HIV-1 Infection in Infants Severely Impairs the Immune Response Induced by Bacille Calmette-Guérin Vaccine

Nazma Mansoor,1,a Thomas J. Scriba,1,a Marwou de Kock,1 Michele Tameris,1 Brian Abel,1 Alana Keyser,1 Francesca Little,2 Andrea Soares,1 Sebastian Gelderbloem,1 Silvia Mlenjeni,1 Lea Denation,1 Anthony Hawkridge,1,2 W. Henry Boom,4 Gilla Kaplan,5 Gregory D. Hussey,1 and Willem A. Hanekom1

1South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine and School of Child and Adolescent Health, University of Cape Town, and 2Aeras Global Tuberculosis Vaccine Foundation, Cape Town, and 3Department of Statistical Sciences, University of Cape Town, Rondebosch, South Africa; 4Tuberculosis Research Unit, Case Western Reserve University and University Hospitals Case Medical Center, Cleveland, Ohio; 5Public Health Research Institute, University of Medicine & Dentistry of New Jersey, Newark

Background. Worldwide, most infants born to mothers infected with human immunodeficiency virus (HIV) receive bacille Calmette-Guérin (BCG) vaccine. Tuberculosis is a major cause of death among infants infected with HIV in sub-Saharan Africa, and it should be prevented. However, BCG may itself cause disease (known as “BCGosis”) in these infants. Information regarding the immunogenicity of BCG is imperative for the risk/benefit assessment of BCG vaccination in HIV-infected infants; however, no such data exist.

Methods. We compared BCG-induced CD4 and CD8 T cell responses, as assessed by flow cytometry, in HIV-infected (n = 20), HIV-exposed but uninfected (n = 25), and HIV-unexposed (n = 23) infants, during their first year of life.

Results. BCG vaccination of the 2 HIV-uninfected groups induced a robust response, which was characterized by CD4 T cells expressing interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and/or interleukin (IL)–2. In contrast, HIV-infected infants demonstrated a markedly lower response throughout the first year of life. These infants also had significantly reduced numbers of polyfunctional CD4 T cells coexpressing IFN-γ, TNF-α, and IL-2, a finding that is thought to indicate T cell quality.

Conclusions. Infection with HIV severely impairs the BCG-specific T cell response during the first year of life. BCG may therefore provide little, if any, vaccine-induced benefit in HIV-infected infants. Considering the significant risk of BCGosis, these data strongly support not giving BCG to HIV-infected infants.

The Mycobacterium bovis bacille Calmette-Guérin (BCG) vaccine is given at birth to almost all infants born in sub-Saharan African countries [1]. Although BCG affords highly variable protection against pulmonary tuberculosis (TB), the vaccine also protects against severe disseminated forms of TB in infancy, with estimated efficacies against meningitis and miliary disease of 73% (range, 67%–79%) and 77% (range, 58%–87%), respectively [1]. However, there is no clear evidence that BCG protects HIV-infected infants against these forms of the disease [2]. It is estimated that, in sub-Saharan Africa, 1500 infants are born with HIV type 1 infection every day [3]. TB is common in this population; in South Africa, the incidence of TB in HIV-infected infants and children was 9.2% in 2004, and the

© 2009 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2009/19907-0010$15.00 DOI: 10.1086/597304
disease remains one of the main identifiable causes of death in this population [4]. Rather than provide protection against severe TB, BCG vaccination of HIV-infected infants may cause disease known as "BCGosis" [5, 6]. BCGosis, which is estimated to affect 110–417 per 100,000 vaccine recipients in South Africa [5], can occur in localized or disseminated forms and can cause significant associated morbidity [7]. To guide a more comprehensive assessment of the risks and benefits associated with BCG vaccination in HIV-infected infants, we assessed whether BCG induces the immune response thought to be required to protect infants against TB.

The immune determinants of vaccination-induced protection against TB are not fully understood. However, the Th helper type 1 (Th1) cytokine response, characterized by interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and interleukin (IL)–2 production, is widely thought to be essential. Recently, we have shown that, in HIV-uninfected infants, BCG induces CD4 and CD8 T cell populations that express combinations of IFN-γ, TNF-α, and IL-2 [8]. These cell populations include "polyfunctional" CD4 T cells (i.e., cells that coexpress all 3 cytokines [8]), which are considered to be good indicators of the quality of the immune response. Although evidence in humans remains inconclusive, data for animals suggest that polyfunctional T cell responses correlate with protection against TB in novel TB vaccine studies (P. Anderson, personal communication). We therefore obtained measurements of these T cell markers as primary outcomes in the present study, in a comparison of HIV-infected and HIV-unexposed, healthy infants.

A secondary aim of the present study was to assess whether immunity induced in HIV-uninfected infants born to HIV-infected mothers was similar to that in HIV-unexposed, healthy infants. There are no data on the efficacy of BCG vaccination in HIV-unexposed, uninfected children. However, multiple studies have shown immunologic changes [9–11] in and increased morbidity [12, 13] among HIV-exposed infants.

**MATERIALS AND METHODS**

**Participants.** Infants born to HIV-infected and HIV-uninfected women and recruited at clinics or maternity wards in the Worcester region of the Western Cape, South Africa, were enrolled between 2003 and 2006. All infants had received documented vaccination with BCG (strain Staten Serum Institute [SSI]) on the first day of life. Maternal HIV status was determined by HIV antibody ELISA, which was performed before study enrollment. The HIV infection status of the infants was determined by viral amplification done at 6 weeks of age. Infants were assigned to 1 of 3 groups: (1) infants born to HIV-uninfected mothers (hereafter known as “HIV− infants”), (2) HIV-uninfected infants born to HIV-infected mothers (hereafter known as “exposed HIV− infants”), or (3) HIV-infected infants (hereafter known as “HIV+ infants”). Antiretroviral therapy (ART) was not routinely available for the treatment of HIV infection during the study period; at that stage, the public health service focused on prevention of mother-to-child transmission.

The protocol was approved by the research ethics committee of the University of Cape Town and by the institutional review board of Rockefeller University (New York, New York) and the University of Medicine & Dentistry of New Jersey (Newark). Good clinical practice and ethics guidelines of the US Department of Health and Human Services and the South African Medical Research Council were followed, and written, informed parental consent was therefore obtained.

**Blood sample collection and processing.** Blood was collected into heparinized tubes when infants were 3, 6, 9, and 12 months of age. This blood was immediately processed at the field site, by use of whole-blood assays that previously had been optimized for this purpose [8, 14]. Whole blood was incubated with BCG and anti-CD28 and anti-CD49d antibodies for 12 h. Blood incubated with costimulatory antibodies alone (i.e., unstimulated blood) served as a negative control. After 7 h, 50–100 μL of plasma was removed and cryopreserved for soluble cytokine analysis. Brefeldin A (10 μg/mL; Sigma) was added for the last 5 h of incubation. After incubation, red blood cells were lysed and white blood cells were fixed with FACS Lysing Solution (BD Biosciences) and then cryopreserved. A small aliquot of heparinized blood was stained for the presence of CD3, CD4, CD8, and CD45. A full blood count and HIV load (for HIV+ infants only) were determined at each time point.

**Antigens and antibodies.** Live BCG was reconstituted from the vaccine vial (SSI) at 1.2 × 10⁶ cfu/mL, as described elsewhere [14]. Staphylococcal enterotoxin B (Sigma) was used as positive control at 10 μg/mL. Anti-CD28 and anti-CD49d antibodies (BD Biosciences) were each used at 0.5 μg/mL. For flow cytometry, the following antibodies were used: anti-CD3 Pacific Blue or phycoerythrin (PE) (UCHT1), anti-CD4 allophycocyanin (SK3), anti-CD45 fluorescein isothiocyanate (FITC) (HI30), anti-CD8 peridinin–chlorophyll protein complex (PerCP)–Cy5.5 or PerCP (SK1), anti–IFN-γ AlexaFlour700 (B27), anti–IL-2 FITC (5344.111), and anti–TNF-α PE-Cy7 (monoclonal antibody 11), all from BD Biosciences.

**Cell counts, staining, and flow cytometric analysis.** Frequencies of CD4 and CD8 lymphocytes were determined by flow cytometry after whole blood was stained for the detection of CD4, CD8, and CD45. These values were used with lymphocyte counts and the results of staining for CD3, to calculate the absolute numbers of CD4 and CD8 T cells per milliliter of blood.

Intracellular cytokines were acquired with the use of an LSR II flow cytometer (BD Biosciences) and were analyzed as described elsewhere [8]. Automated compensation with mouse IgGκ beads and cell controls was applied. Flow cytometric plots are displayed using biexponential scaling. Gating strategies are shown in figure 1, which appears only in the electronic version of the Journal.
Factors, to cope with repeated measurements. This has the effect of imposing a nonzero, equal correlation structure on all measures of a participant. These models were fitted using maximum-likelihood estimation, and, thus, observations made at each time point influenced estimates of treatment or other effects at every time point, including the missing time points, through the specification of the correlation pattern [15]. The numbers of infants in each group at the different time points are listed per analysis in each figure legend, and the total number of infants per group and time point are listed in table 1.

Time was modeled as a continuous (as opposed to categorical) effect, and either linear or quadratic time profiles were fitted. In this way, time profiles were smoothed. All group effects estimated from these models are thus adjusted for time, but we chose not to report effects of time in the tables. Total CD4 (or CD8) cell counts were added to the models to adjust group comparisons for differences in CD4 (and CD8) cell counts (table 2 and figure A2, the latter of which appears in the Appendix, which is available only in the electronic version of the Journal).

Estimated group differences in log-transformed values were exponentiated to determine the proportional differences between groups, with respect to original untransformed measures (table 2). The distributions of the T cell frequency data were extremely skewed, and log transformations did not result in symmetrical distributions. As a result, normal-base linear regression–type models could not be used to model the frequency data. These measurements were thus summarized by group and time point, by use of medians and interquartile ranges, and were compared at each time point by use of the Kruskal-Wallis (for overall effect) and Mann-Whitney U tests. Resulting P values for frequency data should be interpreted conservatively because of the increased chance of false-positive findings resulting from multiple testing. Furthermore, comparison at later time points may be biased as a result of infant dropout.

RESULTS

Infant follow-up and losses. Of 298 infants born to HIV+ mothers, 20 were identified as belonging to the HIV+ group by means of a positive viral amplification test result. The number of infants in the HIV+ group decreased gradually over the 12-month period (table 1). Eight infants died during the study, which was conducted before ART was routinely available for the treatment of HIV infection in South Africa, and 4 infants were lost to follow-up. The median log_{10} plasma viral loads in the group of HIV+ infants at the 4 time points were 5.7, 5.6, 4.9, and 4.9 RNA copies/mL, respectively. Three infants received ART, and their data after initiation of ART were excluded from analysis. Twenty-five of the 278 HIV− infants born to HIV+ mothers were included in the exposed HIV− group. None of these infants were breast-fed. Thirteen of these infants were lost to follow-up during their first year of life. Twenty-three infants born to HIV− mothers were also enrolled in the study. Of these infants, 4 were lost to follow-up. The data analysis approach that was used specifically assessed whether measured outcomes were reliable, regardless of losses to follow-up (see the “Data analysis and modeling” subsection of Materials and Methods).

BCG-specific Th1 cell counts were reduced in HIV+ infants. We measured the BCG-induced immune response in infants by quantifying Th1 cytokine expression in BCG-specific T cells by use of flow cytometry. The BCG-specific response comprised multifunctional CD4 and CD8 T cell populations expressing IFN-γ, TNF-α, and/or IL-2 (figure 1, which appears only in the electronic version of the Journal), as reported elsewhere [8]. In all 3 infant groups, the magnitude of cells producing cytokines

Table 1. The number of participants in each group, by age in months.

<table>
<thead>
<tr>
<th>Group</th>
<th>3 months</th>
<th>6 months</th>
<th>9 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+</td>
<td>20</td>
<td>12</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Exp. HIV−</td>
<td>25</td>
<td>17</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Unexp. HIV−</td>
<td>23</td>
<td>22</td>
<td>22</td>
<td>19</td>
</tr>
</tbody>
</table>

NOTE. Exp. HIV−, exposed HIV uninfected; HIV+, HIV infected; Unexp. HIV−, HIV uninfected.
peaked at 3 months and then waned over the 9-month follow-up (figure 2). Throughout follow-up, the total BCG-specific CD4 T cell response, comprising all cells expressing cytokines, was significantly lower in HIV+ infants than in the other 2 groups (table 2 and figure 2A). This pattern was also seen when the absolute numbers of BCG-specific CD4 T cells that expressed IFN-γ, IL-2, or TNF-α were quantified (table 2 and figure 2B–D). No differences in total cytokine-expressing or IFN-γ–, IL-2–, or TNF-α–expressing CD4 T cells were seen between exposed HIV+ and HIV− infants.

In a complementary analysis, we showed that BCG-specific CD4 T cell loss was independent of CD4 T cell counts. Please see the Appendix, which appears only in the electronic version of the Journal.

**Specific polyfunctional CD4 T cell responses were compromised in HIV+ infants.** We quantified BCG-induced polyfunctional CD4 T cells, which simultaneously express IFN-γ, IL-2, and TNF-α, in the 3 infant groups. The absolute numbers of these cells in HIV+ infants were significantly lower than those noted in the other 2 groups, throughout the first year of life (table 2 and figure 3). By 9 months of age, the depletion of polyfunctional cells was striking in the HIV+ group; these cells were barely detectable in most HIV+ infants. Again, no differences were observed between exposed HIV+ and HIV− infants (table 2 and figure 3).

Next, we compared the proportions of monofunctional (producing 1 cytokine), bifunctional (producing 2 cytokines) and polyfunctional (producing 3 cytokines) CD4 T cells in these infant groups. The BCG-specific CD4 T cell response, which was significantly lower in HIV+ infants than in other infant groups (figure 2), was also predominantly comprised of monofunctional cells (figure 3B). HIV− and exposed HIV− infants displayed markedly higher proportions of bi- and polyfunctional specific CD4 cells than did HIV+ infants (figure 3B).

### Table 2. Differences in absolute T cell responses between infant groups estimated from mixed-effects maximum-likelihood regression models (overall effect).

<table>
<thead>
<tr>
<th>Cell subset, HIV status</th>
<th>Absolute count</th>
<th>Unadjusted for CD4 or CD8 cell counts</th>
<th>Adjusted for CD4 or CD8 cell counts</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD4 cytokine response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. HIV− vs. HIV+</td>
<td>4.032 (2.289–7.315)</td>
<td>&lt;.0001</td>
<td>3.155 (1.800–5.529)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. HIV+</td>
<td>5.479 (3.053–9.836)</td>
<td>&lt;.0001</td>
<td>3.762 (2.098–6.746)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. Exp. HIV−</td>
<td>1.339 (0.815–2.198)</td>
<td>.249</td>
<td>1.192 (0.748–1.900)</td>
<td>.459</td>
<td></td>
</tr>
<tr>
<td>Total IFN-γ CD4 response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. HIV− vs. HIV+</td>
<td>6.056 (2.939–12.478)</td>
<td>&lt;.0001</td>
<td>4.586 (2.232–9.422)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. HIV+</td>
<td>8.628 (4.166–16.912)</td>
<td>&lt;.0001</td>
<td>5.783 (2.740–12.207)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. Exp. HIV−</td>
<td>1.425 (0.772–2.694)</td>
<td>.258</td>
<td>1.261 (0.695–2.289)</td>
<td>.445</td>
<td></td>
</tr>
<tr>
<td>Total IL-2 CD4 response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. HIV− vs. HIV+</td>
<td>4.187 (2.177–8.053)</td>
<td>&lt;.0001</td>
<td>3.142 (1.670–5.918)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. HIV+</td>
<td>6.501 (3.374–12.528)</td>
<td>&lt;.0001</td>
<td>4.267 (2.210–8.248)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. Exp. HIV−</td>
<td>1.553 (0.895–2.694)</td>
<td>.11</td>
<td>1.358 (0.808–2.280)</td>
<td>.248</td>
<td></td>
</tr>
<tr>
<td>Total TNF-α CD4 response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. HIV− vs. HIV+</td>
<td>3.979 (2.168–7.308)</td>
<td>&lt;.0001</td>
<td>3.124 (1.726–5.646)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. HIV+</td>
<td>6.068 (3.294–11.179)</td>
<td>&lt;.0001</td>
<td>4.259 (2.298–7.893)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. Exp. HIV−</td>
<td>1.525 (0.908–2.557)</td>
<td>.11</td>
<td>1.363 (0.834–2.230)</td>
<td>.216</td>
<td></td>
</tr>
<tr>
<td>IFN-γ/IL-2/TNF-α CD4 response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. HIV− vs. HIV+</td>
<td>7.538 (3.099–18.338)</td>
<td>&lt;.0001</td>
<td>5.371 (2.239–12.884)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. HIV+</td>
<td>14.069 (5.737–34.501)</td>
<td>&lt;.0001</td>
<td>8.645 (3.480–21.477)</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. Exp. HIV−</td>
<td>1.866 (0.872–4.003)</td>
<td>.108</td>
<td>1.608 (0.775–3.340)</td>
<td>.202</td>
<td></td>
</tr>
<tr>
<td>Total IFN-γ CD8 response</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. HIV− vs. HIV+</td>
<td>3.190 (1.135–8.844)</td>
<td>.028</td>
<td>3.209 (1.059–9.728)</td>
<td>.039</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. HIV+</td>
<td>2.866 (1.029–7.980)</td>
<td>.044</td>
<td>2.872 (1.020–7.463)</td>
<td>.046</td>
<td></td>
</tr>
<tr>
<td>HIV− vs. Exp. HIV−</td>
<td>0.899 (0.380–2.125)</td>
<td>.808</td>
<td>0.895 (0.364–2.201)</td>
<td>.810</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; Exp. HIV−, exposed HIV uninfected; HIV+, HIV infected; HIV−, HIV uninfected; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

**a** Ratio of means between infant groups.

**b** The 95% CI of the difference ratio.
CD8 T cells did not compensate for the loss of specific CD4 responses in HIV+ infants. We have previously shown that BCG vaccination induces CD8 T cells, although at lower frequencies than CD4 T cells [8]. Specific CD8 T cells mostly express IFN-γ and, to a lesser extent, TNF-α, IL-2, and cytotoxic markers, such as granzymes and perforin [8, 16]. Because the CD4 T cell compartment is compromised in HIV+ infants, we hypothesized that the CD8 T cell response would compensate for this loss. CD8 T cells producing IFN-γ were detectable in most infants, in all groups; however, cells producing TNF-α and IL-2 were very infrequent and mostly undetectable (data not shown). Regression modeling of absolute numbers of BCG-specific IFN-γ-expressing CD8 T cells demonstrated lower responses in the HIV+ group than in the HIV- group (figure 4A). Similar patterns were observed when the frequencies of IFN-γ+ CD8 T cells were analyzed (figure 4B).

BCG-specific secretion of IFN-γ and IL-2 was reduced in HIV+ infants. To confirm the flow cytometry results, we quantified BCG-specific secretion of soluble IFN-γ, IL-2, and TNF-α in plasma. As seen for CD4 T cell cytokine production, the IFN-γ and IL-2 levels at 3 months were significantly lower in HIV+ infants, compared with exposed and unexposed HIV- infants (figure 5A and 5B). Lower IFN-γ and IL-2 values persisted at later time points in the HIV+ group, although these differences were not always statistically significant (figure 5B). TNF-α levels were typically 10-fold higher than IFN-γ and IL-2 levels and were not different between the infant groups, across all time points (figure 5C).

DISCUSSION

To determine whether BCG vaccination is immunogenic in HIV+ infants, we assessed the induction of CD4 and CD8 T cells.
producing Th1 cytokines, a response thought to be required for the control of *Mycobacterium tuberculosis* infection. We found markedly lower numbers of BCG-specific CD4 T cells in HIV+ infants, compared with HIV− infants, 3 months after vaccination of newborns. Furthermore, these numbers dwindled to virtually undetectable levels later during the first year of life. We also showed that the ability of CD4 T cells to simultaneously express IFN-γ, IL-2, and TNF-α, which is thought to indicate the “quality” of the immune response, was diminished in HIV+ infants, compared with nonexposed infants. In addition, we found that the relatively low BCG-induced CD8 T cell response was also lower in HIV+ infants than in HIV− infants, and we therefore could not compensate for the loss of BCG-specific CD4 T cell immunity. Overall, our results demonstrate that the BCG-induced adaptive immune response is severely impaired and, by 9 months, virtually absent in infants with HIV infection.

The severely impaired T cell response in HIV+ infants suggests that BCG may afford very little, if any, vaccine-induced benefit. We cannot exclude the possibility that immune mechanisms other than T cells, such as antibody responses, could be involved in BCG-induced protection. However, overwhelming evidence supports the central importance of the immune function that we examined. Considering the significant risk of BCG disease developing in this vulnerable group, our data support the recommendation by the World Health Organization (WHO) Global Advisory Committee on Vaccine Safety that BCG should not be administered to infants who are known to be HIV+ [17]. BCG vaccination of HIV+ infants remains critically important to the prevention of disseminated disease [1]. The WHO Committee recommends that vaccination of infants born to HIV+ mothers be delayed until the presence of infection has been excluded by a viral amplification test, usually at 6 weeks of age [17]. Unfortunately, in most developing countries, there is no guarantee that infants will return to the clinic for this test, or the test may not be routinely available. In these settings, the recommendation remains to vaccinate all infants at birth [17].

Two questions emerge from the WHO recommendations. First, is BCG immunogenic in exposed HIV− infants? Second, what are the effects of delaying vaccination beyond the immediate newborn period in this population? Regarding the first question, multiple studies have suggested that, in HIV− infants, maternal HIV infection may adversely influence immune responses to various microorganisms [10–13]. Increased global T cell activation in infants exposed to HIV has also been shown [9–11], and these factors may contribute to the increased mortality and morbidity reported in this population [12, 13]. We therefore hypothesized that BCG-induced immunity in exposed HIV− infants would be inferior to that in infants who were not exposed to HIV. However, we found no difference in immunity between these 2 infant groups. Our data imply that the BCG-induced immune response in exposed HIV− infants appears to be normal, and that this group would benefit from vaccination. Our findings are in agreement with another study showing that there were no significant differences in the BCG-specific release of IFN-γ, as measured by ELISA in 7-day whole-blood assays per-

![Figure 3](https://academic.oup.com/jid/article-abstract/199/7/982/857985)
formed for exposed HIV− infants, compared with HIV+ infants, at 6 weeks of age [11]. However, when these investigators used purified protein derivative as antigen for the same analysis, a lower IFN-γ response was observed in exposed HIV− infants. We are currently addressing the second question posed above, by comparing longitudinal immunity induced when exposed HIV− infants are vaccinated at birth and at 10 weeks of age.

The present study is also the first to describe the kinetics of the BCG-induced immune response in infants in some detail. Given our preselected time points, the CD4 T cell response peaked at 3 months after vaccination and waned over time. However, BCG-induced protection against disseminated forms of TB persists until at least 5 years of age [1]. This finding may imply that, after contraction of the peak response measured at 3 months of age, central memory T cells may migrate from the blood to the lymph nodes or specific organs, resulting in a quantitatively lower response that is measurable in peripheral blood. These observations underline the importance of studies of BCG-induced immune correlates of protection.

Interestingly, the proportion of CD4 T cells coexpressing 2 or 3 cytokines decreased over time in all groups. The presence of these polyfunctional T cells may indicate the “quality” of the immune response, as in HIV infection the presence of HIV-specific polyfunctional T cells is associated with a better clinical outcome [18, 19]. Similar observations have been made in murine models of Leishmania infection [18, 19] and M. tuberculosis infection [19] (P. Anderson, personal communication). A similar evolution from a high to a low proportion of polyfunctional T cells was reported for Ag85A-specific CD4 T cells after vaccination of healthy adults with a novel TB vaccine, MVA85A [20]. A polyfunctional response may therefore reflect an “effector” response soon after antigen exposure, whereas maturation of T cells may be associated with differential cytokine profiles. Alternatively, polyfunctional T cells may have redistributed to lymph nodes or specific organs.

Findings from T cell analysis were confirmed when soluble IFN-γ and IL-2 were measured in plasma; both levels were significantly decreased in the HIV+ infant group. The comparable soluble TNF-α levels between the different infant groups are likely the result of TNF-α production by BCG-reactive innate cells, such as monocytes, neutrophils, and NK cells. Indeed, soluble TNF-α concentrations exceeded IFN-γ and IL-2 concentrations by ~10-fold, suggesting the existence of an additional source of this cytokine.

The significant dropout rates in the HIV+ infant group, which primarily were due to death, constituted a limitation of the present study. These death rates are consistent with those observed in other African infant cohorts not receiving ART [21]. We therefore employed maximum-likelihood mixed-effects linear regression modeling, which is designed to cope with missing data points. These models generated results similar to those observed when less sophisticated, nonparametric tests were applied. Another limitation was that no data were available from infants not vaccinated with BCG. Such data would confirm that T cell responses are absent in BCG-naive infants and, thus, that our assay system detects specific T cells. Because BCG is rou-
tinely given to all South African infants on the first day of life, analysis of infants not vaccinated with BCG was not possible. However, some authors of the present study conducted another study, in which an assay identical to the one used in the present study was utilized; in that other study, no BCG-specific T cell responses were detected in BCG-naive infants from South Africa (B. M. N. Kagina and W.A.H., unpublished data).

We did not prospectively address covariates that could have affected infant immunity, such as maternal viral load and immune status, maternal therapy, infant nutritional status, and additional infections. Regardless, the BCG-specific T cell responses in HIV+ infants remained strikingly different in the 2 control infant groups.

In conclusion, our findings suggest that BCG vaccination may not protect HIV+ infants against TB. How can protection against TB then be achieved in HIV+ infants? One approach may be to initiate highly active antiretroviral therapy as early as possible for these infants and thereafter vaccinate them with BCG. Evidence is emerging that the use of early ART for HIV+ infants significantly decreases mortality, compared with initiation of treatment when indicated by clinical or CD4 T cell criteria [22–24]. Moreover, the course of BCG-related illnesses, including BCG-associated immune reconstitution inflammatory syndrome, in HIV+ infants is less severe when treatment is initiated early [22, 24]. Regardless, the approach of BCG vaccination after early ART would have to be studied formally. An alternative approach is to institute isoniazid prophylaxis in all HIV+ infants. This intervention has been shown to significantly reduce mortality and the incidence of TB in HIV+ infants and children [4]. Many questions surrounding this intervention remain unanswered,
such as the effects of isoniazid on drug resistance in populations. Early isoniazid prophylaxis is therefore the focus of large trials currently under way. Ultimately, a novel TB vaccine, which is safer and hopefully more effective than BCG in HIV+ infants, will constitute the most sustainable intervention.

Acknowledgments

We thank the infants and mothers who took part in this study, their families, and the support of the excellent team at our field site.

References

APPENDIX

EFFECT OF CD4 T CELL COUNT ON OBSERVED RESULTS

It is well recognized that HIV-infected (HIV⁺) persons experience a loss of peripheral CD4 T cells. This is also true for infants; however, there is wide variability in the absolute counts over the first year of life, and the range may often overlap with the CD4 cell counts of healthy children [25]. Our cohort also showed a large degree of overlap in the interquartile range of the CD4 cell count. No differences were seen between the infant groups at 3 months, and the differences seen at 6 and 9 months were small (figure A1B). CD4/CD8 T cell ratios have been reported to be a better predictor of HIV infection over the first year of life [26]. In our cohort, throughout the first year of life, HIV⁺ infants displayed significantly reduced CD4/CD8 ratios, compared with the other 2 infant groups (figure A1D). The lack of striking differences in absolute CD4 cell counts between the groups made it unlikely that the markedly lower absolute numbers of BCG-specific CD4 T cells in HIV⁺ infants were the result of lower numbers of CD4 T cells. Despite this, we used 2 approaches to exclude the possibility of this confounder.

First, we adjusted for CD4 cell counts in the random-effects maximum-likelihood regression model. Adjustment for CD4 cell counts did not significantly change the mean cytokine responses for the infant groups (figure A2). Differences in the BCG-specific total cytokine, polyfunctional, total IFN-γ, total IL-2, and total TNF-α CD4 T cell responses were still highly significant between HIV⁺ infants and the other 2 infant groups, after adjustment for CD4 cell counts (table 2).

Second, we compared the frequencies of BCG-specific CD4 T cell cytokine responses, which are independent of the CD4 T cell count, between the 3 infant groups. Nonparametric tests applied at each time point in the analysis showed that BCG-specific polyfunctional, total IFN-γ, total IL-2, and total TNF-α CD4 T cell frequencies were significantly lower at 3 months of age in HIV⁺ infants than in HIV⁻ infants (figure A3). This finding was consistent throughout follow-up, although the differences were not significant at all time points (figure A3E–H). BCG-specific CD4 T cell frequencies were not different in exposed HIV⁻ and HIV⁺ infants.

References

Figure A1. Absolute lymphocyte (A) and CD4 (B) and CD8 (C) lymphocyte counts, as well as CD4/CD8 lymphocyte ratios (D), at each time point and for each infant group. Differences between groups were assessed using the Kruskal-Wallis test (for overall effect) and the Mann-Whitney U test (P values are shown below each plot).
Figure A2. The mean absolute CD4 T cell count before and after adjustment for CD4 T cell counts estimated with a mixed-effects maximum-likelihood regression model. A, Total interferon (IFN)–γ+ CD4 T cells. B, Total interleukin (IL)–2+ CD4 T cells. C, Total tumor necrosis factor (TNF)–α+ CD4 T cells. D, Polyfunctional CD4 T cells. For each group, the nos. of infants in this analysis are identical to those in figure 2.
Figure A3. Frequencies of bacille Calmette-Guérin (BCG)–specific CD4 T cells, defined by intracellular expression of cytokines at 3 months after vaccination, in each infant group (A–D), and the median frequency of CD4 T cells producing cytokines in each infant group at each time point (E–H).

A and E, Polyfunctional CD4 T cells. B and F, Total CD4 T cells producing interferon (IFN)–γ. C and G, Total CD4 T cells producing interleukin (IL)–2. D and H, Total CD4 T cells producing tumor necrosis factor (TNF)–α. Differences between groups were assessed using the Kruskal-Wallis test (for overall effect) and the Mann-Whitney U test (P values are shown below each plot). A–H, Nos. of HIV-infected (HIV+), exposed HIV-negative (Exp. HIV–), and HIV-unexposed (HIV–) infants per group were 19, 25, and 23 at 3 months; 12, 17, and 22 at 6 months; 8, 15, and 22 at 9 months; and 5, 12, and 17 at 12 months, respectively.
Figure 1. Flow cytometric analysis of bacille Calmette-Guérin (BCG)-specific T cell cytokine production. Representative dot plots for a single infant are shown. 

A, Gating strategy used to identify CD4 and CD8 T cells. White cells from whole blood were acquired, and cell doublets were excluded using forward scatter-area versus -height parameters (left). T cells were then selected from singlets by gating on CD3<sup>H11001</sup> cells with low granularity (middle); CD4 and CD8 T cells were then selected (right). Expression of interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α by CD4 T cells (B) and CD8 T cells (C) was then measured.