CD4 and CD8 T-Cell Responses to Mycobacterial Antigens in African Children

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Rationale: The current tuberculosis (TB) vaccine, bacille Calmette-Guérin (BCG), does not provide adequate protection against TB disease in children. Furthermore, more efficacious TB vaccines are needed for children with immunodeficiencies such as HIV infection, who are at highest risk of disease.

Objectives: To characterize mycobacteria-specific T cells in children who might benefit from vaccination against TB, focusing on responses to antigens contained in novel TB vaccines.

Methods: Whole blood was collected from three groups of BCG-vaccinated children: HIV-seronegative children receiving TB treatment ($n = 30$), HIV-infected children ($n = 30$), and HIV-unexposed healthy children ($n = 30$). Blood was stimulated with Ag85B and TB10.4, or purified protein derivative, and T-cell cytokine production by CD4 and CD8 was determined by flow cytometry. The memory phenotype of antigen-specific CD4 and CD8 T cells was also determined.

Measurements and Main Results: Mycobacteria-specific CD4 and CD8 T-cell responses were detectable in all three groups of children. Children receiving TB treatment had significantly higher frequencies of antigen-specific CD4 T cells compared with HIV-infected children ($P = 0.0176$). No significant differences in magnitude, function, or phenotype of specific T cells were observed in HIV-infected children compared with healthy control subjects. CD4 T cells expressing IFN- γ , IL-2, or both expressed a CD45RA⁺CCR7⁺CD27⁺ effector memory phenotype. Mycobacteria-specific CD8 T cells expressed mostly IFN- γ in all groups of children; these cells expressed CD45RA⁺CCR7⁺CD27⁺ or CD45RA⁺CCR7⁺CD27⁺ effector memory phenotypes.

Conclusions: Mycobacteria-specific T-cell responses could be demonstrated in all groups of children, suggesting that the responses could be boosted by new TB vaccines currently in clinical trials.

Keywords: pediatric; HIV-1; tuberculosis; vaccine; T cells

One million children are estimated to develop tuberculosis (TB) globally every year (1). Compared with adults, children have a higher risk of severe TB disease and death (2, 3). HIV infection markedly increases this risk (4), and infected children have increased mortality (5) compared with HIV-negative children (6).

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

HIV-infected children are at greater risk of developing tuberculosis (TB) disease, and might benefit from vaccination with novel TB vaccines. However, little is known about the effect of HIV infection on function and phenotype of T-cell responses to mycobacterial antigens in children.

What This Study Adds to the Field

This study compares CD4 and CD8 T-cell cytokine expression and memory phenotype in children after *in vitro* stimulation with mycobacterial antigens, also contained in novel anti-TB vaccines that are currently undergoing clinical trials. We report higher frequencies of CD4 T cells, expressing IFN- γ , IL-2, or both, in children with TB, but no differences in memory phenotype between children with and without HIV infection.

The only licensed vaccine against TB, bacille Calmette-Guérin (BCG), affords variable and mostly poor protection against pulmonary TB (7). Despite this, BCG protects against severe childhood forms of TB, such as miliary TB and TB meningitis (7). There is no evidence that BCG has any protective effect in HIV-infected children; rather, BCG may cause disease in this population (8, 9). A more efficacious vaccine that is safe and protective in HIV-infected children is urgently needed (10).

Successful vaccination requires long-lived immunological memory that confers protection against infection or disease. CD4 T cells are critical, because impairment of CD4 responses, as seen in HIV infection, leads to increased susceptibility to TB (11). CD8 T cells may also be important for protection (12–15). TB vaccine-induced CD4 and CD8 T-cell responses have therefore been major readouts in clinical trials, but mycobacteria-specific immunity has not been studied in detail in HIV-infected patients (16), particularly not in children. In the absence of validated correlates of protective immunity, IFN- γ is the most commonly measured cytokine, because it has been shown to be associated with protection: individuals with genetic defects in the IFN- γ (or IL-12) pathways suffer from increased susceptibility to mycobacterial disease (17–21). IL-2 may also be important as it is required for secondary expansion of memory T cells (22, 23), and thus for generation of long-term protective immunity. Furthermore, polyfunctional T cells, which coexpress two or more cytokines, have been associated with more effective control of murine intracellular infections (24), including *Mycobacterium tuberculosis* (25), but convincing human data are currently lacking.

T-cell cytokine expression has been linked with cell-surface expression of markers of memory phenotype in viral infections in mice (26) and humans (27). CD45RA, which is lost by T cells on priming, differentiates naive and memory (antigen-experienced) T cells. Within this antigen-experienced population, expression of CCR7 defines central memory T cells, which express IL-2 and are able to home to lymphoid organs. Although these cells are believed to lack immediate effector function, they are long lived and proliferate rapidly on antigen reencounter. By contrast, effector memory T cells, which lack CCR7 expression, predominantly express effector cytokines, such as IFN- γ (26, 27). These populations can be further dissected according to their degree of differentiation, based on the expression level of CD27. This costimulatory marker is sequentially lost during T-cell differentiation (28).

To date, very few studies have analyzed mycobacteria-specific T-cell immunity in detail in children beyond infancy (29). It is unknown whether HIV-infected children can mount similar T-cell responses to healthy children on vaccination with novel TB vaccines. To delineate the type of responses that might be present even before vaccination with novel TB vaccines, we characterized the function and memory phenotype of mycobacteria-specific T cells in African children with and without HIV infection. Some of the results of this study have been previously reported in the form of an abstract (30).

METHODS

Study Participants

This study was approved by the Research Ethics Committees of the University of Cape Town, South Africa (REC 081/2006) and Imperial College London, UK (02/GB/23E).

After informed consent, three groups of children who received BCG vaccination at birth were enrolled:

1. Healthy children (HC) born to HIV-negative mothers were recruited from community clinics, routine immunization clinics, and prebooking clinics for planned surgery at Red Cross Children's Hospital, Cape Town. Children with a past history of TB or any evidence of acute or chronic health problems were excluded. This group served to determine the background of responses to the chosen mycobacterial antigens in a BCG-vaccinated but HIV-negative cohort.
2. HIV-seronegative children diagnosed with TB, who had been receiving standard anti-TB therapy for a minimum of 2 months, were recruited from TB clinics. TB diagnosis was either by culture confirmation (definite TB) or by the combination of suggestive signs and symptoms, a history of TB exposure, abnormal radiology, positive tuberculin skin test response (>10 mm), and response to TB therapy (probable TB). This group served to determine T-cell responses to mycobacterial antigens in children with expected immunological memory due to the presence of TB. Children with a minimum of 2 months of anti-TB therapy were included.
3. Children with perinatally acquired HIV-1 infection, about to start triple-combination antiretroviral therapy (cART) were identified by clinicians and/or the research nurse at HIV community clinics, at pretreatment meetings, or at the Red Cross Children's Hospital. All children were receiving regular follow-up and fulfilled either clinical or immunological criteria for starting cART according to World Health Organization guidelines (31). None of the children had previously received cART or treatment for TB.

Any HIV-infected children who were acutely unwell or had proven TB or other active opportunistic infections were excluded. Children with a history of rapid progression of HIV disease, who required multiple hospital admissions, or who were under the age of 3 months, were also excluded.

Blood Collection and Stimulations

Venous blood was collected and processed within 2 hours. A whole-blood intracellular cytokine staining assay, as previously described (32), was performed. Briefly, whole blood was stimulated for 7 hours with the recombinant proteins Ag85B and TB10.4 (added together due to limited blood volumes, 10 μ g/ml each) or *M. tuberculosis* purified protein derivative (PPD, 20 μ g/ml). All antigens were from Statens Serum Institute, Copenhagen, Denmark. Unstimulated blood served as negative control and staphylococcal enterotoxin B (SEB, at 10 μ g/ml; Sigma, UK) as positive control. Brefeldin A (Sigma; 10 μ g/ml) was added for the last five hours of incubation. After incubation, 2 mM ethylenediaminetetraacetic acid was added, and red blood cells were lysed and white blood cells fixed with fluorescence-activated cell sorter lysing solution (BD Biosciences, Cowley, UK). The fixed cells were cryopreserved in liquid nitrogen.

Surface Phenotypic and Intracellular Cytokine Staining

Cryopreserved cells were thawed and permeabilized using Perm/Wash Solution (BD Biosciences). Cells were stained at 4°C, first with surface antibodies for 30 minutes and then with intracellular antibodies for 1 hour. The following combinations of antibodies were used: anti-CD3 Pacific Blue (clone UCHT1), anti-CD8 PerCP-Cy5.5 (SK1), anti-CD27 PE (MT271), anti-CD45RA PE-Cy7 (L48), anti-IFN- γ AlexaFluor700 (B27), anti-IL-2 fluorescein isothiocyanate (5344.111; all from BD Biosciences), anti-CD4 QD605 (S3.5, Invitrogen), and anti-CCR7 APC (150503, R&D Systems, UK). A minimum of 100,000 CD3⁺ T cells were acquired on a LSR II flow cytometer (BD Biosciences).

Data Analysis

Flow cytometry data were analyzed using FlowJo v8.8.2 (Tree Star), Pestle (v1.6.1), and Spice (v4.9) (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Results were expressed as the frequency of positive events above the negative control (unstimulated sample). Memory phenotypes of cytokine-producing cells were only analyzed if they exceeded 20 events.

Statistical analysis was performed using GraphPad Prism v5.0. Differences between groups were calculated using either Mann-Whitney or Kruskal-Wallis analysis of variance. Correlations were calculated by nonparametric Spearman test. All tests were two-tailed, and a value of $P < 0.05$ was considered significant.

RESULTS

Participant Recruitment and Demographics

Children were recruited from clinics in African communities from TB-endemic areas in the Western Cape region. Demographic details of all groups are shown in Table 1.

TABLE 1. DEMOGRAPHIC DETAILS OF STUDY GROUPS

Demography	Healthy Control Subjects (n = 30)	TB Diseased (n = 30)	HIV Infected (n = 30)
Male	17	17	15
Ethnicity			
Black African	27	27	26
Mixed race	1	3	4
White	2	0	0
Age, yr	3.0 (<1–5)	3.0 (<1–4)	3.5 (<1–6)
<1 yr	2	4	6
1–5 yr	23	22	16
6–12 yr	5	3	8
>12 yr	0	1	0
CD4 absolute count (cells/ μ l)	N/A	N/A	531 (284–1,259)
CD4 percentage	N/A	N/A	17.85 (12.93–21.45)
Viral load (log ₁₀ RNA copies/ml)	N/A	N/A	4.88 (4.30–5.76)

Definition of abbreviations: IQR = interquartile range; TB = tuberculosis. Values given are n or median (IQR); N/A: not applicable.

All children had routinely received BCG vaccination at birth as part of the Expanded Program on Immunization in South Africa. All children enrolled into the TB group (TB) were receiving standard treatment for TB with isoniazid, rifampicin and pyrazinamide for a median of 4 months (range: 2–6 mo). Twenty-nine children had pulmonary TB, and one child had TB of the spine. In seven cases, TB had been microbiologically confirmed by positive culture. In the other 23 cases TB was diagnosed based on epidemiological history, signs and symptoms consistent with TB, abnormal chest radiograph/radiology, a positive tuberculin skin test (induration >10 mm), and clinical improvement after start of TB treatment.

Any children with reported exposure to an active TB case in their household were excluded from the HC and HIV groups. All HIV-infected children had advanced HIV disease, fulfilling clinical and/or immunological criteria for starting cART, according to World Health Organization guidelines (31). The median CD4 count of these HIV-infected children (531 cells/ μ l) is well below CD4 counts reported for age-matched healthy African children from Uganda (1,517 cells/ μ l) (33).

Frequency of Mycobacteria-specific CD4 and CD8 T-Cell Responses

We measured the frequency of Ag85B/TB10.4 or PPD-specific T cells in whole blood by intracellular cytokine staining (Figure 1).

Although fewer CD3⁺ cells were available for analysis in HIV-infected children (median [interquartile range (IQR)]: 154,670 [104,156–294,199]), compared with the number of CD3⁺ T cells from children in the HC (median [IQR]: 254,468 [165,733–345,048]; $P = 0.0261$) and TB (median [IQR]: 247,026 [165,000–393,612]; $P = 0.0361$) groups, mycobacteria-specific CD4 and CD8 T cells were readily detectable in all three groups of children. Frequencies of these specific cells were observed in a remarkably broad range, between 0 and 10.36% (Figure 2). Total cytokine-expressing (IFN- γ or IL-2, or both) Ag85B/TB10.4-specific CD4 T-cell frequencies were significantly lower in HIV-infected children compared with the TB group (Figure 2A). There were no differences in specific T-cell frequencies between HIV-infected children and children in the HC group (Figure 2A). Frequencies of PPD-specific CD4 T cells, expressing total cytokine or total IFN- γ , were also significantly higher in the TB group compared with both the HC and HIV-infected groups (Figure 2B).

There was no difference in IFN- γ -expressing Ag85B/TB10.4-specific CD8 T cells between the three groups (Figure 2C). Total cytokine-producing Ag85B/TB10.4-specific CD4 T-cell frequencies in all groups of children correlated with cytokine-producing CD8 T-cell frequencies (HC: $r = 0.7193$, $P < 0.0001$; TB: $r = 0.8370$, $P < 0.0001$; and HIV: $r = 0.7393$, $P < 0.0001$; data not shown). We also investigated the relationships between age or time on treatment with the frequencies of specific T cells. We observed an inverse correlation between age and the magnitude of the total Ag85B/TB10.4-specific CD4 T-cell response in healthy children (HC: $r = -0.5157$, $P = 0.0035$). This was not observed in the other two groups (TB: $r = -0.1759$, $P = 0.3526$; HIV: $r = -0.3351$, $P = 0.0703$). Frequencies of specific CD8 T cells also did not correlate with age in the three groups. Time on TB treatment did not correlate with the magnitude of the CD4 or CD8 T-cell response (CD4: $r = 0.0273$, $P = 0.8860$; CD8: $r = 0.0846$, $P = 0.6565$).

Quality of the Specific T-Cell Response

To further characterize the quality of the T-cell response, we assessed combinations of cytokine expression among antigen-specific T cells. Overall, Ag85B/TB10.4-specific cells expressing

IL-2 alone composed the predominant subset of specific CD4 T cells (Figures 3A and 3B), whereas IFN- γ -expressing and IFN- γ /IL-2-coexpressing cells composed similarly sized subsets (Figures 3A and 3B). PPD-specific CD4 T cells were more polyfunctional, with IFN- γ /IL-2 coexpressing cells composing approximately 50% of the total response (Figure 3B).

By contrast, Ag85B/TB10.4-specific CD8 T cells expressed almost exclusively IFN- γ (Figures 3C and 3D). The PPD-specific CD8 T-cell response was too low to reliably characterize polyfunctionality (data not shown).

We also compared the cytokine profiles of Ag85B/TB10.4-specific CD4 T cells between the three groups of children. HIV-infected children had significantly lower frequencies of bifunctional CD4 T cells than children with TB (median [IQR], HIV: 0.02 [0.00–0.12]; TB: 0.15 [0.03–0.41]; $P = 0.0015$; Figure 3A). CD4 T cells expressing IFN- γ alone were also significantly less frequent in HIV-infected children compared with children with TB (median [IQR], HIV: 0.01 [0.01–0.15]; TB: 0.09 [0.02–0.23]; $P = 0.029$; Figure 3A).

Despite the low frequencies of Ag85B/TB10.4-specific CD8 T cells producing IL-2 alone or coexpressing IFN- γ and IL-2, these subsets were significantly less frequent in HIV-infected children compared with the TB group (median [IQR], HIV: 0.00 [0.00–0.01]; TB: 0.01 [0.00–0.07]; $P = 0.0243$, IL-2 alone; and HIV: 0.00 [0.00–0.01]; TB: 0.01 [0.00–0.04]; $P = 0.0397$, IFN- γ and IL-2 coexpression; Figure 3C). IL-2-expressing monofunctional CD8 T-cell frequencies were also lower in the HIV-infected group compared with healthy control subjects (median [IQR], HIV: 0.00 [0.00–0.01]; HC: 0.02 [0.00–0.06]; $P = 0.0096$; Figure 3C).

Correlation of Frequencies of Antigen-specific Cells with Total CD4 and CD8 Counts

Antigen-specific T-cell responses after stimulation with mycobacterial antigens were highly correlated between CD4 and CD8 T cells (Spearman $\rho = 0.786$, $P < 0.0001$), independent of the group of children studied.

In the HIV-positive children, the routinely conducted CD4 counts (measured as absolute or in percentage) correlated well with CD8 T-cell counts (Spearman $\rho = 0.78$, $P < 0.0001$), but not with viral load (Spearman $\rho = 0.14$, $P = 0.43$, data not shown).

Antigen-specific CD4 and CD8 T Cells Express an Effector Memory Phenotype

To examine the link between cytokine expression profile and memory phenotype, we analyzed the expression of the phenotypic markers, CD45RA, CD27, and CCR7, on Ag85B/TB10.4-specific CD4 and CD8 T cells expressing IFN- γ and/or IL-2, from healthy children (Figure 4A). CD4 T cells producing IFN- γ or IFN- γ and IL-2 predominantly expressed a CD45RA⁺CCR7⁺ effector memory phenotype. Both presence and absence of the differentiation marker, CD27, was observed on these effector memory cells (Figure 4B). IL-2-producing T cells were significantly more likely to express CD45RA⁺CCR7⁺CD27⁺ compared with IFN- γ ⁺IL-2⁺ ($P = 0.0002$) or IFN- γ ⁺ ($P = 0.0046$) producing CD4 T cells. A larger proportion of IFN- γ ⁺IL-2⁺ ($P = 0.02$) and IFN- γ ⁺ ($P = 0.0098$) CD4 T cells expressed the effector memory phenotype CD45RA⁺CCR7⁺CD27⁺, compared with IL-2⁺ CD4 T cells.

Significantly higher proportions of IFN- γ ⁺IL-2⁺ CD4 T cells expressed an effector memory phenotype (CD45RA⁺CCR7⁺CD27^{+/+}) compared with the non-cytokine-producing total CD4 T cells ($P < 0.0001$, Figure 4B). A small subset of specific CD4 T cells also expressed a CD45RA⁺CCR7⁺CD27⁺ pheno-

Figure 1. Functional characterization of Ag-specific T-cell cytokine expression by intracellular cytokine staining and flow cytometry. (A) Representative density plots from a single healthy child showing the gating strategy used to identify CD4 and CD8 T cells from whole blood. From left to right, *upper plots*, cell doublets were excluded using forward scatter/side scatter (FSC/SSC)-area and FSC-height parameters. Small lymphocytes were then selected. *Lower plots*, selection of T cells by gating on CD3⁺ cells, followed by selection of CD4 and CD8 T cells. (B) Representative plots showing IL-2 and IFN- γ expression by unstimulated (Nil) or Ag85B/TB10.4-stimulated CD4 or CD8 T cells. FITC = fluorescein isothiocyanate.

type, which in CD8 cells has been termed terminally differentiated effector memory cells (34).

In antigen-specific IFN- γ ⁺ CD8 T cells this CD45RA⁺CCR7⁻CD27⁺ terminally differentiated phenotype predominated (Figure 4C). This phenotype was distinct from that expressed on non-cytokine-producing, total CD8 T-cell population, which was primarily CD45RA⁺CCR7⁺CD27⁺ and composed a significantly higher proportion of the total CD8 population compared with antigen-specific IFN- γ ⁺ T cells ($P < 0.0001$, Figure 4C). Specific CD8 T cells also expressed the CD45RA⁻CCR7⁻ phenotype characteristic of effector memory T cells, albeit at lower proportions compared with the

CD45RA⁺CCR7⁻ phenotype (Figure 4C). Approximately half of these IFN- γ -producing effector memory cells expressed CD27.

T-Cell Memory Phenotypes Are Comparable in Healthy and Diseased Children

To analyze whether HIV infection or TB disease might affect the memory phenotypes of mycobacteria-specific cells, we compared the phenotypes of mycobacteria-specific CD4 and CD8 T cells between all three groups (Figure 5). No significant differences in memory phenotype were observed among Ag85B/TB10.4-specific CD4 cells expressing IFN- γ (Figure 5A) or IL-2 (Figure 5B). Similarly, the memory phenotypes of

Figure 2. Mycobacteria-specific T-cell responses measured by intracellular cytokine staining and flow cytometry in whole blood from the three groups of children. Each symbol represents an individual, and for each plot the median is represented by the horizontal line. (A) Frequencies of Ag85B/TB10.4-specific, total cytokine⁺ (IFN- γ ⁺ and/or IL-2⁺) CD4 T cells, total IFN- γ ⁺, or total IL-2⁺ CD4 T cells. (B) Frequencies of purified protein derivative (PPD)-specific CD4 T cells. (C) Frequencies of CD8 T cells expressing IFN- γ ⁺ after stimulation with Ag85B/TB10.4 (left) or PPD (right) or total IL-2⁺ CD4 T cells. Differences between any two groups were calculated using the Mann Whitney U test. HC = healthy control subjects; HIV = children with HIV-1 infection; TB = children with tuberculosis.

IFN- γ -expressing CD8 T cells were also not different between the three groups (Figure 5C).

DISCUSSION

Little is known about the adaptive immune response to *M. tuberculosis* in children from a setting of very high TB incidence. We characterized and compared mycobacteria-specific CD4 and CD8 T-cell responses in healthy children, children with TB, and antiretroviral-naïve, HIV-1-infected children. Five major points emerged from our study: (1) mycobacterial antigens, which are included in novel TB vaccines, were found to be highly recognized as recall antigens in our populations; (2) mycobacteria-specific CD4 and CD8 T cells were readily detected at surprisingly high magnitudes in children from all groups; (3) the frequencies of observed specific T-cell responses

covered a remarkably broad range; (4) no significant differences in magnitude, function, or phenotype of specific T cells were observed in HIV-infected children compared with healthy control subjects, but evidence of active TB significantly enhances the magnitude of the responses; and (5) mycobacteria-specific CD4 and CD8 T cells express an effector memory phenotype.

We detected mycobacteria-specific CD4 and CD8 T-cell responses at high frequencies in children from all three groups, even in the HIV-infected group. These responses covered a remarkably broad range, extending from 0% up to 10%. Whether a high magnitude of T-cell responses is associated with enhanced protection is not clear. Transfer of early secretory antigenic target (ESAT)-6-specific memory Th1 cells to recipient mice before *M. tuberculosis* challenge showed that higher numbers of specific T cells confer better protection than lower numbers (35). However, numerous other animal and human studies have

Figure 3. Analysis of the quality of mycobacteria-specific T cells measured by intracellular cytokine staining and flow cytometry in whole blood from the three groups of children. Each symbol represents an individual and for each plot, the median is represented by the horizontal line. (A) Frequencies of bifunctional or monofunctional Ag85B/TB10.4-specific CD4 T cells. (B) Pie charts representing the median proportions of cells coexpressing IFN- γ and/or IL-2, among the total CD4 T-cell response, after stimulation with Ag85B/TB10.4 or purified protein derivative (PPD). (C) Frequencies of bifunctional or monofunctional Ag85B/TB10.4-specific CD8 T cells. (D) Pie charts representing the median proportions of cells coexpressing IFN- γ and/or IL-2, among the total CD8 T-cell response, after stimulation with Ag85B/TB10.4. Differences between any two groups were calculated using the Mann Whitney *U* test. HC = healthy control subjects; HIV = children with HIV-1 infection; TB = children with tuberculosis.

shown that the CD4 T-cell IFN- γ response does not necessarily correlate with protection but may rather reflect bacterial load or degree of inflammation (25, 36–40). This is also consistent with our finding of the highest T-cell responses in TB-affected children. Our data show that a substantial proportion of children have mycobacteria-specific T-cell populations, expressing IFN- γ and/or IL-2, which may be enhanced by heterologous boosting vaccines. Whether such responses correlate with protection from diseases remains to be further validated in the human host.

We chose TB10.4 and Ag85B as mycobacterial recall antigens for the T-cell assays in our study. These antigens have recently been incorporated into a subunit vaccine in the form of a fusion protein, Ag85B-TB10.4, formulated with the adjuvant IC31 (41). TB10.4 and Ag85B are expressed in a wide range of mycobacterial species and are commonly recognized in *M. tuberculosis*-infected and BCG-vaccinated individuals (42). Our data suggest their high immunodominance in children, including children with HIV infection, for the first time. We were surprised by the magnitude of the responses and conducted control experiments using peptides of the same antigens, which revealed highly correlated results (data not shown). We therefore do not think that our findings are the result of any experimental artifact.

Furthermore, the magnitude of CD8 responses surprised us, although these antigens have previously been shown to induce high levels of CD8 responses in animal models (43). Our findings are encouraging news for the formulation of vaccines incorporating these antigens, because they appear to be able to induce both CD4 and CD8 responses. Detection of CD8 responses using recombinant proteins as recall antigens was most likely a result of cross-priming, also observed in the context of BCG vaccination of infants (44).

Vaccine boosting may have different outcomes in BCG-primed, *M. tuberculosis*-uninfected, and latently infected children. To date, few data are available on the character of T-cell

responses in older children, who are more likely to have been exposed to TB. Most studies in children have been performed after BCG vaccination in infants (44–46). These studies report lower frequencies of specific CD4 and also CD8 T cells in infants to those observed here in older children. This could reflect a less-differentiated infant immune system. Naive T cells constitute a larger proportion of the T-cell compartment in infants compared with older children (47). Because the readout for intracellular cytokine assays is a frequency of T cells, the larger proportion of naive cells may serve to lower the proportion of specific memory cells. In addition, the differences between our results and the infant studies may also reflect the use of different antigens. Furthermore, it is possible that the wide age range in our study may have confounded our results, although children were age matched between the groups.

The finding that frequencies of mycobacteria-specific T-cell responses in HIV-infected and -uninfected children were not significantly different was unexpected. Lower mycobacteria-specific CD4 T-cell responses have been observed in HIV-infected adults when compared with HIV-uninfected control subjects (48, 49). We recently also showed that BCG-specific T-cell responses were markedly impaired in HIV-infected infants during the first year of life (50) and that blood of children with HIV was more permissive of mycobacterial growth *in vitro*, associated with lower levels of IFN- γ compared with HIV-negative children (29). The reason for the similar frequencies between HIV-negative and HIV-positive children observed here is currently unclear. However, given the high rate of mortality in untreated, HIV-infected children in Africa—35% in children in the first year of life and 52.5% up to 2 years of age (51)—our inclusion of HIV-infected children, with a mean age of 3.7 years, may have introduced a selection bias for children with slower HIV progression and better-preserved immune function. The wide range of responses observed in our study

Figure 4. Memory phenotype of mycobacteria-specific T cells in healthy children. (A) Flow cytometric analysis of CCR7, CD45RA, and CD27 expression on total CD4 T cells (red background) and Ag85B/TB10.4-specific CD4 T cells, expressing IFN- γ (black dots, upper plots) or IL-2 (black dots, lower plots). (B) Relative proportion of memory phenotype marker coexpression by the indicated cytokine-producing CD4 T-cell populations, or the total CD4 T-cell population, after stimulation of whole blood with Ag85B/TB10.4. (C) Relative proportion of memory phenotype marker coexpression by IFN- γ ⁺ CD8 T cells, or the total CD8 T cell population, after stimulation of whole blood with Ag85B/TB10.4.

Figure 5. Comparison of Ag85B/TB10.4-specific T-cell memory phenotype between the three groups of children. Relative proportions of memory phenotype marker coexpression by (A) IFN- γ ⁺ CD4 T cells, or (B) IL-2⁺ CD4 T cells for the different groups. (C) Relative proportions of memory phenotype marker coexpression by IFN- γ ⁺ CD8 T cells. No significant differences were observed. HC = healthy control subjects; HIV = children with HIV-1 infection; TB = children with tuberculosis.

may also have led to less statistical power for detecting differences between the groups. Regardless, our data suggest that heterologous vaccine boosting may enhance T-cell responses in at least a proportion of HIV-infected children. Development of vaccines that augment T-cell immunity in this vulnerable group is particularly important.

We found that children with TB had significantly higher mycobacteria-specific CD4 and CD8 T-cell responses compared with HIV-infected children but not compared with the healthy control subjects. This is likely to reflect disease-associated inflammation and/or high levels of antigen exposure. This contrasts with work showing that T-cell responses in patients with active pulmonary TB express lower levels of Th1 cytokines, such as

IFN- γ and IL-2 (52). However, these previously published studies did not examine cytokine production at the single-cell level.

The wide age range of participants in our study groups presents a study limitation. Because it is known that different ages are associated with differences in T-cell maturation and differentiation (47), this is likely to be an important contributor to the large range of mycobacteria-specific T-cell frequencies observed. Ideally, a larger sample size would have allowed stratification of children by age, but this was beyond the scope of this study. We observed an inverse correlation between age and the magnitude of the CD4 T-cell response in healthy children. Multiple factors may affect the magnitude of response, including time since BCG vaccination and the natural waning of the proportion of naive T cells with age (47). Additional studies are required to understand this association.

Another caveat in our study was the variation in time on treatment and time since diagnosis of children in the TB group; however, there was no correlation with frequency of cytokine-producing T cells. Based on studies of culture conversion under treatment and longitudinal observations of immune responses using IFN- γ release assays (53), we reasoned that a significant decline in antigenic load occurs during the early phase of TB treatment with more inherent variability than at later stages and that the longevity of effector responses is yet to be better defined. We therefore decided to enroll into the TB group those children who were already well established on their course of treatment. Another limitation was the unknown *M. tuberculosis* infection status in the HIV-infected and healthy control groups. Latent infection may have a marked effect on the magnitude and phenotype of T cell responses and analysis of infection status as a covariate could have been additionally informative.

The aim of vaccination is the induction of immunological memory. It is therefore essential to better understand memory profiles in children with and without comorbidity who might receive such vaccines, and we chose to extensively investigate the memory profiles of the antigen-specific T cells, because such data in older children are lacking.

Central memory T cells, along with IL-2 expression, are believed to be the optimal phenotype for long-lived protective immunity after infection or vaccination (26). We report here that the Ag85B/TB10.4-specific T-cell response was characterized by an effector memory phenotype. This is in agreement with memory phenotypes reported previously on mycobacteria-specific T cells in adults (54), children (55), and infants (44, 45). Both presence and absence of the differentiation marker, CD27, was observed on these effector memory CD4 and CD8 T cells, suggesting an intermediate to an advanced stage of differentiation. This consistently observed effector memory phenotype suggests high levels of exposure to mycobacterial antigens as seen in chronic viral infections (27), either through exposure to environmental mycobacteria, *M. tuberculosis*, or, less likely, persistent antigens after BCG vaccination. Alternatively, mycobacteria-specific T cells may exhibit alternative differentiation or maturation pathways to those defined in chronic viral models (27). Along these lines, a considerable proportion of mycobacteria-specific T cells express Th1 cytokines, but show a “naive” CD45RA⁺CCR7⁺ phenotype. Such T-cell phenotypes have previously been reported in other studies of antimycobacterial T cells by us and others (44, 45, 54, 56). We previously suggested that this CD45RA⁺CCR7⁺ population reflects early differentiation into Ag-specific cells, before losing CD45RA expression (44). However, this phenotype is not observed in human viral infections (27). Additional studies are required to investigate this further.

Whether mycobacteria-specific T-cell responses in HIV-infected children change during cART should be addressed in

a longitudinal study following children during the course of cART, because further optimization strategies for vaccination of this extremely vulnerable population could be guided by findings of additional reconstitution of memory populations deemed to be central to the vaccine response.

In summary, we report substantial frequencies of mycobacteria-specific effector CD4 and CD8 T-cell responses, which may be augmented by boost vaccines, even in HIV-infected children. These findings are encouraging in the context of ongoing trials of novel TB vaccines.

Conflict of Interest Statement: N.G.T.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.J.W. holds a patent that has been received or pending from Institut Pasteur, United States 10/994,191, confirmation number 1197 "Recombinant adenylate cyclase of *Bordetella* sp for diagnostic and immunomonitoring uses, method of diagnosing or immunomonitoring using said recombinant adenylate cyclase, and kit for diagnosis using said recombinant adenylate cyclase, and kit for diagnosis." P.A. is a full-time employee of Statens Serum Institut and is the named inventor of patents on TB vaccine and diagnostic antigens; all rights are assigned to Statens Serum Institut. W.A.H. received up to \$1,000 from GlaxoSmithKline Bio in consultancy fees and up to \$1,000 from NFID in lecture fees. B.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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