

Delaying BCG Vaccination Until 8 Weeks of Age Results in Robust BCG-Specific T-Cell Responses in HIV-Exposed Infants

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(See the editorial commentary by Kay and Blish on pages 335–7.)

Background. BCG vaccination prevents disseminated tuberculosis in children, but it is contraindicated for persons with human immunodeficiency virus (HIV) infection because it can result in severe disease in this population. In tuberculosis-endemic regions, BCG vaccine is administered soon after birth, before in utero and peripartum HIV infection is excluded. We therefore assessed the immunogenicity of BCG vaccine in HIV-exposed infants who received BCG at birth or at 8 weeks of age.

Methods. HIV-exposed, uninfected infants were randomly assigned to receive BCG vaccination at birth (the early vaccination arm) or 8 weeks of age (the delayed vaccination arm). BCG-specific proliferative and intracellular cytokine responses were assessed in 28 infants per arm at 6, 8, and 14 weeks of life.

Results. There was no difference in BCG-specific T-cell proliferation between the study arms 6 weeks after vaccination. However, at 14 weeks of age, the frequency of interferon γ -expressing CD4⁺ T cells and multifunctional BCG-specific responses in the delayed vaccinated arm were significantly higher than those in the early vaccination arm ($P = .021$ and $P = .011$, respectively).

Conclusions. The immunogenicity of BCG vaccination in HIV-exposed, uninfected infants is not compromised when delayed until 8 weeks of age and results in robust BCG-specific T-cell responses at 14 weeks of age. These findings support further evaluation of this modified BCG vaccination strategy for HIV-exposed infants.

Clinical Trials Registration. NCT02062580.

Keywords. BCG vaccination; T-cell proliferation; intracellular cytokines; maternal HIV; infants.

Tuberculosis is a major global health problem, particularly burdensome in sub-Saharan Africa. According to the World Health Organization (WHO) report on tuberculosis, there were nearly 9 million new cases and

1.3 million tuberculosis-related deaths in 2012 [1]. BCG vaccine, composed of a live, attenuated *Mycobacterium bovis* strain, is administered to almost 100 million children annually and remains the only licensed vaccine for tuberculosis prevention [2]. BCG induces strong T-helper type 1 (Th1) responses, even in neonates [2], who have Th2-biased immune system, and protects against severe forms of childhood tuberculosis, such as tuberculous meningitis and miliary tuberculosis in immunocompetent children [3].

In regions of high tuberculosis endemicity, such as South Africa, BCG vaccine is administered soon after birth. However, the vaccine has been associated with serious adverse events in children, such as BCG-induced immune reconstitution inflammatory syndrome after

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initiation of combined antiretroviral therapy (cART) and disseminated BCG disease in HIV-infected infants in the absence of cART [4, 5]. The WHO now recommends that known HIV infection in infants is a contraindication to BCG vaccination [6, 7]. Since infant HIV status is not known at birth, when BCG is typically administered, routine BCG vaccination continues in most countries, including South Africa [8].

Recent studies demonstrated reduced interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) production by HIV-exposed infants in response to BCG vaccination, suggesting that there may be decreased immunogenicity in HIV-exposed infants even in the absence of HIV infection [9, 10]. Kagina et al recently demonstrated that delaying BCG vaccination from birth to 10 weeks of age in HIV-unexposed South African infants resulted in an enhanced memory T-cell response [11]. On the other hand, Burl et al demonstrated that delaying BCG vaccination from birth to 18 weeks of age led to decreased Th1 responses in HIV-unexposed Gambian infants [12]. We hypothesized that administering BCG vaccination to HIV-exposed infants at 8 weeks of age would allow for the exclusion of HIV infection without compromising BCG vaccine-induced immune responses. Therefore, we performed a randomized trial to evaluate CD4⁺ and CD8⁺ T-cell BCG-specific proliferation and cytokine responses in HIV-exposed infants who received BCG at birth or delayed BCG vaccination at 8 weeks of age.

METHODS

Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki [13]. The University of Cape Town and Stellenbosch University research ethics committees and the University of Washington Institutional Review Board approved the study. All mothers provided written informed consent.

Recruitment of Participants and Vaccination

HIV-exposed infants were recruited within a few hours after birth from a community health center in Khayelitsha, Western Cape Province, South Africa, an informal settlement with an antenatal maternal HIV infection prevalence of 30.1% [14]. In this setting, all mothers are offered voluntary counseling and HIV testing at the time of antenatal care registration and again at 32 weeks gestation, with polymerase chain reaction (PCR) analysis performed for HIV DNA detection in infants at 6 weeks of age and again at either 1 year of age or 6 weeks after breast-feeding cessation. Exclusive feeding choices are encouraged. The Western Cape has one of the highest tuberculosis rates globally, and Khayelitsha has a tuberculosis notification rate of 1389 cases/100 000 population [14].

Eligibility criteria included an HIV-infected mother, a birth weight of >2.4 kg, an uncomplicated pregnancy or labor, vaginal delivery, no known close tuberculosis contacts, and term gestation (>36 weeks). Infants were additionally excluded if they

tested positive for HIV DNA by PCR at birth or on routine testing at 6 weeks of age.

Following receipt of consent from mothers, infants underwent PCR analysis for detection of HIV DNA. Infants were randomly assigned via a computer-generated list to receive BCG vaccine (Danish strain 1331; Statens Serum Institute, intradermal [SSI]) as soon as HIV DNA PCR results were available (on days 2–4 of age; the early vaccination arm) or at 8 weeks of age (the delayed vaccination arm). Infants were vaccinated only if they tested negative for HIV DNA by PCR at birth; those in the delayed arm received BCG vaccine at 8 weeks of age only if they also tested negative for HIV DNA by routine PCR at 6 weeks of age. Infants in both arms received all other routine vaccines according to the South African Expanded Program on Immunization schedule [15].

Whole-Blood Culture Assay

The methods for whole-blood assays were adapted from those previously described [16]. Briefly, 1–3 mL of whole-blood specimens were collected from infants at birth and ages 6, 8, and 14 weeks into a preservative-free heparinized tube and transported to the laboratory within 6 hours. Whole blood was mixed in a 1:10 dilution with warm Roswell Park Memorial Institute 1640 medium, plated into a 24-well culture plate, and incubated at 37°C in 5% CO₂ with 1×10^5 colony-forming units/mL of Danish BCG and a negative control (medium alone). Supernatants were removed after incubation for 24 hours, staphylococcal enterotoxin B (SEB) added to the positive control well, and cells were incubated at 37°C for a further 5 days [16]. On day 6, supernatants were collected from culture, and phorbol 12-myristate 13-acetate (PMA) and ionomycin were added, along with brefeldin A, for the last 4 hours of incubation. Cells were harvested using ethylenediaminetetraacetic acid; red blood cells were lysed, and white blood cells were stained with Pacific Blue Live/Dead stain, fixed (FACS Lysing Solution), and cryopreserved in a 10% dimethyl sulfoxide freezing solution for storage at –80°C until analysis.

Ki-67 Proliferation and Intracellular Cytokine Staining Assay

Fixed cryopreserved cells were thawed, permeabilized, and stained for cellular markers, using intracellular cytokine staining, and for proliferation, using Ki-67 [16] with optimized volumes of fluorescence-conjugated antibodies. Expression was measured by multiparameter flow cytometry (BD LSR Fortessa, BD Biosciences, San Jose, CA), using the following monoclonal antibodies for phenotypic and/or intracellular cytokine staining: anti-CD3–allophycocyanin (APC)–cyanine 7 (Cy7; UCHT1), anti-IFN- γ –Alexa Fluor 700 (B27), and anti-interleukin 17 (IL-17)–phycoerythrin–Cy7 (BL168) from Biolegend; anti-CD8–peridinin chlorophyll protein–Cy5.5 (SK1), anti-Ki-67–fluorescein isothiocyanate (B56), and anti-interleukin 1 (IL-2)–APC (5344.111), from BD Biosciences; and VIVID–Pacific Blue (Invitrogen). Samples were acquired on a BD LSR Fortessa flow cytometer.

Data Analysis

Postacquisition compensation and subsequent analysis was performed using FlowJo V9.4.7 (Tree Star, Ashland, OR). Cell doublets were excluded using forward-scatter-area versus forward-scatter-height parameters (Supplementary Figure 1). Live CD3⁺ that were CD8⁺ or CD8⁻ (for CD4⁺ cells since PMA and Ionomycin degrades CD4 protein) were gated. CD3⁺ cells, CD8⁺ versus CD8⁻ cells (in place of CD4⁺ cells, owing to PMA-ionomycin-mediated CD4 degradation in T cells) were gated [17]. CD8⁻ and CD8⁺ cells expressing Ki67⁺ were then quantified. Further, cells expressing IFN- γ , IL-2, and IL-17 were gated within Ki67⁺CD8⁻ and Ki67⁺CD8⁺ cells (Supplementary Figure 1). Fluorescence minus one controls were used to define gates where expression was continuous. Boolean gating was used to quantify cells expressing a single or a combination of multiple cytokines. Data were analyzed with Pestle V1.7 software (Vaccine Research Center, National Institutes of Health), for background subtraction of the negative control responses from that of specific antigens, and Spice V5.22 software, for analysis of multiple-cytokine expression by CD8⁻ and CD8⁺ T cells. Background subtraction of the proliferating cells in the medium alone was performed for proliferative responses. To calculate net cytokine responses, cytokine-expressing Ki-67⁺ cells were subtracted as a proportion of the total number of CD4⁺ or CD8⁺ cells.

The sample size and power calculations were based on the comparison of the median number of CD4⁺Ki67⁺ cells per microliter in 6-week-old HIV-unexposed infants in response to tetanus toxoid, calculated using previously collected data. A sample size of 28 infants per arm allowed 80% power to detect a 5% difference in the number of CD4⁺Ki67⁺ cells per microliter (2-tailed test at a *P* value of .05). Thus, a subset of 28 infants per arm per time point was randomly selected for further T-cell proliferation and cytokine expression analysis in a blinded fashion. Overall, 20 of 168 samples were excluded from further analysis on the basis of the following exclusion criteria: the positive control responses to SEB were below the cutoff (defined as the median value plus 3 median absolute deviations from the negative control value; 10 of 20 samples), and the number of Ki67⁺ proliferating cells was less than the cutoff of <20 (10 of 20 samples).

Graphs of Ki-67 proliferation and cytokine expression levels were generated using GraphPad Prism 5, after subtraction of the Ki-67⁺ cell frequency (ie, the negative control background). Mann–Whitney nonparametric testing was used to compare proliferation and cytokine expression levels between infants in the early arm and those in the delayed arm. The correlations between CD4⁺ Ki-67 proliferative responses before and after vaccination were analyzed using Spearman rank correlation in Stata 10 (StataCorp, College Station, TX). Pestle and Spice (Vaccine Research Center, National Institutes of Health) were used to examine expression of multiple cytokines (polyfunctionality). The overall difference in polyfunctionality was compared using 2-sided analysis of variance (ANOVA).

RESULTS

Study Participants and Characteristics

A total of 149 HIV-exposed infants were recruited between June 2010 and December 2012 (Supplementary Figure 2). Two infants died of conditions unrelated to tuberculosis. Five infants had HIV infection (3.4% transmission rate), of whom only 1 tested negative for HIV at birth, was assigned to the early arm, and therefore received BCG vaccine. Two infants were excluded because of protocol violations (ie, they inadvertently received BCG vaccine at birth). Careful evaluation revealed no tuberculosis signs or symptoms in any infant, and only 1 infant required isoniazid preventive therapy because of a known tuberculosis contact, after tuberculosis was excluded. Therefore, 140 infants were eligible for randomization, and 122 infants completed the study (Supplementary Figure 2). Based on the power calculations, 28 infants per arm were randomly selected from among these 122 infants at each time point. There were no differences in baseline characteristics, including birth weight (3.12 vs 3.05; *P* = .407), maternal CD4⁺ T-cell count (388 cells/mm³ vs 358 cells/mm³; *P* = .103), breast-feeding (17% vs 10%; *P* = .469), sex (*P* = .140), and median gestational age (39 weeks vs 39 weeks; *P* = .653), between the early and delayed arms.

Delayed BCG Vaccination Does Not Affect T-Cell Proliferative Responses to BCG Stimulation

CD4⁺ T-cell proliferation in the early and delayed arms at weeks 6, 8, and 14 weeks of age was evaluated using Ki-67, a cell cycle marker. As expected, at 6 and 8 weeks of age, infants in the early group had higher CD4⁺ T-cell proliferation in response to BCG vaccine than those in the delayed group (*P* < .001 for both comparisons; Figure 1A). At week 14 of age (ie, 6 weeks and 14 weeks after vaccination for the delayed arm and the early arm, respectively), infants in both arms had similar proliferative CD4⁺ T-cell responses (21.5% and 23%; *P* = .968; Figure 1B). At 6 weeks after vaccination (ie, week 6 of age for infants vaccinated at birth and week 14 of age for infants vaccinated at week 8), no significant difference was observed in CD4⁺ T-cell proliferation (20.5% vs 22.4%; *P* = .881; Figure 1C).

Similar to findings for CD4⁺ T cells, CD8⁺ T-cell proliferation in response to BCG vaccine was higher in the early group at 6 and 8 weeks of age (*P* = .036 and *P* = .018, respectively; Figure 1D). At week 14, there was no difference in CD8⁺ T-cell proliferative responses to BCG vaccine between the 2 arms (*P* = .672; Figure 1E). When CD8⁺ T-cell proliferation was compared at 6 weeks after vaccination (ie, week 6 of age for infants vaccinated at birth and week 14 of age for infants vaccinated at week 8), no significant difference was observed (11.18% vs 5.10%; *P* = .120; Figure 1F).

It is possible that preexisting BCG responses (presumably from environmental mycobacteria or maternal *Mycobacterium*

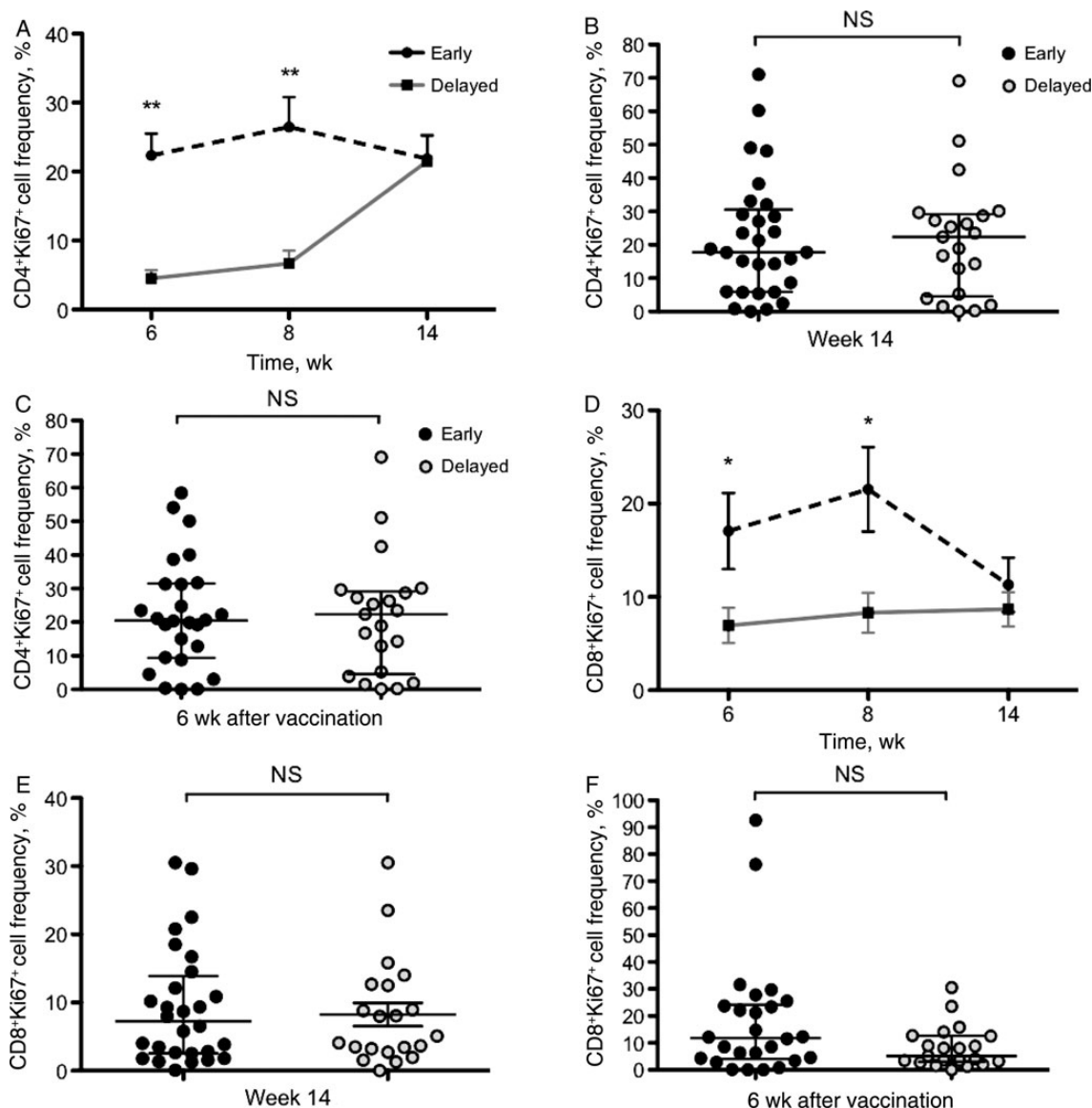


Figure 1. CD4⁺ and CD8⁺ T-cell proliferation in response to BCG stimulation among infants who received BCG vaccine at birth (the early group) or 8 weeks of age (the delayed group). *A*, Longitudinal assessment of the median frequency of CD4⁺Ki67⁺ cells. Whiskers represent interquartile ranges. *B*, Frequency of CD4⁺ T cells expressing Ki67⁺ in the early group (black) and the delayed group (gray) at 14 weeks of age. Lines represent medians, and whiskers represent interquartile ranges. *C*, Frequency of CD4⁺ T cells expressing Ki67⁺ 6 weeks after vaccination (ie, at 6 weeks of age in the early group [black] and 14 weeks of age in the delayed group [gray]). *D*, Longitudinal assessment of the median frequency of CD8⁺Ki67⁺ cells. Whiskers represent interquartile ranges. *E*, Frequency of CD8⁺ T cells expressing Ki67⁺ in the early group (black) and the delayed group (gray) at 14 weeks of age. *F*, Frequency of CD8⁺ T cells expressing Ki67⁺ 6 weeks after vaccination (ie, at 6 weeks of age in the early group [black] and 14 weeks of age in the delayed group [gray]). Lines represent medians, and whiskers represent interquartile ranges. * $P < .05$ and ** $P < .01$. Abbreviation: NS, nonsignificant.

tuberculosis infection) may influence postvaccination responses in infants who received delayed vaccination, although it is unlikely that these infants were exposed to environmental mycobacteria before 8 weeks of age. Few infants had sizable responses to BCG before vaccination, and there was no correlation between BCG-specific responses before vaccination in the delayed arm at week 6 of age and their responses after vaccination, at week 14 of age ($r = 0.027$; $P = .946$). Similarly, CD4⁺ T-cell proliferative

responses at week 8 of age did not correlate with CD4⁺ proliferative responses at week 14 of age ($r = 0.393$; $P = .261$).

Delaying BCG Vaccination Results in Higher CD4⁺ IFN- γ Responses to BCG Stimulation at 14 Weeks of Age

IFN- γ , IL-2, and IL-17 expression in proliferating CD4⁺ T cells was evaluated in the early and delayed groups at weeks 6, 8, and 14 of age. Ki67⁺ cells produced greater levels of cytokines than

Ki67⁺ cells following PMA/ionomycin restimulation on day 6. As expected, infants who received BCG vaccine at birth had a higher proportion of CD4⁺ T cells secreting IFN- γ and IL-2 at 6 and 8 weeks of age ($P < .001$; Figure 2A and 2B). At week 14 of age, however, infants in the delayed arm had a larger proportion of CD4⁺ T cells expressing IFN- γ ($P = .021$; Figure 2A), compared with infants in the early arm, but there was no difference in IL-2 expression (Figure 2B). Similarly, no differences in IL-17 expression were observed between the 2 groups at 14 weeks of age (Figure 2C). Consistent with other studies [16], IL-17 responses to BCG was low in these young infants.

Delaying BCG Vaccination Has No Effect on CD8⁺ Cytokine Responses to BCG Stimulation at 14 Weeks of Age

IFN- γ , IL-2, and IL-17 expression among CD8⁺ T cells was also assessed in the early and delayed groups at 6, 8, and 14 weeks of age (Supplementary Figure 3). Infants who received BCG vaccine at birth had a higher proportion of CD8⁺ T cells expressing

IFN- γ at weeks 6 and 8 of age than those in the delayed group ($P = .049$ and $P < .001$, respectively), but no difference was observed at week 14 of age. Infants who received BCG vaccine at birth had a higher proportion of BCG-specific CD8⁺ T cells expressing IL-2 ($P = .028$) and IL-17 ($P = .009$) at week 6 of age. However, there was no difference in IL-2- or IL-17-expressing CD8⁺ T cells between the 2 arms at weeks 8 and 14 of age.

Delaying BCG Vaccination Has No Effect on CD4⁺ Cytokine Responses but Decreases CD8⁺ IL-2 Expression to BCG Stimulation at 6 Weeks After Vaccination

Since the differences noted at 14 weeks of age could be due to the age at vaccination or the duration of time since vaccination, T-cell cytokine responses 6 weeks after vaccination in the early arm (ie, at week 6 of age) and the delayed arm (ie, at week 14 of age) were compared (Figure 3). There were no significant differences in CD4⁺ T-cell expression of IFN- γ , IL-2, or IL-17 6 weeks after vaccination between the early and delayed arms

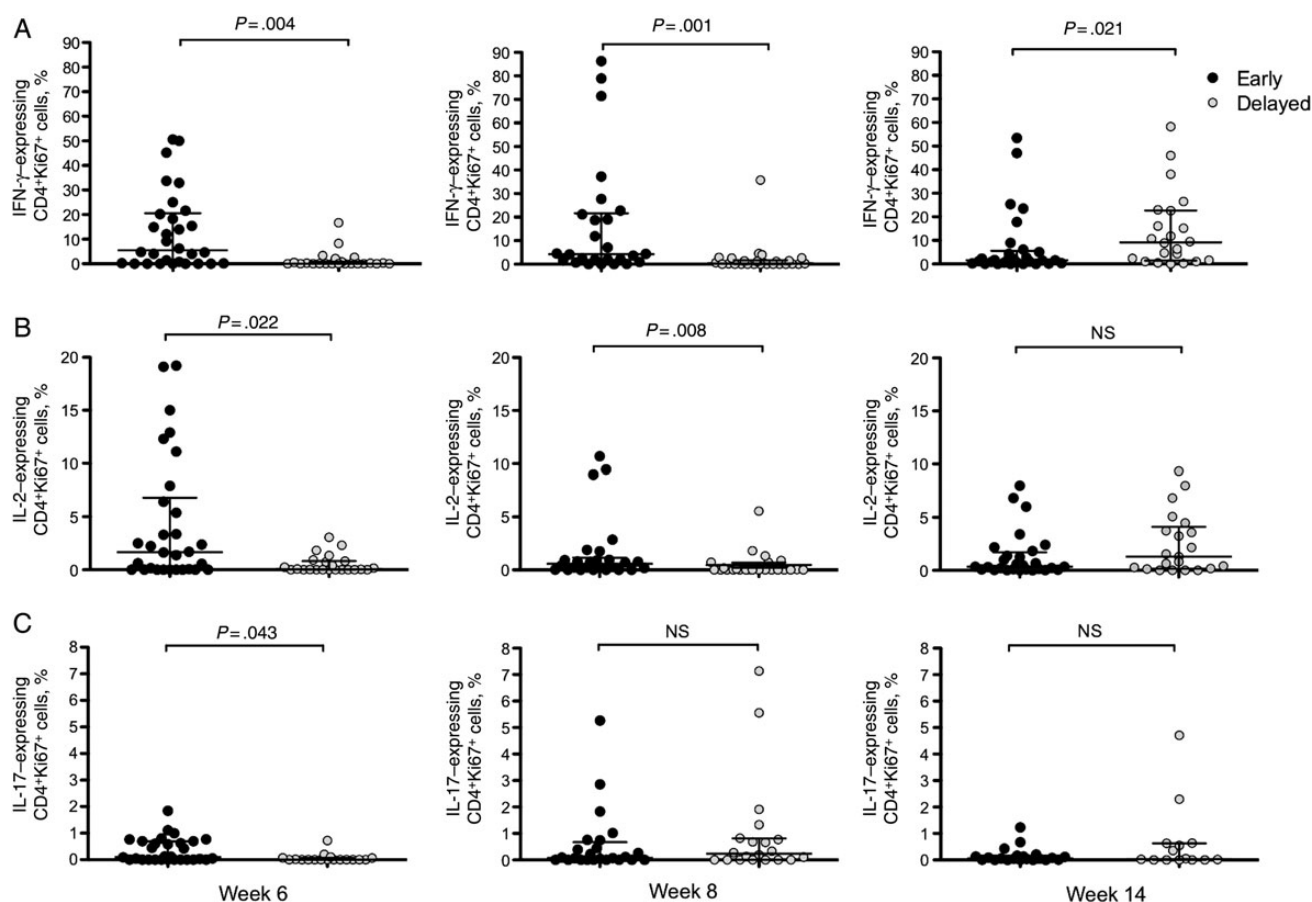


Figure 2. Frequency of proliferating CD4⁺ T-cell intracellular cytokine production among infants who received BCG vaccine at birth (the early group) or 8 weeks of age (the delayed group). *A*, Frequency of CD4⁺Ki67⁺ cells expressing interferon γ (IFN- γ) in the early group (black) and the delayed group (gray). *B*, Frequency of CD4⁺Ki67⁺ cells expressing interleukin 2 (IL-2) in the early group (black) and the delayed group (gray). *C*, Frequency of CD4⁺Ki67⁺ cells expressing interleukin 17 (IL-17) in the early group (black) and the delayed group (gray). Frequencies reported are calculated on the basis of the total number of CD4⁺ T cells. Abbreviation: NS, not significant.

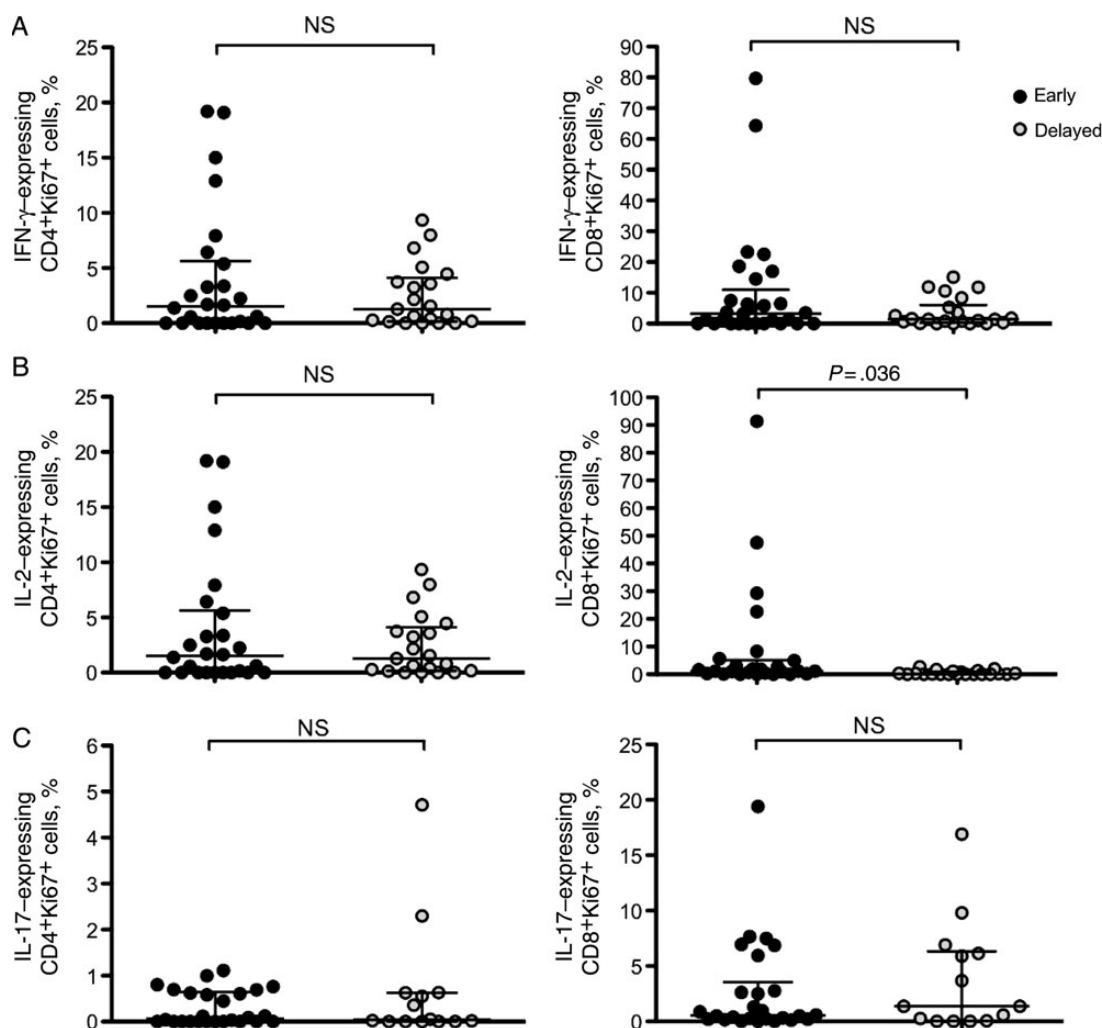


Figure 3. Frequency of proliferating T-cell intracellular cytokine production 6 weeks after vaccination among infants who received BCG vaccine at birth (the early group) or 8 weeks of age (the delayed group). Frequency of CD4⁺ Ki67⁺ and CD8⁺ Ki67⁺ cells expressing interferon γ (IFN- γ ; A), interleukin 2 (IL-2; B), and interleukin 17 (IL-17; C) in the early group (black) and the delayed group (gray) 6 weeks after vaccination (ie, week 6 of age for the early group and week 14 of age for the delayed group). Frequencies reported are of total CD4⁺ T cells and total of CD8⁺ cells. Lines represent medians and whiskers interquartile ranges. Abbreviation: NS, nonsignificant.

(Figure 3A). However, CD8⁺ T cells from infants in the early arm expressed significantly higher IL-2 levels than those from infants in the delayed arm ($P = .036$; Figure 3B).

Delaying BCG Vaccination Increases the Cytokine Functionality of the CD4⁺ and CD8⁺ T-Cell Response to BCG Stimulation at 14 Weeks of Age

We then compared the functionality of proliferating cells by comparing the frequencies of cells making 0 cytokines, any single cytokine, or any combination of 2 or 3 cytokines between infants in the delayed arm and those in the early arm at 14 weeks of age (Figure 4). Overall, CD4⁺ T cells from infants in the delayed arm were significantly more functional in response to BCG stimulation than those in the early arm (Figure 4A;

$P = .011$, by ANOVA). Comparison of the different specific single or multiple cytokine combinations revealed that infants in the delayed arm had significantly higher proportions of proliferating CD4⁺ T cells that produced at least 1 cytokine in response to BCG vaccination and significantly more cells single-positive for IFN- γ than infants in the early arm ($P = .039$ and $.035$, respectively; Figure 4B). The majority of the BCG-specific CD4⁺ T cells from infants in the early arm made none of the measured cytokines.

Comparison of the frequencies of proliferating CD8⁺ T cells that any of one, or a combination of more than one cytokine at 14 weeks (Figure 5), CD8⁺ T cells from the delayed BCG arm also had higher functionality in terms of cytokine responses than those in the early arm (Figure 5A; ANOVA $P = .047$).

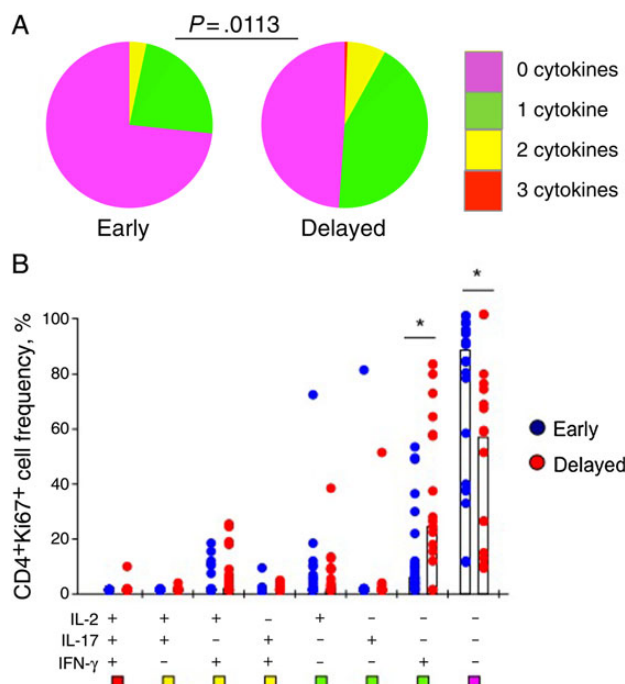


Figure 4. Proliferating CD4⁺ T cells making any combination of cytokine among infants who received BCG vaccine at birth (the early group) or 8 weeks of age (the delayed group). *A*, Pie chart of proliferating cells making 0 cytokines (pink), any single cytokine (green), any combination of 2 cytokines (yellow), or all 3 cytokines (red) in the early group (left) and the delayed group (right). *B*, Frequency of CD4⁺Ki67⁺ T cells making the specific cytokines alone or in combination in the early group (blue) and the delayed group (red). Bars represent medians. **P* < .05. Abbreviations: IFN, interferon; IL-2, interleukin 2; IL-17, interleukin 17.

However, when comparing each individual cytokine or combination of cytokines individually, there were no significant differences between early and delayed BCG vaccines (Figure 5*B*).

Finally, when the functionality of cytokine responses 6 weeks after vaccination was compared (at 6 weeks of age in the early and 14 weeks of age in the delayed arms), there were no differences in overall functionality of T cells in response to BCG vaccine (*P* = .729 for CD4⁺ T cells and *P* = .313 for CD8⁺ T cells; Figure 6). There were also no differences in expression of individual or combinations of cytokines by either CD4⁺ or CD8⁺ T cells (data not shown).

DISCUSSION

This study was designed to evaluate a potential vaccination strategy for HIV-exposed infants that would allow for the implementation of the WHO guidelines, which recommend against administration of BCG vaccine to HIV-infected infants. To our knowledge, this is the first study to examine the effects of delaying BCG vaccination on T-cell responses in HIV-exposed infants, a highly relevant group for such a vaccination strategy.

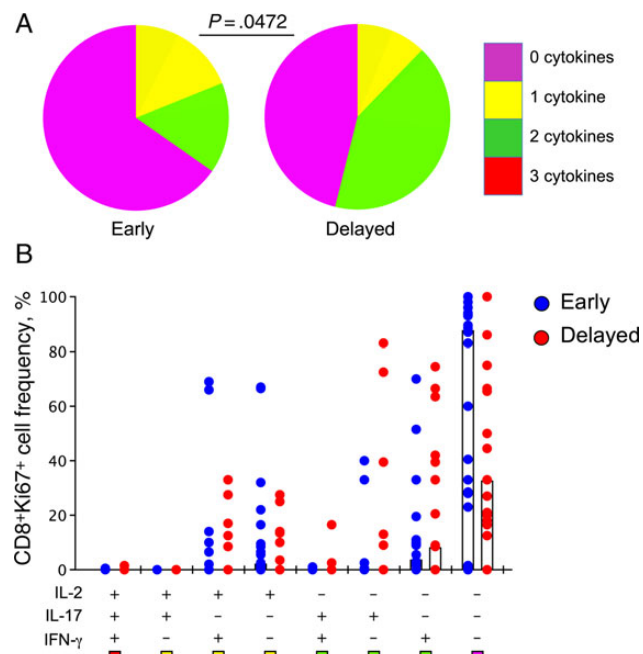


Figure 5. Proliferating CD8⁺ T cells making any combination of cytokines among infants who received BCG vaccine at birth (the early group) or 8 weeks of age (the delayed group). *A*, Pie chart of proliferating cells making 0 cytokines (pink), any single cytokine (green), any combination of 2 cytokines (yellow), or all 3 cytokines (red) in the early group (left) and the delayed group (right). *B*, Frequency of CD8⁺Ki67⁺ T cells making the specific cytokines alone or in combination in the early group (blue) and the delayed group (red). Bars represent medians. Abbreviations: IFN, interferon; IL-2, interleukin 2; IL-17, interleukin 17.

Our findings demonstrate that delaying BCG vaccination from birth to 8 weeks of age does not affect vaccine-specific CD4⁺ and CD8⁺ T-cell proliferation measured at 14 weeks of age in HIV-exposed, uninfected infants.

Delaying BCG vaccination, however, resulted in greater overall functionality of CD4⁺ and CD8⁺ T-cell cytokine responses at 14 weeks. The majority of the differences appear to be due to fewer proliferating cells expressing no measurable levels of cytokines and to a conversely higher frequency of cells producing any one of the cytokines measured. Some evidence suggests that HIV-exposed, uninfected infants may have different Th1 cytokine profiles to BCG vaccination than unexposed infants [9, 10, 18, 19], but very little is known about the effects of HIV-exposure on immune responses to delayed BCG vaccination in these infants. We found that, at 14 weeks of age, HIV-exposed infants who received BCG vaccine at 8 weeks of age had significantly higher frequencies of IFN-γ-secreting CD4⁺ T cells, compared with infants vaccinated at birth. Although the exact immune correlates of protection against tuberculosis remain unknown, IFN-γ-secreting CD4⁺ T cells play an important role in protection against tuberculosis [20, 21]. In addition,

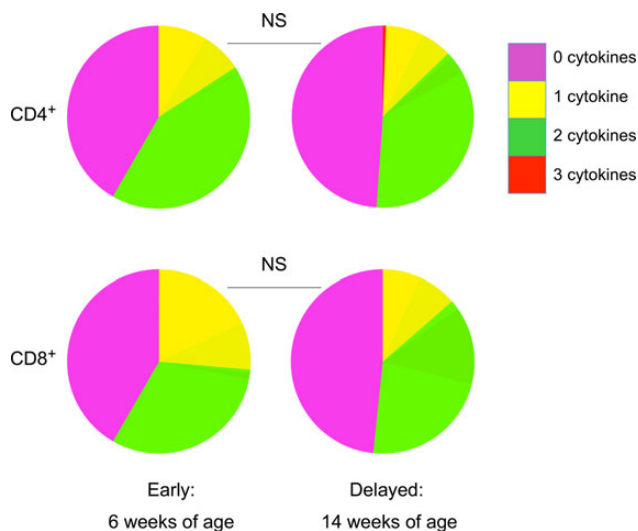


Figure 6. Polyfunctionality of T-cell cytokine responses 6 weeks after vaccination among infants who received BCG vaccine at birth (the early group) or 8 weeks of age (the delayed group). Pie chart of proliferating CD4⁺ (top) and CD8⁺ (bottom) T cells making 0 cytokines (pink), any single cytokine (green), any combination of 2 cytokines (yellow), or all 3 cytokines (red) in the early group (left) and the delayed group (right) 6 weeks after vaccination (ie, at 6 weeks of age for the early group and 14 weeks for the delayed group). Abbreviation: NS, nonsignificant.

higher frequencies of polyfunctional CD4⁺ T cells are recognized as a better quality of response, although such frequencies do not always correlate with protection [22, 23]. Thus, higher frequencies of BCG-specific IFN- γ -secreting CD4⁺ T cells and higher overall functional CD4⁺ T cells observed in the delayed arm at 14 weeks of age could be considered more-optimal responses than at the same time point in the early arm.

Neonates are characterized by defects in IL-12 production and consequent IFN- γ stimulation and, therefore, Th2-skewed responses [24–26]. Since Th1 responses improve with age [25], higher frequencies of BCG-specific IFN- γ -expressing CD4⁺ T cells in infants given BCG vaccine at 8 weeks after birth might be expected. On the other hand, this finding could also be due to the shorter period between vaccination and the time of assay in the delayed arm. To address this, responses 6 weeks after vaccination were compared. The only difference identified in this comparison was higher IL-2 production by CD8⁺ T cells in the early arm. Regardless of the reason, cytokine responses in the delayed group were improved at 14 weeks of age, when infants might begin to be exposed to *M. tuberculosis*.

Previous studies of delayed BCG vaccination in HIV-unexposed infants have shown variable results. In a study from Gambia, delaying BCG vaccination in HIV-unexposed infants to 2 and 4.5 months of age resulted in lower IFN- γ and IL-17 responses in the group for which vaccination was delayed, compared with responses in the group vaccinated at birth [2, 8]. However, similar to our findings in HIV-exposed infants,

Kagina et al found that, in South African HIV-unexposed infants, BCG-specific IFN- γ -expressing CD4⁺ T-cell frequencies 10 weeks after vaccination were significantly higher in the group for which vaccination was delayed, compared with the group vaccinated at birth [11].

It is possible that restimulation of BCG-specific, proliferating cells with PMA/ionomycin may not exactly mimic BCG-specific responses. However, because the use of live, attenuated BCG was an important element of this assay, and since BCG cannot be processed in the presence of BFA, we used the current assay. Soares et al measured intracellular IFN- γ and IL-2 expression in cells from infants who received BCG vaccine after short-term BCG stimulation, and the cells displayed similar cytokine kinetics to those reported here, albeit at lower frequencies [27]. Furthermore, in a study of early versus delayed BCG vaccination in HIV-unexposed infants, this same group stimulated whole blood with BCG and costimulatory antibodies, anti-CD28 and anti-CD49d, and the cytokine profiles identified in the early and delayed groups were similar to our data [11].

Our study is limited by the lack of long-term follow-up. However, we show clear effects of delaying BCG vaccination at 14 weeks of age, a time when HIV-exposed infants living in high-burden tuberculosis settings are at high risk of exposure to *M. tuberculosis* and are exquisitely vulnerable to the development of tuberculosis [28]. Although clinical end points were not part of this study, no cases of tuberculosis were diagnosed during follow-up in either arm, despite active contact and symptom screening. Although HIV-exposed infants have higher rates of tuberculosis exposure and disease than HIV-unexposed infants [29], prior studies of HIV-unexposed infants in this setting have found low *M. tuberculosis* infection rates before 8 weeks of age [11], suggesting that a delay in BCG vaccination until this age may be safe.

In settings such as South Africa, where the burden of tuberculosis and HIV infection is high, BCG vaccine is still routinely administered at birth to infants born to HIV-infected mothers. For optimal safety, BCG vaccine should be administered only after the HIV status is confirmed to be negative, and HIV infection is excluded. Our results suggest that delaying BCG vaccination to 8 weeks of age in HIV-exposed, uninfected infants does not negatively influence vaccine immunogenicity and that this strategy may even improve Th1 responses in HIV-exposed, uninfected infants. Thus, delaying BCG vaccination should be explored further as a viable alternative for HIV-exposed infants in settings with a high tuberculosis burden. Future studies need to further explore safety and programmatic issues related to loss to follow-up should BCG be delayed in HIV-exposed infants.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The

posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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