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J Immunol 2010; 185:2620-2628; Prepublished online 19 July 2010; doi: 10.4049/jimmunol.1000552
http://www.jimmunol.org/content/185/4/2620
Delaying Bacillus Calmette-Guérin Vaccination from Birth to 4 1/2 Months of Age Reduces Postvaccination Th1 and IL-17 Responses but Leads to Comparable Mycobacterial Responses at 9 Months of Age

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Bacillus Calmette-Guérin (BCG) vaccine is the only licensed vaccine against tuberculosis, yet its protective efficacy is highly variable between different geographical regions. We hypothesized that exposure to nontuberculous mycobacteria attenuates BCG immunogenicity by inducing mycobacterial-specific regulatory T cells (Tregs). Gambian neonates were recruited at birth and randomized to receive BCG vaccination either at birth or at 4 1/2 mo. Mycobacterial immune responses were assessed at birth, 4 1/2, and 9 mo of age. At 4 1/2 mo of age the BCG naive individuals had detectable mycobacterial responses, including increased IL-10 production suggesting environmental priming. Vaccination at birth significantly enhanced Th1, Th2, IL-6, IL-17, and Treg responses in mycobacterial cultures at 4 1/2 mo compared with the BCG naive group. Analyzing results at 4 1/2 mo postvaccination revealed lower IFN-γ, IL-6, and IL-17 responses in the delayed BCG vaccine group compared with those vaccinated at birth, but this did not relate to Treg levels prevaccination. When comparing responses pre- and post-BCG vaccination in the delayed vaccine group, there was no priming of mycobacterial IL-17. Mycobacterial responses waned over 9 mo in those vaccinated at birth, leading to comparable mycobacterial immunity in both groups at 9 mo of age. Overall, these data suggest that vaccination at birth induces a broad Th1/Th2/IL-17/Treg mycobacterial response but the Th1/Th-17 response was reduced when delaying the vaccine. The evidence did not suggest that mycobacterial specific naturally occurring Tregs accounted for this attenuated immunogenicity. The Journal of Immunology, 2010, 185: 2620–2628.

The protective efficacy of bacillus Calmette-Guérin (BCG), the only licensed vaccine against tuberculosis (TB), varies from 0–83% partially dependent on geographical location; BCG efficacy is reduced in populations that live in rural areas closer to the equator (1, 2). Despite this, the BCG vaccine is still thought to provide protection against severe extrapulmonary forms of TB in childhood (3) and may reduce the severity of lesions in adult TB patients (4). In countries that are at high risk for TB the World Health Organization recommends BCG vaccine is given at birth or on first contact with healthcare workers, although there is little immunological evidence to determine when best protection is achieved (5). The Gambia is considered a high-risk setting with a TB incidence in 2007 of 258 new cases per 100,000 people per year compared with the U.K. incidence of 15 new cases per 100,000 people/year (6). Vaccination with BCG at birth in The Gambia induces quantitatively and qualitatively similar Th1 responses as vaccination in adulthood, whereas many other vaccines (HBV, DTP) induce Th2 responses when given early in life (7–9).

Several theories have been proposed to explain the variation in BCG efficacy throughout the world, including differences in dose (10), strains of BCG vaccine (11–13), age of vaccination (14), methodological practices (10, 15), and genetic factors (16). However, one widely accepted hypothesis is that natural immunity to nontuberculous mycobacteria (NTM) in the environment renders BCG vaccine less effective or masks its protective effect (17–20). In support of this, epidemiological studies have illustrated greater BCG efficacy in neonates who are vaccinated prior to NTM exposure (1, 21–23), and greater efficacy in vaccine trials where TST positive individuals have been excluded (24). It is also evident that in areas of high exposure to NTM, BCG vaccine is particularly ineffective (2, 25–27). Many animal models also support this theory (28–35). However, previous studies that have delayed the BCG vaccine in human infants have found similar IFN-γ responses to purified protein derivative (PPD), increased TST reactivity and increased polyfunctional T cells after in vitro stimulation with BCG vaccine (36–40), although BCG efficacy was not determined in these studies.

Reduced efficiency of BCG vaccine may be explained by over attenuation of the vaccine through negative regulatory immune mechanisms. Indeed, it has long been recognized that suppressor responses can be induced by BCG vaccine (41, 42).
naturally occurring regulatory T cells (nTregs) (43) and IL-10 production (possibly by inducible adaptive Tregs) have been shown to play a role in immune suppression in mycobacterial infection (44–48).

We hypothesized that exposure to NTM during the first 4 1/2 mo of life would attenuate the response to subsequent BCG vaccination, and that mycobacterial-specific Tregs may be involved in this reduced immunogenicity. Our results showed responses to mycobacterial Ags were detectable in BCG naive individuals at 4 1/2 mo of age indicating mycobacterial exposure and priming prior to BCG vaccination. Reduced mycobacterial proinflammatory responses were apparent in the delayed vaccine group at the same time point post-BCG vaccination than those vaccinated at birth, but nTregs did not appear to be involved. However, by 9 mo of age mycobacterial responses in both vaccine groups were comparable due to waning of the response when vaccinated at birth.

Materials and Methods

Study population

This study was approved by the Joint Gambia Government/Medical Research Council Ethics Committee and the London School of Hygiene and Tropical Medicine Ethics Committee. Gambian newborns were recruited on delivery over a 2-yr period at Sukuta Hospital. Exclusion criteria included low birth weight (<2.5 kg), twin pregnancy, intercurrent infection, maternal exposure to TB within the household or close TB contact at time of recruitment, or likelihood to move out of the study area during the 9-mo study period. Newborns were blocked into four blocks of 20 into one of two groups according to the BCG vaccination schedule: group 1 was vaccinated with BCG at birth (0.05 ml intradermally at the deltoid region of the left arm; Russian strain, Serum Institute of India); and group 2 was vaccinated with BCG at 4 1/2 mo of age indicating mycobacterial exposure and priming prior to BCG vaccination. The estimated seroprevalence of 2–4% among adults and 0.1% among children was considered significantly different from the 10–50 ml cord blood was collected into heparinized syringes at birth (0.05 ml intradermally at the deltoid region of the left arm; Russian strain, Serum Institute of India); and group 2 was vaccinated with BCG at 4 1/2 mo of age in the same way as group 1 and with the same batch of vaccine. All other childhood vaccines were administered according to the recommended Extended Program of Immunization schedule (50). Further venous blood samples of 1 ml/kg (maximum 5 ml) were collected at each mof study and measured. In case of low birth weight (<2.5 kg), twin pregnancy, intercurrent infection, maternal exposure to TB within the household or close TB contact at time of recruitment, or likelihood to move out of the study area during the 9-mo study period. Newborns were blocked into four blocks of 20 into one of two groups according to the BCG vaccination schedule: group 1 was vaccinated with BCG at birth (0.05 ml intradermally at the deltoid region of the left arm; Russian strain, Serum Institute of India); and group 2 was vaccinated with BCG at 4 1/2 mo of age in the same way as group 1 and with the same batch of vaccine. All other childhood vaccines were administered according to the recommended Extended Program of Immunization schedule (50). Further venous blood samples of 1 ml/kg (maximum 5 ml) were collected at 4 1/2 and 9 mo of age and a TB questionnaire was completed to assess for TB exposure. For all individuals, blood was collected prior to BCG vaccination. Subjects were followed up in the first week of life, and then monthly throughout the study. Any child with suspected exposure to TB was monitored closely. Any subject or close contact with a confirmed case of TB was referred to the TB clinic for further treatment. Infants found to be exposed to TB prior to BCG vaccination in group 2 were immediately dropped from the study and monitored closely for TB disease.

Cell culture conditions

From each blood sample, 100 µl whole blood was used to phenotype the cellular profile by flow cytometry (ex vivo). Five hundred microliters whole blood was cultured for 5 d with each of the following Ags: PPD of tuberculin (25 µg/ml; Statem Serum Institute, Copenhagen, Denmark), live BCG (2–66 × 10^4 CFU units/ml [Serum Institute of India, Pune, India; same batch as used for vaccination], ESAT-6/CFP-10 fusion protein [10 µg/ml; kind gift from Dr. Michel Klein, Leiden, The Netherlands, purified as described previously and confirmed to be endotoxin free (51)], Staphylococcal enterotoxin B [SEB] 5 µg/ml; Sigma Aldrich, U.K.), and an unstimulated negative control. After 24 h, the cultures were diluted 1:5 with serum-free RPMI 1640 media containing 2% penicillin/streptomycin (10,000 U/ml) and 1% t-glutamine. After 5 d of culture, 100 µl supernatant was collected and stored at −20°C, whereas cells were immediately phenotyped by flow cytometry.

Cell phenotyping

RBCs were lysed with 1× RBC lysing buffer (BD Biosciences, France). The cells were then washed in FACS buffer (0.5% BSA, 0.1% EDTA, 0.1% sodium azide in PBS) and stained with cell surface Abs (CD4 PE, clone SK3, CD4 allophycocyanin, clone SK3, CD8 PerCP, clone SK1, CD25 FITC, clone M-A251) (BD Pharmingen, France) in 100 µl FACS buffer for 30 mins at 4°C before permeabilization for intracellular staining (ICS). ICS for FOXP3 (allophycocyanin clone PCH101, eBioscience, San Diego, CA) was performed as per manufacturer’s instructions using all reagents from the FOXP3 staining kit (eBioscience) and resuspended in 150 µl fix buffer (2% formalin in PBS). The remaining intracellular markers (IL-10, PE, clone RM4-5 and Ki-67, PerCP, clone M4A4, eBioscience, CA) were used according to manufacturer’s instructions (BD Bioscience) and resuspended in 150 µl fix buffer. Ki-67 was used as a surrogate marker of proliferation representing cells that have already proliferated, are currently proliferating or are about to proliferate. Ab titrations and isotype controls were optimized previously under appropriate study conditions. FOXP3 expression is known to be transiently upregulated in effector T cells after activation, but levels then decline and remaining FOXP3+ cells are thought to represent true Tregs (52). During optimization experiments upregulation of FOXP3 was found to peak on day 1 of culture, and reduce by 5 d (data not shown) leaving a FOXP3+ population that is thought to represent mainly true Tregs. Due to the difficulties in defining the CD25+ population, we chose to define CD4+CD25+FOXP3+ T cells as an nTreg population.

Cytometric bead array

Supernatants were spun at 1500 rpm for 2 min to pellet any precipitation in the sample. All samples stimulated with SEB were diluted 1:2 with media containing 10% serum. IFN-γ, IL-10, IL-13, IL-6, and IL-17 cytokine production (Th1 cytokine kit, Bio-Rad, Hercules, CA) was analyzed for all samples. IL-13 was used as a marker of Th2 activity as levels of IL-4 were too low to detect in most cases. The bead array assays were performed according to manufacturer’s instructions using serial 1/4 dilutions of the standard in RPMI 1640 media containing 10% human AB serum and 50 µl samples and analyzed on a Bio-Plex 200 Suspension Array system (Bio-Rad). Standard curve outliers were eliminated by identifying samples where the coefficient of variance (%CV) >10% and observed/expected × 100 was outside the range of 100 ± 20. Cytokine concentrations below the level of detection, namely, recorded as OOR< were calculated as zero in the analysis. All values that were greater than the highest range within the standard curve (OOR>) were repeated after further dilutions with media containing 10% human AB serum.

Data collection and verification

Flow cytometric data were analyzed using a template previously created during optimization experiments using Flow Jo software (TreeStar, Ashland, OR) with minor sample specific modifications. Data points/samples were eliminated if any of the following problems arose: Extended Program of Immunization vaccine less than 7 d prior to blood collection, cord blood was clotted, cultures were contaminated, flow cytometry Abs did not work optimally, and the positive control (SEB) did not induce a response in vitro and acquisition of <1000 cells.

Statistical analysis

For most values the data were not normally distributed and therefore nonparametric testing was used throughout. Cross-sectional comparisons between groups at each time point were assessed using a two-sided Mann-Whitney U test at 95% significance. All paired analyses (i.e., unstimulated compared with stimulated samples or longitudinal data) were performed using two-sided Wilcoxon matched paired tests at a 95% significance level (p < 0.009–0.050; **p < 0.001–0.010; ***p < 0.001). Correlations were analyzed using Spearman’s nonparametric correlation coefficient. Bonferroni corrections were used according to the number of parameters assessed and the number of times a dataset was analyzed. Data were analyzed using R version 2.9.2 (R The Foundation, official part of the FRS’s GNU project) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

Results

Cohort characteristics

A total of 103 healthy neonates were recruited into the study at birth; 53 were randomly assigned into group 1 (BCG vaccinated at birth) and 50 were randomly assigned into group 2 (vaccinated at 4 1/2 mo of age). Of the 103 subjects, 90 were followed up at 4 1/2 mo (87.3%) and 87 at 9 mo (84.4%) (Fig. 1). Overall the cohort characteristics were similar for both groups (Supplemental Table 1).
All maternal placental biopsies were negative for placental malaria suggesting none of the children in this study had been exposed to malarial parasites in utero. Recent studies in The Gambia have shown very low levels of helminth infection, between 0–3% in adult populations (53) suggesting helminth infection is unlikely to be a confounding factor to the study. None of the children had confirmed TB. Overall, the characteristics of this study cohort were similar to previous studies in Sukuta (54), suggesting that this group of children are representative of the Sukuta community.

BCG vaccination does not affect the phenotype of ex vivo T cell populations

Immune cells from whole blood without stimulation (ex vivo) were phenotyped at birth (cord blood), 4 1/2, and 9 mo of age by flow cytometry with a panel of Abs that defined activated T cells (%CD4+ that coexpress CD25) and nTregs (%CD4+ that coexpress CD25 and FOXP3). Ex vivo T cell populations were comparable across age groups (mean activated T cells 6.9%, mean nTregs 0.83%). Comparing BCG vaccinated (group 1) to unvaccinated (group 2) infants at 4 1/2 mo of age did not reveal any significant difference in ex vivo populations (Fig. 2A, 2B). Similarly, these ex vivo populations were comparable between groups both at 9 mo of age and 4 1/2 mo post-BCG vaccination (i.e., group 1 at 4 1/2 mo of age compared with group 2 at 9 mo of age) (data not shown).

Mycobacterial specific reactivity in BCG naive infants at 4 1/2 mo of age

At 4 1/2 mo of age the BCG naive (group 2) individuals had detectable mycobacterial responses, including activated T cell responses (CD4+CD25+ and CD4+Ki-67+), production of IFN-γ, IL-13, IL-17, and IL-6, and suppressive responses including nTregs (CD4+CD25+FOXP3+) and IL-10 production (both from CD4+ T cells and from the supernatant of cultures) (Fig. 3A–I, Supplemental Table II). Thus, memory responses to mycobacterial Ags were present at 4 1/2 mo of age prior to BCG vaccination. PPD responses were robust in group 2, with lesser reactivity to BCG, and very low level reactivity to the Mycobacterium tuberculosis-specific Ag ESAT-6/CFP-10 fusion protein. Because PPD induced stronger responses than the other mycobacterial Ags, we focused much of the subsequent analysis on PPD responses.

BCG vaccination at birth induces an activated, proinflammatory, and regulatory mycobacterial response at 4 1/2 mo of age

After subtraction of the background unstimulated (net) response most PPD-induced immune parameters were higher at 4 1/2 mo of age in the vaccinated group compared with BCG naive individuals, including proliferation of CD4+ T cells, CD4+CD25+ activated T cells, CD4+CD25+FOXP3+ nTregs and production of the cytokines IFN-γ, IL-13, IL-6 and IL-17 (Fig. 4A–F, Supplemental Fig. 1A–D). However, similar frequencies of IL-10 producing CD4+ T cells or IL-10 secreted into the supernatants of PPD cultures were found between groups (data not shown). All individuals included in the analysis responded to SEB and these responses were comparable between the vaccination groups at each age and between ages (data not shown). We noted that the cytokine responses were heterogeneous, even in the unstimulated cultures. Interindividual variation has been previously described in Gambian infant studies with as great as 10-fold differences in mycobacterial and PHA responses observed (55).

Delaying the BCG vaccine induces activation of T cells but alters the quality of the immune response

We hypothesized exposure to NTM during the first 4 1/2 mo of life may attenuate BCG immunogenicity via the induction of regulatory T cells. Comparisons between groups at 9 mo of age revealed no significant differences for any of the parameters studied (Fig. 5A–G). However, comparing with an equivalent time point

FIGURE 1. Number of subjects recruited and followed up over 9 mo.

FIGURE 2. Ex vivo T cell populations at 4 1/2 mo of age. A total of 100 µl whole blood was phenotyped with Abs to CD4, CD25, and FoxP3. A. Activated T cells (CD4+CD25+) and (B) nTregs (CD4+CD25+FoxP3+) at 4 1/2 mo of age. A Mann-Whitney U test at 5% significance was used to compare groups. Median values are represented with a black line. Group 1, n = 48; group 2, n = 39.
postvaccination (i.e., 4 1/2 mo of age in group 1 and 9 mo of age in group 2) showed several important differences. Production of the cytokines IFN-γ, IL-6, and IL-17 was significantly lower in the delayed vaccine group (group 2) compared with those vaccinated at birth (group 1, Fig. 6A–C), whereas PPD induced activated T cells, nTregs, IL-10, and IL-13 responses were comparable between groups, (data not shown), however, in BCG cultures, the delayed vaccine group produced more IL-10 than those vaccinated at birth (Fig. 6D).

In group 2 activated T cells, IFN-γ, IL-13, and IL-6 were increased in PPD cultures compared with responses prevaccination (p = 0.001, p = 0.010, p < 0.0001, p = 0.003,) (Fig. 5A, 5C–E), whereas nTregs, IL-10 (T cell production and in culture supernatants), and IL-17 did not alter (p = 0.061; p = 0.371; p = 0.218; p = 0.334, respectively) (data not shown). This suggests that delaying the BCG vaccine narrowed the breadth of PPD specific responses compared with vaccinating at birth.

We had hypothesized that mycobacterial-induced Tregs may be responsible for reduced BCG immunogenicity; however, there was no correlation between nTregs or IL-10 at 4 1/2 mo of age and IFN-γ responses at 9 mo of age in group 2 (nTregs: r = 0.1026, p = 0.5699; IL-10: r = 0.0848, p = 0.6127; CD4⁺IL-10⁺:

![FIGURE 3. Mycobacterial-specific responses in BCG naive individuals at 4 1/2 mo of age. Five hundred microliters whole blood was cultured with each of the following Ags, PPD, BCG, and EC (ESAT-6/CFP-10 fusion protein), for 5 d. A. Activated T cells (CD4⁺ CD25⁺), (B) nTregs (CD4⁺ CD25⁺FOXP3⁺), (C) CD4⁺Ki-67⁺, (D) IFN-γ, (E) IL-13, (F) IL-6, (G) IL-17, (H) IL-10 from supernatants of cultures, and (I) IL-10 production from intracellular staining of CD4⁺ T cells in response to PPD are shown for group 2 BCG naive donors at 4 1/2 mo of age prior to vaccination (n = 36–43). Median values are presented as a black line. A Wilcoxon nonparametric paired test was applied at 5% significance to compare unstimulated with stimulated cultures. *p = 0.009–0.050; **p = 0.001–0.010; ***p < 0.001; #not significant after Bonferroni correction for multiple testing.]

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**FIGURE 4.** In vitro responses to PPD at 4 1/2 mo of age in BCG vaccinated and BCG naive (unvaccinated) children. Five hundred microliters whole blood was cultured with the following Ag, PPD, for 5 d. Comparisons between group 1 (vaccinated) and group 2 (unvaccinated) after subtracting the background for (A) activated T cells (CD4+CD25+), (B) nTregs (CD4+CD25+FOXP3+), (C) IFN-γ, (D) IL-13, (E) IL-6, (F) IL-17 was made using a Mann-Whitney U test at 5% significance. Median values are presented as a black line. Group 1, n = 40; group 2, n = 36.

We also found no correlation for IFN-γ responses to PPD between pre- (4 1/2 mo) and postvaccination (9 mo) in group 2 ($r = 0.2021$, $p = 0.2228$), nor indeed for any of the other parameters tested in this group.

**Waning of Th1 and IL-17 responses 9 mo after BCG vaccination**

The persistence of the mycobacterial memory response was investigated by comparing responses at 4 1/2 and 9 mo of age in those vaccinated at birth (group 1) (Fig. 5A–G). PPD induced nTregs, IFN-γ, IL-6, and IL-17 production were all reduced at 9 mo compared with 4 1/2 mo ($p = 0.0463$; $p = 0.0089$; $p = 0.0408$; $p = 0.0200$, respectively), although IL-6 and nTregs were not significantly reduced after correcting for multiple testing (Fig. 5A–G). By contrast, the frequency of activated T cells, the Th2 cytokine IL-13, and the regulatory cytokine IL-10 (CD4+ T cell production and in the culture supernatants) in response to PPD all remained comparable from 4 1/2 to 9 mo ($p = 0.2383$; $p = 0.5074$; $p = 0.7294$; $p = 0.3310$, respectively) suggesting persistence of the Th2/regulatory response at 9 mo of age. This waning of the response 9 mo postvaccination at birth led to similar immune responses in group 1 and group 2 at 9 mo of age. Interestingly, there was an overall positive correlation between IFN-γ responses to PPD at 4 1/2 and 9 mo of age ($r = 0.4313$, $p = 0.0039$) suggesting that the Th1 response observed at 4 1/2 mo of age in those vaccinated at birth may predict the longer-term response to BCG vaccination.

**Discussion**

Overall, our results show that delaying the BCG vaccine to 4 1/2 mo of age did not affect the mycobacterial responses at 9 mo of age compared with vaccination at birth. However, a waning of responses was observed for many of the parameters studied 9 mo after vaccination at birth. A comparison of immunological parameters at the same time point (4 1/2 mo) post-BCG vaccination (i.e., 4 1/2 mo of age in group 1 and 9 mo of age in group 2) demonstrated that delaying BCG from birth to 4 1/2 mo of age led to a reduction in IFN-γ, IL-6, and IL-17 production to PPD in vitro, but a similar IL-13 (Th2)/regulatory T cell immune profile compared with vaccination at birth. It is possible that mycobacterial immunity in group 2 would similarly wane further by 9 mo postvaccination, although this was not assessed in this study. Th1 responses (and possibly Th17) are thought to be protective against intracellular...
organisms, and thus the lower responses in group 2 infants could be detrimental.

Cattle studies have shown that presensitization to NTM for 5 mo, followed by BCG vaccination reduces the protective effect of BCG (56). In support of this, a recent study has shown that IL-17 responses in adult humans to the MVA85A vaccine were reduced in individuals that had pre-existing responses to mycobacterial Ags (57). However, many human studies have shown similar IFN-γ responses after delaying the BCG vaccine to 8 or 10 wk of age in Gambian and South African infants, respectively (36, 37), and increased IFN-γ and polyfunctional T cells in 10-wk-old BCG vaccinated South African infants (38). Differences in immunological assay conditions, length of vaccine delay and sample sizes make direct comparisons between these studies complicated. The South African study examined T cell production of IFN-γ in response to BCG (38). T cell production of IFN-γ was not determined in the other studies or our study and therefore could not be compared, but it is possible that T cell activity may increase in the delayed group as a consequence of a more developed immune system at the age of vaccination. It would be interesting to observe responses in delayed vaccine studies in communities that have less NTM exposure to compare responses after delaying BCG.

We had hypothesized that exposure to NTM prior to BCG vaccination would lead to the induction of Tregs, which, would in turn, reduce the immune response to BCG vaccination. At 4 1/2 mo of age the BCG naive infants responded to mycobacterial Ags with increased activated and regulatory T cells in vitro. Four and a half months after BCG vaccination in this group, PPD induced IFN-γ,

![Longitudinal responses to PPD over time.](image)

FIGURE 5. Longitudinal responses to PPD over time. Longitudinal (A) CD4+CD25+, (B) CD4+CD25+FOXP3+, (C) IFN-γ, (D) IL-13, (E) IL-6, (F) IL-17, and (G) IL-10 responses to PPD after 5 d of culture in group 1 (gray bars) and group 2 (white bars). Unstimulated values were subtracted from stimulated values. Black bar represents the median value of the data. Comparisons between ages within groups was calculated using Wilcoxon non-parametric paired test at 5% significance and comparisons between groups at each age were calculated using a Mann-Whitney U test at 5% significance. All comparisons for IL-6 and IL-17 were calculated using Mann-Whitney U test at 5% significance due to a lower sample size for these cytokines at birth. *p = 0.009–0.050; **p = 0.001–0.010; ***p < 0.001; "p not significant after Bonferroni correction for multiple testing.
IL-6, and IL-17 were significantly lower than 4 1/2 mo after vaccination at birth. There was, however, no correlation between nTregs or IL-10 reactivity to PPD at 4 1/2 mo of age prior to BCG and any of the immune parameters postvaccination. This would suggest nTregs and IL-10 at the time of vaccination are not directly responsible for the reduced proinflammatory responses observed in the delayed vaccine group. It is possible that qualitative properties of Tregs and/or IL-10, not measured in this study, may influence BCG immunoreactivity. An unvaccinated control group at 9 mo would further confirm whether longer exposure to NTM has the same effect, but this would be difficult to justify ethically in a high TB incidence setting.

IL-10 present in PPD cultures prior to vaccination in the delayed BCG group was not enhanced after BCG vaccination. In fact unvaccinated children at 4 1/2 mo of age had greater levels of IL-10 in BCG cultures than the BCG vaccinated individuals, suggesting that mycobacterial induced IL-10 could play a role in attenuating IL-10 production after adult BCG vaccination peaked at 8 wk post-BCG vaccination and declined by 12 mo (48), whereas IL-10 peaked at 2 wk and declined by 8 wk (48). We may therefore have missed the BCG-induced peak IL-10 responses when examining reactivity 18 wk postvaccination.

Black et al. (27, 59) proposed that the difference between pre- and post-BCG vaccination responses to mycobacterial Ags was a better predictor of BCG efficacy than the absolute values post vaccine. Lower IFN-γ responses to PPD prior to BCG correlated with good IFN-γ responses to PPD after vaccination (60). Our results could support the notion that exposure to NTM prior to BCG vaccination alters the quality of mycobacterial specific immunity induced by subsequent BCG vaccination. The delayed vaccine group had PPD-induced IFN-γ and IL-6 responses prior to BCG vaccination that were boosted after immunization, but these responses were less than that induced after vaccination at birth. Several animal studies suggest IL-6 plays a role in cell-mediated antimycobacterial immunity (61, 62) and can render pathogen-specific T cells refractory to the suppressive activity of Tregs (63). Thus, the reduced IL-6 production postvaccination in the delayed vaccine group may result in induction of mycobacterial-specific T cells with a greater sensitivity to Tregs that could lead to suppression of IFN-γ production. In contrast, IL-17, nTregs, and IL-10 were not boosted at all when comparing pre- and postvaccination in the delayed vaccine group, and activation of CD4+ T cells remained similar. Recent human studies have shown induction of IL-17 after BCG vaccination of U.K. infants (64) and reduced levels of CD4+ T cell production of IL-17 in TB patients (65, 66) suggesting that Th17 responses may be protective against TB. Lower levels of IL-6 induction in the delayed BCG vaccine group may contribute to the lack of IL-17 induction, and lead to a reduced IFN-γ response, both of which may lead to reduced protection against TB. Further studies are required to clarify the roles of BCG induced IL-6 and IL-17 in protection from TB.

Similar levels of IL-13 were induced after BCG vaccination at birth or at 4 1/2 mo of age. Although there was a waning of the IFN-γ response 9 mo after BCG vaccination at birth, IL-13 responses persisted supporting the widely accepted paradigm of a Th2 bias, following priming in early life (67). This has also been shown in U.K. adolescents where there was a waning of IFN-γ responses to PPD from 3 mo to 1 y and 1 y to 3 y after receiving BCG vaccination (68). This could be associated with differences in the kinetics/longevity of Th1 and Th2 cells. Indeed, mice experiments suggest that Th1 cells have increased susceptibility to cell death after restimulation compared with Th2 cells (69). It is worth noting that IL-13, a Th2 cytokine produced by T cells, can also be produced by, and directly affect, the function of macrophages, NKT cells, mast cells, and basophils (70). Concentrations of IL-13 are increased in TB patients (71), and Rook (72) suggests that in developing countries IL-13 and IL-4 may undermine Th1 immunity and drive induction of alternative macrophages. The stable IL-13 response at 9 mo alongside reduced IFN-γ responses in group 2 may indicate reduced TB protection in these infants.

Several animal studies have shown that BCG vaccination on a background of prior exposure to M. avium provided less protection than on a background of more distantly related strains such as M. vaccae (34, 35, 73). A study in The Gambia using mycobacterial skin testing to various environmental mycobacterial Ags showed that M. intracellulare (a strain of M. avium) is the predominant NTM species eliciting immune reactivity in The Gambia (81.2% of BCG naive 6–18 y olds had induration >3 mm (74). Reactivity to common NTM may account for the mycobacterial specific reactivity observed in BCG naive infants at 4 1/2 mo of age. These may in turn reduce proinflammatory responses after
vaccination in this group. Unfortunately, the skin test Ags tested in previous Gambian studies are no longer available for human testing. In addition, the limitations of blood volume collected made it difficult to include further mycobacterial Ags that would have helped to determine the influence of exposure to specific NTM. Other explanations that could account for the differences between groups include age-related changes in the immune system or the effect of recently administered vaccines given as part of the intensive EPI schedule in The Gambia.

It was surprising to find IFN-γ, IL-6, and IL-10 responses to the ESAT-6/CFP-10 fusion protein in BCG naive individuals, although levels were very low compared with the BCG and PPD Ag responses (Supplemental Table II). These low-level responses might indicate TB exposure, but because ESAT-6 and CFP-10 are also present in M. leprae, M. kansaii, M. marinum, M. smegmatis, and M. ulcerans (73, 75) they may also be the result of NTM priming. Previous studies in The Gambia have shown 19% and 28.4% of BCG naive Gambians (aged between 6 and 18 y of age) had skin test reactivity (>3 mm) to M. kansaii and M. marinum, respectively (74). In addition, all BCG naive children had anergic TST responses at 4 1/2 mo (S. Burl, U.I. Adetifa, M. Cox, E. Touray, H. Whittle, H. McShane, S.L. Rowland-Jones, and K.L. Flanagan, submitted for publication) and were intensely followed-up to minimize contact with TB cases, thus were unlikely to be latently infected with M. tuberculosis.

Overall, our studies suggest BCG vaccination on the background of exposure to NTM (group 2) reduces the mycobacteria-induced IFN-γ, IL-6, and IL-17 production, all of which have antmycobacterial properties. We did, however, detect significant levels of mycobacterial-induced IL-10 production (possibly from Tr1 adaptive Tregs) most likely induced by NTM exposure prior to BCG vaccination. We speculate that these might have played a role in attenuation of the BCG stimulated protective response. By contrast, BCG vaccination at birth induced a strong mixed Th1 and Th2 response at 4 1/2 mo, with waning of the Th1 response by 9 mo of age, suggesting that although BCG can induce a strong proinflammatory response in early life, it is transient and the persistent response is predominantly Th2 as has been well described in early life immunity. This waning also resulted in similar responses observed at 9 mo of age between the two groups, indicating that the timing of BCG vaccination did not affect the immune responses to the BCG vaccine at 9 mo of age.

Acknowledgments
We thank the field workers and nurses based at Sukuta Health Centre, in particular Sally Savage. We also acknowledge the support of the mothers and infants without whom this study could not take place. We thank Momo-dou Lamin Fatty and Lady Chilel Sanyang for technical assistance in the laboratory. We also thank Dr. Peter Kelly (Institute of Medical Microbiology and Infectious Diseases, Queen Elizabeth University Hospital) and Dr. Chris Finan (Brighton and Sussex Medical School) for help and advice on the R software used to analyze parts of the data.

Disclosures
The authors have no financial conflicts of interest.

References
isolates from different sources in the south Indian BCG trial area. Indian J. Med. Res. 103: 201–211.


SUPPLEMENTAL TABLE I. Cohort characteristics

SUPPLEMENTAL FIGURE 1. Representative flow cytometry plots of PPD stimulated whole blood. 500 µL whole blood was cultured with PPD for 5 days. BCG vaccinated subjects (A and C) and unvaccinated (B and D) illustrating % CD25+ T cells gated on CD4+ T cells (A and B) and % CD25+FOXP3+ T cells gated on CD4+ T cells (C and D) (unstimulated samples on the left and PPD stimulated samples on the right).

SUPPLEMENTAL TABLE 2. Net cytokine responses for PPD, BCG and ESAT-6/CFP-10 fusion protein at 4½ months of age. Median net responses to PPD, BCG and ESAT-6/CFP-10 fusion protein after 5 days of culture at 4½ months of age in Group 1 (vaccinated at birth) and Group 2 (BCG naïve). Unstimulated values were subtracted from stimulated values. Mann Whitney non-parametric test was applied at 5% significance.
# SUPPLEMENTAL TABLE I. Cohort characteristics

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>GROUP 1</th>
<th></th>
<th>GROUP 2</th>
<th></th>
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<tbody>
<tr>
<td>Gender, %</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>27</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>Ethnicity, %</td>
<td>Mother</td>
<td>Father</td>
<td>Mother</td>
<td>Father</td>
</tr>
<tr>
<td>• Mandinka</td>
<td>39</td>
<td>38</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>• Fula</td>
<td>6</td>
<td>11</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>• Wolof</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>• Other</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Median number of live deliveries</td>
<td>3 (1 – 9)</td>
<td>3 (1 – 9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother’s median age (range)</td>
<td>26 (18 – 45)</td>
<td>26 (18 – 37)</td>
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<td></td>
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<tr>
<td>Time lived in Sukuta, n (%)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>• &lt; 2 yrs</td>
<td>5 (9.4)</td>
<td>1 (2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 2 - 10 yrs</td>
<td>14 (26.4)</td>
<td>17 (34.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &gt; 10 years</td>
<td>10 (18.9)</td>
<td>13 (26.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• always</td>
<td>24 (45.3)</td>
<td>18 (36.7)</td>
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<td></td>
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<tr>
<td>Birth weight, kg (range)</td>
<td>3.2 (2.5 – 4.2)</td>
<td>3.1 (2.5 – 3.9)</td>
<td></td>
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</tr>
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</table>
**SUPPLEMENTAL TABLE 2.** Net cytokine responses for PPD, BCG and ESAT-6/CFP-10 at 4½ months of age

<table>
<thead>
<tr>
<th>Cytokine (pg/mL)</th>
<th>PPD</th>
<th></th>
<th>BCG</th>
<th></th>
<th>ESAT-6/CFP-10 FP</th>
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<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
<td>p value</td>
<td>Group 1</td>
<td>Group 2</td>
<td>p value</td>
</tr>
<tr>
<td>IFNγ</td>
<td>823.7</td>
<td>142.1</td>
<td>&lt; 0.0001</td>
<td>154.2</td>
<td>12.11</td>
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<tr>
<td>IL–6</td>
<td>11,923</td>
<td>1,213</td>
<td>&lt; 0.0001</td>
<td>1,253</td>
<td>147.8</td>
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<td>IL–10</td>
<td>15.57</td>
<td>18.43</td>
<td>0.0816</td>
<td>2.34</td>
<td>2.07</td>
<td>0.8084</td>
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<td>IL–13</td>
<td>280.5</td>
<td>10.83</td>
<td>&lt; 0.0001</td>
<td>31.86</td>
<td>2.83</td>
<td>0.0001</td>
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<tr>
<td>IL–17</td>
<td>14.55</td>
<td>1.53</td>
<td>&lt; 0.0001</td>
<td>0.57</td>
<td>0</td>
<td>0.0975</td>
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