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Bacillus Calmette Guerin Vaccination of Human Newborns Induces a Specific, Functional CD8$^+$ T Cell Response[1]

Rose Ann Murray,* Nazma Mansoor,† Ryhor Harbacheuski,* Jorge Soler,* Virginia Davids,† Andreia Soares,† Anthony Hawkridge,† Gregory D. Hussey,† Holden Maecker,‡ Gilla Kaplan,* and Willem A. Hanekom[2]†

Mounting evidence points to CD8$^+$ T cells playing an important role in protective immunity against *Mycobacterium tuberculosis*. The only available vaccine against tuberculosis, bacillus Calmette Guérin (BCG), has traditionally been viewed not to induce these cells optimally. In this study, we show that vaccination of human newborns with BCG does indeed induce a specific CD8$^+$ T cell response. These cells degranulated or secreted IFN-γ, but not both, when infant blood was incubated with BCG. This stimulation also resulted in proliferation and up-regulation of cytotoxic molecules. Overall, the specific CD8$^+$ T cell response was quantitatively smaller than the BCG-induced CD4$^+$ T cell response. Incubation of whole blood with *M. tuberculosis* also caused CD8$^+$ T cell IFN-γ expression. We conclude that BCG induces a robust CD8$^+$ T cell response, which may contribute to vaccination-induced protection against tuberculosis. ***The Journal of Immunology,* 2006, 177: 5647–5651.**

The World Health Organization declared tuberculosis a global health emergency in 1993. The situation is no less urgent today: one-third of the world's population is infected with *Mycobacterium tuberculosis*, 8.9 million developed tuberculosis disease in 2003, and 1.75 million died (1). Control of the epidemic remains difficult because tuberculosis control programs often lack adequate resources, prevalent HIV infection increases risk of disease, and because multidrug resistance is emerging.

An effective vaccine that prevents lung tuberculosis would be an attractive and sustainable long-term intervention. *Mycobacterium bovis* bacille Calmette Guérin (BCG)[3] remains the only available vaccine. BCG offers variable, but mostly poor, protection against pulmonary tuberculosis, at all ages (2). However, the vaccine is given to newborns soon after birth because ~80% protection is afforded against severe forms of childhood tuberculosis, i.e., miliary disease and meningitis (2). The immune mechanisms underlying this protection are not understood. Only a handful of reports have addressed immunity induced by newborn vaccination with BCG. Among more modern studies, Marchant et al. (3) reported that the vaccine induces a Th1-like response in newborns, characterized by IFN-γ production in CD4$^+$ T cells following incubation of PBMC with soluble purified protein derivative of *M. tuberculosis*. These results were confirmed by Hussey et al. (4), who demonstrated lymphoproliferation and predominant IFN-γ production after incubation of infant PBMC with purified protein derivative or with mycobacteria. Specific cytotoxic activity was also demonstrated; although it may be presumed that CD8$^+$ T cells were responsible for this function, this was not addressed.

The aim of our study was to determine whether BCG vaccination of human newborns induces specific and functional CD8$^+$ T cells. *Mycobacteria* do indeed induce a CD8$^+$ T cell response in humans, as evidenced by multiple reports of immunity induced by BCG vaccination at older ages, and of immunity in persons either latently infected or diseased by *M. tuberculosis* (5–8). However, there is a widely held view that BCG itself induces suboptimal CD8$^+$ T cell responses (9, 10). The overall importance of CD8$^+$ T cells in protective immunity against mycobacteria remains incompletely understood. Multiple reports of murine studies suggest that CD8$^+$ T cells participate in protection (11–14); however, overlap of functions with CD4$^+$ T cells (such as IFN-γ production), dependency on CD4$^+$ T cells (15), and complex kinetics of the induced CD8$^+$ T cell response (13, 16–18) have made an assessment of the relative contribution of the latter T cell subset to protection difficult to dissect. We propose that CD8$^+$ T cells are important in BCG-induced protection against tuberculosis of humans. Our study was the first to directly address this T cell population in the context of newborn vaccination against tuberculosis.

Materials and Methods

Study participants and blood collection

Healthy 10-wk-old infants, routinely vaccinated with intradermal BCG (Danish strain 1331; Statens Serum Institut) at birth, were enrolled from clinics in the Cape Town region of South Africa. Infants were excluded if known to be HIV-infected or born to HIV-infected mothers. Additionally, a rapid HIV test was performed on each infant on enrollment—positive results lead to exclusion. Infants with suspected or confirmed tuberculosis, or in contact with adults with pulmonary tuberculosis, were also excluded. Blood was collected and immediately incubated in whole blood assays (see below and Ref. 19), or PBMC were isolated from the blood and cryopreserved. Protocols for this study were approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey and the

*Public Health Research Institute, Newark, NJ 07103; †South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa; and ‡BD Biosciences, San Jose, CA 95131

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[2] Address correspondence and reprint requests to Dr. Willem A. Hanekom, South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine, Wernher Beit S2.01, University of Cape Town Health Sciences Faculty, Anzio Road, Observatory, 7925 Cape Town, South Africa. E-mail address: Willem@rmh.uct.ac.za

[3] Abbreviations used in this paper: BCG, Bacille Calmette Guérin; WB-ICC, whole blood intracellular cytokine assay; MOI, multiplicity of infection; SEB, staphylococcal enterotoxin B.

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Research Ethics Committee of the University of Cape Town. Ethical guidelines of the U.S. Department of Health and Human Services and the South African Medical Research Council were followed, which included written informed parental consent.

Ags and Abs

Viable BCG (Danish strain 1331) was prepared by adding RPMI 1640 (BioWhittaker) directly to the lyophilized vaccine vial, immediately before use. Virulent *M. tuberculosis* strain H37Rv Pasteur was cultured to log phase in 7H9 medium (Difco) with 0.05% Tween 80 (Sigma-Aldrich) and then stored at −80°C. Immediately before use, H37Rv was thawed, sonicated, and diluted in RPMI 1640. For the whole blood intracellular cytokine assay (WB-ICC; see below), 1.2×10^6 organisms of Danish BCG were incubated with 1 ml of infant blood. For the degranulation assay (see below), PBMC were incubated with BCG or *M. tuberculosis* at a multiplicity of infection (MOI) of 0.1. For the BrdU lymphoproliferation assay (see below), 1×10^5 BCG organisms were incubated with 1 ml of infant blood. For the CFSE lymphoproliferation assay (see below), PBMC were incubated with BCG at an MOI of 0.01. For the cytotoxic marker assay, PBMC were incubated with BCG at an MOI of 0.1. These optimal concentrations of mycobacterial Ags were determined in pilot experiments. As a positive control, staphylococcal enterotoxin B (SEB; Sigma-Aldrich) was incubated at 10 μg/ml in WB-ICC and degranulation assays and PHA (Abbott Murex) at 5.4×10^{-3} mitogenic units/ml in cytotoxic marker and lymphoproliferation assays. The costimulatory Abs anti-CD49d and anti-CD28 (BD Biosciences) were added at 1 μg/ml in the WB-ICC and degranulation assays to enhance the specific response. Fluorescence-conjugated Abs against CD3, CD4, CD8, IFN-γ, CD69, granzyme A, perforin, and BrdU (latter with DNase) were all obtained from BD Biosciences. Anti-CD107ab-allophycocyanin was obtained from H. Maecker (BD Biosciences, San Jose, CA). A fluorescence-conjugated Ab against granzyme B was obtained from Caltag Laboratories.

Whole blood BrdU lymphoproliferation assay

The proliferative capacity of specific $CD8^+$ T cells was evaluated using a whole blood assay. A total of 125 μl of infant blood was diluted to 1.25 ml in RPMI 1640 and incubated at 37°C, in 5% CO_2, for 6 days with BCG, PHA (positive control), or medium (control) alone. On day 6 (day 3 for PHA), BrdU (Sigma-Aldrich) was added at 10 μM for the final 4 h of incubation. Cells were harvested using EDTA (2 mM), lysed, and fixed with FACS Lysing Solution and cryopreserved in 10% DMSO (Sigma-Aldrich) in FCS (Delta Bioproducts) (cryosolution). Fixed cells were thawed, permeabilized (0.1% saponin (Sigma-Aldrich) and 0.1% BSA (Boehringer Mannheim) in PBS (BioWhittaker) "permeabilization solution") and stained with fluorescence-conjugated Abs. Following 2 h of incubation at room temperature in the dark, the cells were washed once in permeabilization solution, pelleted, vortexed at high speed, and finally suspended in 1% paraformaldehyde (Electron Microscopy Sciences) in PBS. Flow cytometric acquisition was performed within 24 h of labeling to determine the frequency of proliferating ($BrdU^+$) $CD8^+$ T cells, with a FACSCalibur (BD Biosciences). Further analysis was conducted with CellQuest software (BD Biosciences).

PBMC (CFSE) lymphoproliferation assay

Cryopreserved PBMC were thawed, washed in serum-free RPMI 1640, and resuspended in RPMI 1640 at 5×10^7 cells/ml. CFSE (Molecular Probes) was added at 5 μM and the cells were incubated for 15 min at 37ºC in 5%CO_2. The stain was then quenched with five times the volume of ice-cold RPMI 1640 with 10% pooled human serum (Western Province Blood Transfusion Service) for 10 min. The cells were washed twice and incubated at 3×10^5 cells in 100 μl/well in a 96-well round-bottom plate. DNase (Sigma-Aldrich) was added at 10U/ml, and the cells were "rested" for 6 h at 37°C in 5% CO_2. BCG, PHA, or medium alone were then added (final well volume 200 μl), and the cultures were incubated for 6 days at 37°C in CO_2. Cells were harvested with 2 mM EDTA, fixed in FACS Lysing Solution, and cryopreserved in this solution at −80°C. Later, cells were thawed, washed in 0.1% BSA in PBS ("FACS wash buffer"), stained with fluorescence-conjugated Abs, washed again, and resuspended in 1% paraformaldehyde in PBS. $CD8^+$ T cell proliferation was measured as CFSE dilution by flow cytometry on a FACSCalibur; further analysis of proliferation was completed with FlowJo software (Tree Star). FlowJo develops a model of proliferation based on a CFSE profile and certain assumptions, such as definition of the undivided population, number of generations expected, and no cell death. The model is used to estimate a percentage of the starting population, which would have divided; this frequency is presented in figures generated from CFSE proliferation data.

WB-ICC assay

BCG-induced $CD8^+$ T cell-specific IFN-γ production, after short-term stimulation, was detected with a WB-ICC assay (19). Briefly, 1 ml of infant blood was incubated at 37°C for 12 h in the presence of BCG, SEB, or medium alone and the costimulatory Abs anti-CD28 and anti-CD49d. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the last 5 h of incubation. Whole blood was harvested with EDTA (2 mM; Sigma-Aldrich), and FACS Lysing Solution (BD Biosciences) was added to simultaneously lyse erythrocytes and fix white blood cells. Fixed cells were cryopreserved in cryosolution as described above. Fixed cells were thawed, permeabilized, and stained with fluorescence-conjugated Abs and analyzed by flow cytometry (FACSCalibur, CellQuest; BD Biosciences), as described previously (19).

Degranulation assay (CD107ab detection)

Thawed PBMC were processed with DNase and rested as described above. BCG, *M. tuberculosis*, SEB, or medium were added, in the presence of costimulatory Abs. Following 13 h of stimulation, brefeldin A (10 μg/ml), monensin (5 μg/ml), and fluorescence-conjugated anti-CD107ab were added. Cells were incubated an additional 5 h, then harvested with 2 mM EDTA, and washed with FACS wash buffer. The cells were then incubated in the dark, on ice, with 0.5 μg/ml ethidium monoazide (EMA; Molecular Probes) for 10 min, followed by exposure to bright light for 10 min. This allowed EMA, which had entered only dead cells, to covalently bind to DNA. Cells were washed with FACS wash buffer and fixed with FACS Lysing Solution before cryopreservation at −80°C. In batch, cells were thawed, permeabilized and stained with fluorescence-conjugated Abs against CD3, CD8 and, in some experiments, IFN-γ. Following a wash in FACS wash buffer, cells were resuspended in 1% paraformaldehyde. $CD8^+$ T cell degranulation was measured by CD107ab expression by flow cytometry with a FACSCalibur; FlowJo software was used for further analysis.

Cytotoxic marker assay

PBMC were thawed and suspended in R10 in 96-well plates, treated with DNase, and rested as described above. BCG, PHA, or medium alone were added as described above. After 72 h, cells were harvested with 2 mM EDTA, fixed with FACS Lysing Solution and cryopreserved at −80°C. Later, cells were thawed, permeabilized, and stained with fluorescence conjugated Abs against CD3, CD8, or CD69 and either granzyme A, granzyme B, or perforin. The expression of these cytotoxic markers in activated ($CD69^+$) and nonactivated ($CD69^{neg}$) $CD8^+$ T cells was assessed by flow cytometry with a FACSCalibur; FlowJo software was used for further analysis.

Statistical considerations

Stem-leaf plots showed that the distribution of most continuous variable results was skewed. Therefore, and because the population sizes were small, differences between groups were evaluated by nonparametric paired Wilcoxon signed ranked test. A p value < 0.05 was considered statistically significant.

Results

Study participants

A total of 64 infants was enrolled. Because of small blood volumes, different assays were performed in different groups of infants. Blood products from 3 infants were excluded from analysis because of a positive plasma ELISA test for HIV.

BCG-specific $CD8^+$ T cells are present in the blood of vaccinated infants

To determine whether newborn BCG vaccination induces specific $CD8^+$ T cells, we assessed $CD8^+$ T cell proliferation following ex vivo incubation of blood or of PBMC with viable BCG. The whole blood assay measured incorporation of BrdU into proliferating cells (Fig. 1*A*), and the PBMC-based assay measured CFSE dilution (Fig. 1*B*). Considerable frequencies of proliferating $CD8^+$ T cells were detected in the infants (Fig. 1, *C* and *D*). The frequency of proliferating $CD8^+$ T cells was lower than that of proliferating $CD4^+$ T cells (Fig. 1, *C* and *D*).

The presence of BCG-induced $CD8^+$ T cells was also evaluated by measuring cell-specific IFN-γ production, measured by flow

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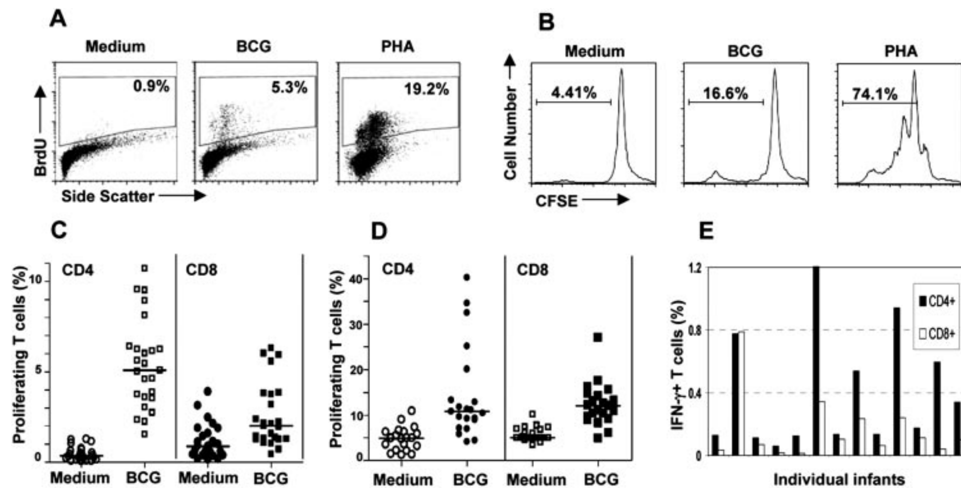

FIGURE 1. *A*, Flow cytometric detection of BrdU$^+$-proliferating T cells in diluted whole blood incubated with medium, BCG or PHA (see *Materials and Methods* for details). Results shown are gated on CD3$^+$CD8$^+$ T cells. *B*, Flow cytometric detection of CFSE$_{low}$-proliferating T cells in PBMC incubated with medium, BCG, or PHA (see *Materials and Methods* for details). Results shown are gated on CD3$^+$CD8$^+$ T cells. *C*, BCG-induced proliferation of CD4$^+$ and CD8$^+$ T cells, as detected by the whole blood BrdU incorporation assay, in 25 10-wk-old infants, vaccinated with BCG at birth. Horizontal bars reflect the median. *D*, BCG-induced proliferation of CD4$^+$ and CD8$^+$ T cells, as detected by the PBMC CFSE assay, in 25 10-wk-old infants, vaccinated with BCG at birth. *E*, CD4$^+$ and CD8$^+$ T cell IFN-γ production, measured by flow cytometry after incubation of whole blood with BCG, in 25 10-wk-old infants, vaccinated with BCG at birth (see *Materials and Methods*). In *B–D*, the difference between results obtained with medium and with BCG was statistically significant, using Wilcoxon signed ranked test: *B*, CD4, $p < 0.0001$, and CD8, $p < 0.001$; *C*, CD4 and CD8, $p < 0.0001$.

cytometry, following 12 h incubation of whole blood with viable BCG. Specific IFN-γ production in CD8$^+$ T cells was detected in all infants (Fig. 1*E*). As for lymphoproliferation, the frequency of IFN-γ$^+$CD8$^+$ T cells was lower than that of IFN-γ-producing CD4$^+$ T cells (Fig. 1*E*).

We concluded that BCG vaccination of human newborns does indeed induce specific CD8$^+$ T cells. The frequency of specific CD8$^+$ T cells is lower than that of BCG-specific CD4$^+$ T cells.

CD8$^+$ T cells degranulate, and up-regulate cytotoxic molecules, in response to BCG

Cytotoxicity is a hallmark function of CD8$^+$ T cells. To assess whether CD8$^+$ T cells induced by newborn vaccination with BCG have cytotoxic potential, we optimized and performed two assays. First, we evaluated BCG-specific cytotoxic degranulation with a PBMC-based, flow cytometric assay. The assay detects specific stimulation-induced expression of LAMP1 and LAMP2 (CD107a and CD107b, respectively), which are exposed on the cell surface upon granule release. Results from this assay have been shown to correlate with more cumbersome chromium release assays (20), which were not possible in our setting, given small infant blood volumes. We modified previously described CD107 assays to exclude dead lymphocytes, by scatter characteristics and by EMA staining (Fig. 2*A*); therefore, excluding false positive CD107ab binding to dead cells. Although assay backgrounds were high, in 19 of 21 infants evaluated, incubation of PBMC with BCG resulted in up-regulation of CD107 expression, reflecting cytotoxic degranulation (Fig. 2*B*).

Whereas results from the short term degranulation assay, described above, might reflect an ex vivo "snapshot" measurement of cytotoxic potential, our second assay measured potential of specific CD8$^+$ T cells to up-regulate expression of the specific cytotoxic effector proteins perforin, granzyme A, and granzyme B 72 h after incubation of PBMC with BCG; this time point was found optimal in pilot experiments. CD8$^+$ T cell-specific production of granzyme B has, in an ELISPOT, been shown to correlate with cytotoxic activity as measured by chromium release assays (21). Because most CD8$^+$ T cells of newborns contain perforin and granzymes at baseline, we decided to optimize our assay to differentiate CD8$^+$ T cells that were activated by specific stimulation with BCG, from those that were not activated in the same culture, to compare cytotoxic protein expression. In preliminary experiments, we found that CD69 expression was the best marker to differentiate activated and nonactivated cells (Fig. 2*C*). In all infants, incubation of PBMC with BCG resulted in up-regulation of expression of perforin, granzyme A, and granzyme B in CD69$^+$CD8$^+$ T cells (Fig. 2*D*).

We concluded that vaccination of human newborns with BCG induces functional, specific CD8$^+$ T cells with cytotoxic potential.

Degranulation and IFN-γ production appear to be discordant among BCG-induced CD8$^+$ T cells

To compare BCG-specific CD8$^+$ T cell degranulation, a marker of cytotoxic capacity, with cytokine-producing potential of these cells, we stained for anti-CD107ab during, and for IFN-γ after, 18 h of incubation of PBMC with BCG (Fig. 3*A*). Dead cells were again excluded by scatter characteristics and EMA staining. A consistent pattern emerged among the vaccinated infants: discordance between degranulation and cytokine production was observed (Fig. 3, *A* and *B*).

We concluded that vaccination of human newborns with BCG induces specific CD8$^+$ T cells that appear to have either degranulation, and therefore cytotoxic, capacity, or cytokine-producing potential, but rarely both.

Virulent M. tuberculosis *induces lesser IFN-γ production and degranulation in BCG-induced CD8$^+$ T cells*

The aim of BCG vaccination is protection against tuberculosis disease. We therefore evaluated whether viable, virulent *M. tuberculosis* as Ag would induce assay results similar to those obtained by using viable BCG. The longer-term lymphoproliferation and cytotoxicity assays could not be performed, as *M. tuberculosis* caused excessive cell death (data not shown). The short-term PBMC-based assay evaluating CD107 and IFN-γ expression could

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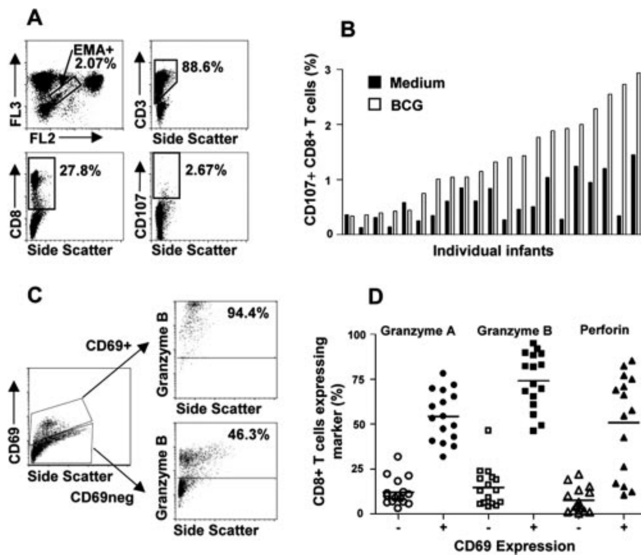

FIGURE 2. *A*, Detection of CD8$^+$ T cell degranulation by surface expression of CD107ab by flow cytometry, following incubation of PBMC with BCG (see *Materials and Methods*). The flow cytometric analysis strategy is shown: after selecting lymphocytes on a forward/side scatter plot (data not shown), dead cells (stained with EMA in the region between FL2 and FL3) were excluded. CD3$^+$ T cells, and then CD8$^+$ T cells, were selected to finally analyze CD107 expression. *B*, CD107ab expression in CD8$^+$ T cells following incubation of PBMC with medium or with BCG, in 21 10-wk-old infants vaccinated with BCG at birth. The difference between results obtained with medium and with BCG was statistically significant, using Wilcoxon signed ranked test ($p < 0.0001$). *C*, Cytotoxic granule expression in CD8$^+$ T cells, 72 h after incubation of PBMC with viable BCG, was detected by flow cytometry. After gating on CD3$^+$CD8$^+$ T cells (data not shown), activated CD8 T cells, defined as CD69$^+$ and nonactivated (CD69$^-$) cells were selected to show intracellular granzyme B expression in these populations. Results from a single infant are shown. *D*, Intracellular expression of the cytotoxic markers granzyme A, granzyme B, and perforin in 25 10-wk-old infants, vaccinated with BCG at birth, in activated and nonactivated CD8$^+$ T cells, following incubation of PBMC with BCG for 72 h. For all markers, the difference between expression in activated and nonactivated CD8$^+$ T cells was statistically significant, using Wilcoxon signed ranked test (granzyme A, $p < 0.001$; granzyme B, $p < 0.0005$; perforin, $p < 0.0001$).

be performed; dead cells were excluded by scatter characteristics and EMA staining. BCG was included as control Ag. *M. tuberculosis* did indeed induce IFN-γ and CD107 expression; however, at levels that were lower than that induced by BCG (Fig. 4). The

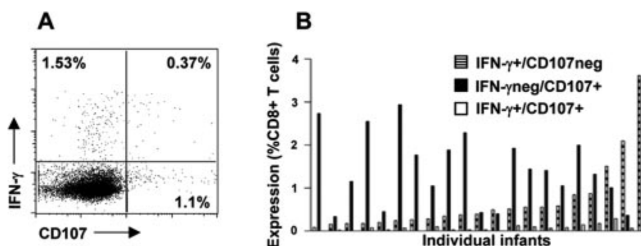

FIGURE 3. *A*, BCG-induced expression of CD107 and of IFN-γ in CD8$^+$ T cells from a 10-wk-old infant, vaccinated with BCG at birth. PBMC were incubated with BCG for 18 h (see *Materials and Methods* for details). Results are shown gated on CD8$^+$ T cells. *B*, BCG-induced expression of CD107, of IFN-γ, or both, in CD8$^+$ T cells from 21 10-wk-old infants, vaccinated with BCG at birth. PBMC were incubated with BCG for 18 h (see *Materials and Methods* for details).

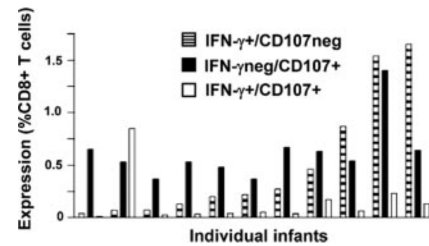

FIGURE 4. *M. tuberculosis*-induced expression of CD107, of IFN-γ, or both, in CD8$^+$ T cells from 11 10-wk-old infants, vaccinated with BCG at birth. PBMC were incubated with *M. tuberculosis* for 18 h (see *Materials and Methods* for details).

pattern of discordance between IFN-γ expressing and CD107 expressing specific CD8$^+$ T cells was again demonstrated (Fig. 4).

We concluded that vaccination of human newborns with BCG induces specific CD8$^+$ T cells that are cross-reactive with virulent *M. tuberculosis*.

Discussion

We show, for the first time, that BCG vaccination of human newborns does induce specific CD8$^+$ T cells. Incubation of whole blood or PBMC from vaccinated infants with BCG resulted in IFN-γ production, degranulation, proliferation, and ultimately increased production of cytotoxic proteins in CD8$^+$ T cells. These results strongly suggest that BCG-induced CD8$^+$ T cells in newborns are functional. Our findings question the poorly substantiated view that BCG does not induce a significant CD8$^+$ T cell response (9, 10).

Our study of the CD8$^+$ T cell response following BCG vaccination was unique in that our participants were naive to other mycobacterial Ag exposure, because of their young age, and because we excluded infants who could have been exposed to adults with tuberculosis disease. Other studies have focused on adults, who were likely to have had at least environmental mycobacterial antigenic exposure. Dockrell's group showed that incubation of PBMC from vaccinated adults with live BCG resulted in up-regulation of the activation markers CD25, HLA-DR, and CD71 on CD8$^+$ T cells (6). Later, they showed presence of specific, MHC class I-restricted CD8$^+$ T cells capable of producing IFN-γ, TNF-α and perforin, and restricted by a range of dominant Ags, in peripheral blood of BCG-vaccinated persons (7). Assays that rely on expansion of PBMC for 6 days were used in these studies.

For our analysis, we chose to use both shorter term, i.e., 18 h assays, which allows a direct ex vivo snapshot of specific CD8$^+$ T cells present in peripheral blood, and longer term, i.e., 3–6 day assays, which rely on expansion of these cells. In the shorter-term assays, we found that specific CD8$^+$ T cells could either produce IFN-γ, or could degranulate, i.e., express CD107 on the surface. The CD107 assay has been reported in a handful of clinical studies only, but our finding suggesting discrepant cytokine production and CD107 expression contrasts with CD8$^+$ T cell function described in HIV-infected persons (22). In the latter setting, peptide Ag stimulation induced cytokine production in the same CD8$^+$ T cells that degranulated. Nevertheless, our results point toward functional heterogeneity in the BCG-induced CD8$^+$ T cell pool, as has been suggested by Dockrell's earlier studies (7).

Results from CD107 assays have been shown to correlate well with those from traditional assays of cytotoxicity, such as chromium release assays (20, 23). However, CD107 assays should be interpreted with caution: it has recently been demonstrated that although multiple subtypes of specific CD8$^+$ T cells may degranulate (express CD107) after specific stimulation, actual cytotoxic

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activity may rather depend on expression of effector molecules, such as the granzymes and perforin (24). We therefore measured expression of these cytotoxic proteins in $CD8^+$ T cells 72 h after incubation of PBMC with BCG. We could demonstrate marked up-regulation of the proteins in BCG-activated $CD8^+$ T cells, strongly suggesting cytotoxic potential.

We also assessed proliferation of $CD8^+$ T cells, as results may reflect expansion of a central memory T cell pool (25). Animal models of viral infection have suggested that this functional T cell pool may be the most critical for long-term protection. We showed that $CD8^+$ T cells of the infants did indeed expand following incubation of PBMC with BCG. It remains to be demonstrated whether this expanded $CD8^+$ T cell pool is associated with protection against tuberculosis disease induced by newborn BCG vaccination; our ongoing and future projects will address this question.

In all assays where BCG-induced $CD4^+$ and $CD8^+$ T cell responses were evaluated side by side, $CD4^+$ T cell responses were quantitatively greater than the $CD8^+$ T cell responses. This is consistent with multiple experimental and human studies of mycobacterial immunity. However, assessment of the relative size of the different specific $CD8^+$ T cell pools induced by BCG should also take into account potentially differential kinetics of these two T cell subsets. In mice, it has been shown that specific $CD8^+$ T cells appear early after *M. tuberculosis* infection, diminish over time, and again increase as bacillary loads increase (17). Similarly, the $CD8^+$ T cell effector molecules perforin and IFN-γ may play distinct roles at different time points after murine infection with *M. tuberculosis* (13, 16, 18). We measured the BCG-induced $CD8^+$ T cell response at 10 wk of age, which is likely to be in the "contraction phase" after newborn vaccination, as the measured $CD4^+$ T cell response at 10 wk of age is quantitatively less than at 6 wk (our unpublished data). It is therefore feasible that, like in mice, quantitative and qualitative differences in the $CD8^+$ T cell response may exist at other time points—longitudinal changes in this response will be a focus of a future study.

Specificity of the $CD8^+$ T cell response, i.e., an assurance that the measured immunity was indeed induced by BCG, would have been supported by evaluation of an unvaccinated control group, or by evaluation of cord blood responses in the same infants. This was not possible in our setting, as delay of BCG vaccination beyond the immediate newborn period is not ethically justifiable. This is because the incidence of tuberculosis disease in infants under 2 years of age exceeds 2%/year, in our study population. Furthermore, collection of cord blood was not possible, because of resource constraints.

In summary, our findings point to the presence of a complex mycobacteria-specific $CD8^+$ T cell pool following BCG vaccination of newborn. In studies to follow, we will address the kinetics of this $CD8^+$ T cell response, and whether specific components of the response correlate with BCG vaccination-induced protection against childhood tuberculosis.

Disclosures

The authors have no financial conflict of interest.

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