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# Selective reduction of IFN-γ single positive mycobacteria-specific CD4+ T cells in HIV-1 infected individuals with latent tuberculosis infection

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SUMMARY

HIV-1 is recognized to increase the risk for tuberculosis even before CD4+ T cell deficiency is profound. To better understand how HIV-1 alters immunity to latent tuberculosis, we compared the magnitude and functional profile of mycobacteria-specific CD4+ T cells between HIV-uninfected and HIV-infected individuals, using flow cytometry. In HIV-1 infection, IFN-γ single positive mycobacteria-specific CD4+ T cells were decreased, while the frequency of polyfunctional cells (IFN-γ+IL-2+TNF-α+) remained unchanged. Moreover, the proportion of IFN-γ single positive cells correlated inversely with viral replication. Our results suggest that HIV-1 affects mycobacteria-specific cells differentially, depending on their functional capacity.



## 1. Introduction

It is estimated that a third of the world's population is latently infected with *Mycobacterium tuberculosis* (Mtb). While in HIV-uninfected persons the risk of progression from latent to active tuberculosis (TB) is 2–10% in a lifetime, it increases up to 5–10% annual risk in HIV-infected individuals. In order to understand the mechanisms involved in the maintenance and/or impairment of TB latency, it is therefore of interest to define in detail the extent to which HIV affects TB immune responses. The most obvious immune defect caused by HIV is a progressive reduction in absolute CD4+ T cell numbers that correlates with increasing TB disease risk [1], attesting to the critical role of CD4+ T cells for efficient immune responses. Several studies provide compelling evidence that HIV decreases the frequency of peripheral Mtb-specific CD4+ T cells even during the early phase of HIV infection [2–4]. It has been proposed that, notwithstanding overall CD4+ T cell depletion, HIV may also induce qualitative changes in CD4+ T cell function, further weakening protective immune responses to Mtb. Alteration of the polyfunctional capacity [5,6], memory profile [7] and lineage differentiation [8] of Mtb-specific CD4+ T cells have been reported. Most work has reported the effect of HIV on TB immune response during active TB; and fewer studies have compared the attributes of mycobacterial-specific CD4+ T cells in the context of latent tuberculosis infection in HIV-uninfected and HIV-infected persons [2,3,8,9].

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To better understand the specific qualitative and quantitative deficits affecting immunity in latent TB during HIV infection, we compared the magnitude, functional and memory profiles of CD4+ T cell responses to distinct mycobacterial antigens (ESAT-6/CFP-10 peptide pool, purified protein derivative (PPD) or Bacille Calmette Guérin (BCG)) in HIV-uninfected individuals and antiretroviral therapy naïve HIV-infected individuals with well-preserved CD4 counts.

## 2. Material and methods

### 2.1. Study subjects

Study participants (n = 49) were recruited from Khayelitsha in Cape Town, South Africa, 25 of whom were HIV-uninfected (median age: 23, 60% female), and 24 were HIV-infected and antiretroviral therapy naïve (median age: 31; 96% female; median CD4 count: 625, IQR [545–786]; median HIV viral load: 7788, IQR [3251–17623]). A summary of the participants' clinical characteristics is presented in Table 1. All participants were Mtb-sensitized, as defined by a positive IFN-γ release assay (Quantiferon™ in-tube, Cellestis). None of the participants reported active TB within the eight years prior to their participation in this study. Of note, whilst gender and age distribution differed between the two groups, no significant differences in the magnitude or profile of mycobacteria-specific CD4+ T cells were observed between males and females, or according to age (data not shown).

### 2.2. Blood collection, whole blood stimulation and staining

Peripheral blood was collected in sodium heparin tubes and processed within 4 h of collection. A whole blood assay was performed according to the protocol optimized by Hanekom et al. [10]. Briefly, whole blood (250 μl) was incubated at 37 °C for a total of 12 h with mycobacterial antigens, namely ESAT-6/CFP-10 peptide pool (4 μg/ml), PPD (20 μg/ml, SSI) or BCG (MOI of 4, *Mycobacterium bovis* Danish strain 1331, SSI), in the presence of anti-CD28 and anti-CD49d antibodies. Lyophilized BCG vaccine was resuspended in 250 μl RPMI (Sigma), from which 15 μl (~$3 \times 10^5$ organisms) was added to 250 μl of whole blood; this equates to an average multiplicity of infection (MOI) of 4 organisms per monocyte. Brefeldin A was added 7 h after the onset of the stimulation, as per Hanekom et al. [10], as prolonged incubation prior to Brefeldin A addition improved the detection of cytokine responses to complex mycobacterial antigens. Non-stimulated (NS) cells were incubated with CD28 and CD49d antibodies only. At the end of the incubation, red blood cells were lysed with Alternative Lysing solution (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 1 mM $Na_4EDTA$). Cells was subsequently stained with ViViD (Molecular Probes), fixed with FACS Lysing Buffer (BD) and cryopreserved in 10% DMSO in FCS for later batch staining. After thawing, cells were washed, surface stained with CD4-PE-Cy5.5 (S3.5; Invitrogen), CD8-Qdot705 (3B5; Invitrogen), CD27-PE-Cy5 (1A4CD27; R&D Systems) and CD45RO-ECD (UCHL1; R&D Systems), permeablized and stained intracellularly with CD3-APC-H7 (SK7, BD), IFN-γ-Alexa700 (B27, BD), IL-2-APC (MQ1-17H12, BD), IL-17-Alexa488 (N49-653, BD) and TNF-α-PE-Cy7 (MAb11; eBiosciences).

### 2.3. Data analyses and statistics

Cells were acquired on a BD Fortessa and analyzed using FlowJo (TreeStar) and Pestle and Spice software. A positive cytokine response was defined as at least twice the background and data are reported after background subtraction. The gating strategy is presented in Supplemental Figure 1. For statistical analysis of Spice data, we used the statistic tools integrated in the software, where the applied test has the ability to compare multi-component measurements by reducing the comparison to a single test rather than comparing individual components that would require a correction for multiple comparisons [11]. All other statistical comparisons were performed in GraphPad Prism. Univariate statistics were applied and no adjustments were made for multiple comparisons. Non-parametric tests were used for all comparisons (Mann–Whitney U, Wilcoxon Signed Rank or Kruskal–Wallis ANOVA tests). Correlations were performed using the Spearman Rank test. A *P*-value of $<0.05$ was considered statistically significant.

### 2.4. Ethical approval

Ethical approval for the study was obtained from the University of Cape Town Research Ethics Committee (158/2010). All participants provided written informed consent.

## 3. Results

We compared the frequency, polyfunctional and memory profile of ESAT-6/CFP-10-, PPD- and BCG-responding CD4+ T cells in Mtb-sensitized HIV-uninfected individuals (n = 25) and HIV-infected subjects (n = 24) with well-preserved CD4 counts (median 625 cells/$mm^3$). In order to evaluate the impact of HIV infection on mycobacteria-specific responses, it was important to first define and compare the profile of mycobacteria-specific CD4+ T cells in response to distinct antigen formulations commonly used to assess TB immune responses, such direct comparisons not having been reported to date. Thus, we first compared the magnitude and polyfunctional profile of mycobacteria-specific CD4+ T cells in HIV-uninfected individuals in response to an ESAT-6/CFP-10 peptide pool, Mtb PPD or BCG. Figure 1A shows representative plots of cytokine production (IFN-γ, IL-17, IL-2 and TNF-α) in CD4+ T cells in response to ESAT-6/CFP-10, PPD or BCG in one HIV-uninfected participant. All individuals tested had a positive response to PPD; 22 and 23 individuals had a detectable response to ESAT-6/CFP-10 and BCG, respectively (Figure 1B). The overall median frequencies

**Table 1**
Clinical characteristics of study participants.

| | HIV-uninfected | HIV-infected | *P*-values* |
|---|---|---|---|
| Number of participants | 25 | 24 | |
| Median CD4 count [IQR]† | 813 [675–933] | 625 [545–786] | p = 0.003 |
| Median HIV viral load [IQR]§ | na | 7788 [3251–17,623] | na |
| Interferon-γ release assay [IQR]** | 7.4 [1.1–10.5] | 3.0 [1.1–10] | p = 0.73 |

* *P*-values were calculated using a Mann–Whitney t-test.
† CD4 count, measured using a Flow-CARE™ PLG CD4 test (Beckman Coulter), is expressed as cells/$mm^3$.
§ HIV viral load, determined using an Abbott m2000 RealTime HIV-1 assay, is expressed as RNA copies/ml.
** Interferon-γ release assay results are expressed in IU/ml (background subtracted).