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Polyfunctional CD4 T-cells correlate with *in vitro* mycobacterial growth inhibition following *Mycobacterium bovis* BCG-vaccination of infants

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ABSTRACT

Background: Vaccination with Bacillus Calmette Guerin (BCG) protects infants against childhood tuberculosis however the immune mechanisms involved are not well understood. Further elucidation of the infant immune response to BCG will aid with the identification of immune correlates of protection against tuberculosis and with the design of new improved vaccines. The purpose of this study was to investigate BCG-induced CD4+ T-cell responses in blood samples from infants for cytokine secretion profiles thought to be important for protection against tuberculosis and compare these to PBMC-mediated *in vitro* mycobacterial growth inhibition.

Methods: Blood from BCG-vaccinated or unvaccinated infants was stimulated overnight with *Mycobacterium tuberculosis* (*M. tb*) purified protein derivative (PPD) or controls and intracellular cytokine staining and flow cytometry used to measure CD4+ T-cell responses. PBMC cryopreserved at the time of sample collection were thawed and incubated with live BCG for four days following which inhibition of BCG growth was determined.

Results: PPD-specific IFNγ+TNFα+IL-2+CD4+ T-cells represented the dominant T-cell response at 4 months and 1 year after infant BCG. These responses were undetectable in age-matched unvaccinated infants. IL-17+ CD4+ T-cells were significantly more frequent in vaccinated infants at 4 months but not at 1-year post-BCG. PBMC-mediated inhibition of mycobacterial growth was significantly enhanced at 4 months post-BCG as compared to unvaccinated controls. In an analysis of all samples with both datasets available, mycobacterial growth inhibition correlated significantly with the frequency of polyfunctional (IFNγ+TNFα+IL-2+) CD4+ T-cells.

Conclusions: These data suggest that BCG vaccination of infants induces specific polyfunctional T-helper-1 and T-helper-17 responses and the ability, in the PBMC compartment, to inhibit the growth of mycobacteria *in vitro*. We also demonstrate that polyfunctional T-helper-1 cells may play a role in growth inhibition as evidenced by a significant correlation between the two.

1. Introduction

A major challenge in the field of tuberculosis (TB) vaccinology is the need for a better understanding of how Bacillus Calmette-Guerin (BCG), the only licensed vaccine in use against TB, confers protection in some populations and settings but not others [1–3]. Knowledge of the mechanisms underlying this difference is vital for the ongoing search for a better vaccine. One TB vaccine candidate that had proved efficacious in pre-clinical animal models [4,5] did not give infants significant protection from TB disease [6]. In that clinical trial, the vaccine, MVA85A, was administered to infants already vaccinated at birth with BCG with the aim of enhancing any BCG-mediated protection. That this did not occur has prompted the suggestion that the protection afforded by BCG alone may be difficult to improve upon and as such, its mechanisms of action deserve greater attention if this is to happen [7].

Our group has previously provided an immunological description of the different responses to BCG in United Kingdom (UK) and African populations in which the efficacy of BCG differs. Malawian adolescents displayed high baseline immune responses

Abbreviations: BCG, Bacillus Calmette-Guerin; PPD, purified protein derivative; SEB, Staphylococcus enterotoxin B; TB, tuberculosis; NTM, non-tuberculous mycobacteria; IQR, interquartile range; ICS, intracellular cytokine staining; MGIA, mycobacterial growth inhibition assay.

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to mycobacterial antigen and BCG did not enhance these. In the UK, adolescents displayed low baseline responses which were increased significantly following BCG [8]. These observations were consistent with the hypothesis that exposure to non-tuberculous mycobacteria (NTM) was masking the response to BCG [9]. In subsequent reports we expanded this description to infants where, although pre-exposure to NTM was unlikely given the very young age of the study group, differences in immune response were still observed between the two settings. UK infants made stronger T helper (Th)-1-type responses including IFNγ whereas Malawian infants made stronger Th-2 and regulatory-type responses [10]. Although Th-1 responses, including cytokines such as IFNγ and antigen-specific CD4+ T-cells, have been reported not to correlate with protection against, or risk of, TB disease [11–15], they remain a focus of interest for TB vaccinologists due to contrasting evidence where these cells do seem to correlate with protection against TB [16] as well as the strong evidence in favour of their importance based on studies of patients with IFNγ signaling deficiencies [17,18] or with HIV infection and low CD4 counts [19] who are both susceptible to TB.

A recent development in the field of TB biomarker discovery has been the establishment of techniques that allow the measurement of mycobacterial growth inhibition *in vitro*. This assessment of the capacity of a compartment such as whole blood or peripheral blood mononuclear cells (PBMC) to inhibit the growth of mycobacteria represents an unbiased surrogate marker of protection against TB and allows for investigations into the potential roles of immune mechanisms of interest in mediating this "protective" effect [20–23].

In light of the recent evidence [24] that BCG vaccination in high latitude, low NTM settings such as the UK might provide important information regarding BCG-induced protective immunity, we hypothesized that the cytokine immune responses we previously described in UK infants would originate from antigen-specific helper T-cells. We report here our findings that BCG vaccination does indeed induce long lasting, polyfunctional Th-1 cellular responses as well as a more transient Th-17 response in UK infants. We also show that BCG vaccination induces PBMC-mediated mycobacterial growth inhibition and that polyfunctional Th-1 responses may play a role in this.

2. Materials and methods

2.1. Study participants and sample collection

Healthy infants, born in the UK to mothers with no history of chronic illness including HIV infection, were recruited after written informed consent was obtained from parents. Depending upon local health service policy, infants either received a single dose of intradermal BCG (BCG Vaccine Danish Strain 1331, Staten Serum Institute, Copenhagen, Denmark) at community clinics (median vaccination age: 5.6 weeks, interquartile range (IQR): 4.3–8.0 weeks) or did not receive BCG. Heparinised venous blood samples were obtained from infants at two time points selected to provide data at distinct intervals (4 months and 1 year) following vaccination or at age-matched time points in unvaccinated infants. Due to variations in availabilities of infant participants and parents, actual median sampling time points were 15.3 weeks (IQR: 14.4–18.0) and 56.3 weeks (IQR: 54.9–58.0). Ethical approval for the study was granted by the National Research Ethics Service Committee London-East (11/LO/0363) and by the Ethics Committee of the London School of Hygiene and Tropical Medicine.

2.2. Diluted whole blood intracellular cytokine staining (ICS) assay

Venous blood (0.5 mL) was diluted 1:1 with warm Iscove's Modified Dulbecco's Medium (Lonza, Belgium) in 15 mL centrifuge tubes (Corning Inc. NY). Diluted blood was incubated alone (medium only negative control), with 10 μg/ml *M. tb* PPD batch RT50 for *in vitro* use; Statens Serum Institute, Copenhagen, Denmark) or with 5 μg/ml staphylococcus enterotoxin B (SEB; Sigma, UK) as a positive control. Co-stimulatory antibodies (2 μg/ml each of anti-CD28 and anti-CD49d (BD Biosciences, Oxford, UK) [25]) were added to all antigen and control tubes. Assay tubes were incubated with loose lids for 2 h at 37 °C after which 3 μg/ml of brefeldin A (Sigma, UK) was added to all tubes which were then incubated for a further 18 h at 37 °C. The average time between venipuncture and the initiation of ICS assay stimulations was 3 h 16 min (SD: 56 min). Following incubations diluted blood was incubated with 10 volumes of 1x PharmLyse solution (BD Biosciences, Oxford, UK) at room temperature for 10 min to lyse red blood cells, centrifuged and washed with 3 mL PBS. Prior to permeabilisation, pelleted cells were surface stained with Vivid live/dead reagent (Molecular Probes), anti-CD4-APC-H7 (BD Biosciences), anti-CD19-efluor450 and anti-CD14-efluor450 (eBiosciences) for 30 min at 4 °C. After washing in PBS + 0.1% BSA + 0.01% sodium azide, cells were permeabilised with Cytofix/Cytoperm reagent (BD Biosciences) at 4 °C for 20 min, washed in Perm Wash buffer (BD Biosciences) and stained with anti-CD3-Horizon-V500, anti-IL-2-FITC, anti-TNFα-PE-Cy7 (BD Biosciences), anti-IL-17-efluor660, anti-IL-10-PE (eBiosciences) and anti-IFNγ-PerCP-Cy5.5 (Biolegend) for 30 min at room temperature. Cells were finally resuspended in 250 μL 1% paraformaldehyde (Sigma, UK) and filtered prior to acquisition. Data was acquired using an LSRII flow cytometer (BD Biosciences) configured with 3 lasers and 10 detectors and FACSDiva acquisition software (BD Biosciences). Compensation was performed using tubes of CompBeads (BD Biosciences) individually stained with each fluorophor and compensation matrices were calculated with FACSDiva.

2.3. PBMC cryopreservation and in vitro mycobacterial growth inhibition assay

For infant blood samples where enough material was available following whole blood ICS assays, PBMC were prepared by density centrifugation and cryopreserved. Briefly, blood diluted 1/3 in HBSS (Invitrogen) was layered over Histopaque 1077 (Sigma) and centrifuged at 800*g* for 20 min. The PBMC layer was transferred to a fresh tube, washed 3 times with HBSS, frozen in RPMI 1640 (Invitrogen) with 20% foetal bovine serum (FBS; Invitrogen) and 10% dimethylsulfoxide (Sigma) and stored at −80 °C for 24 h before transfer to liquid nitrogen. On thawing, cells were rested for two hours at 37 °C in RPMI 1640 with 10% FBS and 10 units/ml of benzonase (Novagen), then washed and re-suspended in RPMI 1640 with 25 mM HEPES (Sigma) supplemented with 2 mM L-glutamine and 10% filtered, heat-inactivated, pooled human AB serum (Sigma). PBMC (1×10^6) were added to 2 ml screw-cap microtubes (Sarstedt, Germany) with a pre-determined, optimal quantity of BCG Danish that equated to 862 cfu and made up to a final volume of 600 μl. Tubes were incubated at 37 °C with 360° rotation for 96 h. Following incubations, cells and remaining BCG were pelleted and cells lysed by incubation in sterile water with vortexing. BCG from a single tube were then transferred into a corresponding MGIT tube and time to positivity determined using a MGIT 960 (Becton Dickinson). As all assays were carried out simultaneously in a single batch, direct-to-MGIT controls were not used, as the calculation of relative growth was not required.

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