Vaccine 34 (2016) 5298–5305

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

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

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ARTICLE INFO

Article history:
Received 15 June 2016
Received in revised form 15 August 2016
Accepted 2 September 2016
Available online 9 September 2016

Keywords:
BCG
T-cells
Cytokines
Infants
Polyfunctional
Mycobacterial growth inhibition

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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http://dx.doi.org/10.1016/j.vaccine.2016.09.002
0264-410X/

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.

2.4. Data analysis, management and statistical analysis

ICS flow cytometric data was analysed using FlowJo software version 9 (TreeStar Inc.) and Spice version 5 [26]. Samples were gated sequentially on singlet, live, CD14-CD19-, lymphoid, CD3 + CD4+ cells and negative control stimulation tubes were used to set cytokine gates (Supplementary Fig. 1). Median cytokine responses in negative control tubes, as a percentage of the gated CD4+ T-cell population, were as follows: IFNγ – 0.003%; TNFα – 0.002%; IL-2 – 0.002%; IL-17 – 0.002%; IL-10 – 0.003%. Median cytokine responses in positive control tubes (SEB-stimulated) were as follows: IFNγ – 0.16%; TNFα – 2.05%; IL-2 – 1.77%; IL-17 – 0.11%; IL-10 – 0.06% (Supplementary Fig. 2). Cytokine responses reported for all stimuli are after subtraction of background values measured in un-stimulated tubes. The median number of CD4+ T-cell events acquired for all tubes was 298,895 (IQR: 239875–312526).

All mycobacteria growth inhibition assays were carried out in duplicate. For each tube, time to positivity in hours was converted to log CFU of bacteria using a previously determined growth curve for the stock of BCG used.

All statistical comparisons between the magnitudes of ICS and growth inhibition responses between vaccinated and unvaccinated groups were made using the Mann-Whitney *U* test. Associations between growth inhibition and ICS responses were measured using Spearman's rank correlation coefficient.

3. Results

3.1. BCG-vaccination of infants at 6 weeks of age induces antigen-specific, polyfunctional Th-1 cells that persist up to at least 1 year post-vaccination

In order to assess the ability of infant BCG vaccination to activate cytokine-secreting helper T-cells we used a diluted whole blood ICS assay and PPD stimulation to measure the frequency of mycobacteria antigen-specific CD4+ T-cell responders at an early and a later time point following vaccination. CD4+ T-cells expressing IFNγ, TNFα and IL-2 were detectable at 4 months and 1 year after vaccination but not in age-matched samples from unvaccinated infants (Fig. 1A–C). The application of Boolean gating to determine the pattern of cytokine co-expression revealed that at both 4 months and 1 year, IFNγ+TNFα+IL-2+polyfunctional T-cells formed the dominant responder population in BCG-vaccinated infants. The overall responder profile, which also included TNFα +IL-2+ double positive and TNFα+ single positive populations, was almost identical at both time points after vaccination (Fig. 1D). These data support the conclusion that BCG vaccination of UK infants activates an antigen-specific, polyfunctional Th-1 response that is still detectable one year after vaccination.

3.2. Antigen-specific Th-17 cells are increased in vaccinated infants at 4 months post-vaccination compared to unvaccinated controls

In addition to IFNγ, TNFα and IL-2, we also examined CD4+ T-cells for antigen-specific, IL-17 and IL-10 responses. Although we could not detect any IL-10 response with this protocol at either time point (data not shown), we did observe a significantly greater frequency of IL-17+ CD4+ T-cells in vaccinated compared to unvaccinated infants at 4 months (median responses of 0.011% IL-17+ and 0.002% IL-17+ CD4 T-cell respectively; $p = 0.0045$) but not at 1 year (median responses of 0.007% IL-17 + and 0.006% IL-17+ CD4 T-cell respectively; $p = 0.894$; Fig. 2A–C). In order to determine whether IL-17+ CD4+ T-cells were secreting other cytokines, we restricted analysis to all possible IL-17+ signatures (Fig. 2D), which revealed that the dominant secretion profile was IL-17+ single positive events. Although the response profile was similar at both time points, there was a significant reduction in the frequency of two less prominent populations at 1 year, namely IL-17+TNFα+IL-2+ triple positive and IL-17+IL-2+ double positive cells ($p = 0.03$ and $p = 0.009$ respectively; Fig. 2D). These data support the conclusion that BCG vaccination of UK infants activates an antigen-specific Th-17 response that, although prominent at 4 months post-BCG, at 1-year post-BCG has reduced to a magnitude that is indistinguishable from that seen in unvaccinated infants.

3.3. PBMC compartment of vaccinated infants displays an increased capacity to inhibit the in vitro growth of BCG mycobacteria at 4 months post-vaccination

In parallel to diluted whole blood ICS assays, where sample volume permitted, PBMC were also prepared from infant blood samples and cryopreserved. These PBMC were later thawed and tested for the capacity to inhibit mycobacterial growth *in vitro* with an assay used as a surrogate marker for protective immune responses against *Mycobacterium tuberculosis in vivo*. Growth of BCG mycobacteria was significantly inhibited by PBMC from BCG-vaccinated infants at 4 months. The median log CFU measured after 4-day culture was 3.2 as compared to 5.1 in unvaccinated infants ($p < 0.001$; Fig. 3A). The difference in median log CFU at 12 months post-vaccination between vaccinated and age-matched unvaccinated infants however was not significant (4.2 and 5.1 respectively; $p = 0.294$; Fig. 3B). As samples from vaccinated and unvaccinated infants at both time points exhibited a range of log CFU values that represented the varying degrees of mycobacterial inhibition, we compared these data with matching frequencies of polyfunctional (IFNγ+TNFα+IL-2+) and Th-17 (IL-17+) CD4 T-cells for those samples where both MGIA and ICS data were available. In order to avoid including dependent variables in the correlation analysis, where data was available for one infant at both time points, only the 4-month data was included. There was a significant degree of inverse correlation between polyfunctional CD4+ T-cell frequency and log CFU (Spearman r value = −0.709; $p = 0.013$; Fig. 3C) but not between Th-17 CD4 T-cell frequency and log CFU (Spearman r value = −0.399; $p = 0.198$; Fig. 3D) as measured after growth inhibition assays.

In summary, BCG vaccination of UK infants induces polyfunctional and Th-17 CD4 T-cells and bestows upon the PBMC compartment the capacity to inhibit mycobacterial growth. Further to these observations, there is an association between the magnitude of the polyfunctional T-cell response and the level of mycobacterial inhibition detected *in vitro*.

4. Discussion

We have measured a population of PPD-specific, CD4+ T-cells that demonstrates polyfunctional (IFNγ+TNFα+IL-2+) capabilities in infants who have received BCG vaccination at 6 weeks of age. This population was detectable at 4 months and 1 year post-vaccination, comprised the most abundant population as compared to other possible secretion profiles at both time points and was absent in unvaccinated infants. This result complements previous immune analyses we have carried out on similar infant cohorts where secreted cytokine responses, including IFNγ, TNFα and IL-2, were observed in vaccinated but not unvaccinated infants following PPD stimulation of diluted whole blood [27,28], and partly answers the question as to which cells were secreting cytokines in those studies. A number of papers have consistently described a more heterogeneous T-helper response in terms of the cytokine secretion profiles detected following BCG in infants

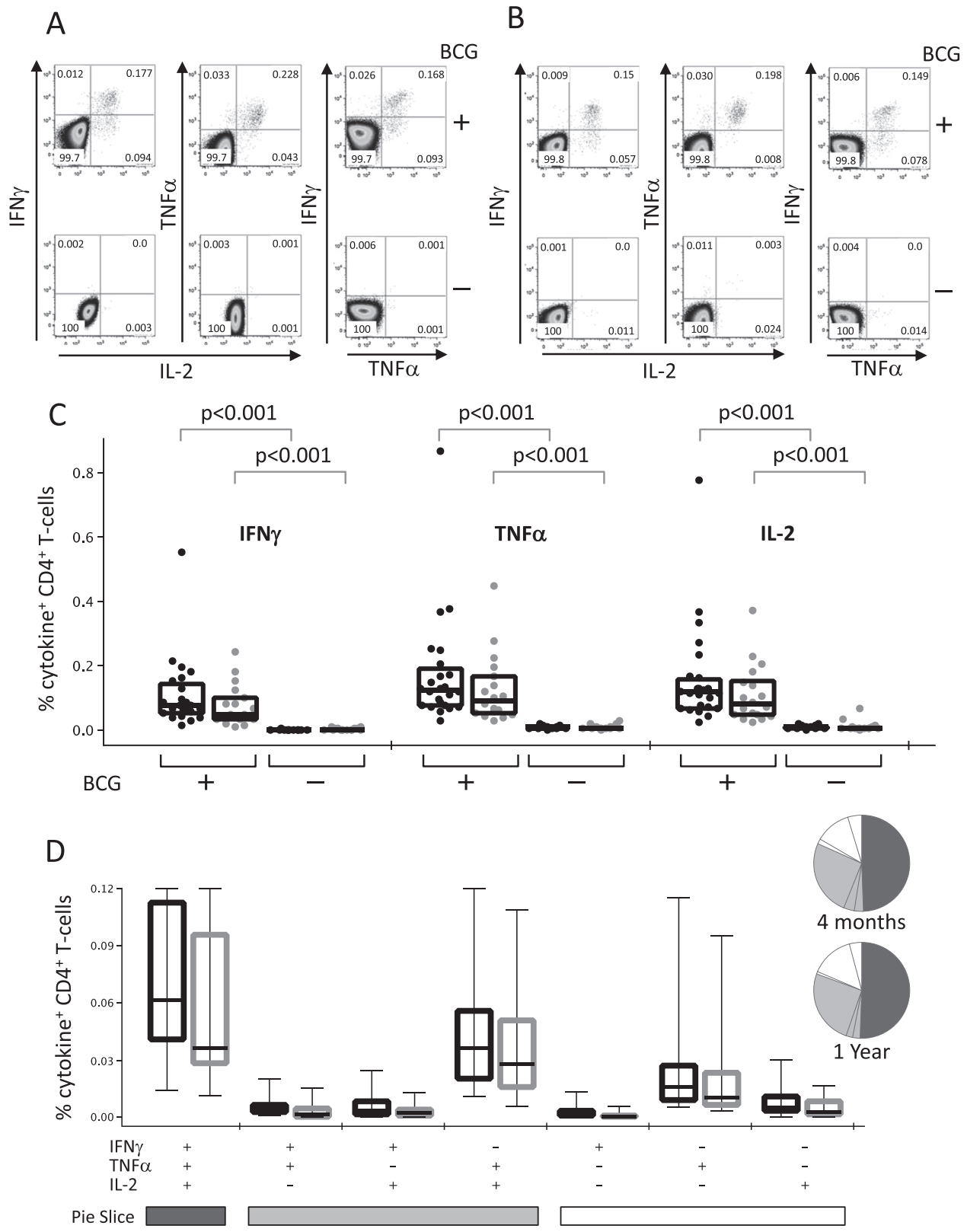

Fig. 1. Persistent, polyfunctional T helper-1 cells are the dominant, PPD-specific CD4+ T-cell population in BCG-vaccinated infants. Venous blood samples obtained 4 months and 1-year post-BCG from vaccinated infants or from age-matched, unvaccinated infants were stimulated with PPD overnight and CD4+ T-cells assessed for cytokine secretion by intracellular staining. An example of IFNγ, TNFα and IL-2 co-staining is shown for 4 month (A) and 1 year (B) samples from a BCG vaccinated (+) and an unvaccinated (−) infant and cumulative composite, single cytokine expression data (C) for vaccinated and unvaccinated infants at 4 months (dark grey circles) and 1 year (light grey circles). (D) The polyfunctionality of CD4+ T-cell cytokine responses in BCG-vaccinated infants at 4 months (dark grey bars) and 1 year (light grey bars). All composite data plots display boxes showing medians and interquartile ranges. Whiskers, where included, show the data range. The Mann-Whitney *U* test was used to determine significance. Sample numbers are n = 24 (BCG-vaccinated infants at 4 months post-BCG); n = 18 (BCG-vaccinated infants at 1 year post-BCG); n = 12 (BCG-unvaccinated infants at 4 month time point) and n = 15 (BCG-unvaccinated infants at 1 year time point).

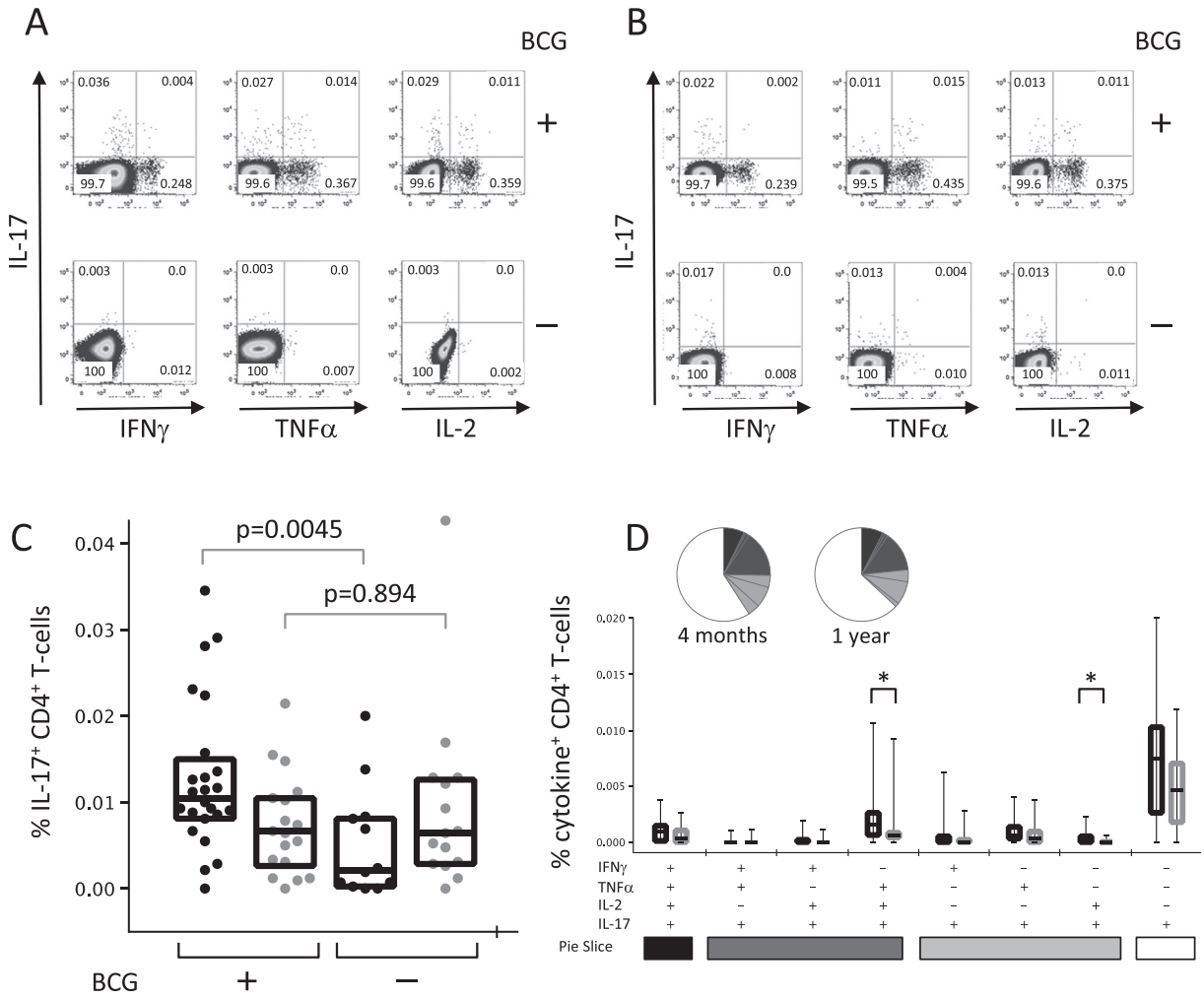

Fig. 2. PPD-specific T helper-17 response in BCG-vaccinated infants. An example of IL-17, IFNγ, TNFα and IL-2 co-staining is shown for 4 month (A) and 1 year (B) samples from a vaccinated (+) and an unvaccinated (−) infant. (C) Cumulative, composite IL-17 expression data for vaccinated and unvaccinated infants at 4 months (dark grey circles) and 1 year (light grey circles). (D) The polyfunctionality of all IL-17-expressing CD4+ T-cell phenotypes in BCG vaccinated infants at 4 months (dark grey bars) and 1 year (light grey bars). Sample numbers are n = 24 (BCG-vaccinated infants at 4 months post-BCG); n = 18 (BCG-vaccinated infants at 1 year post-BCG); n = 12 (BCG-unvaccinated infants at 4 month time point) and n = 15 (BCG-unvaccinated infants at 1 year time point). Statistics for comparisons between vaccinated and unvaccinated infants are Mann-Whitney *U* tests. ∗ indicates $p < 0.05$.

[15,29–31]. Although IFNγ+TNFα+IL-2+ triple positive cells are usually detectable in those studies, it is often single cytokine producers, most notably IFNγ+ Th-1 cells, which are most frequent. In contrast, we did not detect any single IFNγ producers and in fact, the only population that displayed any IFNγ positivity was expressing all three cytokines. The studies referred to above were carried out in Africa, therefore it is possible that these differences in response could be due to the fact that our study was carried out in a northern European setting and so infant participants comprised a different ethnic population who were exposed to a contrasting set of environmental factors. Another possible explanation for the difference between these results and those from the African settings could be differences in stimulation protocols, most notably the use of live BCG mycobacteria as a stimulant in the African studies and our use of PPD. In addition to this study, others who have used PPD as a stimulant to investigate T-cell responses following BCG vaccination have also reported prominent polyfunctional responses in mice and humans [32,33].

Clearly, the implication of observing a persistent, polyfunctional population of Th-1 cells that is very specific to having received the BCG vaccine, is that these cells are in some way involved in the protection that BCG affords these infants against childhood TB [2]. One of the first flow cytometric descriptions of polyfunctional, cytokine-producing T-cells was in the context of the protection they conferred to mice following efficacious vaccination against *Leishmania major* [34]. The field of TB has produced mixed data regarding the role of polyfunctional cells in immune protection. Retrospective analysis of samples taken 10 weeks after BCG vaccination at birth showed no difference in polyfunctional Th-1 cell frequency when infants who eventually contracted TB disease were compared to those who were exposed but remained disease-free during a 2 year follow-up period [15]. Another recent example of polyfunctional Th-1 cells failing to bestow protection against TB was when no significant protective effect was demonstrated following administration of novel TB vaccine MVA85A to infants despite this vaccine's ability to boost antigen-specific, IFNγ+TNFα+IL-2+CD4 T-cells [6]. Evidently, polyfunctional Th-1 cells do not correlate with risk of TB disease or protection, respectively, in these studies carried out in African settings, however, there are other circumstances where polyfunctional T-cell responses are associated with groups who have proved resistant to the development of TB disease; namely HIV-positive patients after anti-retroviral therapy with higher CD4 counts or those with lower viral loads [35,36]. The relatively low TB incidence in the UK

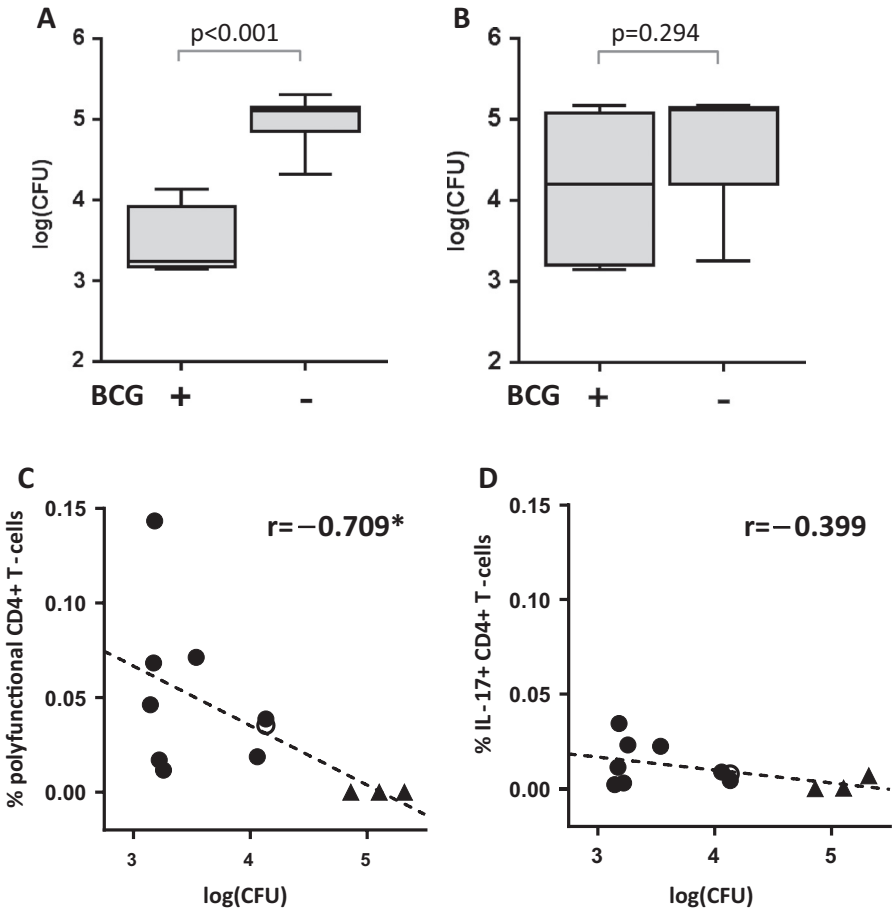

Fig. 3. Increased capacity to inhibit the *in vitro* growth of mycobacteria in PBMC from BCG-vaccinated infants that correlates with polyfunctional T-cell response. Cryopreserved PBMC prepared from venous blood samples obtained 4 months and 1 year post-BCG from vaccinated infants or from age-matched, unvaccinated infants were recovered and incubated with BCG for 4 days after which remaining mycobacteria were quantified by BACTEC MGIT tubes. Inhibition of BCG growth is indicated by the log CFU of remaining mycobacteria after incubation with PBMC obtained at the 4 month (A) or 12 month (B) time point from BCG-vaccinated (+) or BCG-unvaccinated (−) infants. Sample numbers are n = 8 (BCG-vaccinated infants at 4 months post-BCG); n = 8 (BCG-vaccinated infants at 1 year post-BCG); n = 7 (BCG-unvaccinated infants at 4 month time point) and n = 7 (BCG-unvaccinated infants at 1 year time point). For all infant samples where growth inhibition and ICS data was available (n = 12), scatter plots of log CFU versus frequency of polyfunctional CD4+ T-cells (C) or versus IL-17+CD4+T-cells (D) were drawn. Solid symbols indicate 4-month samples; open symbols indicate 12-month samples; circles indicate samples from vaccinated infants; triangles indicate samples from unvaccinated infants. The Mann-Whitney *U* test was used to compare growth inhibition between vaccinated and un-vaccinated groups. Spearman's rank correlation coefficient was calculated between growth inhibition and ICS frequencies and significant correlations ($p < 0.05$) are indicated (∗).

combined with the design of our study means it is not possible to determine whether the Th-1 population we describe here correlates inversely or directly with risk or protection respectively, although it seems that the field would benefit greatly from an assessment of the protective capacity of polyfunctional T-cells in a low NTM, high latitude setting.

In mice, IL-17 responses in the lung were shown to precede an influx of protective Th-1 cells following vaccination and subsequent infection with *M. tb* [37] and furthermore, the IL-23/IL-17 pathway was necessary following BCG vaccination for the effective generation of a Th-1 response specifically to overcome an otherwise inhibitory IL-10 response [38]. In addition to polyfunctional Th-1 cells, we also report here a population of PPD-specific, IL-17 + CD4+ CD3+ cells that are present at a significantly greater frequency in BCG-vaccinated infants at 4 months post-BCG, as compared to age-matched, unvaccinated infants. Again, this is consistent with our previous reports of PPD-specific, IL-17 responses detectable in BCG-vaccinated infants [27,28]. The current data provide evidence that the source of at least some of this IL-17 is antigen-specific Th-17 cells. Unlike Th-1 cellular profiles, Th-17 cells have been less frequently reported in infant BCG studies, partly due to the more recent emergence of IL-17 as a cytokine of interest. Where a Th-17 response was sought, in one report it was not detectable above background levels in control stimulations [39], however in others it was detectable but did not correlate with risk of TB disease in South African infants [15], nor did it differ in Ugandan infants who received BCG at birth as compared to those who received it at 6 weeks of age [31]. We have previously described the complete absence of a PPD-specific, secreted IFNγ response in unvaccinated UK infants up to 15 months of age [40] and describe here a concomitant absence of IFNγ+Th-1 cells in a similar cohort. This very low background immune responsiveness to PPD extends to IL-17 and has allowed us to distinguish an otherwise low frequency Th-17 response at 4 months in vaccinated infants. However, this differential response at 4 months post-BCG did not extend to samples taken at 1-year post-BCG. It is interesting to note that whilst the frequency of the Th-17 response in BCG-vaccinated infants is lower at 12 months than at 4 months, the inverse is true of unvaccinated infants. It has been shown that there is a strong bias towards the development of Th-17 in neonates [41]. In our study, it may be that this propensity is being illustrated in two ways. Firstly, in the large increase in PPD-specific Th-17 responses due to BCG vaccination that gradually wanes by 12 months. Secondly, in the less pronounced increase

in Th-17 responses in BCG unvaccinated infants. That the latter responses were stimulated by PPD suggests that either these infants have been exposed to environmental mycobacteria or that there is some cross-reactivity in these Th-17 responses.

We did not detect any IL-10 responses in this study. Although we previously detected PPD-induced IL-10 in BCG vaccinated infants it was in diluted whole blood culture supernatants after 7 days using multiplex bead array. [27,28]. In this study we used a much shorter stimulation period (18 h) and ICS as a detection method and these may be a less sensitive than is required for IL-10. Another possible explanation is that we restricted our analysis to $CD4^+$ T-cells in this study and may be missing IL-10 responses from cells such as monocytes.

In the absence of a TB disease or infection outcome in our cohort we employed instead an *in vitro* mycobacteria growth inhibition assay (MGIA) as a surrogate marker of immune resistance. There is much interest in developing functional assays such as the MGIA as a surrogate marker of protection in order to facilitate the testing of novel TB vaccines. The aim is that the MGIA will eventually circumvent the need for long, expensive follow up periods with TB disease or infection as an endpoint. An additional advantage is that the unbiased nature of the MGIA means that prior knowledge as to which component of the immune system is responsible for any protective effect is not necessary. In this study, we found that the PBMC compartment of vaccinated infants contained the ability to inhibit the growth of BCG mycobacteria *in vitro* to a greater extent than that of unvaccinated infants in samples taken at 4 months. However by 12-month post-BCG there was no difference between the vaccinated and unvaccinated groups. A previous study has shown that PBMC from adults with a history of BCG vaccination inhibit the growth of BCG mycobacteria *in vitro* to a greater extent than PBMC from adults without a history of BCG vaccination and that the capacity to inhibit mycobacterial growth can be induced in the BCG 'naïve' adults with subsequent, primary vaccination [42]. In another study using a different version of the growth inhibition assay involving luminescent BCG and whole blood samples, neonatal BCG vaccination induced a significant degree of mycobacterial growth inhibition that persisted for up to 6 months when compared to growth inhibition in pre-vaccination samples [43]. Based on the data available, it is difficult to speculate as to why adults who have had BCG many years previously may display significantly more growth inhibition than adults with no history of BCG where as in this study, the difference between vaccinated and unvaccinated infants lost statistical significance by 12 months post vaccination. The interquartile range of the 12-month post-BCG growth inhibition data was much greater in the vaccinated group. This may be due to the relatively small sample size and a bigger study with more infants might allow smaller differences between groups to be detectable that are not here. It should be noted however that in the Fletcher et al study [42], adults receiving primary BCG vaccination that displayed significant PBMC-mediated growth inhibition at 4 weeks post-vaccination had returned to baseline levels by 6 months post-vaccination which is more consistent with the findings we present here.

The data presented here support the hypothesis that PPD-specific polyfunctional CD4 T-cells secreting IFNγ, TNFα and IL-2 play a role in mycobacterial growth inhibition *in vitro*. It is interesting that three previous studies [21,42,43] did not show any correlation between PPD-specific IFNγ responses measured by ELISA or ELISpot and growth inhibition. To our knowledge, this is the first time growth inhibition and polyfunctional CD4 T-cells have been compared in infants and that a correlation is detected might indicate that measuring these cells is revealing a mechanism that looking at IFNγ alone might be missing.

Our data involves a correlation between ICS responses measured in diluted whole blood and MGIA responses measured in PBMC, two different cellular compartments. We cannot rule out possible implications (and limitations) of this, for example, the presence of polymorphonuclear cells in ICS assays and their absence in MGIA or the use of non-autologous pooled human serum in MGIA. It would be worth, in future, repeating this experiment using a whole blood version of the growth inhibition assay to complement the whole blood ICS in order to investigate the possible impact of this.

In conclusion, the present study expands our previous description of cytokine and chemokine immune responses in BCG vaccinated UK infants to reveal a prominent, long-lived and mainly polyfunctional Th-1 response together with the induction of antigen-specific Th-17 cells and that the magnitude of polyfunctional Th-1 cells correlates with *in vitro* mycobacterial growth inhibition and may play a role in this effect.

Conflicts of interest

None.

Funding sources

This work was supported by the European Commission within the 7th framework program (FP7) NEWTBVAC (Grant No. HEALTH-F3-2009-241745), by the European Tuberculosis Vaccine Initiative (Grant No. TBVI-12 NORAD) and by the European Commission within Horizon2020 TBVAC2020 (Grant No. H2020 PHC-643381).

Acknowledgments

We wish to thank all the infants and parents who participated in this study; the staff at NHS Redbridge and NHS West Essex particularly Dr. Catherine Webb and Dr. Paul Roberts and the project phlebotomist Mrs Shakuntala Patel.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.09.002.

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