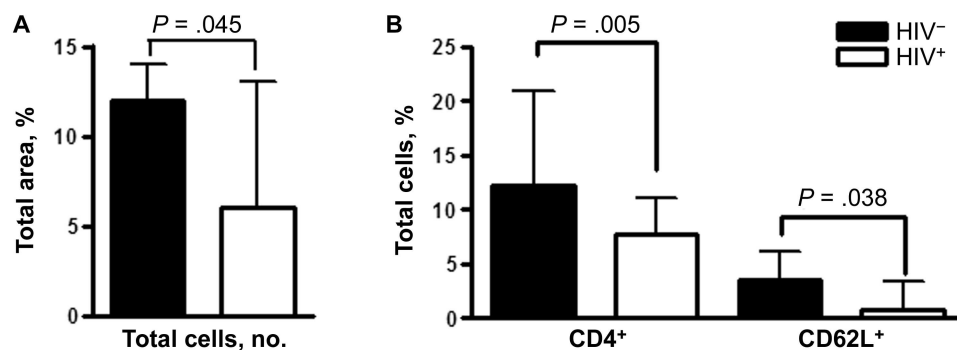
Typical skin specimens obtained from both a reactive and a nonreactive TST site and immunohistochemically stained with anti-CD45RO monoclonal antibodies are shown in figure 2. The total cellular infiltrate, expressed as the median percentage of the cell area measured within the total area of the biopsy specimen was lower at 6.0% (IQR, 5.0%–13.0%) in HIV-1-infected persons than at 12.0% (IQR, 9.0%–14.0%) in HIV-1-uninfected persons ( $P = .045$ ) (figure 3). In HIV-1-infected persons, this difference was entirely due to a lower number of individuals who did not react in the TST (4.5% [IQR, 3%–6%] vs. 11.0% [IQR, 6.5%–15.5%] in reactive persons;  $P = .008$ ) (table 3). The frequency of CD4-positive skin cells was lower in HIV-1-infected persons than in HIV-1-uninfected persons (median, 7.7% [IQR, 3.0%–11%] vs. 12.3% [IQR, 8.0%–20.9%];  $P = .005$ ) (figure 3). The difference was reduced but remained significant when only reactive biopsy specimens were considered (median, 12.6% [IQR 9.4%–23.3%] in HIV-1-uninfected persons vs. 9.2% [IQR, 4.4%–11.0%] in HIV-1-infected persons;  $P = .022$ ). A

similar trend was seen for the frequency of CD62L-positive cells, which was lower in HIV-1-infected persons (median, 0.7% [IQR, 0.6%–3.5%] vs. 3.5% [IQR, 1.2%–6.1%];  $P = .037$ ). This difference again remained significant when only reactive biopsy specimens were considered (median, 0.7% [IQR, 0.5%–3.4%] in HIV-1-infected persons vs. 3.5% [IQR, 1.2%–6.1%] in HIV-1-uninfected persons;  $P = .046$ ).

Taken together, these results suggest that the cell population causing the infiltrate in the TST reaction in HIV-1-infected individuals is different from that in the HIV-1-uninfected individuals. Indeed, HIV-1-infected persons had a higher median proportion of cells positive for HLA-DR<sup>+</sup> cells than did HIV-1-uninfected persons: 37.8% (IQR, 31.0%–43.1%) vs. 26.2% (IQR, 16.4%–34.7%;  $P = .046$ ). Unreactive HIV-1-infected persons had lower median values (22.9%; IQR, 13.7%–41.2%), but this difference did not attain significance. When TST-unreactive and TST-reactive HIV-1-infected groups were compared, decreased numbers of CD45RA<sup>+</sup> cells (median, 1.7 cells [IQR, 0.1–3.5 cells] vs. 4.4 cells [IQR, 2.9–7.8 cells];  $P = .036$ ) and CD45RO<sup>+</sup> cells (median, 5.7 cells [IQR, 1.7–10.0 cells] vs. 16.0 cells [IQR, 13.8–23.8 cells];  $P = .003$ ) were also noted in TST-unreactive persons (figure 4).



**Figure 2.** Examples of a microscopic view of samples of tuberculin T test (TST)-reactive (A) and TST-unreactive (B) skin obtained from HIV-1-infected persons and immunohistochemically stained with monoclonal antibodies against CD45RO (original magnification,  $\times 220$ ). The TST-unreactive specimen had a lower concentration of the total cellular infiltration and, especially, of CD45RO<sup>+</sup> memory T cells.



**Figure 3.** A, Cellular infiltrates in the area of the tuberculin T test (TST) reaction of HIV-1-uninfected persons vs. HIV-1-infected persons, expressed as the percentage of the cell area within the total area of the skin specimen obtained 48 h after tuberculin inoculation. HIV-1-infected persons had lower nos. of cells infiltrating the TST reaction site than did HIV-1-uninfected persons ( $P = .045$ ). B, Distribution of cellular infiltrates in immunohistochemically stained biopsy specimens obtained from the TST reaction sites of HIV-1-uninfected vs. HIV-1-infected persons relative to the total no. of infiltrating cells. Specimens obtained from HIV-1-infected persons had significantly lower frequencies of cells staining positive for CD4 ( $P = .005$ ) and CD62L ( $P = .038$ ).

Overall, in the entire patient group, the total cell area relative to the total area of the section (Spearman's correlation coefficient,  $r = 0.559$ ;  $P = .000$ ), the number of infiltrating CD4<sup>+</sup> cells (Spearman's correlation coefficient,  $r = 0.406$ ;  $P = .013$ ), the number of CD1a<sup>+</sup> cells (Spearman's correlation coefficient,

$r = 0.362$ ;  $P = .030$ ), and the number of infiltrating CD45RO<sup>+</sup> cells (Spearman's correlation coefficient,  $r = 0.365$ ;  $P = .029$ ) were positively correlated with the size of the TST induration. In the HIV-1-infected group, the total number of infiltrating cells (Spearman's correlation coefficient,  $r = 0.76$ ;  $P = .001$ ) and

**Table 3.** Analysis of the phenotype of cells in skin specimens obtained from tuberculin skin test (TST) reaction sites of subjects, by HIV-1 status and TST reaction status.

Marker	HIV-1 status <sup>a</sup>				
	Uninfected		Infected		
	All (n = 22)	Reactive (n = 21)	All (n = 15)	Reactive (n = 9)	Unreactive (n = 6)
Total % of all rows	12 (9.0–14.0) <sup>a</sup>	13 (10–14)	6.0 (5.0–13.0)	11 (6.5–16.0) <sup>b</sup>	4.5 (3.0–6.0)
CD4 <sup>+</sup>	12 (8.0–21.0) <sup>c</sup>	13 (9.4–23.0) <sup>d</sup>	7.7 (3.0–11.0)	9.2 (4.4–11.0)	4.8 (2.0–12.0)
CD8 <sup>+</sup>	4.9 (2.4–13.0)	5.1 (2.9–14.0)	5.5 (2.3–8.0)	5.8 (1.8–13.0)	4.1 (2.0–7.2)
CD28 <sup>+</sup>	3.8 (1.4–8.0)	3.4 (1.2–8.8)	5.6 (2.6–13.0)	5.0 (2.9–10.0)	6.0 (2.1–23.0)
IFN- $\gamma$ <sup>+</sup>	3.1 (1.7–6.0)	3.1 (1.2–6.1)	3.4 (1.9–6.4)	3.8 (2.5–6.4)	2.4 (1.9–6.8)
HLA-DR <sup>+</sup>	26 (15–35)	26 (18–34) <sup>e</sup>	35 (19–43)	38 (31–43)	23 (14–41)
CD1a <sup>+</sup>	3.6 (0.9–8.6)	5.9 (1.6–9.3)	4.2 (2.1–7.0)	5.3 (3.3–7.0)	2.1 (1.2–5.7)
CD45RA <sup>+</sup>	5.3 (2.5–15.0)	5.3 (2.5–12.0)	3.0 (1.3–6.4)	4.4 (2.9–7.8) <sup>f</sup>	1.7 (0.1–3.5)
CD45RO <sup>+</sup>	16 (13–23)	16 (13–23)	14 (7.3–18.0)	16 (14–24) <sup>g</sup>	5.7 (1.2–12.0)
CD62L <sup>+</sup>	3.5 (1.2–6.1) <sup>h</sup>	3.5 (1.2–6.1) <sup>i</sup>	0.7 (0.6–3.5)	0.7 (0.5–3.4)	1.5 (0.7–3.1)
Granulysin <sup>+</sup>	0.6 (0.3–1.0)	0.6 (0.2–1.0)	0.5 (0.4–1.0)	0.5 (0.4–0.9)	0.7 (0.3–1.0)
FoxP3 <sup>+</sup>	0.3 (0.1–0.8)	0.3 (0.1–0.6)	0.5 (0.2–0.8)	0.7 (0.3–1.0)	0.2 (0.1–0.8)

**NOTE.** Data are the median % (interquartile range [IQR]) of the positive cells within the cell area of the section for all rows except the "Total % of all cells" row, for which data are the median % (IQR) of the cell area measured within the total section. IFN, interferon.

<sup>a</sup>  $P = .045$ , compared with all HIV-1-infected persons.

<sup>b</sup>  $P = .008$ , compared with TST-unreactive HIV-1-infected persons.

<sup>c</sup>  $P = .005$ , compared with all HIV-1-infected persons.

<sup>d</sup>  $P = .022$ , compared with TST-reactive HIV-1-infected persons.

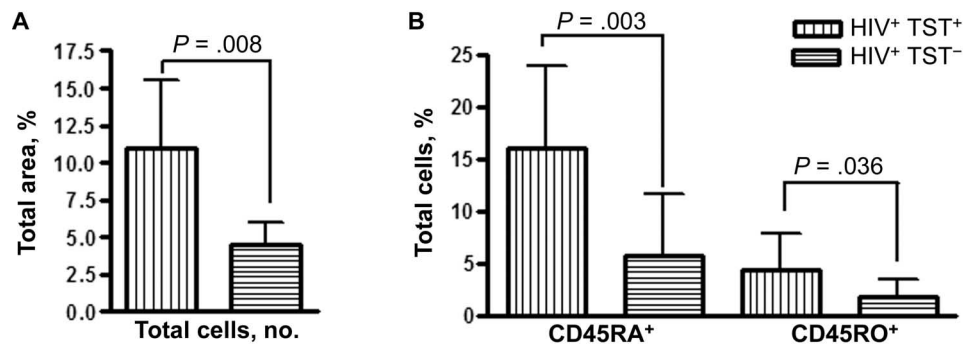
<sup>e</sup>  $P = .037$ , compared with TST-reactive HIV-1-infected persons.

<sup>f</sup>  $P = 0.036$ , compared with TST-unreactive HIV-1-infected persons.

<sup>g</sup>  $P = .003$ , compared with TST-unreactive HIV-1-infected persons.

<sup>h</sup>  $P = .038$ , compared with all HIV-1-infected persons.

<sup>i</sup>  $P = .046$ , compared with the TST-reactive HIV-1-infected persons.



**Figure 4.** A, Cellular infiltrates in the tuberculin T test (TST) reaction site of HIV-1-infected persons with positive TST reactions vs. HIV-1-infected persons with negative TST reactions, expressed as the percentage of the cell area within the total area of the skin specimen being obtained 48 h after tuberculin inoculation (TST cutoff, >5 mm of induration). HIV-1-infected TST-unreactive persons had lower concentrations of cellular infiltrates at the TST reaction site than did HIV-1-infected TST-reactive persons ( $P = .008$ ). B, Distribution of cellular infiltrates in immunohistochemically stained biopsy specimens from TST reaction sites on the skin of HIV-1-infected TST-reactive persons vs. HIV-1-infected TST-unreactive persons relative to the total no. of infiltrating cells. Samples obtained from HIV-1-infected TST-unreactive persons had significantly lower frequencies of cells staining positive for CD45RO ( $P = .003$ ) and CD45RA ( $P = .036$ ).

the number of CD45RO<sup>+</sup> cells (Spearman's correlation coefficient,  $r = 0.67$ ;  $P = .006$ ) were positively correlated with the size of the TST induration. No correlations were observed between the distribution of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD1a<sup>+</sup> at the TST site, compared with PPD-uninfiltrated skin.

## DISCUSSION

A reduced reaction to the TST is observed in the majority of persons with advanced HIV-1 infection and presumptive latent TB infection [3, 18–22]. In a cohort of persons from a community where TB is endemic (estimated incidence rate, 1612 cases/100,000 persons) [10], ~40% of HIV-1-infected persons did not have a reaction to the TST, whereas >90% of HIV-1-uninfected persons from the same community had a reaction. Previous investigations conducted by TST survey and by both commercial and “in-house” IFN- $\gamma$  release assays among inhabitants of the same community [9, 10] revealed that ~80% of all adults living in Khayelitsha Township are latently infected with *M. tuberculosis*.

By immunohistochemical staining of skin biopsy specimens obtained from the TST area, as well as by computerized image analysis, we confirmed that TST induration is caused by a localized lymphocytic cell infiltrate rather than by inflammatory edema of the skin [23]. In the early stages of the delayed-type hypersensitivity response to tuberculin (after 6 h), an accumulation of T lymphocytes and monocytes around small blood vessels is noted that, later, also infiltrates the interstitial dermis and epidermis [23, 24]. Previous studies reported an increase in the number of activated macrophages, Langerhans cells, and interdigitating cells after intradermal introduction of tuberculin [25, 26], with the maximum no. of infiltrates reached after 48–72 h. In agreement with earlier studies for which in situ digital image analysis was not available [24–27], we found a predominance of

CD4 T cells in the TST reaction area, with a CD4<sup>+</sup>:CD8<sup>+</sup> T cell ratio of ~2:1. The composition of cellular infiltrates in the delayed-type hypersensitivity reaction to tuberculin has not previously been analyzed in detail [16, 17], but this is the first study in humans to show that the cells causing the TST induration are predominantly CD4<sup>+</sup> cells with a memory CD45RO<sup>+</sup> phenotype. There was no correlation between preexisting numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, or CD1a<sup>+</sup> T cells in healthy skin and the size of the TST induration that suggested recruitment of T cells rather than local T cell expansion.

In contrast to previous findings that associated reduced TST reactivity mainly with the numbers of circulating CD4<sup>+</sup> T cells [2, 3, 20, 28], we observed that the size of the TST reaction diameter was not correlated with the numbers of circulating CD4 cells but, rather, was negatively correlated with the numbers of circulating CD4<sup>+</sup>FoxP3<sup>+</sup> T cells. CD4<sup>+</sup>FoxP3<sup>+</sup> T cells are known to be expanded in chronic HIV infection [29–31], and high expression of FoxP3 is associated with the severity of HIV disease [32–34]. The numbers of circulating CD4<sup>+</sup>FoxP3<sup>+</sup> T cells also have been associated with the suppression of *M. tuberculosis*-specific immune responses in active TB [7, 8, 35]. It has been demonstrated that these cells proliferate and expand at sites of *M. tuberculosis* infection [36] and that high frequencies of CD4<sup>+</sup>CD25<sup>+</sup> T cells and high levels of FoxP3 expression suppress otherwise efficient T cell responses to *M. tuberculosis* and the production of IFN- $\gamma$  in active TB [8, 37–40]. Our data extend these observations suggesting that CD4<sup>+</sup>FoxP3<sup>+</sup> T cells may contribute to the suppression of antigenic specific responses to tuberculin in the TST. Although we did not directly investigate the immunosuppressive properties of the cells staining positive for FoxP3, we suggest that they may play a role in the regulation of the response to the antigen challenge, because it previously has been shown that the function and activity of reg-

ulatory T cells are marked by FoxP3 expression [6]. Thus, our findings are in agreement with previous reports that the impaired antigen-specific T cell response of HIV-infected persons was not correlated to the number of circulating CD4 T cells alone. Indeed, impairment of antigen-specific responses is observed in the early stages of HIV infection, before CD4 depletion is profound [41].

Our study focused on cells likely to influence the TST, rather than naive cells. However, a previous study documented that the proportion of naive CD8 cells is decreased in HIV-1 infection [42], a finding that we reproduced in the present study (table 2). The same study also documented a reduction in the proportion of naive CD4 cells as HIV-1 infection progressed. We did not document such a decrease. This difference between studies may relate to undoubted differences in pathogen exposure between California and South Africa, sex (most of our patients were female), and previous receipt of antiretroviral therapy (all our patients were antiretroviral naive). We did corroborate (Spearman's correlation coefficient,  $r = 0.91$ ;  $P < .0001$ ) the finding of Roederer et al. [42] that indicated that the absolute number of naive cells is strongly associated with the absolute CD4 cell count (data not shown).

The results of the present study have increased understanding of the immune mechanisms of the TST and the distribution of T cell subsets in the TST area in humans. A limitation of the study is that lack of a reaction to the TST may have arisen because of a lack of exposure to TB, although overall rates of positive TST results or positive RD1-based ELISPOT results were very high (table 1), consistent with our previous observations in this community [9–11]. This community has no certain test to ascertain a lack of exposure to TB in HIV-1-infected persons. Lack of infection cannot be inferred either from an absence of a TST reaction or a lack of an in vitro reaction to RD1-encoded antigens, because we not infrequently encounter patients with active disease who have negative results of both assays.

To strengthen our findings, it will be necessary to conduct a larger study that perhaps investigates secretion of chemokines or other activity markers. However, to our knowledge, this is the largest study to date that has compared immunophenotypes in peripheral blood and at the site of tuberculin injection in HIV-1-infected and –uninfected persons. This is also the first study to investigate the role of different T cell populations in the peripheral blood and at the site of the TST, as well as the influence of HIV infection in humans. We found that a positive TST reaction was characterized by a local accumulation of predominantly CD45RO<sup>+</sup> memory T cells. Reduced TST reactivity was related to the number of circulating CD4<sup>+</sup>FoxP3<sup>+</sup> T cells rather than to the number of total CD4<sup>+</sup> T cells in the blood.

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