

# Specific T Cell Frequency and Cytokine Expression Profile Do Not Correlate with Protection against Tuberculosis after Bacillus Calmette-Guérin Vaccination of Newborns

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**Rationale:** Immunogenicity of new tuberculosis (TB) vaccines is commonly assessed by measuring the frequency and cytokine expression profile of T cells.

**Objectives:** We tested whether this outcome correlates with protection against childhood TB disease after newborn vaccination with bacillus Calmette-Guérin (BCG).

**Methods:** Whole blood from 10-week-old infants, routinely vaccinated with BCG at birth, was incubated with BCG for 12 hours, followed by cryopreservation for intracellular cytokine analysis. Infants were followed for 2 years to identify those who developed culture-positive TB—these infants were regarded as not protected against TB. Infants who did not develop TB disease despite exposure to TB in the household, and another group of randomly selected infants who were never evaluated for TB, were also identified—these groups were regarded as protected against TB. Cells from these groups were thawed, and CD4, CD8, and  $\gamma\delta$  T cell-specific expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-17 measured by flow cytometry.

**Measurements and Main Results:** A total of 5,662 infants were enrolled; 29 unprotected and two groups of 55 protected infants were identified. There was no difference in frequencies of BCG-specific CD4, CD8, and  $\gamma\delta$  T cells between the three groups of infants. Although BCG induced complex patterns of intracellular cytokine expression, there were no differences between protected and unprotected infants.

**Conclusions:** The frequency and cytokine profile of mycobacteria-specific T cells did not correlate with protection against TB. Critical components of immunity against *Mycobacterium tuberculosis*, such as CD4 T cell IFN- $\gamma$  production, may not necessarily translate into immune correlates of protection against TB disease.

**Keywords:** mycobacteria immunity; pediatric settings

Tuberculosis (TB) kills 1.7 million people worldwide each year (1). The current TB vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), affords approximately 80% protection against severe forms of childhood TB (2, 3). However, BCG's protection against pulmonary TB, particularly in adults,

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Correlates of protection against tuberculosis (TB) remain unknown; hence, knowledge on the subject is incomplete.

### What This Study Adds to the Field

The study emphasizes the need to explore beyond the classical immune markers thought to be important for protection against TB in humans.

is highly variable and mostly poor (4). Adults with lung TB spread the disease; new, better TB vaccines that target pulmonary disease are therefore needed urgently.

Our knowledge of immune correlates of protection against TB remains incomplete. Consequently, assessment of immunogenicity of TB vaccines may, at best, be a measure of vaccine take. Current evaluation of vaccine-induced immunity focuses on immunity essential for protection against TB. For example, experimental and clinical evidence support a critical role for CD4 T cells (5, 6), particularly IFN- $\gamma$  production by these cells (7, 8), in protection against TB. This outcome is, therefore, the most commonly measured when determining vaccine take. Because important roles for other type-1 cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and IL-2 (9–11), and for CD8 T cells (12–14), in protection against TB have also been described, all these markers are commonly measured together, by multiparameter flow cytometry after short-term stimulation of whole blood or peripheral blood mononuclear cells (PBMCs) (15–21). Experimental animal studies assessing the efficacy of novel TB vaccines have reported an association between mycobacteria-specific polyfunctional T cells that coexpress IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 at the site of the infection and protection against TB (22, 23). These findings have stimulated much interest in evaluating this subset of T cells in clinical trials.

Our aim was to assess whether these markers correlate with protection against childhood TB after newborn vaccination with BCG. We complemented this assessment by also determining whether expression of IL-17 correlates with protection. Memory T helper (Th) 17 cells are present in peripheral blood of persons exposed to mycobacteria (24); experimental evidence supports a role for these cells in the induction of chemokine release in the lung, resulting in Th1 cell recruitment (25). Furthermore, the magnitude of IL-17 response has been shown to correlate with the clinical outcome of *Mycobacterium tuberculosis* (*M.tb*) infection (26). We also wished to determine whether  $\gamma\delta$  T cell

activation correlates with protection. Our interest to evaluate  $\gamma\delta$  T cells was based on potent mycobacteria-specific activation of  $\gamma\delta$  T cells in 7-month-old infants who had received BCG vaccine at birth (27), and on experimental evidence that suggests an important role in protection against TB, possibly by activating antigen-presenting cells to prime T cell responses (28).

Some of the results of these studies have been previously reported in the form of a poster presentation (29).

## METHODS

### Participant Enrollment

We enrolled participants at the South African Tuberculosis Vaccine Initiative field site in the Worcester area, near Cape Town, South Africa. This area has one of the highest TB incidence rates in the world, documented to be in excess of 2,000/100,000/year in children under 2 years of age (30). The study was nested within a randomized controlled trial (30), which aimed to determine whether intradermal or percutaneous delivery of Japanese BCG at birth resulted in equivalent protection against TB. The following were exclusion criteria at 10 weeks of age: mother known to be infected with human immunodeficiency virus; BCG not received by infant within 24 hours of birth; significant perinatal complications in the infant; any acute or chronic disease in the infant at the time of enrollment; clinically apparent anemia in the infant; and household contact with any person with TB disease, or any person who was coughing. The study was conducted according to the U.S. Department of Health and Human Services and Good Clinical Practice guidelines, and included protocol approval by the University of Cape Town Research Ethics Committee and written informed consent from the parent or legal guardian.

### Blood Collection, Stimulation, and Cryopreservation

At 10 weeks of age, heparinized blood was collected from all infants, and 1 ml was immediately incubated with BCG (SSI strain,  $1.2 \times 10^6$  organisms/ml), as previously described (31). Medium alone served as negative control, whereas staphylococcal enterotoxin B (10  $\mu\text{g/ml}$ ; Sigma-Aldrich, St. Louis, MO) was used as positive control. The costimulatory antibodies, anti-CD28 and anti-CD49d (1  $\mu\text{g/ml}$  each; BD Biosciences, San Jose, CA), were added to all conditions, as this results in enhancement of specific responses (31). Blood was incubated for 7 hours at 37°C. Brefeldin-A was then added, followed by incubation for an additional 5 hours. Cells were then harvested, fixed, and cryopreserved as previously described (31).

### Participant Follow Up and Evaluation

Participants were followed for 2 years. Community-wide passive surveillance systems identified patients with TB disease and children with symptoms suggestive of TB disease, or from households where an adult had TB disease (30). Children who fell into the latter two categories were followed actively; among these, participants who never developed TB over the 2-year follow-up period were classified as household controls, whereas participants who developed TB disease over this period were classified as TB cases. Community controls were randomly selected from the passive surveillance arm, and were never evaluated for TB. The parent study (30) reports the detailed criteria employed to detect all cases of TB disease among participants up to the age of 2 years. All infants who had symptoms compatible with TB disease, or who had contact with an adult with TB disease, were admitted to a dedicated research ward for clinical examination, chest radiography, tuberculin skin testing, two early-morning gastric aspirations, and two sputum inductions for *M.tb* smear and culture (30). All infants admitted to the research ward were also tested for human immunodeficiency virus infection: a positive antibody test resulted in exclusion.

### Intracellular Cytokine Staining and Multiparameter Flow Cytometry

Cryopreserved cells from the protected and unprotected groups only (see subsequent data analysis here) were thawed, washed, and permeabilized with Perm/wash solution (BD Biosciences). Cells were then incubated at

4°C for 1 hour with fluorescence-conjugated antibodies directed against surface antigens and intracellular cytokines. The following antibodies were used: anti-CD3 Pac Blue (clone UCHT1); anti-CD8 Cy5.5PerCP (SK-1); anti- $\gamma\delta$  T cell receptor allophycocyanin (B1); anti-TNF- $\alpha$  Cy7PE (Mab11); anti-IFN- $\gamma$  Alexa 700 (B27); anti-IL-2 fluorescein isothiocyanate (5,344.111) (all from BD Biosciences, San Jose, CA); anti-CD4 QDot605 (S3.5; Invitrogen, Eugene, OR); and anti-IL-17 phycoerythrin (eBio64CAP17; eBioscience, San Diego, CA). Cells were acquired on a LSR II flow cytometer (BD Biosciences) configured with 3 lasers and 10 detectors, with FACS Diva 6.1 software (San Jose, CA). Optimal photomultiplier tube settings were established for this study before sample analysis. Cytometer setting and tracking beads (BD Biosciences) were used to record the target median fluorescence intensity (MFI) values for the baseline settings, and these calibrations were performed each day before sample acquisition. Compensation settings were set with anti-mouse kappa-beads (BD Biosciences) labeled with the respective fluorochrome-conjugated antibodies. Flowjo version 8.8.4 (Treestar, Ashland, OR) was used to compensate and to analyze the flow cytometric data. Boolean gating was applied to generate combinations of cytokine-expressing CD4 and CD8 T cell subsets.

### Data Analysis

Flow cytometric analysis was compared between three groups of infants: (1) those who ultimately developed culture-confirmed TB disease—regarded as not protected against TB and termed “TB cases”; (2) those who were evaluated for TB because of household exposure to an adult with TB disease, but found not to have TB—regarded as protected against TB and termed “household controls”; and (3) a randomly selected group, subjects who had no known exposure to an adult with TB disease, or symptoms compatible with TB disease, and were therefore never evaluated for TB—this was the second group regarded as protected against TB and termed “community controls.” A total of 20 TB cases were excluded from the analysis due to insufficient sample volumes. The microbiological, clinical, and radiological features of the excluded TB cases were comparable to those of the included TB cases (data not shown).

For flow cytometric analysis of cytokine-expressing cells, frequencies of cytokine expression from the negative control (i.e., blood incubated with costimulatory antibodies alone) were subtracted from the BCG-specific responses. Participants were excluded from the analysis for any of the following reasons: (1) a positive control (staphylococcal enterotoxin B) response less than the median plus 3 median average deviations of the negative control; (2) frequencies in the negative control and in the BCG sample in a similar range, suggesting possible contamination of the negative control; and (3) number of CD3 T cell events counted less than 75,000.

For the analysis of MFI of the BCG-stimulated samples, further exclusions occurred if results from participants did not meet any of the following conditions: (1) ratio of BCG to unstimulated frequencies greater than 2; (2) frequencies of BCG-specific cells greater than 0.01%; and (3) number of positive events in the BCG-stimulated sample greater than 20. The Kruskal-Wallis test was used to assess differences between the three groups, and when differences had a *P* value less than 0.05, a Mann-Whitney U test was used to assess differences between individual groups. A *P* value less than 0.05 was considered significant.

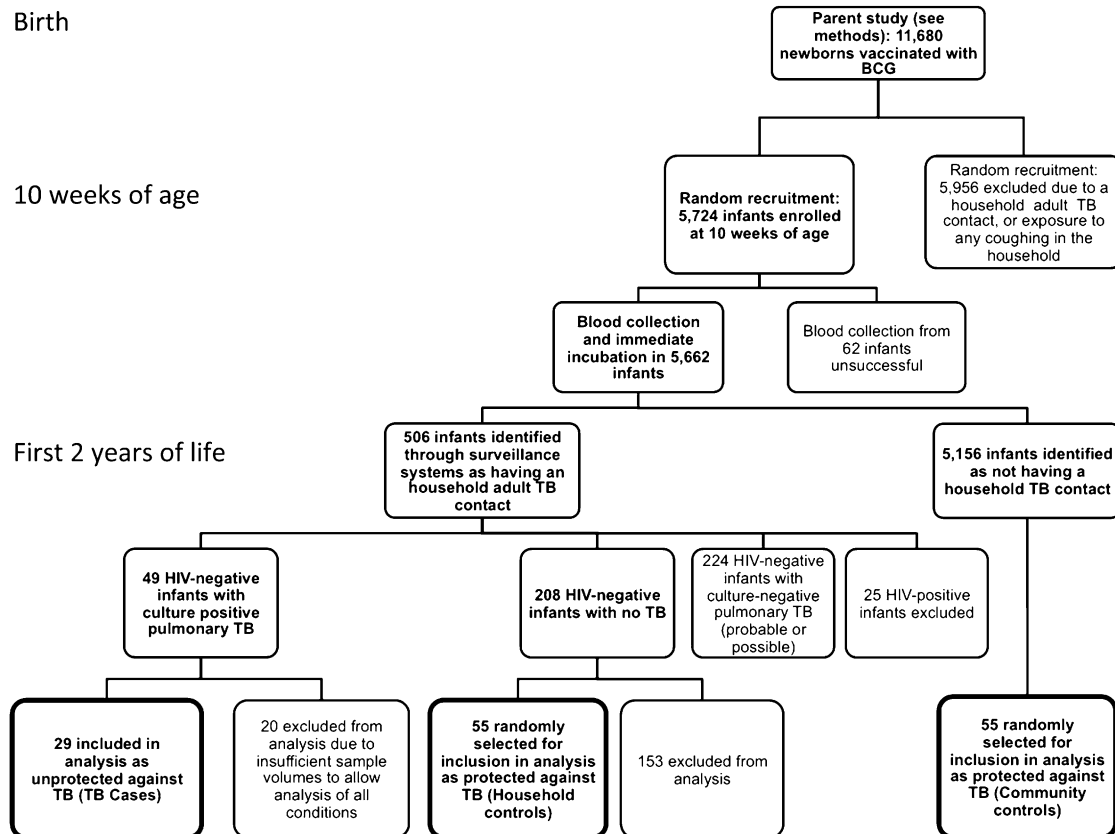
## RESULTS

### Study Participants

A total of 5,724 infants routinely vaccinated with BCG at birth were randomly enrolled from the parent cohort of 11,680 infants (30). Identification of the three infant groups, with clinical exclusions, is shown in Figure 1.

### Frequency and Cytokine Profile of BCG-Specific CD4 T Cells

We used an intracellular cytokine assay to evaluate the frequency and cytokine profiles of specific T cells: the flow cytometric gating strategy is shown in Figure 2. A median of 409,077 (154,687–802,636) CD3 T cells were evaluated. Results from seven participants were excluded from analysis, because the



**Figure 1.** Recruitment and enrollment of participants into the study.

inclusion criteria for analysis were not met (*see* Table E1A in the online supplement). There were no differences in frequencies of CD4 T cells expressing any cytokine, or IFN- $\gamma$ , TNF- $\alpha$ , IL-2, or IL-17 individually, between the three groups of infants (Figure 3A). When expression patterns of cytokines on an individual cell level were evaluated, no differences in polyfunctional CD4 T cells coexpressing IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 together, or CD4 T cells expressing any other combination of the four cytokines, could be shown (Figure 3B). We concluded that, with this assay system at 10 weeks of age, frequency of cytokine profiles of BCG-specific CD4 T cell responses did not correlate with protection against TB.

#### Frequency and Cytokine Profile of BCG-Specific CD8 T Cells

The frequency of specific CD8 T cells and pattern of activation of these cells did not differ between cases and controls (Figures 4A and 4B). In contrast to CD4 T cell responses, where a complex pattern of BCG-specific subsets coexpressing cytokines was shown, most CD8 T cells expressed IFN- $\gamma$ , whereas expression of TNF- $\alpha$  or IL-2 or -17 was very low or undetectable (Figure 4A).

#### BCG-Induced $\gamma\delta$ T Cell Responses

The  $\gamma\delta$  T cells induced by BCG stimulation almost exclusively produced IFN- $\gamma$ ; no differences were seen between the three groups (Figure 5). A very small number of  $\gamma\delta$  T cells expressed TNF- $\alpha$ ; again, there were no differences between the groups (Figure 5).

#### Median Fluorescent Intensity of Cytokine Expression in BCG-Specific T Cells

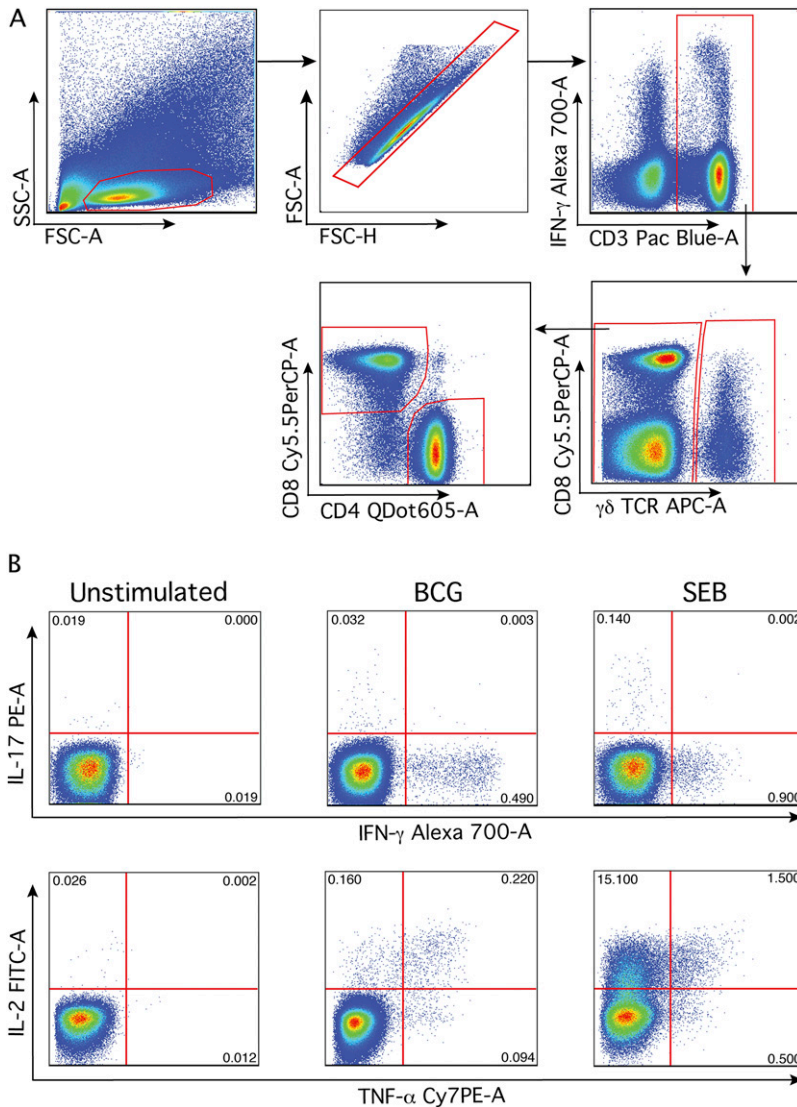
MFI of cytokine expression has been suggested as a useful readout of quality of the T cell response, because the intensity

of cytokine expression appears to be highest in polyfunctional T cells (32). We therefore compared the MFI of cytokine expression of BCG-induced CD4, CD8, and  $\gamma\delta$  T cells between the three groups. Participants were excluded from analysis if the previously described inclusion criteria were not fulfilled for the flow cytometric data (Tables E1B–E1G). We found no differences in any of the MFI values evaluated between the TB cases and the control groups (CD4 T cell MFI is shown in Figure E1; other data not shown).

#### DISCUSSION

We report that, after newborn BCG vaccination, the magnitude and profile of cytokine expression of BCG-specific CD4 and CD8 T cells did not correlate with protection against childhood TB. Importantly, there were no differences in polyfunctional BCG-specific CD4 T cells, which coexpress IFN- $\gamma$ , TNF- $\alpha$ , and IL-2. We also confirm production of IFN- $\gamma$  by  $\gamma\delta$  T cells when whole blood is stimulated with BCG; however, expression of this cytokine by this subset was not associated with protection against childhood TB.

Multiple experimental studies have shown that the Th1 cytokines, IFN- $\gamma$  and TNF- $\alpha$ , are required for immunity against *M.tb* infection and disease (10, 11, 33). This is supported by findings from experimental TB vaccine studies that evaluated biomarkers of protection (34–36). For example, in a heterologous prime-boost strategy with BCG followed by adenovirus-expressing Ad85A, Forbes and colleagues (23) reported a correlation between magnitude of polyfunctional Ad85A-specific Th1 responses in the lungs after *M.tb* challenge and protection against disease. Transfer of early secretory antigenic target (ESAT)-6-specific Th1 memory cells to recipient mice before *M.tb* challenge enhanced protection, suggesting the



**Figure 2.** Gating strategy Flow cytometric analysis of bacillus Calmette-Guérin (BCG)-induced T cell cytokine production. Whole blood was incubated with BCG for 12 hours. Representative dot plots from a single participant are shown. (A) Gating strategy used to identify CD4, CD8, and  $\gamma\delta$  T cells. From left to right, leukocytes from whole blood were acquired and cell doublets excluded with forward scatter area versus forward scatter height parameters. T cells were identified by assessing CD3 expression against IFN- $\gamma$  expression, which enables inclusion of any T cells that may have down-regulated CD3 expression upon activation. Subsequently, CD3<sup>+</sup> T cells were differentiated into conventional T cells, which did not express  $\gamma\delta$  T cell receptor, and  $\gamma\delta$  T cells, by assessing  $\gamma\delta$  expression against CD8 expression. Finally, the conventional T cell population was divided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (B) Representative dot plots of cytokine coexpression patterns in CD4 T cells from unstimulated, BCG, and staphylococcal enterotoxin B (SEB)-stimulated conditions. APC = allophycocyanin; Cy5.5PerCP-A = cyanine peridinin chlorophyll protein; FSC = forward scatter.

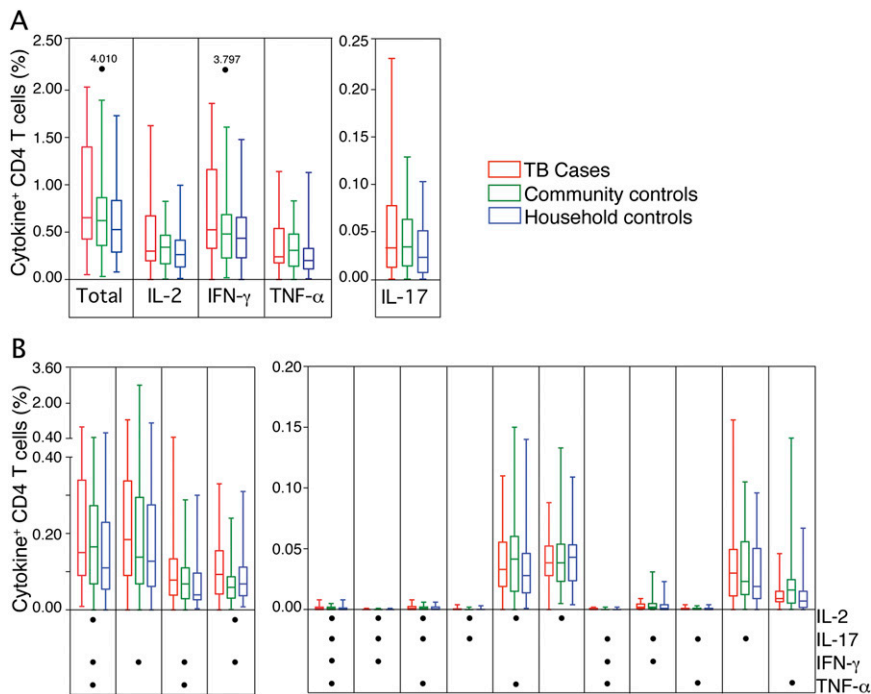
importance of the quantity of antigen-specific T cells at the disease site (37). In contrast, multiple other experimental studies have shown that IFN- $\gamma$  production at the disease site does not correlate with protection against TB; rather, expression of the cytokine may be a marker of the magnitude of the inflammatory response (38–40). For example, Mittrucker and colleagues (38) reported no correlation of BCG-induced T cell responses and protection in a mouse TB challenge model. Furthermore, in a clinical study, Sutherland and colleagues (41) reported that patients with TB disease had a higher polyfunctional CD4<sup>+</sup> T cell response after overnight stimulation of whole blood with ESAT-6 and purified protein derivative compared with healthy individuals with a positive tuberculin skin test.

In our study, we did not measure IFN- $\gamma$  at a disease site—this is not possible in healthy 10-week-old infants—but in peripheral blood. We found no association between the frequency of BCG-specific Th1 cells and protection against TB. This finding is of particular importance, because this peripheral blood outcome is used to assess vaccine-induced immunity in most clinical trials of new TB vaccines. The latter studies often focus on the quality of the CD4 T cell response, with the hypothesis that polyfunctionality (i.e., combined expression of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 by individual cells) is a marker of protective immunity. The interest to evaluate

mycobacteria-specific polyfunctional T cells is based on observations from experimental mouse models of protection against other intracellular organisms, such as *Leishmania major* (32), and, to a limited extent, from animal studies of novel TB vaccination (23). We showed no correlation between polyfunctional BCG-specific CD4 T cell responses in peripheral blood and protection against TB. In an experimental mouse model with *L. major* infection, Darrah and colleagues (32) reported that MFI of cytokine expression could be used as an additional measure of quality of the T cell response, as polyfunctional cells had the highest MFI of cytokine expression. In this study, we assessed the MFI of BCG-specific T cell cytokine expression, and showed no association with protection against TB.

We evaluated IL-17 expression in CD4 T cells based on evidence that this cytokine plays a protective role against TB (25, 26). This is the first study to demonstrate induction of BCG-specific IL-17 cells in infants after BCG vaccination at birth; however, frequencies of BCG-specific Th17 cells did not correlate with protection against TB.

We investigated CD8 T cell responses as possible correlates of protection, based on the important role of this subset suggested by recent experimental and clinical studies (13, 14). For example, Chen and colleagues (13) reported that depletion of CD8 T cells in BCG-vaccinated rhesus macaques led to



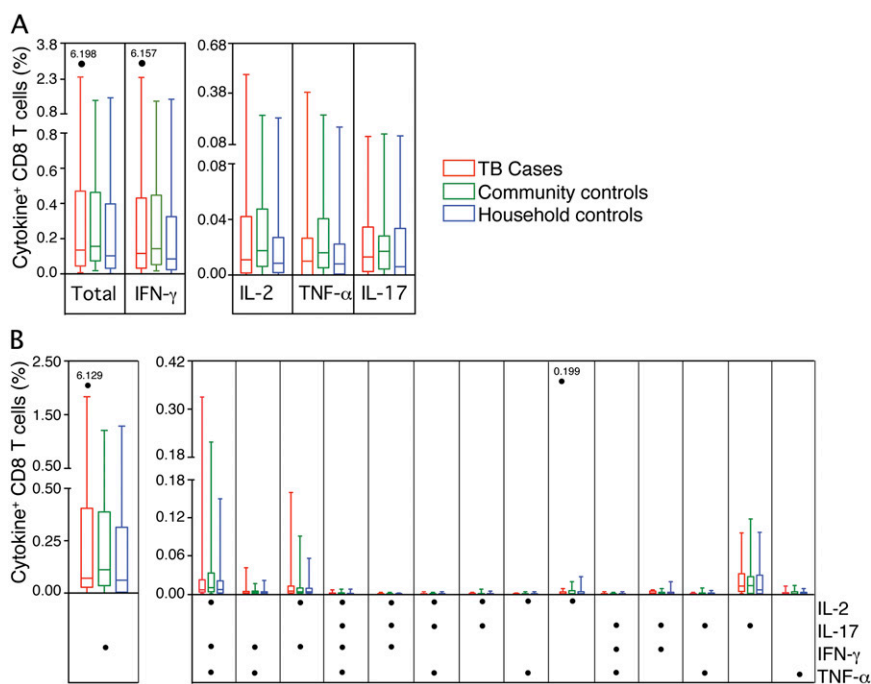
**Figure 3.** Frequency and cytokine expression profile of bacillus Calmette-Guérin (BCG)-specific CD4 T cells. (A) Frequencies of total, IL-2, IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and IL-17 cytokine-expressing CD4 T cells, as detected by an intracellular cytokine assay after incubation of whole blood with BCG. Total cytokine frequencies incorporate all cytokine-positive CD4 T cells. One participant had a very high total and IFN- $\gamma$  CD4 T cell response (4.010 and 3.797%, respectively), and these values are shown individually on the graph. (B) Frequencies of distinct subsets of specific CD4 T cells, based on combinations of cytokine expression. After selecting CD4 T cells, boolean gating was used to generate 15 distinct cytokine-expressing subsets. The Kruskal-Wallis test was used to assess differences between the groups of infants. None of the parameters assessed was different between groups (all:  $P > 0.05$ ). TB = tuberculosis.

a decrease in induced immunity upon subsequent challenge of the animals with *M.tb*. Furthermore, Bruns and colleagues (14) showed that patients undergoing anti-TNF therapy had decreased antimicrobial activity against *M.tb* due to diminished numbers of antigen-specific effector memory CD8 T cells, with an associated increased incidence of TB disease. We observed no differences when comparing specific CD8 T cell responses between protected and unprotected infants.

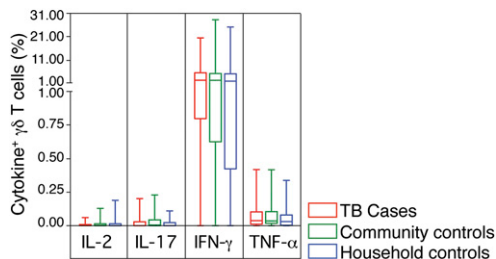
We assessed  $\gamma\delta$  T cell responses based on a report that only the  $\gamma\delta$  T cells expanded from PBMCs of purified protein derivative-positive donors incubated with BCG, and not  $\gamma\delta$  T cells expanded by phosphoantigen, were able to inhibit growth of *M.tb* in autologous macrophages (42). However, we found no

association between the frequency of BCG-induced  $\gamma\delta$  T cells and protection against TB in our study.

Our results strongly suggest that aspects of BCG-specific CD4 and CD8 T cell immunity, or  $\gamma\delta$  T cell immunity, measured in this whole blood assay at 10 weeks of age, may not correlate with protection against TB. We cannot exclude the possibility that these outcomes, measured at another time point after newborn BCG vaccination, or with different antigens or assay systems, might correlate with protection. An infant biomarker study of this size ( $n = 5,662$ ) has not been reported to date; the magnitude of the project required that we limit our blood collection to one practical time point, before *M.tb* infection, and at an age before significant exposure to environmental mycobacteria. This was



**Figure 4.** Frequency and cytokine expression profile of bacillus Calmette-Guérin (BCG)-specific CD8 T cells. (A) Frequencies of total, IL-2, IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and IL-17 cytokine-expressing CD8 T cells, as detected by an intracellular cytokine assay after incubation of whole blood with BCG. Total cytokine frequencies incorporate all cytokine-positive CD8 T cells. One participant had a very high total and IFN- $\gamma$  CD4 T cell response (6.198 and 6.157%, respectively), and these values are shown individually on the graph. (B) Frequencies of distinct subsets of specific CD8 T cells, based on combinations of cytokine expression. After selecting CD8 T cells, boolean gating was used to generate 15 distinct cytokine-expressing subsets. One participant had a very high IL-2 CD4 T cell response (0.199%), and this value is shown individually on the graph. The Kruskal-Wallis test was used to assess differences between the groups of infants. None of the parameters assessed was different between groups (all:  $P > 0.05$ ).



**Figure 5.** *Bacillus Calmette-Guérin* (BCG)-induced  $\gamma\delta$  T cell responses. Frequencies of  $\gamma\delta$  T cells expressing cytokines after incubation of whole blood with BCG. The Kruskal-Wallis test was used to assess differences between the groups of infants. None of the parameters assessed was different between groups (all:  $P > 0.05$ ). TNF = tumor necrosis factor.

also the reason for selecting a single viable bacterial antigen that can be processed for recognition by a wide range of lymphocytes. The results generated by using BCG as an antigen are likely to be specific, as we have recently demonstrated that responses with BCG in a whole blood assay are detectable at 10 weeks of age only in infants who have been vaccinated at birth, and not in unvaccinated infants (43). Regardless, individual mycobacterial antigens might yield different results. Furthermore, the T cell response to mycobacteria is complex (44–46), and involves cytotoxic activity (46–48), for example, in addition to cytokine production. These additional aspects of T cell immunity might correlate with outcome, whereas routine vaccine take measurements focus on cytokine production, using a short-term whole blood assay. Similarly, innate host responses may also be important. We propose that biomarkers of protection against TB may only be unraveled when multiple host factors are examined together in a system biology approach. Ongoing, complementary studies will address whether biomarkers of protection against TB may be identified through other approaches. These include measuring soluble levels of cytokines, chemokines, and growth factors after 7-hour incubation of whole blood with BCG, the cytotoxic, proliferative, and cytokine-expressing capacity of T cells after incubation of PBMCs with BCG for 3–6 days, and gene expression profiles in PBMCs incubated with BCG for 12 hours.

Overall, our results strongly suggest caution when interpreting T cell immune markers commonly evaluated in new TB vaccine clinical trials. More importantly, our results indicate that protective immunity against *M.tb* may be very complex, and suggest a need to look beyond the classical Th1 immunity when assessing the efficacy of novel TB vaccines in clinical trials.

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