The Journal of Infectious Diseases

MAJOR ARTICLE

Human Immunodeficiency Virus Infection Impairs Th1 and Th17 *Mycobacterium tuberculosis*–Specific T-Cell Responses

Lyle W. Murray,[1,2] Iman Satti,[3] Jodi Meyerowitz,[1] Matthew Jones,[1] Christian B. Willberg,[1,4] James E. Ussher,[1,5] Dominique Goedhals,[6] Jacob Hurst,[1,7] Rodney E. Phillips,[1,7] Helen McShane,[3] Cloete van Vuuren,[8,a] and John Frater[1,4,7,a]

[1]Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, United Kingdom; [2]Department of Internal Medicine, University of the Witwatersrand, Johannesburg, South Africa; [3]Jenner Institute, University of Oxford, and [4]Oxford National Institute of Health Research Biomedical Research Centre, United Kingdom; [5]Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand; [6]Department of Medical Microbiology and Virology, National Health Laboratory Service/University of the Free State, Bloemfontein, South Africa; [7]Oxford Martin School, Oxford, United Kingdom; and [8]Division of Infectious Diseases, Department of Internal Medicine, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa

Background. Human immunodeficiency virus (HIV)–infected individuals have a higher risk of developing active tuberculosis (TB) than HIV-uninfected individuals, but the mechanisms underpinning this are unclear. We hypothesized that depletion of specific components of *Mycobacterium tuberculosis* (*Mtb*)–specific $CD4^+$ and $CD8^+$ T-cell responses contributed to this increased risk.

Methods. *Mtb*-specific T-cell responses in 147 HIV-infected and 44 HIV-uninfected control subjects in a TB-endemic setting in Bloemfontein, South Africa, were evaluated. Using a whole-blood flow cytometry assay, we measured expression of interferon gamma, tumor necrosis factor alpha, interleukin 2, and interleukin 17 in $CD4^+$ and $CD8^+$ T cells in response to *Mtb* antigens (PPD, ESAT-6/CFP-10 [EC], and DosR regulon-encoded α-crystallin [Rv2031c]).

Results. Fewer HIV-infected individuals had detectable $CD4^+$ and $CD8^+$ T-cell responses to PPD and Rv2031c than HIV-uninfected subjects. *Mtb*-specific T cells showed distinct patterns of cytokine expression comprising both Th1 (CD4 and CD8) and Th17 (CD4) cytokines, the latter at highest frequency for Rv2031c. Th17 antigen-specific responses to all antigens tested were specifically impaired in HIV-infected individuals.

Conclusions. HIV-associated impairment of $CD4^+$ and $CD8^+$ *Mtb*-specific T-cell responses is antigen specific, particularly impacting responses to PPD and Rv2031c. Preferential depletion of Th17 cytokine-expressing $CD4^+$ T cells suggests this T-cell subset may be key to TB susceptibility in HIV-infected individuals.

Keywords. HIV; *Mycobacterium tuberculosis*; immune responses; Th1; Th17.

Infection with human immunodeficiency virus (HIV) predisposes individuals to active tuberculosis (TB), resulting in a global HIV/TB coinfection epidemic that disproportionately affects sub-Saharan Africa [1–3]. The key components of protective mycobacterial immunity remain incompletely defined. *Mycobacterium tuberculosis* (*Mtb*)–specific T cells play an important role in protection against TB, with the Th1 $CD4^+$ T-cell response believed to be critical [4, 5]. HIV coinfection is associated with early and preferential depletion of Th1 *Mtb*-specific $CD4^+$ T cells, possibly explaining the increased TB susceptibility before the onset of profound $CD4^+$ T-cell loss [6, 7]. In addition to quantitative *Mtb*-specific $CD4^+$ T-cell loss, HIV infection induces functional changes to the Th1 T-cell response, including the suppression of T-cell proliferation and cytokine secretion, and reduced polyfunctionality in both peripheral blood and the lung [8–12].

Mtb-specific Th1 $CD4^+$ T-cell responses to the best-described TB antigens (*Mtb* purified protein derivative [PPD], early secreted antigenic target 6 kDa [ESAT-6, Rv3875], and culture filtrate protein 10 kDa [CFP-10, Rv3874]) are not sufficient for protection and are poor correlates of TB risk [13–15]. There is therefore a need to explore other *Mtb*-specific antigens, such as those of the DosR regulon (ie, 16-kDa α-crystallin [Rv2031c]) [16]) expressed during in vitro conditions of hypoxia, low pH, and high nitric oxide intermediates believed to exist in *Mtb* granulomata during latent *Mtb* infection (LTBI) [17, 18].

Mtb-specific Th17 $CD4^+$ T cells also contribute to the *Mtb*-specific $CD4^+$ T-cell response, although the effect of HIV on this subset is not well understood [19]. Animal studies suggest a role for *Mtb*-specific $CD8^+$ T-cell responses [20–22], although their importance in humans is controversial, but with some evidence for a preserved if poorly functional response [23, 24]. Evidence from mouse models suggests that loss of

Received 18 September 2017; editorial decision 19 January 2018; accepted 9 March 2018; published online March 13, 2018.

Presented in part: Keystone Symposia Conference, Santa Fe, New Mexico, 22–27 January 2015.

[a]C. v. V. and J. F. contributed equally to this work.

Correspondence: J. Frater, MBBS, PhD, FRCP, Peter Medawar Building for Pathogen Research, University of Oxford, Oxford OX1 3SY, UK (john.frater@ndm.ox.ac.uk).

The Journal of Infectious Diseases® 2018;217:1782–92

DOI: 10.1093/infdis/jiy052

interferon gamma (IFN-γ) from $CD4^+$ T cells may negatively impact $CD8^+$ T-cell responses, although this is yet to be shown in humans [25].

Here, we explore the effect of HIV infection on *Mtb*-specific Th1 and Th17 $CD4^+$ and $CD8^+$ T-cell cytokine responses in a well-characterized South African cohort. Responses to cytomegalovirus (CMV) are used as a comparator.

MATERIALS AND METHODS

Study Site and Participants

Participants were recruited from antiretroviral therapy (ART) initiation sites in Bloemfontein, Free State Province, South Africa at Heidedal, Mangaung University of the Free State Community Partnership Programme, and National Hospital clinics. HIV-infected individuals were ART-naive and recruitment was based on $CD4^+$ T-cell counts to reflect varied levels of disease progression. Exclusion criteria included active TB, current AIDS-defining illness, pregnancy, previous ART, current use of immunosuppressive medication, and age <18 years. HIV-uninfected individuals were recruited from the same healthcare facilities. The study was approved by the University of the Free State Ethics Committee (ETOVS 171/08). Participants were aged 18 years or older, were informed in their own language, and provided written consent.

Collection and Processing of Blood Samples

Within 2 hours of venipuncture, 1 mL of heparinized whole blood was incubated with antigen at 37°C, with anti-CD28 and anti-CD49d antibodies (Becton Dickinson) [26]. Stimulatory antigens were as follows: positive control (staphylococcal enterotoxin B [SEB], 5 μg/mL; Sigma-Aldrich), negative control (co-stimulatory antibodies alone), *Mtb* PPD (20 μg/mL; Statens Serum Institut), ESAT-6 and CFP-10 recombinant proteins in a single combined stimulation designated "EC" (ESAT-6, 10 μg/mL; CFP-10, 10 μg/mL; Lionex), Rv2031c recombinant protein (10 μg/mL; Lionex, Germany), and CMV lysate (5 μg/mL, Virusys). The *Mtb* recombinant proteins were expressed and purified from *Escherichia coli*, and had purity of >95% and endotoxin contents <25 IU/mg (Limulus Amebocyte Lysate assay). Brefeldin A (5 μg/mL; Sigma-Aldrich) was added after 6 hours and further incubated for 5 hours. Red blood cells were lysed and white cells fixed before long-term storage in liquid nitrogen.

Flow Cytometry and Intracellular Cytokine Staining

Cryopreserved white cells were stained as previously described [26]. The following conjugated monoclonal antibodies directed at cell-surface markers and intracellular cytokines were used: CD3-eFluor 450 (clone UCHT1), IFN-γ–PE/Cy7 (clone 4S.B3), and tumor necrosis factor alpha (TNF-α)–PerCP/Cy5.5 (clone MAb11) (eBioscience); CD4-APC (clone RPA-T4), CD14-APC/Cy7 (clone HCD14), CD19-APC-Cy7 (clone HIB19), and IL-17 AlexaFluor 488 (clone BL168) (BioLegend); and CD8-VioGreen (clone BW135/80) and IL-2-PE (clone N7.48A) (Miltenyi Biotec).

Stained cells were acquired on a MACSQuant flow cytometer (Miltenyi Biotec) and all events were captured. At least 50 000 events were captured for each sample included in the analysis. Data were analyzed using FlowJo version X (Tree Star). The gating strategy is shown in Supplementary Figure 1.

Boolean cytokine combination gates were created using FlowJo version X and analyzed with PESTLE (version 1.7; National Institutes of Health [NIH]) and SPICE software (version 5.35; NIH). A positive T-cell cytokine response was defined as a frequency of total cytokine-positive $CD4^+$ and/or $CD8^+$ T cells at least 0.1% of total $CD4^+/CD8^+$ T cells after background subtraction and at least twice the frequency of total cytokine-positive $CD4^+/CD8^+$ T cells in the negative control. As opposed to *Mtb*, the genome of attenuated *Mycobacterium bovis* BCG lacks the RD1 region encoding the genes for the proteins ESAT-6 and CFP-10. In addition, Rv2031c and components of PPD can be found in mycobacteria other than *Mtb*. Therefore, as per accepted convention, we classified individuals with LTBI only if demonstrating a cytokine-positive T-cell response to ESAT-6 and CFP-10 as used in combination in our assay.

Statistical Analyses

Standard statistical approaches for parametric and nonparametric data were used as appropriate. Specific tests are detailed in the results and include Fisher exact *t* tests, Kruskal–Wallis tests, and Mann–Whitney tests (Prism 5 for Mac OS X version 5.0a; GraphPad). Partial permutation analyses were calculated using SPICE (version 5.35; NIH). Multivariate logistic regression was performed using SPSS (version 22; IBM).

RESULTS

Participant Characteristics

Forty-four HIV-uninfected and 147 HIV-infected individuals were studied (Table 1). Of the latter, $CD4^+$ T-cell counts ranged from ≤200 cells/μL (n = 51), to 201–350 cells/μL (n = 48), to >350 cells/μL (n = 48). Fourteen HIV-infected individuals had a history of active TB; there were no previous TB cases in the HIV-uninfected group. All were believed to have received BCG vaccination in childhood, although only 20 (45%) HIV-uninfected and 89 (61%) HIV-infected individuals had a BCG vaccination scar. HIV-infected subjects were stratified by $CD4^+$ T-cell count.

Impairment of PPD and Rv2031c-Specific $CD4^+$ T-Cell Responses in HIV-Infected Individuals

As expected, median total $CD4^+$ T-cell counts were significantly lower in HIV-infected than uninfected individuals, whilst $CD8^+$ T-cell counts were higher ($P < .001$ for all, Mann–Whitney test) (Table 1). The median CD4:CD8 ratio of 1.63 (interquartile range, 1.02–2.23) in the HIV-uninfected group was as expected for healthy African individuals [27, 28], but was significantly reduced in HIV-infected individuals (Table 1).

Table 1. Demographics of Study Participants

Characteristic	HIV Uninfected (n = 44)	HIV Infected Total (n = 147)	HIV Infected CD4 >350 Cells/μL (n = 48)	HIV Infected CD4 201–350 Cells/μL (n = 48)	HIV Infected CD4 ≤200 Cells/μL (n = 51)
Sex, No. (%)					
Female	20 (45)	96 (65)	37 (77)	34 (71)	25 (49)
Male	24 (55)	51 (35)	11 (23)	14 (29)	26 (51)
Age, y	24 (20–32)	36 (30–44)	36 (31–44)	37 (29–46)	36 (31–44)
CD4 count, cells/μL	879 (686–1054)	254 (178–379)	473 (380–596)	260 (231–295)	155 (120–183)
CD8 count, cells/μL	586 (409–923)	946 (673–1542)	1234 (806–1986)	925 (715–1447)	821 (492–1206)
CD4:CD8 ratio	1.63 (1.03–2.23)	0.27 (0.17–0.42)	0.51 (0.30–0.77)	0.27 (0.18–0.39)	0.17 (0.12–0.26)
Viral load, RNA copies/mL	NA	48 173 (11 490–161 087)	15 306 (6914–64 855)	58 963 (16 423–181 851)	139 402 (34 425–456 048)
BMI , kg/m^2	24.3 (22.7–27.8)	24.9 (22.3–28.7)	26.5 (22.7–31.5)	24.9 (21.0–28.1)	24.9 (21.0–28.2)
Previous TB, No. (%)	0 (0)	14 (10)	6 (13)	5 (10)	3 (6)
BCG scar, No. (%)	20 (45)	89 (61)	31 (65)	25 (52)	33 (65)

Data are expressed as median (interquartile range) unless otherwise indicated.

Abbreviations: BMI, body mass index; HIV, human immunodeficiency virus; NA, not applicable; TB, tuberculosis.

We examined the effect of HIV infection on *Mtb* (EC, PPD, and Rv2031c) and CMV-specific CD4$^+$ T-cell cytokine responses. In the HIV-uninfected control group, a cytokine-positive response to EC was present in 21 of 44 (47.7%) participants, reflecting the proportion with LTBI, and similar to other sub-Saharan African populations (Table 2) [29, 30]. This was not significantly different to the proportion of HIV-infected participants responding to EC (58/146 [39.7%], P = .385) (Table 2). However, the proportions of individuals with CD4$^+$ T-cell responses to PPD and Rv2031c were significantly lower in the HIV-infected group compared with healthy controls (66.4% vs 86.4%, P = .013; and 38.6% vs 56.8%, P = .038, respectively) (Table 2). Similar proportions of HIV-infected and uninfected participants responded to CMV lysate (91.8% vs 97.6%, P = .304; Table 2).

We next compared the frequencies of cytokine-positive CD4$^+$ T cells responding to *Mtb* and CMV antigens in individuals across different CD4$^+$ T-cell strata (≤200, 200–350, and >350 cells/μL) (Figure 1). The frequencies of total CD4$^+$ T cells responding to PPD and Rv2031c were significantly lower in HIV-infected individuals compared with uninfected controls (P < .001 for both; Figure 1B and 1C). CD4$^+$ T-cell responses to PPD were reduced in participants with CD4$^+$ T-cell counts >200 cells/μL, including those with CD4$^+$ T-cell counts >350 cells/μL, indicating specific depletion of PPD-specific CD4$^+$ T cells despite relatively preserved total CD4$^+$ T-cell counts (Figure 1B). Reduced frequencies of Rv2031c-specific CD4$^+$ T cells between HIV-uninfected and HIV-infected subjects were more evident at lower CD4$^+$ T-cell counts, with relative preservation >350 cells/μL (Figure 1C). Interestingly, the lack of significant differences in the proportions of HIV-infected and HIV-uninfected subjects responding to CMV and EC (Table 2) was also reflected in similar frequencies of CD4$^+$ T-cell responses to these antigens in these groups (Figure 1A and 1D).

Mtb and CMV-Specific T-Cell Responses Exhibit Distinct Patterns of Cytokine Expression

As the loss of antigen-specific CD4$^+$ T-cell responses following HIV infection has been associated with different patterns of cytokine expression, we characterized and compared the cytokine expression patterns of each antigen-specific CD4$^+$ T-cell population in our cohort [31]. To set a comparator group, we first measured the relative contributions of IFN-γ, TNF-α, IL-2, and IL-17 to the total CD4$^+$ T-cell response within the HIV-uninfected control group.

Th1 cytokines (IFN-γ, TNF-α, and IL-2) dominated the CD4$^+$ T-cell cytokine responses to both EC and PPD, with all 3

Table 2. Comparison of Cytokine-Positive CD4$^+$ Responders Between Human Immunodeficiency Virus (HIV)–Infected and HIV-Uninfected Subjects

Antigen	CD4 Responders HIV-Uninfected	CD4 Responders HIV-Infected	CD4 Responders *P* Value[a]	CD8 Responders HIV Uninfected	CD8 Responders HIV Infected (%)	CD8 Responders *P* Value[a]
EC	21/44 (47.7)	58/146 (39.7)	.385	10/44 (22.7)	22/146 (15.1)	.250
PPD	38/44 (86.4)	97/146 (66.4)	**.013**	17/44 (38.6)	21/146 (14.4)	**.001**
Rv2031c	25/44 (56.8)	56/145 (38.6)	**.038**	22/44 (50.0)	46/145 (31.7)	**.032**
CMV	41/42 (97.6)	135/147 (91.8)	.304	26/42 (61.9)	110/147 (74.8)	.120

Values in bold denote *P* Value <.05.

Abbreviations: CMV, cytomegalovirus; EC, early secreted antigenic target 6 kDa/culture filtrate protein 10 kDa; PPD, purified protein derivative; Rv2031c, 16-kDa α-crystallin lysate.

[a]Fisher exact test.

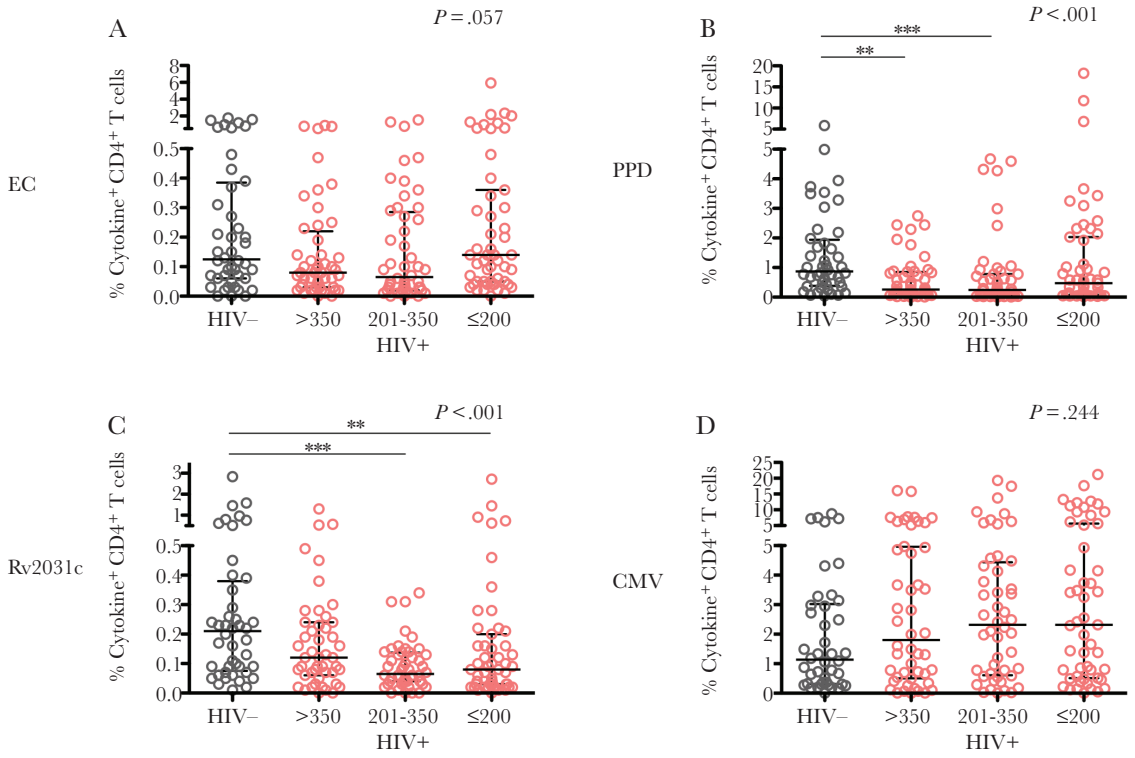


Figure 1. Reduced purified protein derivative– and 16-kDa α-crystallin–specific CD4 T-cell frequencies in human immunodeficiency virus (HIV)–infected individuals. Antigen-specific CD4 T-cell frequencies were compared between the HIV-uninfected and HIV-infected subjects within different CD4 T-cell count strata. The data are shown as total cytokine-positive CD4 T-cell frequencies as measured using flow cytometry and therefore include cells expressing any of the 4 cytokines measured singly or in combination. The horizontal bars indicate the median and interquartile range. *P* values on the top right of each panel indicate Kruskal–Wallis *P* values. Significant differences between individual groups were tested using Dunn multiple comparisons test. $^{**}P < .01$, $^{***}P < .001$. Abbreviations: CMV, cytomegalovirus; EC, early secreted antigenic target 6 kDa and culture filtrate protein 10 kDa; HIV, human immunodeficiency virus; PPD, purified protein derivative; Rv2031c, 16-kDa α-crystallin.

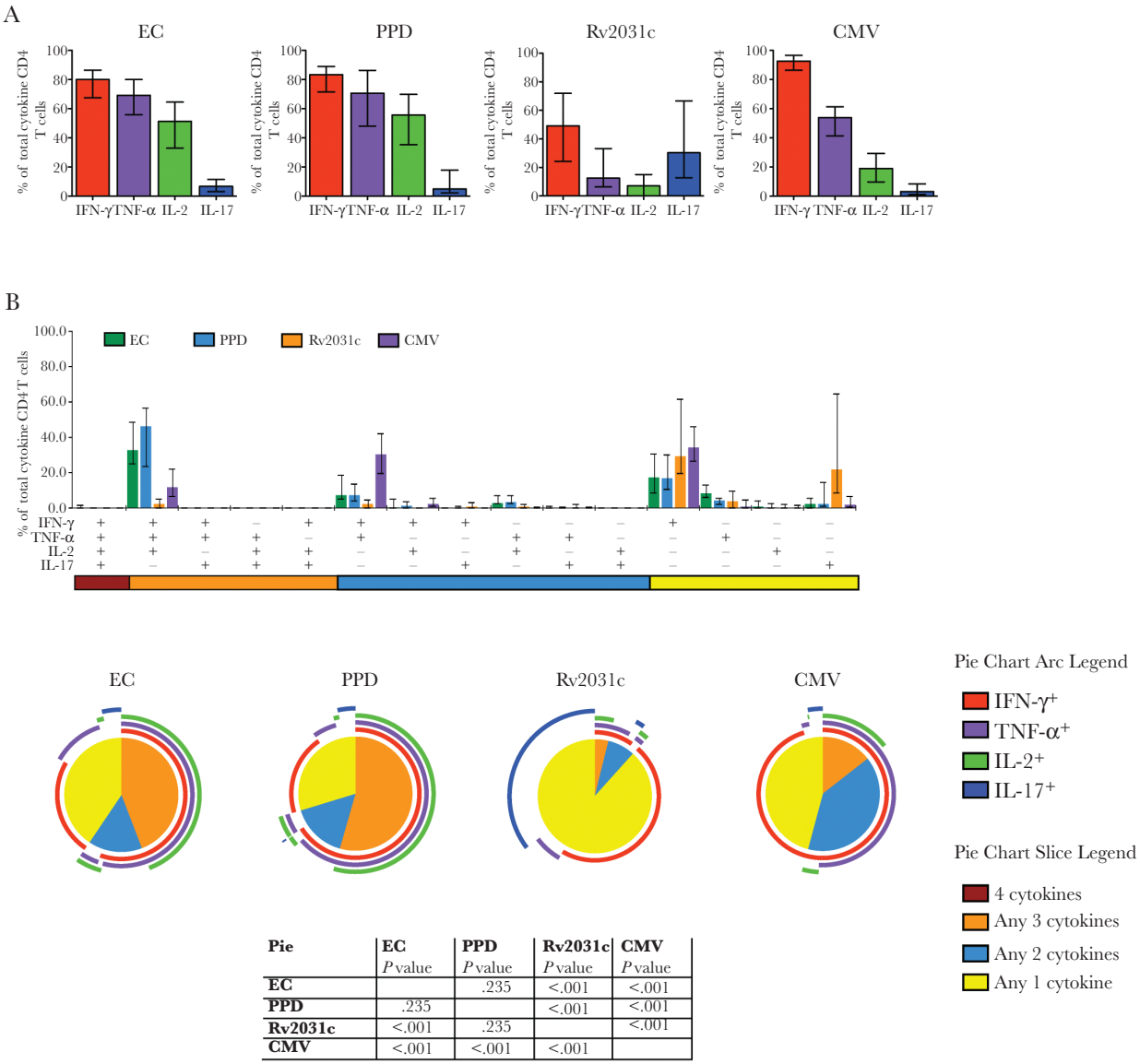
expressed by >50% of cytokine-expressing antigen-specific cells in responding participants (Figure 2A). Rv2031c also induced Th1 cytokines, dominated by IFN-γ, but with increased production of IL-17 (30% of cytokine-expressing cells), which was also seen for EC or PPD, but to a much lesser extent (7% and 5%, respectively). The CMV-specific CD4+ T-cell responses showed a similar hierarchy as EC and PPD, but with decreased IL-2 expression and very low IL-17 expression (Figure 2A). For all antigen responses, there was very little coexpression of Th1 cytokines and IL-17 (Figure 2B). Interestingly, despite EC and PPD-specific CD4+ T-cell responses showing markedly different patterns of depletion in HIV-infected subjects, their cytokine expression profiles were similar in HIV-uninfected control individuals ($P = .235$; Figure 2B).

Mtb- and CMV-Specific Th17 CD4+ T-Cell Responses Are Significantly Depleted in HIV-Infected Individuals

We then characterized CD4+ T-cell cytokine responses in the HIV-infected participants to determine if the observed HIV-associated loss of antigen-specific responses would be reflected in changes to particular cytokine-expressing CD4+ T-cell subpopulations. Specifically, we were interested in the different patterns of Th1 and Th17 cytokine expression. As before, Th1 CD4+ T-cell responses were defined by the expression of IFN-γ, TNF-α, and IL-2 alone or in combination, whereas Th17 CD4+ responses were defined by expression of IL-17 alone.

Among EC-specific CD4+ T cells, only those cells expressing IL-2 and IL-17 were reduced compared with uninfected controls ($P = .036$ and $P = .037$, respectively), whereas those expressing IFN-γ and TNF-α were preserved (Figure 3A). For PPD, which showed the greatest proportionate decrease in CD4+ T-cell frequencies between HIV-infected and -uninfected participants, there was significant reduction of CD4+ T cells expressing all cytokines representing both Th1 and Th17 responses ($P \leq .001$ for all comparisons tested) (Figure 3B). Interestingly, the impact of HIV infection on the CD4+ T-cell response to Rv2031c was manifested in a significant loss of both IFN-γ– and IL-17–expressing CD4+ T cells, the 2 most dominant components of the response in HIV-uninfected controls (Figure 3C).

The CMV-specific response was also impacted by HIV infection, with HIV-infected participants making statistically significantly larger TNF-α responses (Figure 3D). In contrast, the median frequency of IL-17–positive CMV-specific CD4+ T cells was reduced ($P = .005$) (Figure 3D). In summary, the Th17 components of all antigen-specific CD4+ T-cell responses were significantly depleted in HIV-infected



Pie	EC	PPD	Rv2031c	CMV
	P value	*P* value	*P* value	*P* value
EC		.235	<.001	<.001
PPD	.235		<.001	<.001
Rv2031c	<.001	.235		<.001
CMV	<.001	<.001	<.001	

Figure 2. *Mycobacterium tuberculosis*– and cytomegalovirus (CMV)–specific CD4+ T cells exhibit distinct cytokine expression profiles in human immunodeficiency virus (HIV)–uninfected controls. Expression of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 2 (IL-2), and interleukin 17 (IL-17) in HIV-uninfected antigen responders was assessed using flow cytometry (early secreted antigenic target 6 kDa and culture filtrate protein 10 kDa [EC], n = 21; purified protein derivative [PPD], n = 38; 16-kDa α-crystallin [Rv2031c], n = 25; CMV, n = 41). *A*, The fraction of total cytokine-positive CD4+ T cells expressing each cytokine is shown. *B*, Values shown are the fraction of total cytokine-positive CD4+ T cells responding to each antigen (colored as per the upper legend) producing the given combination of cytokines (upper panel). Filled bars represent median values and whiskers show the interquartile range. The colored bars under the graph represent the number of cytokines produced by each cell (crimson, 4; orange, 3; blue, 2; yellow, 1). The same colors are used in the pie charts (lower panel), which summarize the proportion of cells expressing 1, 2, 3, or 4 cytokines simultaneously. The arcs surrounding the pie charts indicate the proportion of the responses contributed by each of the single cytokines. *P* values shown in the table at the bottom compare pie charts and are calculated using a partial permutation analysis [50].

subjects, which was not uniformly the case for antigen-specific Th1 CD4+ T-cell responses, suggesting increased susceptibility of antigen-specific Th17 CD4+ T cells to depletion during HIV infection.

Chronic HIV Infection Is Not Associated With Changes in Polyfunctional *Mtb*-Specific CD4+ T-Cell Subsets

Polyfunctional antigen-specific CD4+ and CD8+ T-cell responses to viral antigens have been shown to be protective in humans and animals, but the association is less clear for anti-*Mtb* immunity [32]. Having shown the differential impact of HIV infection on Th1 and Th17 CD4+ T-cell responses, we wished to determine how HIV infection might influence polyfunctionality. We compared the proportion of single (1+), dual (2+), and triple (3+) cytokine-expressing Th1 CD4+ cells responding to EC, PPD, Rv2031c, and CMV (including the positive control, SEB) between HIV-infected and -uninfected participants (Figure 4).

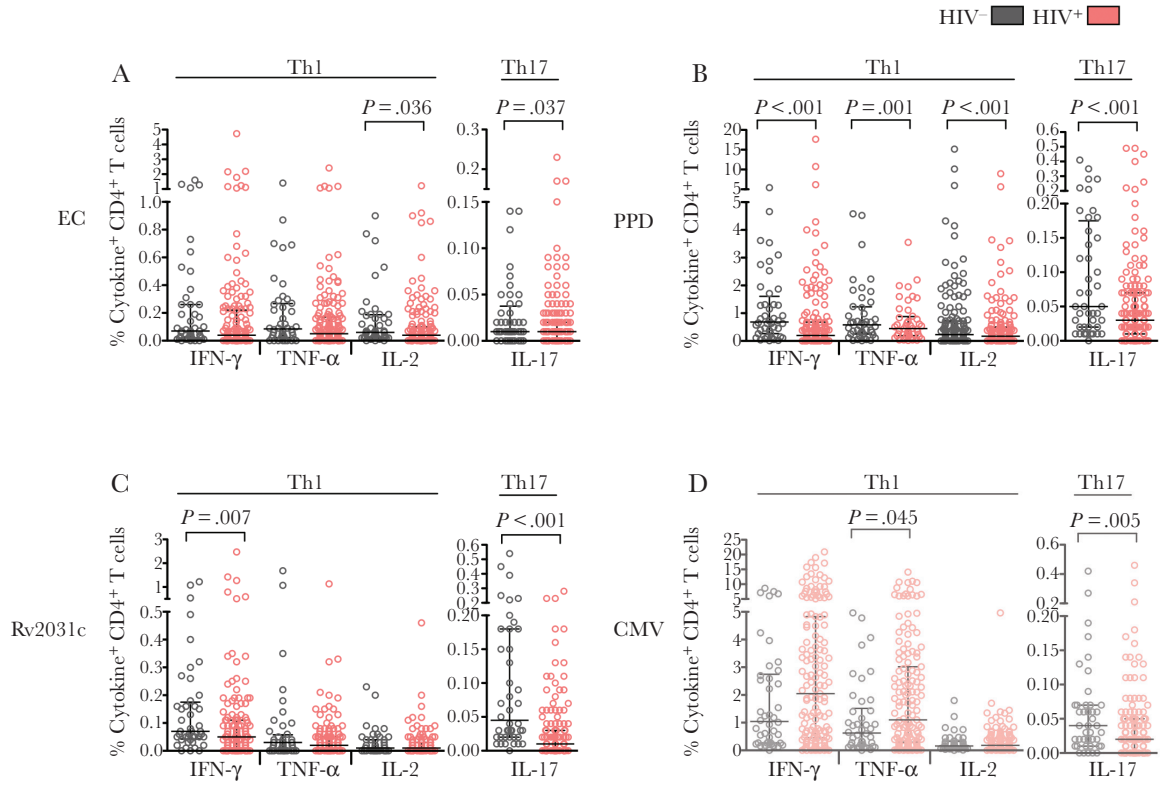


Figure 3. Human immunodeficiency virus (HIV)–infected individuals have impaired Th17 CD4⁺ T-cell responses to all *Mycobacterium tuberculosis* and cytomegalovirus antigens tested, in addition to impairment of certain antigen-specific Th1 CD4⁺ T-cell responses. The frequencies of antigen-specific CD4⁺ T cells expressing each cytokine were compared between HIV-infected and HIV-uninfected individuals. The Th1 CD4⁺ T-cell response included CD4⁺ T cells expressing interferon gamma, tumor necrosis factor alpha, or interleukin 2 alone or in combination while the Th17 response was defined by interleukin 17 expression. Horizontal bars indicate median values and interquartile ranges. *P* values were generated using Mann–Whitney tests. Abbreviations: CMV, cytomegalovirus; EC, early secreted antigenic target 6 kDa and culture filtrate protein 10 kDa; IFN, interferon; IL, interleukin; PPD, purified protein derivative; Rv2031c, 16-kDa α-crystallin; TNF, tumor necrosis factor.

HIV-infected subjects had a significantly smaller proportion of 3+ SEB-stimulated CD4⁺ T cells and a greater proportion of 1+ CD4⁺ T cells. Similarly, there was a reduction in the polyfunctionality of the CMV-specific response, with HIV-infected subjects having relatively smaller proportion of 3+ and larger proportion of 2+ CD4⁺ T cells, compared with controls. However, there were no significant differences between HIV-infected and -uninfected individuals in the proportions of 2+ or 3+ CD4⁺ T cells responding to any of the *Mtb* antigens (Figure 4).

PPD- and Rv2031c-Specific CD8⁺ T-Cell Frequencies Are Reduced in HIV-Infected Individuals

We next characterized the antigen-specific CD8⁺ T-cell cytokine response. The proportions of HIV-infected individuals with detectable CD8⁺ T-cell cytokine responses to PPD and Rv2031c were significantly lower than HIV-uninfected controls ($P = .001$ and $P = .032$, respectively) (Table 2). In a similar pattern to the CD4⁺ T-cell responses, there was no difference in the proportions of EC or CMV CD8⁺ T-cell responders between HIV-infected and HIV-uninfected individuals ($P = .250$ and $P = .120$, respectively; Table 2).

A comparison of the frequencies of cytokine-positive CD8⁺ T cells responding to EC between HIV-uninfected individuals and HIV-infected individuals within different CD4⁺ T-cell count strata did not show any significant differences ($P = .432$; Figure 5A). However, consistent with a decrease in the proportion of individuals making a PPD-specific CD8⁺ response, there was a reduction in PPD-specific cytokine-positive CD8⁺ T-cell frequencies in individuals across all CD4⁺ T-cell strata compared with the HIV-uninfected control group ($P < .001$; Figure 5B). For Rv2031c-specific CD8⁺ T-cell responses, there was evidence for a global decrease in CD8⁺ T-cell frequencies in HIV-infected individuals compared with controls ($P = .043$), but this was not attributable to differences between any particular CD4⁺ T-cell subset (Figure 5C). There was no evidence of an impact of HIV infection on the CMV-specific CD8⁺ cytokine response, even at CD4⁺ T-cell counts ≤200 cells/μL (Figure 5D).

Loss of Antigen-Specific CD8⁺ T-Cell Cytokine Responses to EC and PPD Are Associated With Loss of Antigen-Specific CD4⁺ T-Cell Responses

As CD8⁺ T cells are not frequently infected by HIV, we hypothesized that the impact of HIV infection on the antigen-specific CD8⁺ T-cell cytokine response was secondary to the depletion

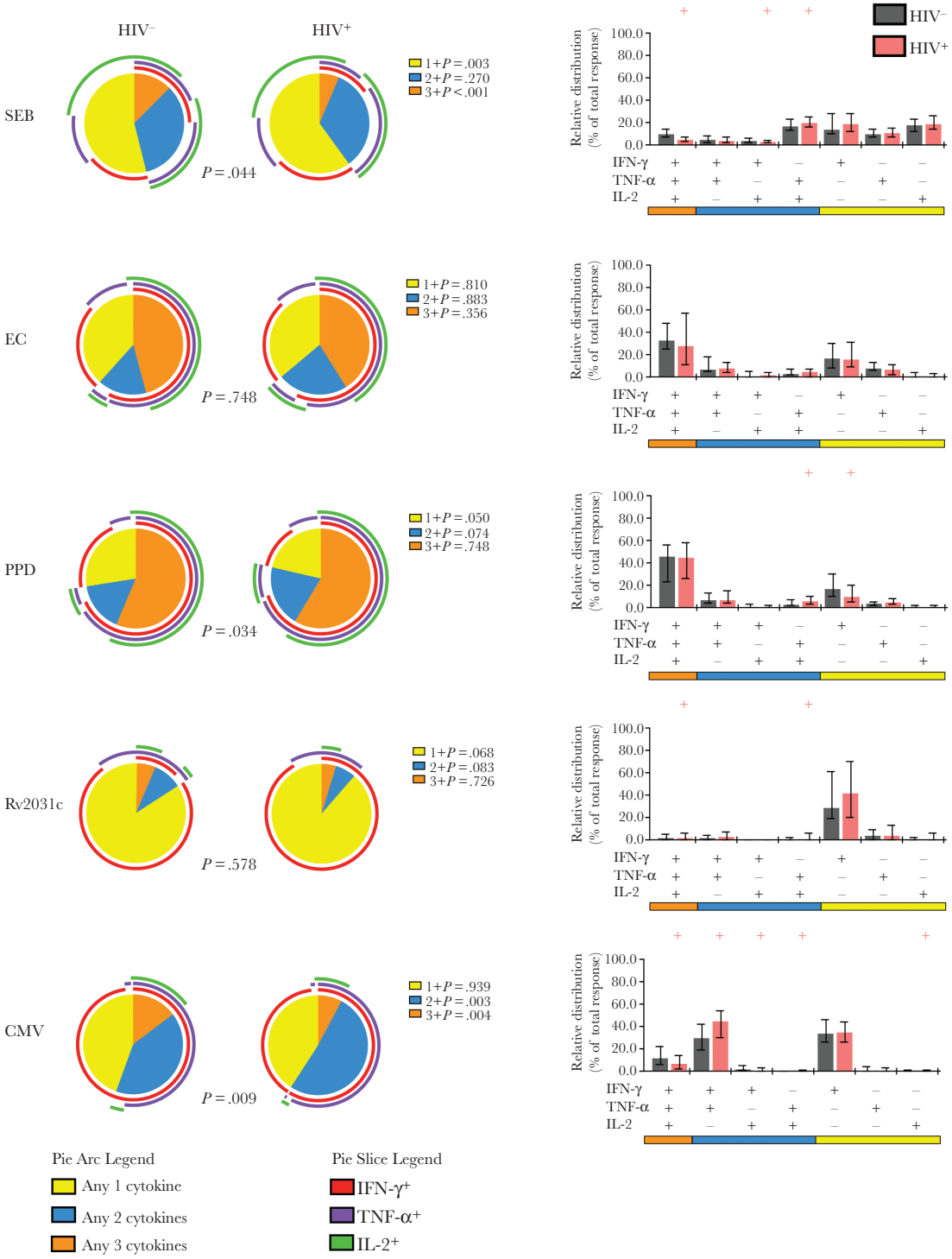


Figure 4. The effect of human immunodeficiency virus (HIV) infection on CD4⁺ T-cell polyfunctionality is variable and antigen-specific. The effect of HIV infection on polyfunctionality of antigen-specific CD4⁺ T-cell populations was assessed through a comparison of proportions of single (1+), dual (2+), and triple (3+) cytokine-expressing CD4⁺ T cells contributing to the total cytokine-positive antigen-specific response, between HIV-infected and HIV-uninfected individuals. Only individuals with a demonstrated cytokine-positive CD4⁺ T-cell response were included in the analysis (staphylococcal enterotoxin B [SEB]: HIV⁻/HIV⁺, n = 44/147; early secreted antigenic target 6 kDa and culture filtrate protein 10 kDa [EC]: HIV⁻/HIV⁺, n = 21/58; purified protein derivative [PPD]: HIV⁻/HIV⁺, n = 38/97; 16-kDa α-crystallin [Rv2031c]: HIV⁻/HIV⁺, n = 25/56; cytomegalovirus [CMV]: HIV⁻/HIV⁺, n = 41/135). Each pie chart summarizes the proportion of cells expressing 1, 2, or 3 cytokines alone or in combination. The arcs surrounding the pie charts indicate the proportion of the responses contributed by each of the single cytokines (interferon gamma [IFN-γ], interleukin 2 [IL-2], tumor necrosis factor alpha [TNF-α]). *P* values shown between pie charts give an overview of different cytokine expression profiles between HIV-uninfected and HIV-infected individuals for each antigen and are calculated using a partial permutation analysis [50]. *P* values shown next to the pie charts compare the proportion of 1+, 2+, or 3+ CD4⁺ T cells in HIV-uninfected and HIV-infected individuals and are calculated using Mann–Whitney test. Data in the right-hand panel are shown as relative distributions of the different cytokine-expressing CD4⁺ T-cell populations within the total response to each antigen. *P* values were calculated using Student *t* tests and are denoted "+" if $P < .05$.

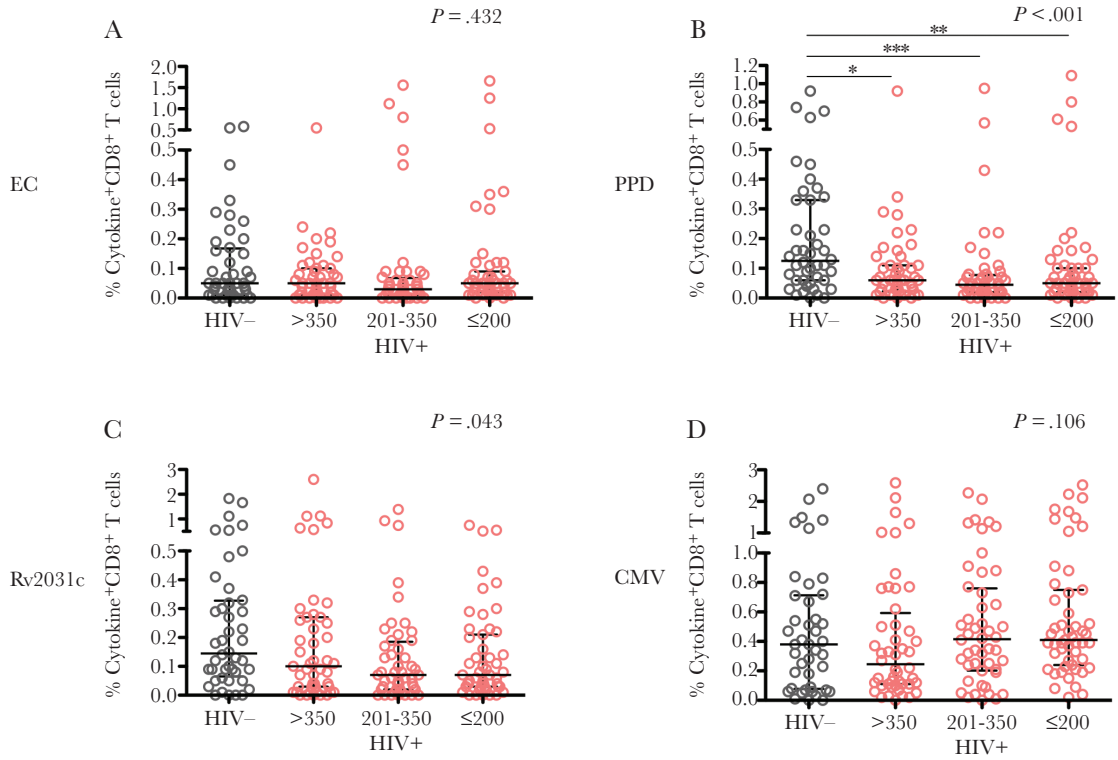


Figure 5. Early secreted antigenic target 6 kDa and culture filtrate protein 10 kDa (EC; *A*), purified protein derivative (PPD; *B*), 16-kDa α-crystallin (Rv2031c; *C*), and cytomegalovirus (CMV; *D*)–specific $CD8^+$ T-cell frequencies in human immunodeficiency virus (HIV)–infected individuals. Antigen-specific $CD8^+$ T-cell frequencies were compared between the HIV-uninfected and HIV-infected subjects within different $CD4^+$ T-cell count strata. The data are shown as total cytokine-positive $CD8^+$ T-cell frequencies and therefore include cells expressing any of the 4 cytokines measured singly or in combination. The horizontal bars indicate the median and interquartile range. *P* values on the top right of each panel indicate Kruskal–Wallis *P* values. Significant differences between individual groups were tested using Dunn multiple comparisons test. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

of $CD4^+$ T-cell help, as has been described in animal models of TB and in human CMV studies [25, 33]. We therefore examined the effect of different variables on the odds of having a detectable $CD8^+$ T-cell cytokine response to each of the antigens tested using multivariate logistic regression (Supplementary Table 1). Individuals included in the analysis had a demonstrated T-cell response to the antigen of interest and variables included total $CD4^+$ T-cell count, total $CD8^+$ T-cell count, HIV plasma viral load, and antigen-specific $CD4^+$ T-cell count. A $CD8^+$ T-cell cytokine response to both EC and PPD was positively associated with antigen-specific $CD4^+$ T-cell magnitudes (odds ratio [OR], 1.7 [95% confidence interval {CI}, 1.2–2.6], $P = .008$; and 1.3 [95% CI, 1.1–1.5], $P < .001$, respectively) but not with total $CD4^+$ T-cell count, total $CD8^+$ T-cell count, or plasma HIV viral load (Supplementary Table 1). Making an Rv2031c-specific $CD8^+$ T-cell response was, however, not associated with an Rv2031c-specific $CD4^+$ T-cell response, but was positively associated with HIV plasma viral load (OR, 2.7 [95% CI, 1.3–5.8], $P = .008$). No factors assessed (including the magnitude of the CMV-specific $CD4^+$ response) significantly affected the likelihood of making a CMV-specific $CD8^+$ response.

DISCUSSION

In this study we have analyzed *Mtb*-specific $CD4^+$ and $CD8^+$ T-cell responses to PPD, ESAT-6/CFP-10, and the *Mtb* DosR regulon antigen Rv2031c, as well as CMV-specific responses in HIV-infected and HIV-uninfected individuals.

We detected cytokine-positive $CD4^+$ and $CD8^+$ T-cell responses to all the antigens tested in both HIV-uninfected controls and HIV-infected individuals. The high proportion of $CD4^+$ and $CD8^+$ T-cell responders to Rv2031c was striking as the frequency of responders has been lower in other studies [34, 35]. This discrepancy may be related to differences in endemic rates of LTBI and exposure to non-TB and environmental mycobacteria between the study populations. In addition, different assay techniques were used to measure Rv2031c-specific $CD4^+$ T-cell responses. Arlehamn et al used cryopreserved PBMCs in an IFN-γ enzyme-linked immunospot assay [34], while Sutherland et al employed a whole-blood IFN-γ enzyme-linked immunosorbent assay [35]. The addition of co-stimulatory antibodies during antigen incubation, as well as the measurement of TNF-α, IL-2, and IL-17 in addition to IFN-γ by flow cytometry in the whole-blood

intracellular cytokine staining assay used in our study, may have contributed to the increased sensitivity we observed [36].

Different rates of $CD4^+$ T-cell decline have previously been shown for *Mtb* and CMV-specific $CD4^+$ T-cell responses [31]. We confirmed this finding and also showed that different *Mtb*-specific $CD4^+$ T-cell populations are depleted to different extents in chronic HIV infection. PPD and Rv2031c-specific $CD4^+$ T cells were depleted in HIV-infected individuals whereas EC-specific $CD4^+$ T cells were maintained. This relative sparing of EC-specific $CD4^+$ T cells has been shown before using IFN-γ release assays (IGRAs) [37–39]. Despite this, EC-based IGRAs have shown suboptimal accuracy for diagnosing LTBI in HIV-infected individuals in some studies and could be enhanced through the inclusion of latency-associated antigens [40]. In contrast, PPD-specific $CD4^+$ T-cell responses measured by IGRA, flow cytometry, and tuberculin skin testing have consistently been shown to be reduced in HIV infection [10, 41, 42]. The differential impact of HIV infection on responses to these 2 antigens is intriguing, particularly considering that the ESAT-6 and CFP-10 antigens are likely to be part of the antigen mix of PPD, although at much lower concentrations. Depletion of PPD-specific $CD4^+$ T cells has been associated with increased IL-2 expression, a less mature phenotype, and reduced production of macrophage inflammatory protein 1β [31]. We found no differences however, in the pattern of cytokine expression between PPD- and EC-specific $CD4^+$ T cells.

We also found a specific loss of $CD4^+$ responses in HIV-infected individuals to Rv2031c, an *Mtb* DosR regulon-encoded antigen. HIV-infected individuals with LTBI also show impairment of responses to heparin-binding hemagglutinin (HBHA), another antigen associated with LTBI, and supporting a role for responses to antigens that are preferentially expressed during conditions of *Mtb* latency [43, 44].

Analysis of cytokine profiles in HIV-uninfected controls provided insight into antigen-specific responses prior to HIV infection. Most notable was the increased IL-17 expression of Rv2031c-specific $CD4^+$ T-cell responses and the relatively decreased IL-2 expression of CMV-specific T-cell responses in comparison with EC and PPD-specific responses. The importance of Th17 CD4 T-cell responses to antigens associated with LTBI is supported by a study by Dreesman et al, which investigated responses to HBHA in children exposed to TB [45]. We also showed that the IL-17–expressing components of all the antigen-specific $CD4^+$ T-cell responses were preferentially depleted in HIV-infected individuals. Although Th17 $CD4^+$ T cells are known to be depleted in HIV infection, this has not been confirmed for *Mtb*-specific Th17 $CD4^+$ T-cell responses [46, 47]. This preferential depletion has been linked to the increased expression of CD4, CXCR4, and CCR6, resulting in enhanced permissiveness to HIV infection [48]. The increased proportion of IL-17–expressing $CD4^+$ T cells within the Rv2031c $CD4^+$ T-cell response may therefore contribute to preferential depletion of this antigen-specific $CD4^+$ T-cell population. Further evidence from a population of HIV-uninfected adolescents with LTBI in South Africa correlated suppression of Th17 responses with progression to active TB, highlighting the importance of Th17 CD4 T-cell responses in LTBI control [49].

Although the importance of polyfunctional $CD4^+$ T cells in control of viral infections has been described, this has not been confirmed in TB. While we show that polyfunctional *Mtb*-specific $CD4^+$ T-cell responses are present in our study participants, we found no evidence to suggest that this was impacted by HIV infection.

The effect of HIV infection on *Mtb*-specific $CD8^+$ T-cell responses has not been examined in detail. Sutherland et al showed that $CD8^+$ T-cell responses were maintained in advanced HIV infection but lost polyfunctionality [24]. We show that $CD8^+$ T-cell cytokine responses to PPD and Rv2031c are significantly impaired in HIV-infected individuals, possibly indicating that loss of *Mtb*-specific $CD8^+$ T-cell cytokine responses may contribute to susceptibility to TB disease in HIV infection. We further determined that impairments of antigen-specific $CD8^+$ T-cell responses to some *Mtb* antigens were associated with loss of antigen-specific $CD4^+$ T-cell help. While effective stimulation of $CD8^+$ T-cell cytokine responses was achieved in this study, the full breadth of these responses may not have been identified due to the use of protein antigens.

Our study is limited by its cross-sectional nature. Ideally, a longitudinal analysis of *Mtb*-specific T-cell perturbations in acute and chronic HIV infection would provide further clarity. In conclusion, our data show that HIV infection is associated with a complex pattern of *Mtb*-specific T-cell impairment affecting both $CD4^+$ and $CD8^+$ T-cell populations, and suggest that depletion of antigen-specific Th1 and Th17 $CD4^+$ T cells may directly lead to TB disease susceptibility compounded by loss of corresponding $CD8^+$ T-cell function.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. The authors thank the laboratory staff at the National Health Laboratory Service Virology Laboratory at Universitas Hospital in Bloemfontein as well as at the Peter Medawar Building for Pathogen Research in Oxford for helping to facilitate this research. We appreciate the generous contributions of all study participants in this study. Boniswa Malefane also deserves special thanks for her significant contributions to making this study possible.

Financial support. This work was supported by a Medical Research Council Senior Fellowship (to J. F.) and a Rhodes Scholarship award (to L. M.).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Bates M, Mudenda V, Mwaba P, Zumla A. Deaths due to respiratory tract infections in Africa: a review of autopsy studies. Curr Opin Pulm Med **2013**; 19:229–37.
2. Getahun H, Gunneberg C, Granich R, Nunn P. HIV infection-associated tuberculosis: the epidemiology and the response. Clin Infect Dis **2010**; 50:S201–7.
3. Harries AD, Zachariah R, Corbett EL, et al. The HIV-associated tuberculosis epidemic—when will we act? Lancet **2010**; 375:1906–19.
4. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med **1993**; 178:2249–54.
5. Harris J, Keane J. How tumour necrosis factor blockers interfere with tuberculosis immunity. Clin Exp Immunol **2010**; 161:1–9.
6. Sonnenberg P, Glynn JR, Fielding K, Murray J, Godfrey-Faussett P, Shearer S. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. J Infect Dis **2005**; 191:150–8.
7. Geldmacher C, Schuetz A, Ngwenyama N, et al. Early depletion of *Mycobacterium tuberculosis*-specific T helper 1 cell responses after HIV-1 infection. J Infect Dis **2008**; 198:1590–8.
8. Zhang M, Gong J, Iyer DV, Jones BE, Modlin RL, Barnes PF. T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection. J Clin Invest **1994**; 94:2435–42.
9. Diedrich CR, Flynn JL. HIV-1/*mycobacterium tuberculosis* coinfection immunology: how does HIV-1 exacerbate tuberculosis? Infect Immun **2011**; 79:1407–17.
10. Day CL, Abrahams DA, Harris LD, et al. HIV-1 infection is associated with depletion and functional impairment of *Mycobacterium tuberculosis*-specific CD4 T cells in individuals with latent tuberculosis infection. J Immunol **2017**; 199:2069–80.
11. Kalsdorf B, Scriba TJ, Wood K, et al. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. Am J Respir Crit Care Med **2009**; 180:1262–70.
12. Jambo KC, Sepako E, Fullerton DG, et al. Bronchoalveolar CD4+ T cell responses to respiratory antigens are impaired in HIV-infected adults. Thorax **2011**; 66:375–82.
13. Kagina BM, Abel B, Scriba TJ, et al. South African Tuberculosis Vaccine Initiative. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guérin vaccination of newborns. Am J Respir Crit Care Med **2010**; 182:1073–9.
14. Abebe F. Is interferon-gamma the right marker for bacille Calmette-Guérin-induced immune protection? The missing link in our understanding of tuberculosis immunology. Clin Exp Immunol **2012**; 169:213–9.
15. Gallegos AM, van Heijst JW, Samstein M, Su X, Pamer EG, Glickman MS. A gamma interferon independent mechanism of CD4 T cell mediated control of *M. tuberculosis* infection in vivo. PLoS Pathog **2011**; 7:e1002052.
16. Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. J Bacteriol **1998**; 180:801–8.
17. Guirado E, Mbawuike U, Keiser TL, et al. Characterization of host and microbial determinants in individuals with latent tuberculosis infection using a human granuloma model. MBio **2015**; 6:e02537–14.
18. Singh S, Saraav I, Sharma S. Immunogenic potential of latency associated antigens against *Mycobacterium tuberculosis*. Vaccine **2014**; 32:712–6.
19. Scriba TJ, Kalsdorf B, Abrahams DA, et al. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. J Immunol **2008**; 180:1962–70.
20. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci U S A **1992**; 89:12013–7.
21. Sousa AO, Mazzaccaro RJ, Russell RG, et al. Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. Proc Natl Acad Sci U S A **2000**; 97:4204–8.
22. Chen CY, Huang D, Wang RC, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS Pathog **2009**; 5:e1000392.
23. Lewinsohn DA, Heinzel AS, Gardner JM, Zhu L, Alderson MR, Lewinsohn DM. *Mycobacterium tuberculosis*-specific CD8+ T cells preferentially recognize heavily infected cells. Am J Respir Crit Care Med **2003**; 168:1346–52.
24. Sutherland JS, Young JM, Peterson KL, et al. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. J Immunol **2010**; 184:6537–44.

25. Green AM, Difazio R, Flynn JL. IFN-γ from CD4 T cells is essential for host survival and enhances CD8 T cell function during *Mycobacterium tuberculosis* infection. J Immunol **2013**; 190:270–7.
26. Hanekom WA, Hughes J, Mavinkurve M, et al. Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies. J Immunol Methods **2004**; 291:185–95.
27. Lugada ES, Mermin J, Kaharuza F, et al. Population-based hematologic and immunologic reference values for a healthy Ugandan population. Clin Diagn Lab Immunol **2004**; 11:29–34.
28. Tsegaye A, Messele T, Tilahun T, et al. Immunohematological reference ranges for adult Ethiopians. Clin Diagn Lab Immunol **1999**; 6:410–4.
29. Mitchell JE, Chetty S, Govender P, et al. Prospective monitoring reveals dynamic levels of T cell immunity to *Mycobacterium tuberculosis* in HIV infected individuals. PLoS One **2012**; 7:e37920.
30. Day CL, Mkhwanazi N, Reddy S, et al. Detection of polyfunctional *Mycobacterium tuberculosis*-specific T cells and association with viral load in HIV-1-infected persons. J Infect Dis **2008**; 197:990–9.
31. Geldmacher C, Ngwenyama N, Schuetz A, et al. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. J Exp Med **2010**; 207:2869–81.
32. Saharia KK, Koup RA. T cell susceptibility to HIV influences outcome of opportunistic infections. Cell **2013**; 155:505–14.
33. Komanduri KV, Donahoe SM, Moretto WJ, et al. Direct measurement of CD4+ and CD8+ T-cell responses to CMV in HIV-1-infected subjects. Virology **2001**; 279:459–70.
34. Arlehamn CS, Sidney J, Henderson R, et al. Dissecting mechanisms of immunodominance to the common tuberculosis antigens ESAT-6, CFP10, Rv2031c (hspX), Rv2654c (TB7.7), and Rv1038c (EsxJ). J Immunol **2012**; 188:5020–31.
35. Sutherland JS, Lalor MK, Black GF, et al. GCGH Biomarkers for TB Consortium. Analysis of host responses to *Mycobacterium tuberculosis* antigens in a multi-site study of subjects with different TB and HIV infection states in sub-Saharan Africa. PLoS One **2013**; 8:e74080.
36. Hanekom WA, Dockrell HM, Ottenhoff TH, et al. Immunological outcomes of new tuberculosis vaccine trials: WHO panel recommendations. PLoS Med **2008**; 5:e145.
37. Chapman AL, Munkanta M, Wilkinson KA, et al. Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of *Mycobacterium tuberculosis*-specific T cells. AIDS **2002**; 16:2285–93.
38. Rangaka MX, Wilkinson KA, Seldon R, et al. Effect of HIV-1 infection on T-cell-based and skin test detection of tuberculosis infection. Am J Respir Crit Care Med **2007**; 175:514–20.
39. Clark SA, Martin SL, Pozniak A, et al. Tuberculosis antigen-specific immune responses can be detected using enzyme-linked immunospot technology in human immunodeficiency virus (HIV)-1 patients with advanced disease. Clin Exp Immunol **2007**; 150:238–44.
40. Wyndham-Thomas C, Dirix V, Schepers K, et al. Contribution of a heparin-binding haemagglutinin interferon-gamma release assay to the detection of *Mycobacterium tuberculosis* infection in HIV-infected patients: comparison with the tuberculin skin test and the QuantiFERON-TB Gold In-tube. BMC Infect Dis **2015**; 15:59.
41. Rangaka MX, Diwakar L, Seldon R, et al. Clinical, immunological, and epidemiological importance of antituberculosis T cell responses in HIV-infected Africans. Clin Infect Dis **2007**; 44:1639–46.
42. Hammond AS, McConkey SJ, Hill PC, et al. Mycobacterial T cell responses in HIV-infected patients with advanced immunosuppression. J Infect Dis **2008**; 197:295–9.
43. Delogu G, Vanini V, Cuzzi G, et al. Lack of response to HBHA in HIV-infected patients with latent tuberculosis infection. Scand J Immunol **2016**; 84:344–52.
44. Chiacchio T, Delogu G, Vanini V, et al. Immune characterization of the HBHA-specific response in *Mycobacterium tuberculosis*–infected patients with or without HIV infection. PLoS One **2017**; 12:e0183846.
45. Dreesman A, Corbière V, Dirix V, et al. Age-stratified T cell responses in children infected with *Mycobacterium tuberculosis*. Front Immunol **2017**; 8:1059.
46. Prendergast A, Prado JG, Kang YH, et al. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. AIDS **2010**; 24:491–502.
47. Hed A El, Khaitan A, Kozhaya L, et al. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. J Infect Dis **2010**; 201:843–54.
48. Alvarez Y, Tuen M, Shen G, et al. Preferential HIV infection of CCR6+ Th17 cells is associated with higher levels of virus receptor expression and lack of CCR5 ligands. J Virol **2013**; 87:10843–54.
49. Scriba TJ, Penn-Nicholson A, Shankar S, et al; ACS Cohort Study Team. Sequential inflammatory processes define human progression from *M. tuberculosis* infection to tuberculosis disease. PLoS Pathog **2017**; 13:e1006687.
50. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A **2011**; 79:167–74.