Effects of Antenatal and Postnatal Environments on CD4 T-Cell Responses to *Mycobacterium bovis* BCG in Healthy Infants in The Gambia

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The *Mycobacterium bovis* BCG vaccine has a poor record of efficacy in low-income tropical settings. Against this background, we evaluated the immune response of infants to mycobacterial antigens over the 2 years following BCG vaccination at birth by measuring the gamma interferon (IFN-γ), interleukin-2 (IL-2), and CD154 responses of CD4 T cells. Similar numbers of cells expressed IFN-γ in infants, 4- to 5-year-old children, and adults, while CD154 was not expressed at comparable levels until the second year of infancy. The IL-2 response remained relatively low in infants, children, and adults but correlated negatively with mother's body mass index and was highest among infants born to Mandinka mothers. Similarly, infants born in the wet season had a stronger CD154 response than those born in the dry season throughout the 2 years of the study. We conclude that the prenatal and perinatal environments have a lasting effect on the response of infants to the BCG vaccine.

*Mycobacterium tuberculosis* is a major cause of infectious disease worldwide, especially in low-income countries. Although about one-third of the world’s population are infected (9), the overwhelming majority do not develop tuberculosis. The mechanisms that underlie the ability or failure to contain *M. tuberculosis* infection remain obscure. It has been suggested that protective immunity depends on containment of the initial infection (10), although it is not clear whether certain individuals are predisposed to be able to do so or what might prevent them from doing so.

Several studies have suggested an influence of the antenatal environment on subsequent ability to mount immune responses and fight disease, as maternal body mass index (BMI) and season of birth influence antibody responses to vaccines (24, 29, 30), thymus development (6, 23), and even mortality (27). The effect of season of birth has been most intensively studied in The Gambia, where there is a marked decrease in energy intake as stored food from the previous harvest becomes scarce during the wet season, which is also a period of intense agricultural labor largely carried out by women. Consequently, there is a marked seasonal fluctuation in energy balance during pregnancy that affects the birth weight of offspring (38).

The only licensed vaccine for tuberculosis is the bacillus Calmette-Guérin strain of *Mycobacterium bovis* (BCG). Protection against all forms of tuberculous disease averages 74% in randomized controlled trials (5) but varies widely from one trial to another (5, 40). The vaccine performs particularly poorly in low-income tropical settings, and trials in Malawi (37) and India (31) indicated no protection against tuberculosis. Genomic variability between different passages of the vaccine strain (2), helminth infection (21), and exposure to other *Mycobacterium* spp. (1) have all been suggested as reasons for BCG’s variable performance. The last suggestion is particularly relevant to low-income settings, as a T-cell response to mycobacterial antigens has been shown in unvaccinated Gambian infants as young as 3 months (45).

There is a certain amount of evidence that the CD4 T-cell population is essential for defense against *M. tuberculosis*, and a CD4 T-cell subpopulation that produces the cytokine gamma interferon (IFN-γ) is associated with protection against *M. tuberculosis* (7, 15, 19, 20, 33, 44). However, patients with active tuberculosis tend to have large numbers of IFN-γ-producing *M. tuberculosis*-specific T cells (15, 36, 46), and therapeutic use of IFN-γ in tuberculosis patients has not been successful (47), implying that deficiencies in IFN-γ alone cannot account for the susceptibility of some individuals to tuberculosis. Administration of interleukin-2 (IL-2) has a better record as a therapy (16–18), which suggests that autologous IL-2 production may be important in protection against tuberculosis.

Expression of CD154 (CD40L) by CD4 T cells enhances the killing activity of monocytes and macrophages in human and mouse models of mycobacterial infection (12, 14) and is depressed in patients with active tuberculosis (42), which suggests another factor involved in protection. While IFN-γ, IL-2, and CD154 probably all play a part in the control of *M. tuberculosis* infection, none clearly correlates with protection from tuberculosis, implying the existence of unidentified mechanisms of protection.

Although CD4 T cells are critical to the control of mycobacterial infections (44), studies on BCG efficacy have necessarily...
focused on the incidence of tuberculosis, and there is little information on the responses to cytomegalovirus (CMV) infection, diagnosed by PCR screening of urine up to the age of 12 months and by serodiagnosis at 24 months of age (26). We conducted a separate analysis on the responses of those infants to BCG. We were only able to find evidence that CMV infection had any effect on responses to mycobacterial antigens (26), since coverage rates in a given level of infant health care were not always possible and so the largest number of samples at any one time point were included, although this was unlikely to be a significant confounder in this study.

The study protocol involved taking blood samples from infants at predetermined time points following CMV infection. As neither CMV status nor recent CMV infection had any effect on responses to mycobacterial antigens (26), we conducted a separate analysis on the responses of those infants to BCG. We identified all samples that had been collected at 3 (12 to 17 weeks), 6 (24 to 39 weeks), 9 (52 to 66 weeks), and 24 (104 to 118 weeks) months of age. Data from 133 infants for at least one time point were included, although longitudinal follow-up was not always possible and so the largest number of samples at any one time point was 87. All infants enrolled in the study were visited quarterly by a study field worker, and none showed signs of CMV-related disease.

Samples were also collected from 25 children aged between 4 and 5 years and from 11 women, aged between 21 and 31 years, sampled 18 months after giving birth in Sukuta Health Centre.

Informed consent was obtained from the mothers and documented by signature or thumb print. The study was approved by the Medical Research Council (MRC)-Gambian Government Joint Ethics Committee.

Immunization with BCG. All the infants were vaccinated with BCG at birth by intradermal injection into the deltoid region of the arm, in accordance with the Gambian expanded program of immunization. The infant welfare cards of the 4- to 5-year-old children were checked at recruitment to ensure that they had received BCG. No such records were available for the adults, but the earliest available records of national coverage report 85% in 1980, and coverage has not dropped below 90% since 1981 (49). It is likely that the coverage rates in a population close to the capital of Banjul were higher than the national average.

The prevalence of tuberculosis in The Gambia at the time of the study was 352 per 100,000 (48). No subjects in the present study showed clinical signs of tuberculosis.

Evaluation of maternal health and socioeconomic status. At the time of recruitment, a structured questionnaire on the living conditions and socioeconomic status of the mother of each infant was filled in by a field worker, who posed the questions to the mother in her first language. The mother was also asked for consent for the field worker to access her records from the antenatal clinic if she had attended it, which included her red blood cell packed cell volume measured 8 to 14 weeks before she gave birth. The mother's BMI was assessed 6 months after she gave birth.

Blood sampling and intracytoplasmic cytokine staining. Blood samples were collected into heparin (Sigma), and peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis-Shield) within 4 h of collection. Infant and child PBMCs were stimulated immediately. It was not logistically possible to test all adult PBMCs, so PBMCs of the mothers were cryopreserved on collection and tested when we identified a subset whose infants did not develop any pathology or abnormalities during the first 2 years of life. The PBMC preparations were resuspended at 2 × 10^6 cells ml^-1 in RPMI containing 100 U ml^-1 penicillin plus 100 μg ml^-1 streptomycin, 10% (vol/vol) fetal bovine serum and treated with either 10 μg ml^-1 of the RT49 preparation of tuberculin purified protein derivative (PPD) (Statens Serum Institut) or 10 μg ml^-1 normal human dermal fibroblast lysate (NHDF) (Viruses) as a negative control, which was required by the CMV study. After 2 h at 37°C
TABLE 1. Indicators of health and socioeconomic status among the mothers of infants recruited into the study

| Variable                      | Median      | Interquartile range | n  |
|-------------------------------|-------------|---------------------|----|
| Infant birth wt (kg)          | 3.0         | 2.7–3.3             | 133|
| Age of mother (yr)            | 25          | 20–30               | 126|
| No. of living children born to same mother | 2          | 0–3                 | 133|
| No. of people living in house | 8           | 5–13                | 130|
| No. of people living in same room as infant | 3          | 2–3                 | 130|
| Mother’s BMI                  | 21.8        | 18.4–24.9           | 40 |
| Mother’s antenatal red blood cell packed cell volume | 10.3       | 10–11.3             | 115|

in the first stage, and a potential factor was considered significant at a P value of <0.05 in the second stage. If more than one factor was included and not all were significant at a P value of <0.05, the least significant was removed from the model and the GEE was recalculated.

Data storage, analysis, and presentation were carried out using MS Access (Microsoft), Stata 8 (Statacorp), and Minitab 15 (Minitab Inc.).

RESULTS

Cohort characteristics. Characteristics of the infants that could be described quantitatively are given in Table 1. A total of 60 of the 133 infants (45%) and 10 of the 25 4- to 5-year-old children (40%) were female. Seasons were divided into a wet season from June to October and a dry season from November to May. Of the 133 births, 72 (54%) took place in the dry season from June to October and a dry season from November to May. Of the 133 births, 72 (54%) took place in the dry season.

The Sukuta community is ethnically diverse, and intermarriage between ethnic groups is commonplace, so that 38 of the 133 infants recruited (28%) had a mother and father from different groups. We classified ethnic groups as defined by the mother. By far the largest group were the Mandinka at 66 (50%) of the 133 infants, while 21 (16%) were Fula, 20 (15%) were Wolof, 10 (7.5%) were Jola, 7 (5.3%) were Serahula, 4 (3.1%) were Manjago, 2 (1.5%) were Serere, and 1 (0.7%) each was Bambara, Karonika, and undeclared ethnicity. Due to the small numbers from many groups, groups other than Mandinka, Fula, and Wolof were classified as “minority groups” for the purposes of analysis.

The dominant response to PPD was IFN-γ. A higher proportion of cells produced IFN-γ than either IL-2 or CD154 at all sample times during the first year of life, although the difference was significant only in infants sampled at 12 months of age (P = 0.0085). The antibody against CD154 became available only after sampling had begun, so few data were collected at the 3-month time point. At 3 and 6 months, few cells produced CD154 in response to PPD, and the medians for those time points are actually negative, indicating fewer CD154-producing cells for the PPD treatment than for the NHDF treatment. By 2 years of age, the CD154 response was similar in magnitude to the specific IFN-γ response, although neither the increase in the CD154 response nor any other age-related changes in the three responses were significantly significant (Table 2).

The median proportion of IL-2-producing cells remained very low throughout the 2 years of study, indicating the generally low level of IL-2 production in response to PPD (Fig. 1; Table 2).

As the bifunctional responses were effectively subsets of each of the three possible types of response, their magnitudes were lower by definition, though variability was as high as when the types of response were considered in isolation from each other. The proportion of cells producing both IFN-γ and CD154 was always greater than the proportion producing any other bifunctional response, probably because of the low IL-2 responses, though differences were significant only at 6 and 12 months of age (P = 0.0001) (Table 3). It was observed that the intensity of expression of IFN-γ and CD154 on bifunctional cells often appeared similar, so that cells that produced relatively high levels of one also tended to produce relatively high levels of the other (Fig. 1). There were no significant age-related differences in any bifunctional response.

TABLE 2. Percentages of PPD-stimulated CD4 T cells expressing IFN-γ, IL-2, or CD154 at each time point

| Age at sampling | Antibody type | Antibody response (%)|
|-----------------|---------------|-----------------------|
|                 | Value after subtraction of the percentage in NHDF-treated CD4 T cells. | n  |
|                 | Median        | Interquartile range   |    |
| 3 mo            | IFN-γ         | 0.024                 | –0.004–0.099 | 29 |
|                 | IL-2          | –0.006                | –0.080–0.023 | 29 |
|                 | CD154         | –0.023                | –0.137–0.039 | 6  |
| 6 mo            | IFN-γ         | 0.045                 | 0.002–0.145  | 56 |
|                 | IL-2          | 0.004                 | –0.044–0.037 | 56 |
|                 | CD154         | –0.101                | –0.422–0.029 | 28 |
| 12 mo           | IFN-γ         | 0.083 a               | 0.008–0.294  | 87 |
|                 | IL-2          | –0.001 b              | –0.061–0.043 | 87 |
|                 | CD154         | 0.022 b               | –0.465–0.099 | 45 |
| 2 yr            | IFN-γ         | 0.074                 | 0.000–0.410  | 22 |
|                 | IL-2          | 0.019                 | –0.090–0.233 | 22 |
|                 | CD154         | 0.089                 | –0.070–0.175 | 11 |
| 4–5 yr          | IFN-γ         | 0.015                 | –0.020–0.093 | 25 |
|                 | IL-2          | 0.023                 | –0.014–0.054 | 25 |
|                 | CD154         | 0.030                 | –0.225–0.075 | 18 |
| 21–31 yr        | IFN-γ         | 0.069                 | 0.000–0.096  | 11 |
|                 | IL-2          | –0.013                | –0.201–0.060 | 11 |
|                 | CD154         | 0.053                 | –0.407–0.338 | 11 |

* Value after subtraction of the percentage in NHDF-treated CD4 T cells.

As three possible types of response were measured, it was also possible to quantify cells that expressed IFN-γ, IL-2, and CD154 simultaneously. However, the specific trifunctional response exceeded 0.05% in only 7 of the 119 samples (5.8%) in which it was measured, so meaningful analyses of the trifunctional response were not possible.

IL-2 responses were highest in Mandinka infants and inversely correlated with maternal BMI. The first stage of the analysis of the effect of the mother’s health and socioeconomic status identified the mother’s BMI and ethnic group as potential predictors of infant IL-2 responses to PPD, and when the two factors were combined in the second-stage analysis, there was an inverse correlation between the mother’s BMI and the infant’s response (P = 0.035) (Fig. 2). However, when ethnic
group was considered independently of BMI, IL-2 responses of Mandinka infants were greater than those of the Wolof ($P = \text{0.032}$) or minority group ($P = \text{0.015}$), although they were not different from those of the Fula infants (Fig. 3).

In order to eliminate the possibility that differences between ethnic groups were not simply a consequence of differences in BMI between ethnic groups, it was confirmed that BMI did not vary significantly between ethnic groups either among the 40 mothers whose infants were considered in the analysis of responses to PPD or among all 361 mothers from the Sukuta cohort for whom BMI data were available.

None of the factors considered affected the IFN-γ response. However, Mandinka infants had a higher proportion of cells that produced both IFN-γ and IL-2 than Wolof infants ($P = \text{0.013}$), although there was no difference between Mandinka and minority infants (Fig. 3) and BMI did not predict the response.

CD154 responses were highest in infants born during the wet season. Neither ethnic group nor BMI had any effect on the expression of CD154, though substantial numbers of cells expressing CD154 did not usually appear in infants under 12 months of age (Table 2). In spite of the low responses at the earlier time points, infants born during the wet season had a significantly higher proportion of CD154-expressing cells than infants born in the dry season when a comparison was carried

### TABLE 3. Percentages of PPD-stimulated CD4 T cells expressing each of the three possible combinations of IFN-γ, IL-2, and CD154 at each time point

| Age at sampling | Antibody type | Antibody response | $n$ |
|-----------------|---------------|-------------------|-----|
|                 |               | Median            | Interquartile range |
| 3 mo            | IFN-γ and IL-2| 0.004             | 0.000–0.021          | 29  |
|                 | IFN-γ and CD154| 0.016             | 0.004–0.026          | 6   |
|                 | IL-2 and CD154| 0.012             | 0.005–0.033          | 6   |
| 6 mo            | IFN-γ and IL-2| 0.006 b           | 0.000–0.027          | 56  |
|                 | IFN-γ and CD154| 0.014 a           | 0.002–0.055          | 28  |
|                 | IL-2 and CD154| 0.013 ab          | 0.000–0.027          | 28  |
| 12 mo           | IFN-γ and IL-2| 0.003 b           | −0.002–0.022         | 87  |
|                 | IFN-γ and CD154| 0.030 a           | 0.004–0.113          | 45  |
|                 | IL-2 and CD154| 0.005 b           | 0.000–0.04           | 45  |
| 2 yr            | IFN-γ and IL-2| 0.017             | 0.007–0.046          | 22  |
|                 | IFN-γ and CD154| 0.029             | 0.006–0.226          | 11  |
|                 | IL-2 and CD154| 0.016             | 0.006–0.035          | 11  |
| 4–5 yr          | IFN-γ and IL-2| 0.007             | −0.003–0.031         | 25  |
|                 | IFN-γ and CD154| 0.014             | −0.013–0.048         | 18  |
|                 | IL-2 and CD154| 0.006             | −0.039–0.026         | 18  |
| 21–31 yr        | IFN-γ and IL-2| 0.000             | −0.044–0.029         | 11  |
|                 | IFN-γ and CD154| 0.024             | −0.040–0.053         | 11  |
|                 | IL-2 and CD154| 0.000             | −0.021–0.019         | 11  |

* Value after subtraction of the percentage in NHDF-treated CD4 T cells. Responses with the same letter were not significantly different, while different letters indicate a $P$ value of $<0.005$. Comparisons were between responses within age groups.

### FIG. 2. Fitted lines indicating the relationship between the mother’s BMI and the infant IL-2 response following Fisher-Yates transformation at 3 months (A), 6 months (B), 12 months (C), and 2 years (D) of age. The relationship is significant at a $P$ value of 0.035 by GEE.

### FIG. 3. Plots of specific IL-2 responses and specific IFN-γ and IL-2 bifunctional responses of infants of different ethnic groups following Fisher-Yates transformation at 3 months (A), 6 months (B), 12 months (C), and 2 years (D) of age. Significances are derived from a GEE that incorporated all ages.
out across all time points \((P = 0.028)\). As there were no 3-month samples collected from infants born in the wet season and the median response at 6 months was actually slightly lower among infants born in the wet season, the difference was evidently driven by differences at 12 and 24 months of age (Fig. 4).

None of the factors considered had any effect on any bifunctional response other than IFN-\(\gamma\) and IL-2. Neither the infant birth weight nor any of the other measures of maternal health or socioeconomic status predicted any differences in any type of response.

For all significant combinations of factors and response, a GEE was also fitted for the negative control values and none were found to be significant, confirming that the relationships between the factors and significant responses were not due to fluctuations in background responses.

**DISCUSSION**

We evaluated the development of CD4 T-cell responses to PPD within the first 2 years of life in infants who received BCG at birth. Production of IFN-\(\gamma\) was the predominant response within the first year, though it was equaled by CD154 in the second year. Production of IL-2 remained very low. Season of birth of the infants, maternal BMI, and ethnicity also influenced the immune response to BCG.

The CD154 response to PPD was very low throughout the first year but rose to levels similar to those of IFN-\(\gamma\) by the time the infants reached 2 years of age. While CD154 has been reported to be a sensitive marker of T-cell response to polyclonal or antigen-specific activation in adults (4, 11), it is relatively poorly expressed by neonatal and infant T cells in response to polyclonal stimuli (8, 34). Data from the same cohort
also showed a poor CD154 response to CMV (26), suggesting that expression of CD154 by infant CD4 T cells is limited in response to antigenic as well as polyclonal stimuli. While studies on viral antigens in adults have indicated that CD154 is expressed on the majority of responding cells (3, 43), Hughes and colleagues (15) reported that it was necessary to consider both CD154 and IFN-γ to evaluate the response to PPD. The finding that only a subset of the CD154-producing cells also expressed IFN-γ even in Gambian adults supports their conclusion.

Infant CD4 T cells are also less able to produce IFN-γ than those of adults, and studies carried out concurrently with the present study showed that both CD4 and CD8 T cells showed relatively poor production of IFN-γ in response to CMV antigens during the first year (25, 26). The similarity in IFN-γ expression in response to PPD by infant and adult CD4 T cells is in clear contrast to the studies on CMV but concurs with the finding that infant responses to PPD are similar to those of adults (22), suggesting a qualitative difference between the mechanisms that underlie the responses to mycobacterial antigens and the more widely studied responses to viral antigens.

Previous studies carried out in The Gambia have found that birth during the wet season is associated with impaired antibody responses to vaccines (29, 30) and a relatively high risk of adult mortality (27, 28), which would suggest impairment of vaccine-induced immunity. However, we found that birth during the wet season and relatively low maternal BMI were associated with increased proportions of CD4 T cells expressing CD154 and IL-2, respectively. Neither this study nor those that preceded it considered T-cell and antibody responses in the same cohort, but these findings suggest that different mechanisms govern the magnitude of antibody responses and cytokine production by CD4 T cells.

Although Sukuta is periurban and the studies on antibody responses took place in a rural area, many women in Sukuta are engaged in agricultural labor and the price of food fluctuates with availability. Consequently, the season of birth, which predicted the infants’ CD154 response, and maternal BMI, which predicted the infants’ IL-2 response, are probably not independent of one another.

The high levels of moisture during the wet season are likely to considerably expand the habitat conducive to the growth of nonpathogenic Mycobacterium spp. and thus increase the level of exposure of pregnant women and infants alike. Also, lower BMIs are typical of poorer women, whose housing is more conducive to exposure, though such accommodation would probably be more crowded and no effect of crowding was found. Whatever the relevant influences are, they persisted for at least the 2 years in which the study was carried out, which indicates that conditions at birth are important for the subsequent development of the immune system.

We excluded infants with a low birth weight in order to restrict the study to relatively healthy infants, while most of the previous studies had included all children born. The different compositions of the cohorts may explain why we found higher responses in infants born in the wet season and infants whose mothers had a relatively low BMI and why these effects were independent of the birth weight of the infants, while the previous studies found inhibition of immune responses associated with birth in the wet season and prenatal undernutrition (27–29, 39).

Mandinka had stronger IFN-γ responses than any of the other ethnic groups, which concurs with the finding that Mandinka in Guinea-Bissau also had stronger tuberculin skin test responses than sympatric ethnic groups (41). Together, these findings suggest that the Mandinka have a particularly robust response to PPD, although they have not been shown to have a risk for tuberculosis that is substantially different than that of other ethnic groups (13).

Differences in the HLA repertoires of the Fula, Wolof, and Mandinka in The Gambia have been found (35), and inheritance of class II HLA genes has been associated with the heritability of the IFN-γ response to PPD (32). However, it has not been established whether the alleles that influenced responses to PPD were the same as those that varied between ethnic groups or whether Gambian Mandinka are more genetically similar to the Mandinga of Guinea-Bissau than to sympatric groups in The Gambia.

Evidence of ethnic differences in immune responses in a community with high rates of intermarriage suggests that sociocultural or economic differences are at least as important as genetic differences, at least in the context of responsiveness to mycobacterial antigens. However, as none of the socioeconomic factors measured in the present study predicted differences in IFN-γ response between ethnic groups, it remains to be established what the important differences may be.

While we have been able to consider several functions of the CD4 T-cell population, which is important in the control of tuberculosis infection (19, 44), the interpretation of the findings is limited as no immunological correlate of protection against disease has been identified. It was necessary to use markers of immune response to the vaccine as an end point, as it was not logistically feasible to recruit a large enough cohort to base the analysis on incidence of tuberculosis disease. A further challenge in the design of the study was that the data were collected according to the requirements of a study intended to address a different hypothesis, so the sensitivity to the effects measured was probably lower than would have been possible with a simple longitudinal sampling regimen based on age. Nevertheless, several of the predictors that we considered exerted a strong enough influence over the infants’ immune responses to be clearly detectable in spite of the limitations imposed by the study design.

In conclusion, we were able to identify some of the factors responsible for the considerable interindividual variability in BCG responses. Given the many factors that affected responses within a relatively small cohort, the large variability in protection afforded by BCG between trials carried out in very different geographical and economic contexts is unsurprising and demonstrates that the antenatal and immediately postnatal environments in which BCG is administered have a lasting influence on the immune response that it induces.

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