Bacillus Calmette-Guérin Vaccination of Human Newborns Induces T Cells with Complex Cytokine and Phenotypic Profiles


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Bacillus Calmette-Guérin Vaccination of Human Newborns Induces T Cells with Complex Cytokine and Phenotypic Profiles


The immune response to vaccination with bacillus Calmette-Guérin (BCG), the only tuberculosis vaccine available, has not been fully characterized. We used multiparameter flow cytometry to examine specific T cell cytokine production and phenotypic profiles in blood from 10-wk-old infants routinely vaccinated with BCG at birth. Ex vivo stimulation of whole blood with BCG for 12 h induced expression of predominantly IFN-γ, IL-2, and TNF-α in CD4+ T cells in seven distinct cytokine combinations. IL-4 and IL-10 expression was detected in CD4+ T cells at low frequencies and only in cells that did not coexpress type 1 cytokines. Specific CD8+ T cells were less frequent than CD4+ T cells and produced mainly IFN-γ and/or IL-2 and less TNF-α, IL-4, and IL-10. Importantly, many mycobacteria-specific CD4+ and CD8+ T cells did not produce IFN-γ. The predominant phenotype of BCG-specific type 1 T cells was that of effector cells, i.e., CD45RA−CCR7−CD27+, which may reflect persistence of Mycobacterium bovis BCG in infants until 10 wk of age. Among five phenotypic patterns of CD4+ T cells, central memory cells were more likely to be IL-2+ and effector cells were more likely to be IFN-γ+. We concluded that neonatal vaccination with BCG induces T cells with a complex pattern of cytokine expression and phenotypes. Measuring IFN-γ production alone underestimates the magnitude and complexity of the host cytokine response to BCG vaccination and may not be an optimal readout in studies of BCG and novel tuberculosis vaccination. The Journal of Immunology, 2008, 180: 3569–3577.

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been reported. We also wished to assess T cell IL-10 expression, since this cytokine is likely to be an important regulator of effector T cell responses against TB (21) and is induced by newborn BCG vaccination (15, 16, 22).

The memory phenotype of T cells induced by BCG vaccination of newborns has not been described. Ag-experienced cells may be categorized based on expression of surface markers (13, 23–26). Central memory cells express CCR7 but not CD45RA and are likely to represent a long-lived population, which expands rapidly in lymph nodes following subsequent Ag encounter (23, 27). In contrast, effector cells are both CCR7– and CD45RA– and act immediately following Ag exposure, but have limited proliferative capacity (13, 23). A third subset, terminally differentiated memory cells are CD45RA+ and CCR7– and the most differentiated subpopulation, based on short telomere length and function (26, 28). Naïve or non-Ag-experienced T cells characteristically express both CD45RA and CCR7 (13, 26, 28). Combination of markers other than CCR7 and CD45RA may also differentiate subsets of Ag-experienced cells. Fritsch et al. (26) recently proposed the phenotypic classification of CD4+ T cell populations based on expression of CD27 and CCR7. Central memory T cells were defined as CD27+ and CCR7+, effectors as CD27+ and CCR7–, and terminally differentiated T cells as CD27– and CCR7–. Our aim was to evaluate expression of all of these markers among Ag-experienced T cells induced by BCG. Although several investigators have characterized mycobacteria-specific immune responses by four-color flow cytometry (29–31), these studies could only measure two cytokines or phenotypic markers at a time and thus most likely underestimated the complexity of the response.

Our hypothesis was that BCG vaccination of newborns would induce both CD4+ and CD8+ T cells capable of producing multiple cytokines and that a central memory phenotype of specific cells would be dominant 10 wk after vaccination. We used an intracellular cytokine assay with multiparameter flow cytometry to comprehensively characterize these variables. To achieve our goals, we established clinical structures and optimized techniques (32) to overcome hurdles common to investigation of immunity in infants.

Materials and Methods

Study participants and blood collection

Healthy 10-wk-old infants, routinely vaccinated intradermally with BCG (Statens Serum Institut, Copenhagen, Denmark) at birth, were enrolled in the Cape Town region of South Africa. This area has a very high TB disease incidence in children 5-year old and younger, exceeding 2% per year in certain areas (32). Infants born to HIV-positive mothers, infants known to be HIV positive, infants with suspected or confirmed TB disease, infants with possible exposure to TB disease and infants with any other active or chronic illnesses at the time of enrollment were excluded. Human participation was according to the U.S. Department of Health and Human Services and good clinical practice guidelines. This included protocol approval by the University of Cape Town Research Ethics Committee and written informed consent. Sodium heparinized blood was collected from each infant. Two different cohorts were enrolled to preserve blood volume collected for assessment with two different flow cytometric protocols (see below): 29 infants to assess cytokine expression of T cells and 27 infants to assess the phenotype of IFN-γ- and IL-2-expressing T cells.

**Ags and Abs**

BCG was reconstituted from the vaccine vial (SSI) at 1.8 × 10^6 organisms/ml as previously described (32). The positive control streptococcal enterotoxin B (SEB; Sigma-Aldrich) was used at 10 μg/ml. The costimulatory Abs anti-CD28 and anti-CD49d (both from BD Biosciences) were examined using the following conjugated Abs: anti-CD3 Amcyan (SK7) and anti-IL-2 Alexa 610-PE (5344.111), both custom-conjugated at BD Biosciences; and anti-CD4 Alexa Fluor 700 (B27) and anti-IL-2 FITC (5344.111), all obtained from BD Biosciences. A separate protocol was used for assessing T cell phenotypes using the following conjugated Abs: anti-CD3 PacBlue (UCHT1), anti-CD4 Cy5.5PerCP (SK3), anti-CD8 Cy5.5PerCP (SK1), anti-CD45RA Cy7PE (L48), anti-CD27 PE (MT271), anti-IFN-γ AlexaFluor 700 (B27) and anti-IL-2 FITC (5344.111), obtained from BD Biosciences, and anti-CCR7 allophycocyanin (150503), obtained from R&D Systems.

**Whole blood intracellular cytokine detection assay**

To determine cell-associated cytokine production, 1 ml of heparinized whole blood was incubated with BCG and anti-CD28 and anti-CD49d as described before (32). Blood incubated with SEB and costimulants, or with costimulatory Abs alone (UNS), served as positive and negative controls, respectively. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added during the last 5 h of incubation to capture cytokines intracellularly. After a total...
incubation of 12 h, RBC were lysed and white blood cells were fixed with FACS Lysing Solution (BD Biosciences), followed by cryopreservation.

**Cell staining and flow cytometric analysis**

To detect intracellular cytokines, cryopreserved cells were thawed, washed in PBS, permeabilized with Perm/Wash solution (BD Biosciences), and incubated at 4°C with fluorescence-conjugated Abs for 1 h. To assess T cell memory phenotypes, a two-step staining method, resulting in optimal staining, was used: cells were thawed, washed in PBS, and permeabilized with Perm/Wash solution and then incubated with surface marker Abs for 1 h at 4°C, followed by an additional hour with Abs specific for intracellular cytokines. Flow cytometric acquisition was completed on a LSRII flow cytometer (BD Biosciences) configured for 3 lasers and 12 detectors. All of the cells in the tube were acquired. Analysis was performed using FACS Diva software (BD Biosciences). Although automated compensation with mouse IgG κ beads was applied, compensation settings were assessed manually after acquisition and adjusted if necessary. Multiparameter panel development (data not shown) included evaluation of appropriate staining controls of Ab and fluorochrome interactions and of spectral overlap using control blood samples (33, 34).

Cutoffs to determine positive cytokine expression in CD4⁺ and CD8⁺ T cells from blood incubated with BCG were set using cells from blood incubated with costimulatory Abs alone (negative control) (Fig. 1A). Angled cutoff lines were necessary for some fluorochromes, because of data spread at higher fluorescence intensities, following instrument compensation (e.g., Fig. 1). SEB was an excellent positive control for induction of all cytokines, except IL-4. IL-4-expressing HICK-2 cells (BD Biosciences), processed per the manufacturer’s protocol, were used as positive control for IL-4. Where distinction between positive and negative surface marker populations was not clear, isotype-matched control Ab staining was used to set cutoffs for phenotypic markers.

**Plasma cytokine detection**

Plasma was collected from the stimulated whole blood after 7 h and cryopreserved. Later, thawed plasma was used to measure levels of IL-2, IL-4, IL-10, and IFN-γ with multiplex beads according to the manufacturer’s instructions (Bio-Rad) and read on a luminometer (Luminex). The range of detection for all cytokines was 1.95–32,000 pg/ml. The optimal plasma dilution for our assay, determined in pilot experiments, was 1/4. Background cytokine levels measured in plasma harvested from unstimulated blood were subtracted from BCG-stimulated blood.

**Statistical considerations**

Negative control (background) values for cytokine expression were not subtracted from BCG-induced responses, because the median backgrounds for all CD4⁺ T cell subsets was 0.001% (range, 0.000–0.01%) and for CD8⁺ T cell subsets was 0.000% (range, 0.000–0.01%). We used an empirical cutoff value of 0.01% as positive: given that a median of 508,509 CD4⁺ T cells and 188,498 CD8⁺ T cells was collected, this cutoff was predicted to be >90% different from background, at an α of 0.05 (35). IL-4 and IL-10 expression was reported with backgrounds (see below). Non-parametric tests were used to compare differences in cytokine expression and phenotypic profiles between CD4⁺ and CD8⁺ T cells. Associations between cellular expression of cytokines and plasma levels of these were assessed by the nonparametric Spearman test. Data were considered statistically significant when \( p < 0.05 \). Statistical analysis was performed using GraphPad Prism 4 software.
Table 1. Association between type 1 cytokine levels in plasma and frequencies of CD4+ or CD8+ T cells expressing these cytokines after incubation of whole blood with BCG

<table>
<thead>
<tr>
<th>Expressing Cytokine</th>
<th>CD4+ T Cells</th>
<th>CD8+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Plasma IFN-γ</td>
<td>0.6793</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma IL-2</td>
<td>0.6441</td>
<td>0.0003</td>
</tr>
<tr>
<td>Plasma TNF-α</td>
<td>0.4034</td>
<td>0.01</td>
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</table>

A Spearman test was used to assess correlation in 29 infants.

Results

**BCG-specific CD4+ and CD8+ T cell type 1 cytokine production**

Intracellular expression of three type 1 cytokines thought to be critical for protective immunity against mycobacteria, IFN-γ, IL-2, and TNF-α (36, 37), was evaluated by incubating blood from 29 BCG-vaccinated infants with BCG for 12 h (Fig. 1). The median frequencies of CD4+ T cells expressing either IFN-γ or IL-2 or TNF-α were similar (Fig. 2A). Lower frequencies of CD8+ T cells expressed IFN-γ, IL-2 (p < 0.05), or TNF-α (p < 0.0001), compared with CD4+ T cells (Fig. 2A). There was a strong positive correlation between the frequencies of IFN-γ- or IL-2- or TNF-α-expressing CD4+ and CD8+ T cells (r = 0.770, r = 0.879, r = 0.760, respectively, all p < 0.0001, Spearman test).

Analysis of simultaneous expression of IFN-γ, IL-2, and TNF-α on a single cell level revealed seven distinct type 1 cytokine-expressing CD4+ T cell populations (Fig. 2B). Among CD8+ T cells, the dominant population expressed IFN-γ only; three other populations were discernable (Fig. 2C). Importantly, a substantial proportion of CD4+ T cells expressing IL-2 and/or TNF-α did not coexpress IFN-γ (Fig. 2D). Similarly, among CD8+ T cells, a proportion of IL-2-expressing T cells also did not coexpress IFN-γ (Fig. 2D). Total type 1 T cell responses were dominated by IFN-γ expression; however, measuring IFN-γ alone did not detect all type 1 cytokine-expressing T cells (Fig. 2E).

All three type 1 cytokines could also be detected in plasma (data not shown). There was a significant correlation between plasma levels of IFN-γ, IL-2, and TNF-α and frequencies of CD4+ T cells producing these cytokines (Table I). Plasma IFN-γ and IL-2 also correlated with frequencies of CD8+ T cells producing IFN-γ and IL-2, respectively (Table I).

Taken together, we concluded that BCG vaccination of newborns induces multiple type 1 T cell subsets defined by expression of distinct cytokine combinations.

**BCG-specific IL-10 and type 2 cytokine production**

The frequency of T cells expressing IL-10 or IL-4 following incubation of whole blood with BCG was low (Fig. 3). Few donors had responses above 0.01%, our cutoff for a positive response, but IL-10 and IL-4 production was consistently above the background expression levels found in blood not incubated with BCG (Fig. 3, B and D). CD4+ T cell expression of both cytokines was slightly higher than that of CD8+ T cells (CD4+ T cell expression shown in Fig. 3; CD8+ T cells: median, 0.004%; range, 0.001–0.024%, expressed IL-10 and median, 0.005%; range, 0.000–0.011%, expressed IL-4). IL-10 and IL-4 were never coexpressed by cells making type 1 cytokines (Fig. 3, A and C). Intracellular IL-4 could be readily detected in HICK-2 cytokine-expressing cells, which served as positive control (Fig. 3C). IL-4 and IL-10 were detected at low levels in plasma of whole blood incubated with BCG (Fig. 4).

We concluded that BCG vaccination of newborns induces low levels of IL-4 and IL-10 expression.

**Phenotypic profiles of specific type 1 T cell subsets**

Studies in other infectious disease models have shown that distinct populations of Ag-experienced T cells may be associated with long-lived protection (38). We therefore examined the phenotypic
profiles of BCG-induced T cells. Specific CD4+ T cells were defined as either IFN-γ- or IL-2-expressing (Fig. 5, A–D); frequencies of cells expressing other cytokines were too low to reliably delineate the phenotype. Five major and distinct Ag-experienced CD4+ T cells subsets could be discerned, based on expression of CD45RA, CCR7, and CD27 (Fig. 6, A–C). By far the most common phenotype of both IFN-γ- and IL-2-expressing CD4+ T cells was CD45RA−CCR7−CD27+ (Fig. 6A), a phenotype that has been reported to be characteristic of effector T cells (13). The second most common phenotype among IFN-γ-expressing CD4+ T cells was CD45RA−CCR7−CD27+, also characteristic of effector T cells (Fig. 6A). Among IL-2-expressing CD4+ T cells, the latter population was significantly less frequent (Fig. 6A), while central memory phenotypes were more common: IL-2+ cells were more likely to be CD45RA+CCR7+CD27+, CD45RA−CCR7−CD27+, or CD45RA−CCR7−CD27+, compared with IFN-γ-expressing CD4+ T cells (Fig. 6A). CD4+ T cells that expressed both IFN-γ and IL-2 were predominantly effector memory cells (CD45RA−CCR7−CD27+ or CD45RA−CCR7−CD27+; Fig. 6B). As a comparison, the expression of phenotypic markers among cytokine-negative CD4+ T cells are depicted in Fig. 6C.

Phenotypes of BCG-specific CD8+ T cells could reliably be detected only for IFN-γ-producing cells, because the frequencies of IL-2-producing CD8+ T cells were too low. CD8+IFN-γ+ T cells also displayed a predominant CD45RA−CCR7−CD27+ effector phenotype (Fig. 6D). Unlike CD4+IFN-γ+ T cells, a central memory population (CD45RA−CCR7−CD27+) was the second

![FIGURE 4.](image)

**FIGURE 4.** Levels of IL-4 and IL-10 in plasma from whole blood incubated with BCG for 7 h. The horizontal line represents the median. Background cytokine levels were subtracted.

![FIGURE 5.](image)

**FIGURE 5.** Phenotype of BCG-specific CD4+ T cells in 10-wk-old infants vaccinated at birth. Ag-specific CD4+ T cells were identified by expression of intracellular IFN-γ (A), IL-2 (B), or both cytokines (C), and the expression of CD45RA, CCR7, and CD27 was determined in each case. The plots illustrate the distribution of cytokine-expressing cells (in color; foreground) in relation to the entire CD4+ T cell population (gray; background, also shown in D).
most common. The expression of phenotypic markers among cytokine-negative CD8^+ T cells are depicted in Fig. 6E.

We concluded that the majority of specific T cells induced by BCG vaccination of newborns has an effector phenotype and that IL-2 expression is more likely to be associated with a central memory phenotype.

Discussion

We showed that BCG vaccination of human newborns induces a diverse set of T cells, delineated by distinct cytokine production and phenotypic profiles. BCG-specific T cells produced mainly type 1 cytokines, as has been demonstrated before (14–16, 20, 22). However, we showed that not only IFN-γ is produced. A considerable number of IFN-γ-negative CD4^+ T cells were present, expressing the other type 1 cytokines IL-2 and TNF-α. Similarly, many CD8^+ T cells produced IL-2 in the absence of IFN-γ. The most commonly used measure of mycobacteria-induced immunity today is IFN-γ production, be this to diagnose latent infection via IFN-γ release assays (39, 40) or to describe human immune responses to novel TB vaccines (41). Recent experimental data suggest that measuring IFN-γ may not correlate with vaccination-induced protection against TB (42–44). This strongly supports measurement of all three cytokines on a cellular level to delineate a mycobacteria-specific type 1 response. This is further supported by our observation that diversity in the T cell cytokine response was predominant in our study population, e.g., in some infants cells that produced one cytokine dominated, whereas in others cells that produced three cytokines dominated. Measuring a single component of the immune response may underestimate the magnitude and complexity of BCG-induced immunity.

A BCG-induced CD8^+ T cell response was readily detectable, as type 1 cytokine-producing cells. Murine studies suggest that...
CD8+ T cells play an important role in control of M. tuberculosis infection and contribute substantially to total IFN-γ production (4). Although a BCG-induced CD8+ T cell response has been described before (14, 45), we now show that the response is characterized by both IFN-γ- and IL-2-producing subsets. An interesting observation was that CD8+ T cells produced very little TNF-α. Smith et al. (45) detected similar frequencies of CD8+ T cells expressing TNF-α and IFN-γ following incubation of PBMC with BCG for 6 days. The contrasting low frequency of TNF-α expression observed in our study could be due to differences in assays, since we measured cytokine expression 12 h after incubation of whole blood with BCG. In longer term assays such as those performed by Smith et al. (45), TNF-α production may be derived from newly differentiated effector T cells, whereas our short-term assay measures cytokine-producing potential directly ex vivo. Our results also contrast with data from HIV-infected adults, whose HIV-specific CD8+ T cells readily express TNF-α in short-term intracellular cytokine assays (46, 47), implying that BCG-specific CD8+ T cells in newborns express little TNF-α.

The memory phenotype of BCG-induced T cells has not been reported. We identified five phenotypically distinct subsets within BCG-specific type 1 T cells, based on expression of CD45RA, CCR7, and CD27. The predominant phenotype of both IFN-γ- and IL-2-expressing CD4+ and CD8+ T cells was CD45RA⁺CCR7⁺CD27⁺. CCR7⁺ effector cells are characteristic of persistent activation of T cells seen in chronic viral infections where Ag is not cleared (48–50). Similarly, increased numbers of effector T cells are observed in children with active TB (51). It is possible that M. bovis BCG had persisted up to 10 wk of age, the time at which blood was collected from infants, resulting in this predominant phenotype.

Distinct differences in phenotypes were observed among CD4+ IFN-γ- and IL-2-expressing cells. IL-2-expressing cells were significantly more likely to have a central memory phenotype, e.g., CD45RA⁻CCR7⁻CD27⁺, compared with IFN-γ-expressing cells. IL-2-expressing T cells induced by BCG vaccination therefore follows patterns similar to those previously described for purified human central memory populations, which are likely to express IL-2 (13). However, our results, from 10 wk after vaccination with BCG, contrast with results obtained 8 wk after vaccination with tetanus toxoid; specific IL-2-expressing central memory cells were present but in a relatively small population, whereas this population was predominant after tetanus vaccination (52). Tetanus toxoid is not a persistent Ag; we therefore hypothesize that viable M. bovis BCG persisted following vaccination, driving differentiation predominantly into IFN-γ-expressing effector cells and preventing differentiation into IL-2-expressing central memory cells. An alternate hypothesis is that continuous exposure to environmental mycobacteria may contribute to chronic immune activation and therefore a predominance of effector T cells. The infants in this study were from a region with high rates of environmental mycobacteria and unpublished data suggest that exposure may occur even within the first 10 wk of life. It has been proposed that exposure to environmental mycobacterial Ags in tropical regions may undermine protective immunity induced by BCG (53). A third hypothesis is that the time point at which we measured the host response was too early for full differentiation into the central memory phenotype. A central memory/IL-2 phenotype has been associated with improved prognosis of chronic human viral infections such as HIV (49) and in experimental settings of chronic intracellular bacterial infection with long-lived protection (38). It remains to be determined whether a central memory phenotype is also associated with successful vaccination against TB.

We found that a significant amount of specific CD4+ and CD8+ T cells had a phenotype traditionally regarded as naive, i.e., CD45RA⁺ and CCR7⁺. In a recent study of children with TB, Caccamo et al. (54) also described this population, identified as specific by MHC class I pentamers of Ag-85A. We propose that this CD45RA⁺CCR7⁺ population reflects early differentiation into Ag-specific cells, before loosing CD45RA expression.

We could detect very low intracellular expression of the type 2 cytokine IL-4 above background values. Expression of type 2 cytokines has been associated with a suboptimal immune response to mycobacteria (55). For example, Ordway et al. (56) showed that long-term control of latent M. tuberculosis infection in humans appeared to be associated with optimal type 1 cytokine production and absence of detectable type 2 cytokine production. They showed that high percentages of IL-4-expressing CD8+ and γδ T cells soon after M. tuberculosis infection were associated with ultimate development of TB disease. The IL-4-expressing cells were detected after incubation of PBMC for 6 days, which contrasted with our 12-h assay; longer term assays may be required to detect these cells in the setting of BCG vaccination of the newborn. Intracellular expression of IL-10 could also be detected in our cohort, was low, and, like IL-4 expression, was never coexpressed with any type 1 cytokine. We propose that the IL-10-expressing T cells were induced regulatory T cells, which are expected to be present at low frequencies. Regulatory T cells control conventional effector immune responses, are induced by infections (57) and likely also by vaccination with BCG.

Our results clearly demonstrate the advantages of complex multiparameter flow cytometric analysis for deciphering a vaccination-induced immune response. However, this technology is not readily available. It is therefore important to note, from our findings, that when detection of IFN-γ or IL-2 is the aim and flow cytometry is not available, plasma levels may serve as surrogates of T cell cytokine production. We showed that plasma levels of type 1 cytokines correlated strongly with intracellular expression despite the fact that non-T cells also have the ability to make these cytokines (58, 59). When only four-color flow cytometry is available, inclusion of all three type 1 cytokines in one-color channel for detecting the total type 1 response may be the most useful. This proposal is supported by observations in HIV infection, where the presence of “polyfunctional” CD8+ T cell populations, i.e., HIV-specific CD8+ T cells that coexpress multiple cytokines, was associated with better clinical outcome (46). The observation in a mouse model of Leishmania major infection that polyfunctional T cell induction is also associated with the best outcome (60) suggests that measurement of these cells may also be important following BCG vaccination, since the mechanisms of immune protection are very similar for Leishmania and mycobacteria. Regardless, measurement of all three type 1 cytokines in one color would still not delineate the complexity of cytokine expression profiles. This may be important, since other studies of HIV infection showed that IL-2 expression, rather than IFN-γ or dual expression, correlated with the best clinical outcome (49). We therefore propose that delineation of multiple cytokine-expressing subsets, individually, will be important to investigate protective immunity against TB, either following natural infection or following vaccination.

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Disclosures
The authors have no financial interest of conflict.

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