Comparable CD4 and CD8 T cell responses and cytokine release after at-birth and delayed BCG immunisation in infants born in Australia

Nicole Ritz [1,2,3], Dan Casalaz [4], Susan Donath [5], Marc Tebruegge [1,2,6], Binita Dutta [2], Tom G. Connell [1,2], Roy Robins-Browne [1,2,7], Warwick J. Britton [8], Willem A. Hanekom [9], Nigel Curtis [1,2]

[1] Department of Paediatrics, The University of Melbourne; and Infectious Diseases Unit, Royal Children's Hospital Melbourne, Parkville, Australia

[2] Infectious Diseases and Microbiology Group, Murdoch Children's Research Institute, Royal Children's Hospital, Australia

[3] University of Basel Children's Hospital Basel, Infectious Diseases Unit and Paediatric Pharmacology, Basel, Switzerland;

[4] Department of Paediatrics, The Mercy Hospital for Women, Heidelberg, Australia

[5] Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute; Royal Children's Hospital Melbourne, Parkville, Australia

[6] Academic Unit of Clinical & Experimental Sciences, Faculty of Medicine, and Institute for Life Sciences, University of Southampton, Southampton, UK

[7] Department of Microbiology and Immunology, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity;

[8] Centenary Institute of Cancer Medicine and Cell Biology and Department of Medicine, University of Sydney, Camperdown, Australia

[9] South African Tuberculosis Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine & Department of Paediatrics and Child Health, University of Cape Town, Cape Town, South Africa

Corresponding author contact information:

Nicole Ritz, MD, PhD

Infectious Diseases and Paediatric Pharmacology

University Children's Hospital Basel

Spitalstrasse 33, CH-4031 Basel

Tel: + 41 61 704 29 94

e-mail: nicole.ritz@unibas.ch

Key words: Bacille Calmette-Guérin; HIV, immune maturation, vaccine, tuberculosis, TB

Conflict of interest

None for all authors.

Abstract

Background

More than 120 million doses of BCG vaccine are administered worldwide each year. Most infants are given BCG at birth in accordance with WHO recommendations. However, the effect of the maturing neonatal immune system on the immune response and protection conferred by BCG remains uncertain. Previous studies investigating the influence of age at immunisation on the immune response induced by BCG have reported conflicting results. This study compared BCG given at birth and at two months of age in infants in Australia.

Methods

Infants born in Melbourne were randomly allocated to immunisation with BCG-Denmark at birth or two months of age. Ten weeks after immunisation, anti-mycobacterial immune responses were measured in a whole blood assay using intracellular cytokine assays and x-MAP multiplex cytokine analysis.

Results

Results from 98 BCG-immunised infants were included in the final analysis. BCG immunisation at birth (n=54) and at 2 months of age (n=44) induced comparable proportions of mycobacteria-specific cytokine-producing CD4 and CD8 T cells, as well as comparable proportions of polyfunctional (TNF+ IL-2+ IFN-g+) CD4 T cells. Concentrations of cytokines in supernatants were also similar in both groups.

Conclusions

Cellular immunity measured 10 weeks after BCG immunisation was similar in infants given BCG at birth and in those given BCG at 2 months of age. Although definitive correlates of protection against TB remain uncertain, these results suggest that

68 delaying BCG immunisation does not confer any immunological advantage in cellular
69 immunity.

Introduction

Bacille-Calmette-Guérin (BCG) is one of the most commonly administered vaccines worldwide [1]. BCG immunisation is most effective for the prevention of severe forms of tuberculosis (TB), with protective efficacy of up to 87% against TB meningitis and miliary TB [2, 3]. These forms of TB are most commonly seen in infants and children less than two years of age. The World Health Organization (WHO) therefore recommends that BCG is given as soon as possible after birth in countries with high TB incidence [4].

However, BCG immunisation soon after birth may not be optimal for two reasons. Firstly, there is the risk of inadvertently immunising infants who are infected with human immunodeficiency virus (HIV). HIV-infected infants are at risk of developing disseminated BCG disease, which is associated with a mortality of up to 75% [5, 6]. As a result, WHO revised their recommendations in 2007 to state that BCG vaccine should not be used in children who are known to be HIV-infected and, in settings with adequate HIV services, to delay BCG immunisation for infants born to mothers known to be HIV-infected until these infants are confirmed to be HIV-uninfected [4]. This recommendation was reinforced in 2010 [7]. Secondly, BCG immunisation administered at birth potentially induces an immune response that is inferior to that provided by immunisation beyond the neonatal period. The human immune system undergoes significant maturational changes in early life [8]. Consequently, the 'immature' immune system of newborns may be less capable of generating protective anti-mycobacterial immune responses after BCG immunisation compared with the immune system of older infants.

Only few studies have investigated the influence of age at immunisation on the immune response and protection against TB induced by BCG. Clinical studies in Canada and Colombia indicate that immunisation after 6 to 12 months of age may be associated with better protective efficacy [9, 10]. More recently, four important studies in Africa comparing the mycobacteria-specific immune response induced by BCG immunisation at birth with delayed immunisation have reported conflicted results [11-14]. In some studies, certain subsets of mycobacteria-specific cytokine-producing CD4 and CD8 T cells were higher in infants with delayed BCG immunisation while in others the same subsets were not different (detailed further in discussion below). In addition, geographical setting plays an important role in the early life immune response in infants. For example, a recently published study comparing the cytokine response of monocytes and dendritic cells in 2-year old children from Canada, Belgium, Ecuador and South Africa showed that children from South Africa had lower interleukin (IL)-6, IL-12, interferon (IFN)-α, IFN-γ, and tumor necrosis factor (TNF) concentrations [15, 16]. The mechanism underlying differing immune responses between populations is uncertain but is likely attributable to host genetics and environmental factors. It is therefore important to study the immune response to early versus delayed BCG immunisation in different settings.

Materials and Methods

Study population and BCG immunisation

Infants were recruited during a related but independent study [17]. Pregnant women attending the antenatal clinic at the Mercy Hospital for Women in Melbourne, Australia were approached if one of the parents was born in a country with high TB incidence (defined as more than 100 cases per 100 000 inhabitants) and planned to

travel to their country of origin within the next five years. This identified infants for whom BCG immunisation is recommended by the Australian Immunisation Guidelines [18]. Written informed consent was obtained from the mother. Exclusion criteria included mothers known to be infected with HIV, premature birth (less than 35 weeks of gestation), birth weight below 2500 grams, and any symptoms or signs of illness. Participants were randomly allocated to be immunised with BCG at birth or at two months of age. BCG vaccine (SSI-1331 from Statens Serum Institute, Copenhagen, Denmark) was given as a 0.05 ml intradermal injection in the left deltoid region using a 26-gauge needle.

The study was approved by the Mercy Health Human Research Ethics Committee (R07/16), and approved as a clinical trial by the Australian Therapeutic Goods Administration (TGA). The trial was registered with the Australian New Zealand Clinical Trials Registry (number ACTRN12608000227392).

Immunological assays

Ten weeks after BCG immunisation (ie at 10 weeks of age in the at birth BCG-immunised group and at 18 weeks of age in the delayed group), up to 6 ml of blood were collected in sodium heparin tubes and stimulation assays were done within two hours of collection. A whole blood intracellular cytokine/cytotoxicity assay was done as previously described [17]. Briefly, blood was incubated with BCG (SSI-1331, Statens Serum Institute), purified protein derivative (PPD; Batch RT50, Statens Serum Institute), heat-killed M. tuberculosis (MTB; H37Rv), staphylococcal enterotoxin B (SEB; Sigma-Aldrich, St. Louis, MO, USA), or medium alone in the presence of co-

stimulatory antibodies CD28 and CD49d (BD Biosciences, San Jose, CA, USA) at 37 °C for 7 hours. Plasma was removed for cryopreservation and the remaining blood was incubated for a further 5 hours with brefeldin A (BfA; Sigma-Aldrich) for intracellular cytokine staining or a combination of BfA, monensin (Sigma-Aldrich) and anti-CD107a-APC (BD Biosciences) for cytotoxicity assays. The remaining blood was then harvested, and cells lysed and fixed in FACS lysing solution (BD Biosciences) before cryopreservation at -80 °C.

For the flow cytometric analyses, thawed samples were permeabilised with Perm2 Solution (BD Biosciences), washed with staining buffer and incubated with the following fluorochrome-conjugated antibodies (all BD Biosciences): anti-CD3 PerCP-Cy5.5 (SK7), anti-CD4 FITC (RPA-T4), anti-CD8 Alexa-700 (RPA-T8), anti-IFN-γ PE-Cy7 (4S.B3), anti-IL-2 PE (MQ1-17H12) and anti-TNF APC (Mab11), and anti-CD107a APC (H4A3). Analysis was done on an LSRII flow cytometer (BD Biosciences) with optimised PMT voltages and standardisation using CST beads (BD Biosciences). Automated compensation was calculated with FACSDiva software (version 6.1, BD Biosciences, San Jose, CA, USA) using stained anti-mouse and anti-rat Ig kappa beads. Flow cytometric analysis was done using FlowJo software (version 8.8.6, TreeStar Inc, Ashland, OR, USA). A hierarchical gating strategy was used to select single cell CD4 and CD8 T cell populations. Gates for cytokine expression and cytotoxic markers for blood stimulated with mycobacterial antigens and SEB were set using the unstimulated control cells. A Boolean combination was used to determine polyfunctional T cells producing more than one cytokine, ie double-positive and triple-positive populations.

Concentrations of cytokines in supernatants were measured using human multiplex bead-based cytokine kits using xMAP technology (Milliplex Human

Cytokine/Chemokine Immunoassay, Millipore Corp, Billerica, MA, USA).

Preliminary experiments were done to determine which cytokine and chemokines are detectable in supernatants from infants after BCG immunisation and the variability of the assay. These included 16 cytokines and chemokines covering the spectrum of Th1, Th2, Th17, regulatory and pro-inflammatory pathways. IL-4, IL-5 and IL-17 were not detectable and not further analysed. Differences between samples run in duplicate were minimal. MIP-1α and MIP-1β results were highly correlated so only MIP-1β was further analysed. EGF, eotaxin, fractalkine, IL-2, IL-10, IL-12 (p40), IL-13, and IFN-γ were analysed in undiluted samples and IL-6, MCP-1, MIP-1β and TNF were analysed in 1:20 diluted samples based on previous optimisation experiments. Standard curves were generated using six dilutions of standards run in duplicate and two controls were included in every run. Samples were run as single assays. Assays were read with a Luminex 200 bioanalyser (Luminex Corp. TX, USA), which was calibrated before each run and set to acquire 50 events per bead. All outcome assessors were blinded to group assignment.

Statistics

All data were analysed after background correction using the unstimulated control sample. A Mann-Whitney U test was used for comparisons between groups. All p-values were interpreted in the light of multiple significance testing; a p-value < 0.01 was considered potentially significant. Statistical analyses were done using STATA 11 software (College Station, TX, USA). Graphs were created using Prism 5 software (Graph Pad Software Inc., La Jolla, CA, USA).

Results

Participant characteristics

A total of 124 infants were randomised to be immunised at birth or at two months of age, of which 102 (82%) returned for the follow-up sample collection. Data from 98 infants (79%) were included in the final analysis (Figure 1). The demographic and baseline characteristics of participants included in the final analysis were comparable between the groups (Table 1).

The proportions of mycobacteria-specific cytokine-producing CD4 and CD8 T cells were comparable in at-birth and delayed BCG immunisation

The proportions of single, double and triple mycobacteria-specific cytokine-producing CD4 T cells were comparable in both groups (Figure 2). Generally, proportions of mycobacteria-specific single cytokine-producing CD4 and CD8 T cells were higher than those of double and triple cytokine-producing CD4 and CD8 T cells. The proportions of cytokine-producing CD4 T cells were lower in the group of infants immunised at two months of age, but none of the comparisons had a p-value ≤ 0.01. In addition, whilst, for example, the proportion of PPD-specific IL-2/TNF double positive CD4 T cells was lower in infants immunised at two months of age, this was not the case when BCG or MTB was used as the in vitro stimulant. Similar to the findings for CD4 T cells, the proportions of cytokine-producing CD8 T cells were similar in both groups (Figure 3). Also, there were no significant differences between both groups in the unstimulated (negative 'nil' control) and SEB-stimulated (positive control) responses (data not shown).

The proportions of mycobacteria-specific cytotoxic CD4 and CD8 T cells were comparable in at-birth and delayed BCG immunisation

CD107-expressing cytotoxic T cells did not produce IFN-γ or IL-2 (data not shown). The proportion of mycobacteria-specific cytotoxic T cells was generally lower in CD8 than in CD4 T cells, and mycobacteria-specific CD107 expression was similar in both groups (Figure 4). There were no significant differences between both groups in the unstimulated (negative control) and SEB-stimulated (positive control) responses (data not shown).

Cytokine expression was comparable in at-birth and delayed BCG immunisation

There were no significant differences in the concentrations of the 12 background-corrected cytokines in supernatants between infants immunised at birth and those with delayed BCG immunisation, regardless of the in vitro stimulatory antigen used (Figure 5). However, in the delayed group, there was a trend towards a higher concentration of IL-12 p40 ($p=0.04$) and a lower concentration of EGF ($p=0.02$) in samples stimulated with MTB. These differences were not seen in samples stimulated with BCG or PPD.

Discussion

This study compared the mycobacteria-specific immune response 10 weeks post immunisation in infants immunised with BCG at birth and those in whom BCG immunisation was delayed until two months of age. To our knowledge, this is the first study to compare the immune response to at-birth and delayed BCG in a setting outside the African continent. Our findings suggest that delaying BCG immunisation from birth to 10 weeks of life does not significantly affect mycobacteria-specific immune responses, assessed by cytokine-producing and cytotoxic T cells and the expression of several cytokines involved in the human anti-mycobacterial immune response. This contrasts with the results of our previous study that compared different BCG vaccine strains given at birth in a separate set of participants, which showed

mycobacteria-specific immune responses were significantly influenced by the BCG vaccine strain administered [17].

Four previous studies have investigated the influence of delayed BCG immunisation, three of which were randomised trials (Table 2). Two studies used the same BCG vaccine strain (BCG-Denmark) as our study given at age 8 and 10 weeks [12, 14]. Both these studies showed higher proportions of IFN-γ/IL-2 double- and/or IFN-γ single-producing CD4 T cells, measured 6 to 14 weeks after immunisation, when BCG was delayed. However, consistent with our findings, both studies showed comparable results for all other cytokine-producing CD4 and CD8 subpopulations, in particular for polyfunctional T cells.

In addition, our study found that delaying BCG immunisation did not affect cytotoxic T cell responses. Only one previous study has investigated the influence of delayed BCG immunisation on cytotoxic T cells [13]. This was a non-randomised study in Uganda that compared cytotoxic T cells by measuring perforin expression at 40 weeks of age. Despite the differences in study design, this study also found no differences in cytotoxic T cells in children immunised at birth compared to children immunised at 6 weeks of age.

When we investigated multiple cytokines that are known to be important in the protective immune response against TB, infants immunised at birth and those immunised at 2 months of age had comparable concentrations in supernatants from whole blood stimulation assays. Two previous studies have investigated the influence of delayed BCG immunisation on the mycobacteria-specific cytokine response, assessing eight cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and IL-17) [11, 13]. In the study in Uganda, the concentration of only one (IL-10) of the six measured

cytokines was higher in the delayed BCG group (measured at 40 weeks of age) [13]. Similarly, in the study in The Gambia, higher IL-10 concentrations were found in infants with delayed BCG compared to those immunised at birth [11]. In addition, in the Gambian study, three cytokines (IFN-γ, IL-6 and IL-17) were lower and one (IL-13) was not different in the delayed group (measured 20 weeks post-immunisation). However, when the cytokines were measured at 40 weeks of age (ie 20 weeks post-immunisation in the delayed BCG group and 40 weeks post immunisation in the at-birth BCG group), cytokine concentrations were no longer significantly different. This suggests that mycobacteria-specific cytokine responses wane after immunisation, which might not be apparent when there is a comparatively short interval between at-birth and delayed BCG, as was the case in our study. Importantly, the BCG vaccine strain used in the Gambian study was BCG-Russia, which induces a significantly different immune response with generally lower proportions of IFN-γ, IL-2 and TNF single and polyfunctional CD4 T cells and lower concentrations of Th1 cytokines compared to BCG Denmark [17].

One important caveat in this study is that the immune response was inevitably measured at a different postnatal age in the two groups, as the interval between BCG immunisation and measurement of the mycobacteria-specific immune response was 10 weeks in both groups. This interval had to be consistent as the time between immunisation and measurement of the immune response influences the detected immune response [11, 19]. As a result of the limited available blood volumes, a number of other potentially important aspects of the immune response, including memory T cell phenotypes, could not be assessed. However, no influence on memory phenotypes was found in the two previous studies that investigated the influence of delayed BCG on this aspect of the mycobacteria-specific immune response [12, 13].

We also did not determine non-conventional T cells, which have recently been recognised to play an important role in the immune response to BCG [20-22]. Another inevitable limitation of our study is that we were not able to compare clinical outcomes (ie protective efficacy) as a result of the low TB incidence in Australia.

Although definitive immunological correlates of protection against TB induced by BCG remain uncertain, our study, together with the four studies done in Africa, suggest that delaying BCG immunisation for several weeks results in a similar anti-mycobacterial immune response as immunisation at birth. The choice of whether to administer BCG at birth or later in infancy should therefore be based on other factors. This will involve balancing the risk of inadvertent immunisation of HIV-infected infants inherent when an at-birth BCG immunisation strategy is used with the risk of reduced coverage or prior TB infection with a delayed BCG immunisation strategy. Finally, the potential beneficial heterologous ('non-specific') effects of BCG [23-27] immunisation at birth also need to be considered when deciding on the timing of routine BCG in settings with high infant mortality.

Funding and Acknowledgements

NR and MT were supported by fellowship awards from the European Society for Paediatric Infectious Diseases and scholarships from The University of Melbourne. NR was supported by the Rozalia Foundation and a grant from the University of Basel; MT is currently supported by a Clinical Lectureship by the U.K. National Institute for Health Research. The study was funded by a project grant from the Australian National Health and Medical Research Council (NHMRC project grant number 546486). It was also supported by grants from the John Burge Trust, the Myer Foundation, The Aranday Foundation, the Nossal Institute for Global Health and the Murdoch Children's Research Institute. The BCG vaccine was kindly provided by the Statens Serum Institut, Copenhagen, Denmark. We thank the infants and their parents for participating in this study.

References

[1] World Health Organization. Reported estimates of BCG coverage. 2014.

[2] Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet. 2006;367:1173-80.

[3] Walker V, Selby G, Wacogne I. Does neonatal BCG vaccination protect against tuberculous meningitis? Arch Dis Child. 2006;91:789-91.

[4] World Health Organization. Revised BCG vaccination guidelines for infants at risk for HIV infection. Wkly Epidemiol Rec. 2007;82:193-6.

[5] Hesseling AC, Rabie H, Marais BJ, Manders M, Lips M, Schaaf HS, et al. Bacille Calmette-Guerin vaccine-induced disease in HIV-infected and HIV-uninfected children. Clin Infect Dis. 2006;42:548-58.

[6] Hesseling AC, Marais BJ, Gie RP, Schaaf HS, Fine PE, Godfrey-Faussett P, et al. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. Vaccine. 2007;25:14-8.

[7] World Health Organization. Use of BCG vaccine in HIV-infected infants. Weekly epidemiological record. 2010;85:32-3.

[8] Marchant A, Kollmann TR. Understanding the ontogeny of the immune system to promote immune-mediated health for life. Frontiers in immunology. 2015;6:77.

[9] Houston S, Fanning A, Soskolne CL, Fraser N. The effectiveness of bacillus Calmette-Guerin (BCG) vaccination against tuberculosis. A case-control study in Treaty Indians, Alberta, Canada. Am J Epidemiol. 1990;131:340-8.

[10] Shapiro C, Cook N, Evans D, Willett W, Fajardo I, Koch-Weser D, et al. A case-control study of BCG and childhood tuberculosis in Cali, Colombia. Int J Epidemiol. 1985;14:441-6.

[11] Burl S, Adetifa UJ, Cox M, Touray E, Ota MO, Marchant A, et al. Delaying Bacillus Calmette-Guerin Vaccination from Birth to 4 1/2 Months of Age Reduces Postvaccination Th1 and IL-17 Responses but Leads to Comparable Mycobacterial Responses at 9 Months of Age. J Immunol. 2010;185:2620-8.

[12] Kagina BM, Abel B, Bowmaker M, Scriba TJ, Gelderbloem S, Smit E, et al. Delaying BCG vaccination from birth to 10 weeks of age may result in an enhanced memory CD4 T cell response. Vaccine. 2009;27:5488-95.

[13] Lutwama F, Kagina BM, Wajja A, Waiswa F, Mansoor N, Kirimunda S, et al. Distinct T-cell responses when BCG vaccination is delayed from birth to 6 weeks of age in Ugandan infants. J Infect Dis. 2014;209:887-97.

[14] Tchakoute CT, Hesseling AC, Kidzeru EB, Gamieldien H, Passmore JA, Jones CE, et al. Delaying BCG Vaccination Until 8 Weeks of Age Results in Robust BCG-Specific T-Cell Responses in HIV-Exposed Infants. J Infect Dis. 2014.

[15] Smolen KK, Cai B, Gelinas L, Fortuno ES, 3rd, Larsen M, Speert DP, et al. Single-cell analysis of innate cytokine responses to pattern recognition receptor stimulation in children across four continents. J Immunol. 2014;193:3003-12.

[16] Smolen KK, Ruck CE, Fortuno ES, 3rd, Ho K, Dimitriu P, Mohn WW, et al. Pattern recognition receptor-mediated cytokine response in infants across 4 continents. J Allergy Clin Immunol. 2014;133:818-26 e4.

[17] Ritz N, Dutta B, Donath S, Casalaz D, Connell TG, Tebruegge M, et al. The influence of bacille Calmette-Guerin vaccine strain on the immune response against tuberculosis: a randomized trial. Am J Respir Crit Care Med. 2012;185:213-22.

[18] Australian Technical Advisory Group on Immunisation. Tuberculosis. Australian Immunisation Handbook. 9 ed2008. p. 297-302.

363 [19] Hanekom WA. The immune response to BCG vaccination of newborns. Annals
364 of the New York Academy of Sciences. 2005;1062:69-78.
365 [20] Mazzola TN, Da Silva MT, Moreno YM, Lima SC, Carniel EF, Morcillo AM, et
366 al. Robust gammadelta+ T cell expansion in infants immunized at birth with BCG
367 vaccine. Vaccine. 2007;25:6313-20.
368 [21] Kagina BM, Abel B, Scriba TJ, Hughes EJ, Keyser A, Soares A, et al. Specific T
369 Cell Frequency and Cytokine Expression Profile do not Correlate with Protection
370 against Tuberculosis, Following BCG Vaccination of Newborns. Am J Respir Crit
371 Care Med. 2010;182:1073-9.
372 [22] Zufferey C, Germano S, Dutta B, Curtis N, Ritz N. The Contribution of Non-
373 Conventional T Cells and NK Cells in the Mycobacterial-Specific IFNgamma
374 Response in Bacille Calmette-Guerin (BCG)-Immunized Infants. PLoS ONE.
375 2013;8:e77334.
376 [23] Flanagan KL, van Crevel R, Curtis N, Shann F, Levy O. Heterologous
377 ("nonspecific") and sex-differential effects of vaccines: epidemiology, clinical trials,
378 and emerging immunologic mechanisms. Clin Infect Dis. 2013;57:283-9.
379 [24] Ritz N, Casalaz D, Hanekom WA, Britton WJ, Dutta B, Donath S, et al. Reply:
380 Bacille Calmette-Guerin vaccine: innate immunity and nonspecific effects. Am J
381 Respir Crit Care Med. 2013;187:779-80.
382 [25] Ritz N, Mui M, Balloch A, Curtis N. Non-specific effect of Bacille Calmette-
383 Guerin vaccine on the immune response to routine immunisations. Vaccine.
384 2013;31:3098-103.
385 [26] World Health Organization. Meeting of the Strategic Advisory Group of Experts
386 on immunization, April 2014 – conclusions and recommendations. 2014. p. 221-36.

387 [27] Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a
388 historical perspective: intervention studies in animal models of infectious diseases.
389 Trans R Soc Trop Med Hyg. 2015;109:287.

390

Table 1 Characteristics of study participants.

		BCG at birth (n = 54)	BCG at 2 months (n = 44)
Gestational age (weeks)	Median IQR	39.7 39.0–40.3	39.9 38.9–40.4
Female	Number Proportion	28 (52%)	28 62%)
Birth-weight (grams)	Median IQR	3325 3015–3523	3355 3025–3477
Age at immunisation (weeks)	Median IQR	0.1 0.1–0.4	10 9.8–10.6
Interval immunisation to follow-up (weeks)	Median IQR	10.0 9.9–10.5	10.1 9.9–10.9
Maternal age (years)	Median IQR	30.1 26.4–34.2	30.1 26.8–35.7

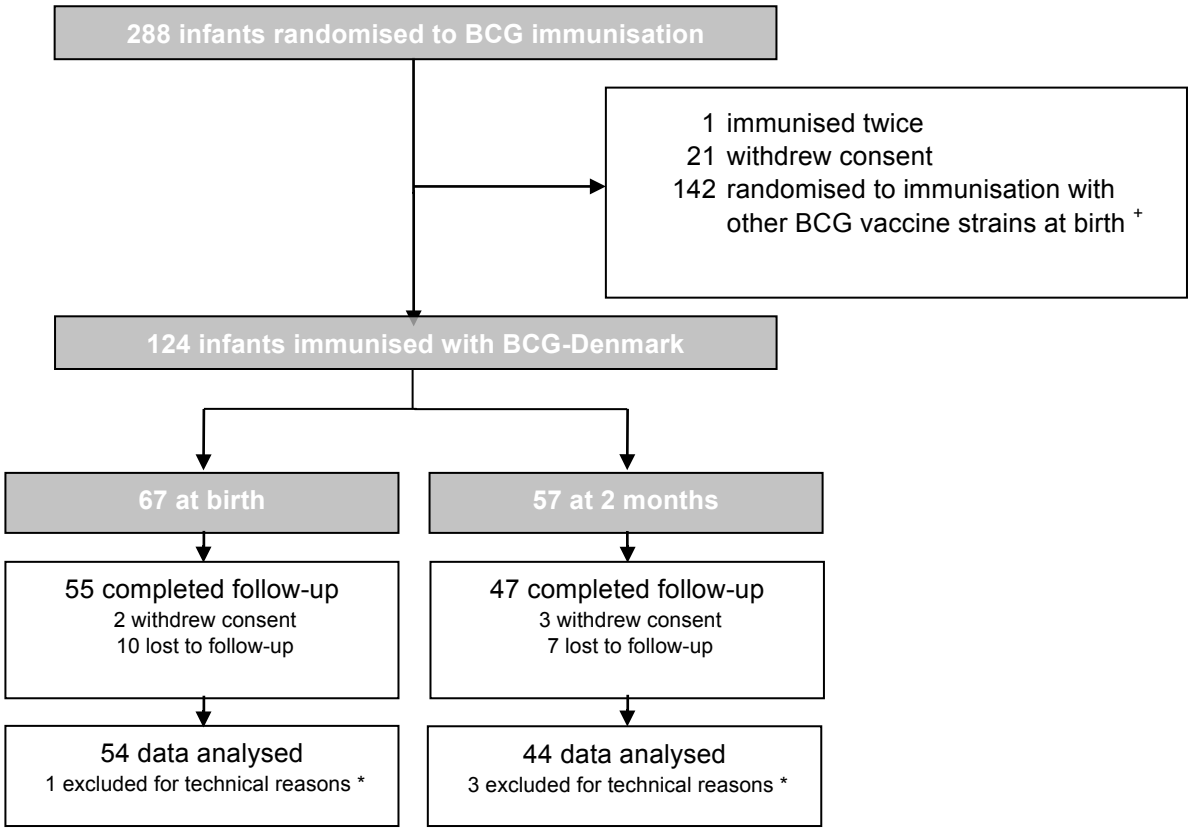
Table 2 Summary of previous studies comparing the mycobacteria-specific immune response in infants immunised with BCG at birth and those whose BCG immunisation was delayed until later in infancy

Ref	Country (year) Study design	Number (immunisation at birth/ delayed)	HIV status	Age at BCG immunisation birth/delayed (weeks)	BCG vaccine strain	Age at blood collection (weeks)	*In vitro* stimulation	Assay	Main findings
[14]	South Africa (2010-2012) RCT	63/59 (only 28 per group analysed at each time point)	HIV-exposed infants	0/8	BCG-Denmark	0, 6 ,8 ,14	BCG-Denmark SEB	6 d whole blood	At 6 weeks *post immunisation*: • BCG-specific CD4 and CD8 T cell proliferation: no difference. • Proportion of BCG-specific CD4 and CD8 polyfunctional T cells: no difference. At 14 weeks *of age*: • IFN-γ-expressing CD4 T cells: higher proportion in delayed group. • IL-2 and IL-17 expressing CD4 T cells: no difference.
[13]	Uganda (ns) Retro-spective	44/40	Mother HIV negative or in a HIV mother to child prevention program	0/6	ns	40	BCG-Denmark PHA	7-12 h and 6 d whole blood	At 40 weeks *of age*: • proportion of proliferating CD4 and CD8 T cells: no difference. . • proportion of proliferating cytokine-producing IFN-γ/IL-2/TNF CD4 T cells: lower in delayed group. • proportion of proliferating TNF-single-producing CD4 T cells: higher in delayed group. • proportion of BCG-specific IFN-γ-producing CD4 and CD8 T cells: lower in delayed group. • polyfunctional CD4 and CD8 T cells T cells: no difference. • proportion of memory phenotype of IFN-γ producing CD4 and CD8 T cells: no difference. • IL-10 in supernatant: higher in delayed group • IFN-γ, IL-2 in supernatant: no difference.

[11]	The Gambia (ns) RCT	46/41	HIV status not assessed	0/20	BCG-Russia	0, 20, 40	PPD BCG Russia ESAT6/CFP-10 SEB	5 d whole blood	At 20 weeks *post immunisation*: • PPD-specific concentrations for IFN-γ, IL-6 and IL-17: lower in delayed group. • BCG-specific IL-10 in supernatant: lower in delayed group • PPD specific IL-10 in supernatant: no difference. At 40 weeks *of age*: • PPD-specific activated, regulatory or proliferating CD4 T cells: no difference. • PPD-specific cytokine concentrations for IFN-γ, IL-6, IL-10, IL-13, IL-17: no difference.
[12]	South Africa (2006-2008) RCT	25/21	Mother documented HIV negative	0/10	BCG-Denmark	8-14 18-28 41-54	Nil BCG Denmark SEB	16 h whole blood	At 10 weeks *post immunisation*: • IFN-γ and IFN-γ /IL-2 producing CD4 T cells: higher in delayed group. At one year *of age*: • IFN-γ /TNF/IL-2, IFN-γ /TNF, TNF/IL-2 and TNF producing CD4 T cells: higher in delayed group. • memory phenotype of cytokine expressing CD4 T cells: no difference.

ns = not specified, h = hour, d = day

Figure 1 Study flow chart showing recruitment and final number of included participants



+ as part of a separate study [17]

* technical problems included: inadequate staining for flow cytometry, and incorrect instrument settings during flow cytometry

Figure 2 Box plots (depicting lower, median and upper quartiles, with Tukey whiskers) showing background-corrected proportions of triple, double and single cytokine-producing CD4 T cells following *in vitro* stimulation with BCG, PPD or MTB (heat-killed whole cell *M. tuberculosis*) measured 10 weeks post immunisation. The p-values for comparisons between infants immunised at birth (white bars) and infants immunised at 2 months (grey bars) are shown above each pair. Note the different scale used for the y-axis in the plots showing single cytokine-producing cells in the third column.

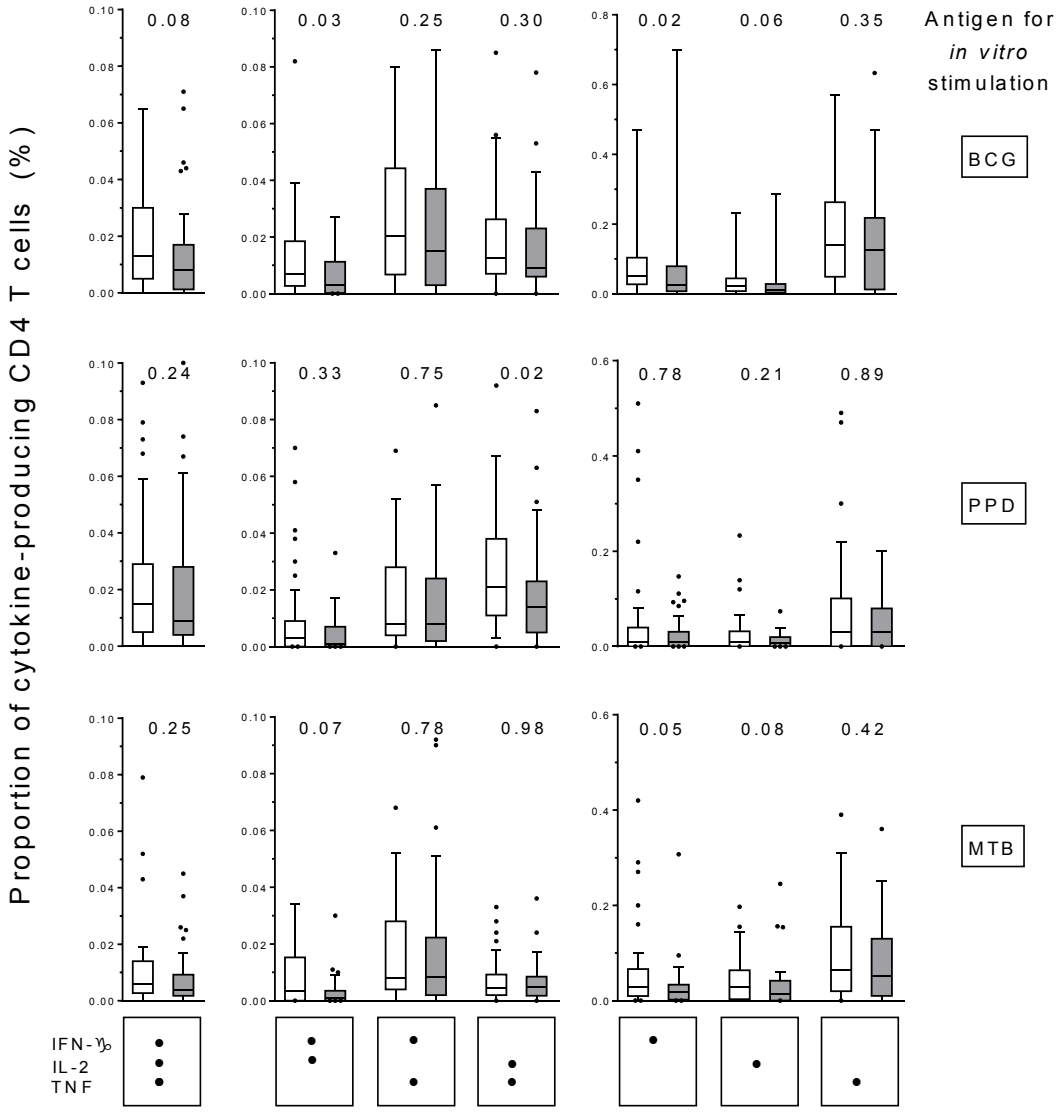


Figure 3 Box plots (depicting lower, median and upper quartiles, with Tukey whiskers) showing background-corrected proportions of triple, double and single cytokine-producing CD8 T cells following in vitro stimulation with BCG, PPD or MTB measured 10 weeks post immunisation. The p-values for comparisons between infants immunised at birth (white bars) and infants immunised at 2 months (grey bars) are shown above each pair. Note the different scale used for the y-axis in the plots showing single cytokine-producing cells in the third column.

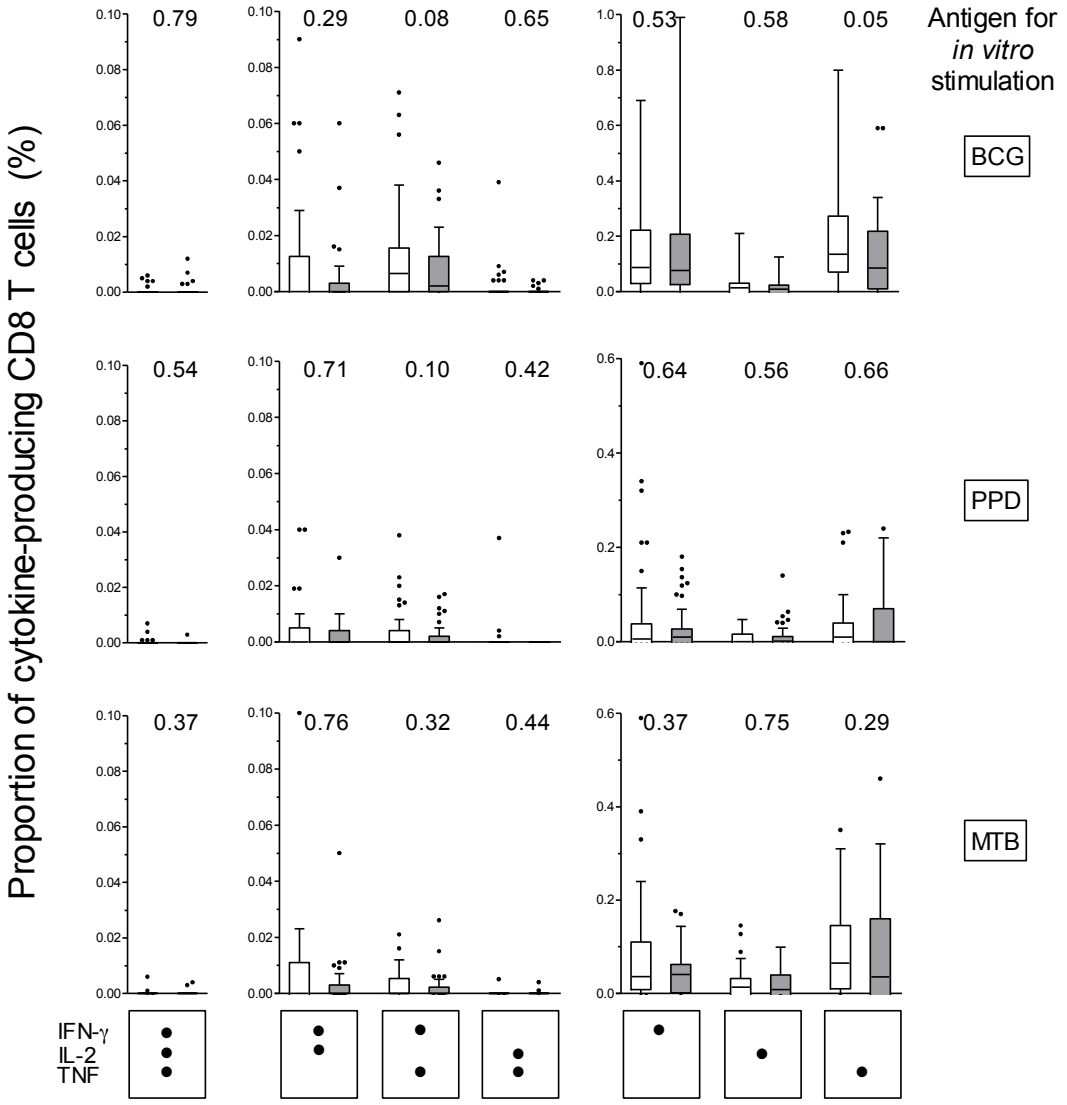


Figure 4 Box plots (depicting lower, median and upper quartiles, with Tukey whiskers) showing proportions of CD107-expressing (cytotoxic) CD4 and CD8 T cells following in vitro stimulation with BCG and MTB measured 10 weeks post immunisation. The p-values for comparisons between infants immunised at birth (white bars) and infants immunised at 2 months (grey bars) are shown above each pair.

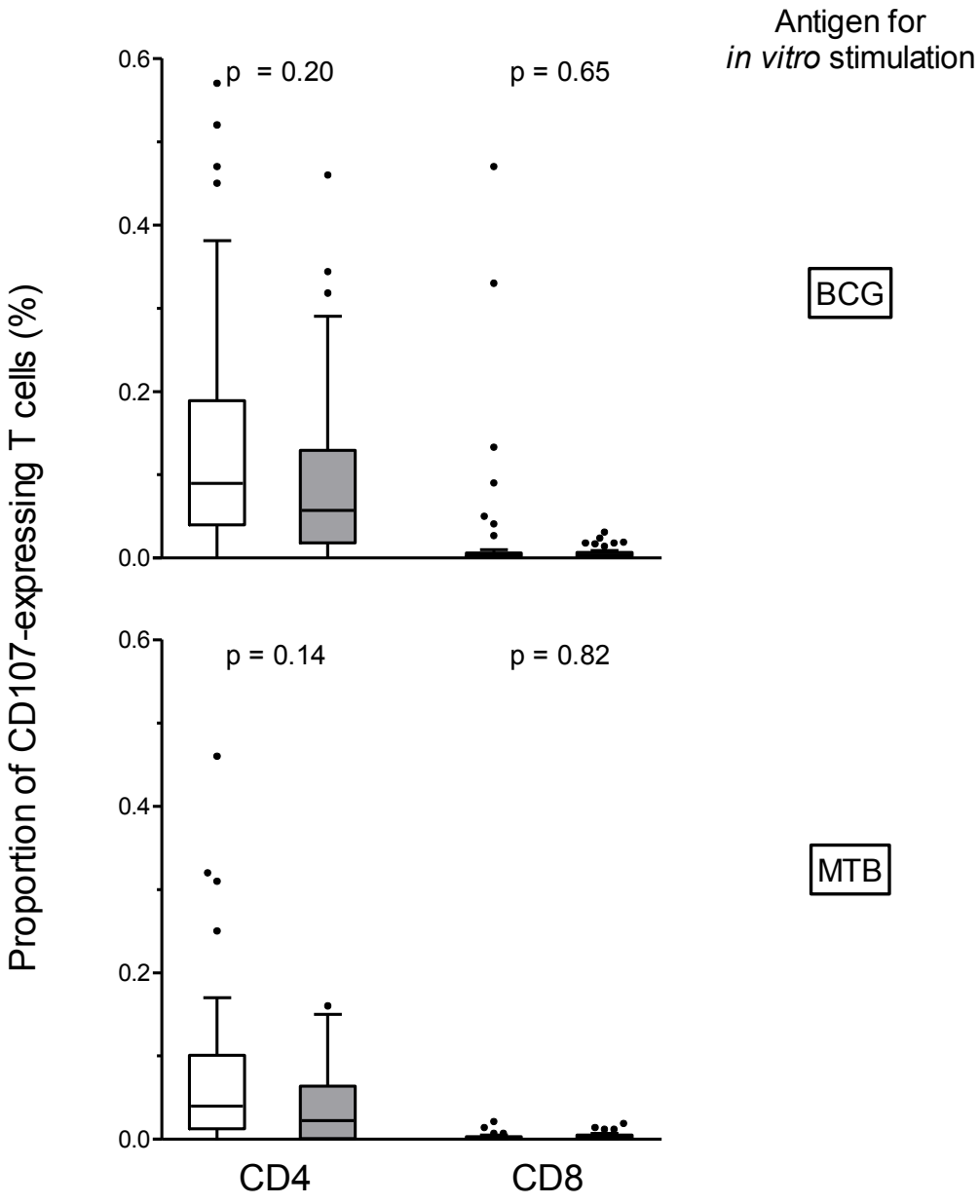
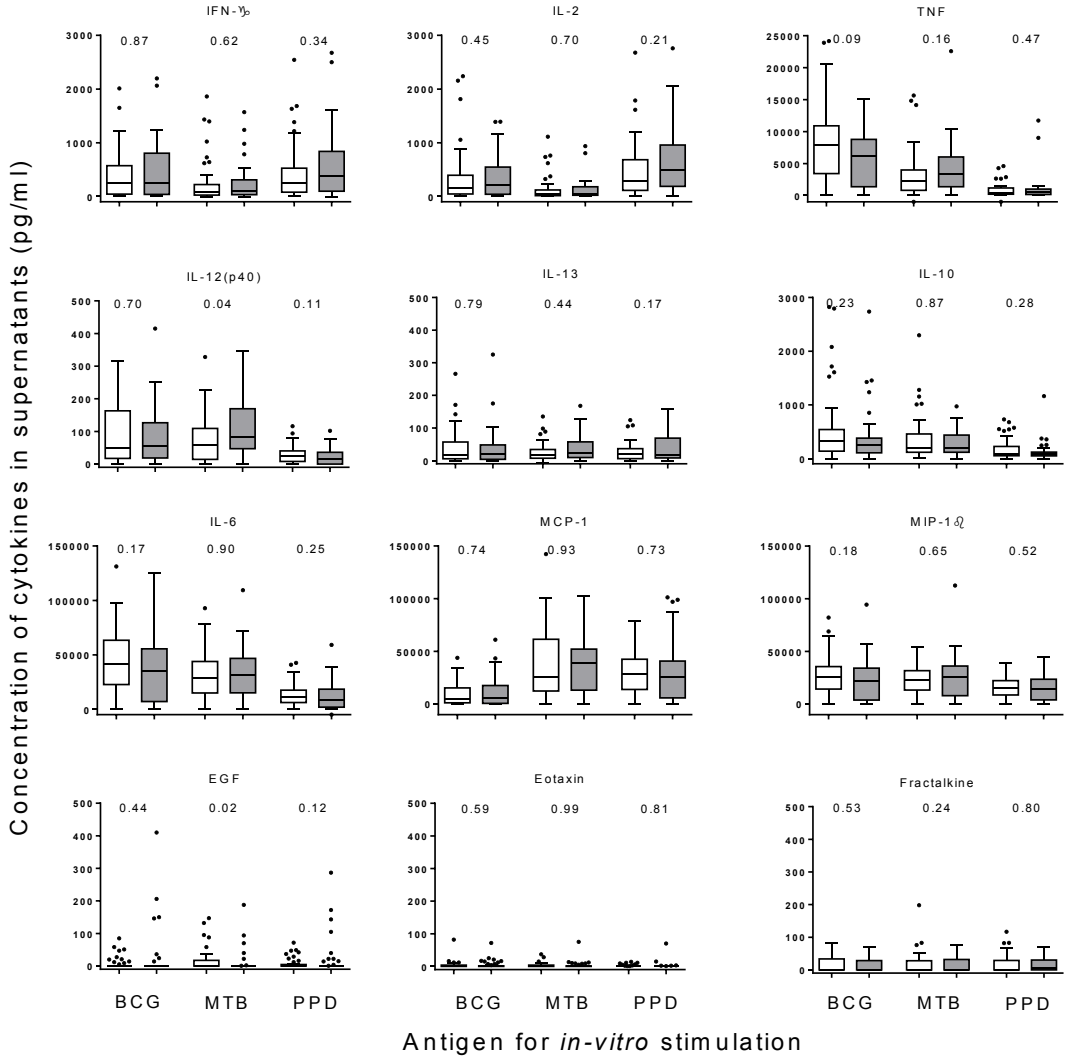
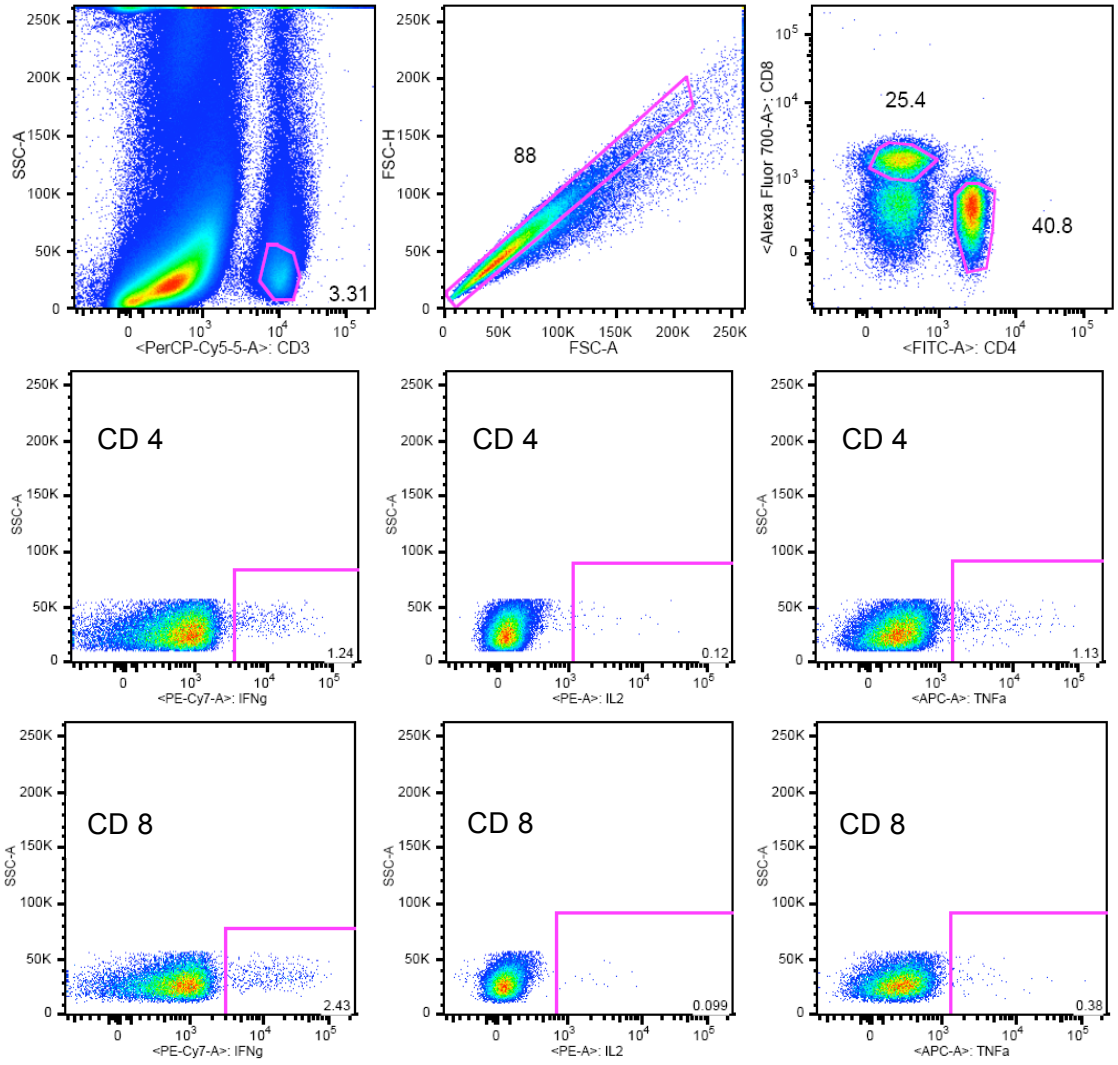


Figure 5 Box plots (depicting lower, median and upper quartiles, with Tukey whiskers) background (nil)-corrected cytokine concentrations in supernatants following *in vitro* stimulation with BCG, MTB and PPD. The p-values for comparisons between infants immunised at birth (white bars) and infants immunised at 2 months (grey bars) are shown above each pair. Note negative values reflect a concentration in the antigen-stimulated sample lower than that in the nil control sample. Background-corrected values with negative concentrations were set to zero.



Supplementary Figure: Gating strategy used to select IFN-γ, IL-2 and TNF producing CD4 and CD8 T cells shown in a BCG-stimulated sample from one individual.



.