# Studies on innate immune responses to *Mycobacterium bovis* BCG

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# *Declaration of one's own work*

*I, **Eglė Butkevičiūtė**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.*

# *Abstract*

Tuberculosis (TB) is a significant global health burden. BCG, the only licenced anti-TB vaccine, provides variable protection against TB but can also provide heterologous benefits.

Trained immunity has been implicated in mediating the heterologous effects of BCG and protection against mycobacterial infections; however, factors mediating these effects have not been fully explored. To examine the innate immune factors with a possible role in the protection against TB and non-mycobacterial infections, this study investigated innate immune responses to BCG and other agents using samples available from healthy adult donors and 2 BCG-vaccinated infant cohorts from the UK and South Africa (SA).

An *in vitro* system for human CD14+ monocyte training with BCG was independently investigated to screen for markers associated with trained immunity and protection against TB. A weaker effect of training on IL-6 and TNFα production upon secondary stimulation was observed compared to previous studies, the extent of which was associated with dose and viability of BCG.

Differences in heterologous and mycobacteria-specific effects of BCG have been observed in different populations, so CD14+ monocyte responses to BCG and toll-like receptor agonists were studied by flow cytometry in PBMCs from UK and SA infants and their gene expression, cytokine profiles and mycobacterial growth inhibition analysed. Although the two cohorts differed, these agents induced similar patterns of innate responses.

Previous studies suggested that trained immunity can contribute to protection against TB, therefore cytokine profiles of whole blood cultures stimulated with BCG or TB vaccine candidates were analysed. Distinct cytokine profiles were detected, predominantly driven by vaccine antigen carriers. A spore-based fusion protein vaccine induced the strongest production of G-CSF, GM-CSF, IL-1β, IL-6 and TNFα, suggesting that TB vaccine candidates

may contribute to antimycobacterial protection via trained immunity and that cytokine profiling can be an effective tool to screen for candidates that may have such properties.

To conclude, this study found some heterologous effects of BCG *in vitro*; similar innate cytokine response patterns in two BCG-vaccinated infant cohorts and suggested that other TB vaccines may also have heterologous effects.

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I don't know if I reach my destination, but at least I will have made my journey.

# *COVID-19 Statement of Impact*

**1. Details on how disruption caused by COVID-19 has impacted the research:**

Because of the COVID-19 pandemic, the work planned for the third year of this project was severely disrupted. Because of the national lockdown implemented in March 2020, laboratories at LSHTM were closed from 23rd March until mid-June 2020, preventing any lab work. I was able to return to the laboratory from June 2020 but the work continued to be affected. Firstly, there were restrictions on the number of people permitted to work in the laboratories and the offices, and so I was not able to work every day.

Secondly, SARS-CoV-2 vaccines were not available until late 2020 and the mass vaccination campaign only began in 2021, with only the individuals in the highest risk groups of severe disease vaccinated first, so not all the staff at LSHTM were able or willing to return to work at Keppel Street. Access to some of the lab equipment that needed additional supervision or assistance was therefore limited, and there were delays in the delivery of some reagents or items.

Thirdly, there have been additional problems with access to the blood samples needed for my project. From June 2020 our original phlebotomist was only available for 2 days a week. She then left and the training of the replacement phlebotomist was delayed because of the COVID-19 pandemic. The availability of donors was also affected because many staff and students were working from home and were less frequently available for donation.

There were also other disturbances.

My primary supervisor also left LSHTM in December 2019, although Dr Hazel Dockrell kindly agreed to take over my supervision. There were plans for my former primary supervisor to visit LSHTM regularly for in person supervision meetings and support for lab work, but only one visit (in January 2020) was possible because of COVID-19-associated restrictions implemented from March 2020.

The recruitment of the BCG-vaccinated and non-vaccinated infant samples in the UK had also been slowed up by earlier disruptions with the global supply of the BCG vaccine and issues with the availability of medical personnel trained to administer this vaccine, resulting in no samples being collected from November 2017 to May 2018. Because of the COVID-19 pandemic, the end of the TBVAC2020 funding and a lack of additional funding, it was not possible to re-open recruitment. While I focused my project on training monocytes *in vitro,* this system is very delicate and sensitive, which also resulted in data collection going slowly in the early phases of the project.

There were also discussions regarding a potential overseas visit to Dr Nigel Curtis's lab in Melbourne for collection of data from unvaccinated and BCG-vaccinated infants but because of the pandemic it was decided to exploit BCG-vaccinated UK and South African infant samples already available and analyses of healthy adult volunteer responses to BCG and other TB vaccine candidates.

**2. A description of how the planned work would have fitted within the thesis' narrative:**

Recruiting more infants in the UK – both the BCG- and the non-vaccinated infants – would have allowed additional analyses of the long-term effects of BCG on pathogen recognition receptors and innate cytokine profiles in infants and increased the statistical power of the analyses. In addition, it would have made the infant dataset more comparable with the work

on primary human monocyte training *in vitro*.

Accessing unvaccinated and BCG-vaccinated infant samples from Dr Nigel Curtis's lab would have allowed investigating how BCG immunisation influences infant innate immune cell surface receptor expression, expression of genes associated with protection against TB, innate cytokine profiles and their ability to inhibit mycobacterial growth in association with these parameters.

Recruiting more adult volunteer donors would have increased the power of the *in vitro* innate immune training analyses and allowed exploring additional features of the *in vitro* trained primary human monocytes, e.g. fluorescent microscopy analyses of what happens to BCG during the training and resting phases in trained monocyte cultures and whether training improves the ability of primary human monocytes to associate with, internalise and kill mycobacteria upon subsequent exposure.

Further recruitment would also have provided samples for additional DNA methylation analyses to investigate how this epigenetic mechanism influences long-term innate immune responses in the vaccinated individuals.

**3. A summary of any decisions / actions taken to mitigate for any work or data collection/analyses that were prevented by COVID-19:**

Because of the timing of the pandemic and the phase the project was in when the pandemic struck, it was decided to focus on the innate immune cell markers, trained immunity-associated cytokine production, expression of genes potentially associated with protection against TB and mycobacterial inhibition in infant PBMC cultures stimulated with BCG or TLR agonists. As previous work in Professor Hazel Dockrell's lab indicated that BCG-vaccinated infants from different populations can respond differentially to PBMC stimulation with mycobacterial

antigens and additional literature suggested that innate immune responses in infants from different populations also differ, it was also decided to focus the analysis on samples available from two cohorts of BCG-vaccinated UK and South African infants.

Some studies have associated the production of such innate cytokines as IL-1β or G-CSF not only with secondary responses to heterologous stimuli in trained primary monocytes cultures or human or murine immunisation models but also with the ability to regulate the innate immune cell populations in the vaccinated individuals. It was therefore decided to also expand the planned analyses by running additional multiplex bead array analyses on adult volunteer whole blood samples stimulated with BCG and novel TB vaccine candidates and compare their innate and adaptive cytokine profiles, investigate individual characteristics and predict which type of immune responses they would be likely to induce if given to animals or humans *in vivo* and whether they could also induce trained immunity or similar responses.

# *Contents*

# *List of Abbreviations*

**Ag85A / Ag85B** – antigens 85A or 85B

**AM** – alveolar macrophage

**APC** – antigen presenting cell

**ATRA** – all-transretinoic acid

**BCG** – Bacille Calmette-Guérin

**BM** – bone marrow

**BMDM** – bone marrow-derived macrophage

**BSA** – bovine serum albumin

***B. subtilis*** – *Bacillus subtilis*

***C. albicans*** – *Candida albicans*

**cAMP** – cyclic adenosine monophosphate

**cDNA** – complementary DNA

**CFU** – colony forming unit

**CNS** – central nervous system

**COVID-19** – coronavirus disease

**DC** – dendritic cell

***D. discoideum*** **–** *Dictyostelium discoideum*

**DMSO** – dimethyl sulfoxide

**DPBSA** – Dulbecco's PBS A

**DTP** – diphtheria, tetanus, pertussis

***E. coli*** – *Escherichia coli*

**EDTA** - Ethylenediaminetetraacetic acid

**ELISA** – enzyme linked immunosorbent assay

**FCS** – foetal calf serum

**FP-1** – fusion protein 1

**γBCG** – irradiation-killed BCG

**H3K4me3** – trimethylation of lysine 4 on histone 3

**HBSS** – Hank's balanced salt solution

**HI ABS** – heat-inactivated human AB serum

**HI FCS** – heat-inactivated foetal calf serum

***H. influenzae*** – *Haemophilus influenzae*

**HIV** – human immunodeficiency virus

**HSC** – haematopoietic stem cell

**IFN** - interferon

**IgA, IgG** – immunoglobulins A or G

**ILC** – innate lymphoid cell

**LAM** - lipoarabinomannan

**LBW** – low-birth-weight

***L. monocytogenes*** – *Listeria monocytogenes*

**lncRNA** – long non-coding RNA

**LPS** – lipopolysaccharide

**LTBI** – latent TB infection

***M. bovis*** – *Mycobacterium bovis*

***M. leprae*** – *Mycobacterium leprae*

***M. marinum*** – *Mycobacterium marinum*

***M. obuense*** – *Mycobacterium obuense*

***M. paratuberculosis*** – *Mycobacterium paratuberculosis*

***M. tuberculosis*** – *Mycobacterium tuberculosis*

**MACS** – magnetic-activated cell sorting

**MALP** – macrophage activating lipopeptide

**mDC** – myeloid dendritic cell

**MDP** – muramyl dipeptide

**MFI** – mean fluorescence intensity

**MGIA** – mycobacterial growth inhibition assay

**MGIT** – mycobacterial growth inhibition tube

**MHC** – major histocompatibility complex

**MMR** – measles, mumps, rubella vaccine

**ML ratio** – monocyte to lymphocyte ratio

**moDC** – monocyte derived dendritic cell

**MOI** – multiplicity of infection

**MPP** – multipotent progenitor cell

**MVA** – modified *Vaccinia* Ankara

**NK** - natural killer cell

**NSE** – non-specific (heterologous) effect

**OADC** - oleic acid, albumin, dextrose and catalase

**OD** – optical density

**OPV** – oral polio vaccine

**oxLDL** – oxidised lipoprotein

**Pam3Cys** or **Pam3CSK4** – N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine

**PAMP** – pathogen associated molecular pattern

**PANTA** – polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin mixture

**PBMC** – peripheral blood mononuclear cell

**PBS** – phosphate buffered saline

**PCA** – principal component analysis

**PCR** – polymerase chain reaction

**pDC** – plasmacytoid dendritic cell

**PDE** – c-di-phosphodiesterase

**PEPG** – peptidoglycan

***P. falciparum*** – *Plasmodium falciparum*

**PHA** - phytohemagglutinine

**PMA** – phorbol myristate acetate

**Poly I:C** – polyinosinic:polycytidilic acid

**PPD** – purified protein derivative

**PRR** – pathogen recognition receptor

**P/S** – penicillin/streptomycin

**qRT-PCR** - quantitative reverse-transcriptase - polymerase chain reaction

**R848** - resiquimod

**RPMI** – Roswell Park Memorial Institute 1640 medium

**RT** – room temperature

**SARS-CoV-2** – severe acute respiratory syndrome coronavirus 2

**SATVI** – South African Tuberculosis Vaccine Initiative

***S. aureus*** – *Staphylococcus aureus*

**SCID** – severe combined immunodeficiency

**SNP** – single nucleotide polymorphism

**SSI** – Statens Serum Institut

***S. pneumoniae*** – *Streptococcus pneumoniae*

***S. pyogenes*** – *Streptococcus pyogenes*

**TB** – tuberculosis

**tBLP** - triacylated bacterial lipopeptide

**TLR** – Toll-like receptor

**TMB** – 3,3´,5,5´-tetramethylbenzidine

**TriDAP** – L-Ala-γ-D-GlumDAP

**TTP** – time-to-positivity

**WBC** – white blood cell

**WHO** – World Health Organization

**WT** – wild-type

**YFV** – yellow fever vaccine

# *List of Tables and Figures*

## Chapter 2:

## Chapter 3:



## Chapter 4:

## Chapter 5:

## Appendix:

**Figure S3.1.** *The growth of BCG on 7H10 agar plates*.

**Table S3.1***. BCG standard concentrations as estimated by growth on 7H10 agar plates*.

**Table S4.1.** *Antibodies and dyes used in FACS staining of infant PBMCs*.

**Table S4.2***. Cytokines investigated in UK and South African infant PBMC response to BCG and TLR agonist analyses*.

**Table S4.3.** *Contribution of host factors to the to the total variation in infant innate immune responses to BCG and TLR agonists as indicated by two-way ANOVA analysis*.

**Table S4.4.** *The effects of BCG and TLR agonists on BCG-vaccinated infant immune responses in the UK and South African cohorts as shown by P-values generated by Šidák's multiple comparisons test*.

**Table S4.5.** *Differences in innate immune responses of the UK male and female infants or stimulated vs unstimulated PBMC cultures as indicated by p-values from Šidák's multiple comparisons test.*

**Table S4.6.** *Infant cytokine responses to BCG vaccination*.

**Table S4.7.** *Population associated differences in cytokine production in BCG-vaccinated infants*.

# **Chapter 1:** Introduction

## 1.1 Incidence and burden of adult and childhood TB

In 1993, the World Health Organization (WHO) declared tuberculosis (TB) a public health emergency (1). Since then, several ambitious strategies have been initiated to reduce the global incidence of TB (2–5). One of them is the End TB strategy, aiming to reduce the number of TB deaths by 90% and TB incidence by 80% by 2030 compared to the rates reported in 2015 (5). This strategy is based on several approaches, focusing on patient-centred TB care and prevention, policies and support systems and intensive research and innovation (5).

Despite the enthusiastic approach, the success of these strategies has been mixed so far. The global burden of TB decreased significantly over the last two decades falling from the 6$^{th}$ leading global cause of death and 1.7 million deaths in HIV-negative individuals in 2000, and in 2019 TB was no longer in the list of the 10 leading global causes of death (6). However, according to the Global TB Report 2021, there were 1.3 million TB deaths in HIV-negative individuals in 2020, with a similar number reported in 2018 and 2019 (1.24 million and 1.21 million respectively) (7–9). Although, before the SARS-CoV-2 pandemic, the incidence of TB per 100,000 individuals had been decreasing stably, at a rate of about 1.5% per year, this rate was lower than the one targeted by the End TB strategy, and TB still remained a significant global health burden (5). The situation may have worsened during the COVID-19 pandemic due to limited access to health care services – it has been estimated that 1.4 million less individuals received TB care in 2020 compared to 2019 and that this may have led to an additional 0.5 million deaths (10).

Therefore, it is possible that despite the global effort to eradicate TB, that this disease will still remain an important public health issue. It has been estimated that around 23% of the global population might be latently infected with *Mycobacterium tuberculosis* (11). In 2019, TB was the 8$^{th}$ leading cause of death in low-income countries and the 7$^{th}$ in lower-middle-income countries (6). Prior to the COVID-19 pandemic, in 2018, 7 million and in 2019, 7.1 million new cases of TB were reported (7,8). During the pandemic, in 2020, the estimated number of new

TB cases was 4.1 million, probably reflecting underreporting due to the lack of access to health services due to the pandemic (10,12).

Although most cases of TB occur in adults, 11% of cases (~1.1 million cases) reported prior to the pandemic in 2018 were detected in children under 15 years of age, with 205,000 deaths, and in 2019, ~1.2 million cases, with 230,000 deaths (7,8). It should be noted though, that depending on the selected modelling approach, these estimates can vary. One study suggested that 239,000 children in this age group may have died because of TB in 2015, 191,000 of these deaths occurring in children under 5 years of age (13). In contrast, the Global Burden of Diseases (GBD) study predicted that both the incidence of TB and associated mortality in children under 15 years of age in 2015 and 2016 might have been lower than estimated by Dodd et al. (13) or by the WHO, with ~690,000 incident cases and ~70,000 annual deaths (14–16). It should be noted that infants and very young children (<5 years of age) are at a higher risk of death due to TB than older children (>5 years of age), especially, if treatment is not available; however, anti-TB treatment significantly reduces the risk of death due to TB (17).

## 1.2 Bacillus Calmette-Guérin (BCG)

### *1.2.1 Background*

Developed in 1921, Bacillus Calmette-Guérin (BCG) is among the most widely used vaccines, either for universal immunisation in settings where TB is endemic, or for selected risk groups, such as travellers to areas where TB is endemic, those at occupational risk or those whose immediate contacts may have been exposed to TB (18). It is estimated by UNICEF to cost around 0.11 USD per dose in 2018, making it an affordable TB control measure, including in lower income countries where TB rates are higher (19).

BCG is a live-attenuated form of *Mycobacterium bovis*, a bovine TB causing acid-fast bacillus from *M*. *tuberculosis* complex, derived by Albert Calmette and Camille Guérin over a series of passage through potato and ox bile (9,20). It was first tested in human neonates soon after it

was developed – in 1921 – and has remained in use since, becoming one of the oldest vaccines still in use (9,20). First used orally, BCG has been shown to be effective against TB when administered intradermally, and this route is now the major way of BCG administration (18,20). Since the 1920s, the BCG had been shared with other laboratories globally where it was cultured independently, with multiple strains developed by the 1960s (9). These strains contain a number of genomic differences (21,22), and their influence on immunological differences in individuals or resistance to TB or other infectious diseases are still an active theme of immunological and epidemiological studies (23–25).

### *1.2.2 Efficacy of the BCG vaccine and associated variation*

Despite being the only licenced human anti-TB vaccine, its broad coverage and over 100-year use, the potential of BCG to control TB is limited by the variability of its anti-tuberculosis effects. The BCG vaccine is generally considered protective when administered in children or young infants (26,27), and has been shown to be effective against severe, disseminated, forms of childhood TB, with an estimated 64-73% and 77-78% efficacy against tuberculous meningitis and disseminated TB respectively (28,29). The protective efficacy of BCG against pulmonary TB in adults, however, is variable, with almost no protection in some populations and efficacy as high as 80% in others (27,30). Multiple factors have been implicated in this variation, including geographical latitude, prior exposure to environmental mycobacteria and consecutive epitope masking, age at BCG vaccination, population genetics or strain of BCG used for immunisation (27,30). Timing of BCG vaccination may also be an important factor contributing to infant protection against TB. In settings with high burden of TB, the WHO recommends vaccinating infants immediately after birth or at the earliest opportunity (31). Unfortunately, that is not always possible, especially in resource-poor settings, where many infants receive their BCG vaccine later (32). It has been shown that even when BCG coverage at birth is as high as 92%, reduction of TB-associated deaths can be relatively small – 2.8%, increasing to 16.5% when BCG coverage at birth reaches 100% (33).

There is some disagreement as to how long the protective effects of BCG can last. Although BCG-induced protection against pulmonary TB is generally considered to wane over time in vaccinated individuals, some studies suggest that the anti-tuberculosis effects of BCG can last for many years. For instance, a systematic review and meta-analysis of studies on the duration of the effects of BCG found that protection against pulmonary or extrapulmonary forms of TB can last for up to 10-15 years (26). There is also evidence that, at least in some populations, protective effects of BCG can last longer. A study in individuals vaccinated with BCG at school age in the United Kingdom detected protective effects of BCG for up to 20 years or longer, with estimated 57% vaccine efficacy at 15-20 years post vaccination (34). Similar duration of the protective effects of BCG was detected in Norway, with 58% vaccine efficacy observed at 10-19 years since immunisation with this vaccine (35). Finally, a study on BCG vaccination in the indigenous people in the United States suggested the protective anti-tubercular effects can last for over 50 years (36). In these studies, vaccine efficacy was also associated with the latitude or distance from the equator or inversely associated with time since vaccination (26,34,35).

### *1.2.3 Immune responses in neonates, infants and adults and risk of TB*

The immune system in neonates and very young infants is polarised differently from that of the adults. It is considered adapted to the sterile environment of the uterus and required to adjust to microbial colonisation occurring over the first few days after birth or later infections, and therefore, primed towards anti-inflammatory responses to prevent disruptive inflammation in a newborn (37,38). Neonatal mice and human cord blood is enriched with immunosuppressive erythroid CD71+ cells that suppress the production of pro-inflammatory cytokines TNFα, IFNγ or IL-17 (39,40). While these cells may help prevent inflammation in the gut of neonatal mice during commensal colonisation, that happens at the expense of resistance to infections, murine pups failing to control the growth of *Listeria monocytogenes* in their spleens or *Bordetella pertussis* in their lungs (39,40). The extent of this CD71+ cell

suppression is context dependent, however, as these cells were found not to inhibit production of IL-12p70 by neonatal monocyte-derived dendritic cells (moDCs) upon Toll-like receptor (TLR) or dectin-1 stimulation (41). Apart from CD71+ cells, there are other, complementary mechanisms to control inflammatory responses in neonates. Macrophages derived from human cord blood produce elevated levels of immunoregulatory cytokine IL-27, inversely correlating with BCG burden in the infected macrophages or IFNγ levels (42). Neonatal plasma was also found to contain high levels of adenosine or cyclic adenosine monophosphate (cAMP), molecules shown to inhibit TNFα production by mononuclear blood cells, both in samples from newborns or adults (43). Overall, already prone to suppression of excessive inflammatory responses, the newborn innate immune system is also more responsive to external anti-inflammatory agents (44).

Apart from enhanced immunoregulatory cell populations and elevated levels of anti-inflammatory cytokines or other soluble mediators, the composition of immune cell populations differs in neonates. Lower frequencies of CD8+ T-cells and CD19+ B-cells and elevated frequencies of CD14+ monocytes are found in cord blood compared to infants at 1-5 years of age or older (45). Changes in cell frequencies over the first year of life are accompanied by changes in DNA methylation patterns (45). Some studies report lower percentages of CD14+CD16+ monocytes in cord blood and reduced TLR4 expression, affecting downstream TNFα responses (46). Neonates have also been shown to have lower frequencies of classical CD14+CD16- monocytes or CD16+CD62L+ granulocytes and higher percentages of intermediate CD14+CD16+ monocytes or inflammatory CD16dimCD62L+ granulocytes compared to healthy adults (47). Of interest, in neonates, frequencies of both the TLR2-expressing immunosuppressive CD16+CD62L- and inflammatory CD16dimCD62L+ granulocytes were elevated, although neonate CD16dimCD62L+ granulocytes were less prone for oxidative burst and their monocytes expressed less HLA-DR than those of the adults (47). While this suggests that neonate neutrophils may be less efficient at killing microorganisms and that their monocytes may be less potent at presenting antigens to T-cells,

monocytes from neonates and adults have been shown to have similar capacity for phagocytosis and killing of *Staphylococcus epidermidis* (48). Apart from innate cell populations, there are differences in newborn and adult lymphocyte populations and responses too. Circulating naïve B-cells in neonates express higher levels of TLR4 and TLR9 compared to B-cells from adults and produce more IL-10 and TNFα upon TLR9 stimulation while producing less IL-6 upon TLR7 stimulation (49). In addition, neonate B-cells are less responsive to CD40 stimulation, less capable of immunoglobulin class-switching, express less CD73 enzyme and produce less adenosine, a molecule important for cell proliferation than cells from the adults (49–51).

Although differences in neonatal and adult cell populations and impaired function of some cell populations suggests high levels of immune response regulation in neonates or young infants, this is not straightforward. Monocyte-derived DCs from neonates produce less IL-23 upon dectin-1 stimulation and lower levels of IL-23 or IL-12p70 upon TLR stimulation compared to those of the adults (41). Studies exploring broader gene signatures in neonates and the adults also find differences in pathways associated with interferon regulatory factor 3 or Type I interferon signalling, expression of the associated genes downregulated in neonates (52). However, neonates produce less IFN-α2, IFNγ, IL-12p70 or TNFα upon TLR stimulation than adults while also producing similar or higher levels of IL-12p40, IL-23, IL-6, IL-1β and IL-10 upon pathogen recognition receptor (PRR) stimulation (53–56), suggesting a complex counterbalance of pro- and anti-inflammatory responses early on after birth. This regulation is particularly important as imbalance of these responses, e.g. by suboptimal IL-12/IL-23p40 responses has been associated with poorer control of such conditions as sepsis, especially in pre-term infants, although inflammatory cytokine responses have been shown to also mature in pre-term infants during the neonatal period and reach similar levels as those of term infants (57,58). It should be noted that in those cases where differential immune responses are found in neonates and other age groups, not only cytokine responses are different but also those of other mediators involved in innate defence. Childhood vaccines containing alum and TLR

agonists were found to induce higher pentraxin 3 responses in whole blood from newborns while alum-containing hepatitis B vaccine stimulated stronger matrix metalloproteinase-9 responses in whole blood from adults (59).

Cytokine profiles in neonates or the adults can also be influenced by the cell types analysed, PRRs stimulated, agonists or their doses used to trigger cytokine responses (53,60–63). For instance, although cord blood generally produces less TNFα, IL-1β or other inflammatory cytokines, different TLR agonists or their combinations can be exploited to trigger equivalent responses in neonatal and adult blood or purified cell populations (61–63). In some cases, neonatal inflammatory responses may be higher than those observed in adults. For instance, group B *Streptococcus* was found to induce stronger TNFα responses in cord blood compared to blood samples from adults, although this difference was abrogated when adherent monocytes were investigated, with TNFα responses in this study partly dependent on complement components (64). Elevated cord blood production of IL-6 was also found in another study comparing adult and newborn cytokine responses to TLR1/2 and TLR2/6 agonists or heat-killed whole microorganisms (43). These differences may also be pathway or receptor dependent as lower TNFα responses were found in cord blood stimulated with triacylated bacterial lipopeptide (tBLP, TLR1/2 agonist), macrophage-activating lipopeptide (MALP, TLR2/6 agonist), lipopolysaccharide (LPS, TLR4 agonist) or imiquimod (TLR7 agonist), although neonate and adult TNFα responses did not differ for TLR7/8 stimulation with R848 (resiquimod) (43,65).

Although differently polarised from that of the adults, to adapt to the microorganism rich environment after birth, the immune system of neonates and infants undergoes not only changes in cell populations over the first year of life but also in its cytokine profiles, skewing towards inflammatory, Th1 or Th17-inducing profiles (37,38). Some of these changes occur quite soon after birth. Human cord blood responds to stimulation with TLR agonists, releasing IFNγ or TNFα, however, production of IFNγ significantly increases over the first month of life (66). Stimulus- and TLR-dependent elevation in IL-1β, TNFα or IFNγ release over the first 2

years of life was also reported in a study exploring cytokine response maturation in Caucasian and Asian infants (67). These changes are associated not only with changes in surface receptor expression, but also cellular signalling. For instance, elevation of TNFα, IL-6 and IL-12 was observed in response to BCG or Pam3Cys stimulation in whole blood from 10 or 36 week old infants and was accompanied by an increase in co-stimulatory molecule CD40 or CD83 expression in monocytes or myeloid DCs (mDCs) and reduced inhibition of NFκB signalling (68), although some of these changes may have been induced by BCG itself (69). Although the general trend is for an increase in TLR-triggered inflammatory cytokine – IL-6, IL-1β or IL-12 – levels over the first year of life, some responses, such as NALP3-dependent production of IL-1β can diminish and be overall lower in adults (70). It should be noted that responsiveness of cytokines produced by monocytes or circulating DCs, such as TNFα, IL-6 or IL-12, varies over one's life, increasing in infants at 1-2 years of age and then decreasing by adulthood, at least for samples stimulated with Pam3Cys or LPS (71). Similar changes happen in young mice – transcription of genes encoding subunits of IL-27 intensifies as mice reach infancy, stabilises when mice reach adolescence and reduces from later adolescence over adulthood (42). Production of some cytokines may also peak at different points in life. Elevated IL-10, IL-6, IL-1β, IL-8 (CXCL8), CCL2, CCL4 and CCL5 responses were associated with neonatal period while infants tended to produce more IFNα or TNFα than neonates or adults (72).

Although it is expected that inflammatory capacity increases with aging, sometimes the pattern is reversed, for instance, alum-induced IL-8 (CXCL8) or MCP-1 responses were found to diminish over the first year of life, increasing afterwards (70). One study in South Africa also found a consistent decrease in whole blood cytokine production upon TLR and NOD1/2 receptor stimulation from 2 weeks until 12 months of age, with the exception of TLR4-mediated cytokine responses, the same trend observed for TNFα, IL-6 and IL-12 in monocyte or conventional DC (cDC) cultures (73). It is not clear why the cytokine production pattern in this study was different from the expected increase (37,38), but it is possible that cytokine profiles

detected at 2 weeks of age are different from those in cord blood samples often used in neonatal immune response studies – due to maturation of infant immune responses or exposure to commensal or other microorganisms. Indeed, some changes in neonatal immune responses happen over the very first few days of life. Populations of basophils, CD56-expressing or CD56dim NK cells, neutrophils or plasmacytoid dendritic cells (pDCs) diminish over the first week of life while myeloid DCs (mDCs) expand (74). These changes are accompanied by a parallel increase in IL-17A, MDC, CXCL10 (IP-10) and IFNγ levels or decrease in G-CSF, CCL5, IL-6 and IL-10 levels, as well as complex transcriptional, proteomic and metabolomic changes (74), including increase in antimicrobial peptides or proteins, such as bactericidal/permeability increasing protein (75).

### *1.2.4 BCG-induced antimycobacterial immune responses in infants*

Differential polarisation of the immune system in neonates or young infants compared to that of the adults is also reflected in their responses to BCG immunisation or *M. tuberculosis* infection. The anti-tuberculosis effects of BCG manifest most strongly in individuals administered this vaccine soon after birth or in childhood and BCG is effective at protecting infants from TB meningitis or disseminated TB (27–29). However, the period of intensive immune response development and changes over the very first year of life corresponds to the period with a significant risk of acquiring and developing TB, with neonates and young infants at the highest risk of TB and related complications. Infants <1 year of age are at the highest risk of developing severe, extrapulmonary forms of TB, such as TB lymphadenopathy, disseminated TB, and TB of the central nervous system (CNS) (76,77). A study in South Africa reported in 2006 that infants <3 years of age comprised 84.2% cases of miliary TB or advanced TB meningitis cases among children <13 years of age (78). Similar findings have been also reported in other settings with high rates of TB, with a study in India reporting TB meningitis corresponding to 50% of extrapulmonary TB cases among paediatric TB patients (79). Diagnosis of pulmonary or disseminated forms of TB can be particularly difficult because

of non-specific presentation of associated symptoms and challenges obtaining microbiological confirmation in infants, whose sputum samples can often be paucibacillary (80–82). In the absence of treatment, childhood TB results in high mortality, especially among infants <5 years of age (17). Preventive therapy can significantly reduce the risk of progression from latent to active disease (83).

Although the correlates of protection against TB are not fully clear, BCG induces Th1- or Th17-polarised immunological memory and this leads to the enhancement of inflammatory TNFα, IL-1β or IFNβ responses required for activation of alveolar macrophages and killing of *M. tuberculosis* (84). More recently, however, other mechanisms have been implicated in early clearance of *M. tuberculosis*, including innate immune responses mediated by macrophages, monocytes, neutrophils and NK cells or non-conventional T-cells and antimycobacterial antibody responses (85). Infants, especially those <1 year of age are more susceptible to TB than adults, and it is believed that a number of differences in the adult and infant immune systems can contribute to higher susceptibility of infants to TB and its complications. Apart from lower inflammatory cytokine responses, reduced numbers of circulating DCs or alveolar macrophages, their impaired chemotactic or microbicidal properties, and diminished cytotoxic T-cell responses are thought to contribute to susceptibility (86). Some studies indicate that although infant alveolar macrophages display similar expression of surface receptors (TLR2, IFNGR1, CD206), antigen presenting molecules (HLA-DR) or the stimulatory molecules (CD40) and similar rates of phagocytosis as those from adults, infant alveolar macrophages may be less capable of killing *M. tuberculosis* (87). In addition, infant alveolar macrophages have been shown to produce more neutrophil attracting chemokines than adult macrophages (87), and neutrophil necrosis can be exploited by *M. tuberculosis* to survive and enhance its spread (88). It is possible that higher levels of immune response regulation in infants can contribute to TB pathology – infants that develop TB disease have been reported to have higher percentages of regulatory T-cells, elevated production of IL-10 and IL-1β, but produce

less TNFα or IFNγ compared to healthy controls (89). External factors, such as co-infection with cytomegalovirus, may also contribute to infant susceptibility to TB (90).

It should be noted, however, that although some responses in infants or neonates are diminished, it is not that they are missing completely, and BCG immunisation can induce or enhance them in the vaccinated infants. BCG triggers IL-12p40 production by neonatal moDCs and Type I interferon production by pDCs (91), and monocytes from BCG-vaccinated infants in Chile demonstrated higher viability in cell cultures and improved short-term uptake of *M. tuberculosis* compared to unvaccinated children, although lower than those adults (92). BCG can also induce cord NK cell mediated IFNγ release and production of IL-10 and IL-12 by CD14+ cord monocytes, and although shown not to stimulate cord CD4+ or CD8+ T-cell responses, BCG induces T-cell responses detectable from at least 10 weeks or more post immunisation, including effector, memory or regulatory cell populations in vaccinated infants (91,93–95).

BCG immunisation does induce memory Th1 responses in neonates that receive this vaccine at birth. At 2 months post BCG immunisation, purified protein derivative (PPD) or *killed M. tuberculosis* induced cell proliferation and IFNγ production in cell cultures from infants that received BCG at birth or at 2 months of age (96). In addition, in BCG-vaccinated infants, CD4+ T-cell IFNγ responses to PPD are comparable to those of adults (97,98). Not only Th1 but also Th2 recall responses are induced in infants that receive neonatal BCG, with detectable IFNγ, IL-13 and IL-5 responses to PPD or other *M. tuberculosis* antigens (99). While IFNγ responses to PPD or polyclonal stimuli were shown to last, for at least 2 years since BCG immunisation, IL-13 or IL-10 responses diminished by this time point (100), although some studies suggest that BCG-induced IFNγ or other inflammatory cytokine producing T-cell populations or cytokine responses diminish over time, in some cases, over the first year of life, in others – later in infancy or adolescence (95,101–103). In other studies, although comparable frequencies of PPD-responsive, IFNγ-producing producing CD4+ T-cells are found in infants and adults, expression of CD154 (CD40L), a co-stimulatory molecule, may be lower over the

first year of life (104), suggesting that some of their functions may be impaired. BCG-specific IFNγ-producing T-cells in vaccinated infants have been associated with protection against TB; however, the implications of this effect are not fully clear as activated HLA-DR+ CD4+ T-cells have been associated with increased risk of TB disease (105) and TB exposed but uninfected children produce less IFNγ than age-matched counterparts with *M. tuberculosis* infection (106).

Apart from helper T-cell responses, BCG also induces CD8+ T-cell responses in infants that receive neonatal BCG, enhancing their proliferation, granzyme, perforin or IFNγ production 10 weeks later, although not all infants develop CD8+ T-cells that contained both IFNγ and cytotoxic molecules (107). Compared to cord blood, increased frequencies of granulysin- or perforin-expressing CD4+ or CD8+ T-cells or NK cells in response to PPD stimulation are detected at 10 weeks after birth in BCG-vaccinated infants, at similar levels compared to PPD-responsive adults, although cord blood on its own also responds to BCG, increasing frequencies of T-cells expressing these cytolytic molecules, even if to a lesser extent (108). Cytotoxic T-lymphocytes from cord blood have been shown to be able to kill BCG or *M. tuberculosis* infected cells to some extent, however, these cytotoxic responses are enhanced by BCG vaccination (109). Although BCG immunisation enhances IFNγ-producing CD8+ T-cell proliferative responses to PPD both in adults and children, cells from the adults may be somewhat more sensitive to PPD stimulation than those from children (98).

Other T-cell populations are also engaged by BCG vaccination. In mice, γδ T-cells were found to contribute to IFNγ or IL-17 responses to *M. tuberculosis* and control of its growth in the lungs of the infected mice (110). Neonatal vaccination induces BCG-specific γδ T-cells in human infants too, enhancing their proliferation in parallel to that of CD4+ or CD8+ T-cells upon *ex vivo* stimulation at 7 months of age (111), although some studies in BCG-vaccinated infants report that IFNγ+ γδ T-cell frequencies increase up to 5 years of age or even later (95). CD4-CD8- γδ T-cells, together with NK cells were identified as the major IFNγ producers in BCG-vaccinated infants (112). Although these cells may contribute to inflammatory responses

against TB, their role in protection against TB in BCG-vaccinated infants is not fully defined. A study in South Africa comparing cytokine-producing T-cell frequencies found no differences in IL-2-, IL-17-, IFNγ- or TNFα- producing γδ T-cell percentages among BCG-vaccinated infants who developed TB in 2 years of follow-up and their community or household controls (113).

Polyfunctional T-cell subsets are also induced in BCG-vaccinated infants. Both double- or triple-functional CD4+ or CD8+ T-cells producing IFNγ and TNFα or IL-2 and TNFα, or all three of the cytokines are detected after BCG vaccination in children or young infants, and their frequencies are comparable to those of adults (94,98,104). There is some evidence that these polyfunctional, proinflammatory cells contribute to BCG-vaccinated infant protection against TB. For instance, some infant BCG immunisation studies found an association between frequencies of PPD-specific, polyfunctional, IFNγ+TNFα+IL-2+ CD4+ T-cells from BCG-vaccinated infants and their ability to inhibit mycobacterial growth *ex vivo* (114). Others, however, suggest that antimycobacterial responses mediated by these cell populations, by themselves, may not be sufficient to protect vaccinated infants from TB. A study in South Africa that followed BCG-vaccinated infants for 2 years, found no differences in BCG-specific single-, bi- or polyfunctional CD4+ or CD8+ T-cell frequencies in samples from those infants that developed TB or BCG-vaccinated house or community controls (113). Another study in Australia detected higher frequencies of ESAT-6-, CFP-10-, PPD- or killed *M. tuberculosis*-responsive TNFα+, IL-17+ and IFNγ+TNFα+ or IL-2+IL-17+ CD4+ T-cells in children with active TB compared to children with LTBI or healthy controls (115). In Korean adults with LTBI, frequencies of CD4+ IFNγ+IL-2+TNFα+ or IL-2+TNFα+ T-cells were also higher than in healthy controls and polyfunctional CD4+ T-cell frequencies did not correlate with mycobacterial growth inhibition (116). In addition, the responses that are thought to be protective against TB are not induced equally in all the BCG-vaccinated children. High and low IFNγ producer phenotypes have been identified in multiple infant BCG immunisation studies in India and South Africa (107,117,118). Together, this suggests that it is also

important to explore other mechanisms or their combinations that may be involved in, contribute to or mediate protection against TB.

Some BCG immunisation studies also suggest that anti-mycobacterial antibodies can contribute to infant protection from TB. BCG vaccination has been shown to modulate B-cell populations in vaccinated infants to some degree (119). Ag85A-specific IgG was associated with infant protection against TB disease in BCG immunisation followed by MVA85A boost trial (105). In another study, QuantiFERON-positive status (indicative of *M. tuberculosis* infection) in infants was inversely associated with total IgG levels, with a similar trend for IgG2 specific for BCG (120). Some studies also report lower IgG responses to lipoarabinomannan (LAM) and mycobacteria in children with disseminated compared to localised TB, suggesting that antimycobacterial antibodies may contribute to control of *M. tuberculosis* dissemination in TB complications (121). ESAT-6- or CFP10-specific IgG was also shown to correlate with inhibition of BCG or *M. tuberculosis* growth *ex vivo*, although its titres and frequency of CD27+CD21+ B-cells were elevated in active TB (122). IgA class of antibodies can also play a role in antimycobacterial responses. Reduction in LAM- or 38kDa-specific serum IgA has also been associated with TB lymphadenitis in children and adolescents (123), and decrease in anti-HrPA (heat-stress-induced ribosome binding protein A) or anti-MDP-1 (mycobacterial DNA-binding protein 1) IgA were associated with severity of pathology of TB (124). Such antibodies can be induced by BCG vaccination, and sera from BCG-vaccinated individuals containing antibodies against arabinomannan or other mycobacterial components have been shown to improve neutrophil, macrophage or THP-1 cell association with and internalisation of BCG, inhibition of its growth or even enhance CD4+ or CD8+ IFNγ+ T-cell proliferation in co-cultures with DCs (125–127). It is therefore possible that antimycobacterial antibodies may play a larger role in protection against TB or its complications than thought previously.

Immune responses to mycobacterial components in BCG-vaccinated infants are not limited to Th1, Th17 or IFNγ responses or antibody-responses, but also involve broad cytokine, chemokine and growth factor profiles (128–130), suggesting that responses of other cell

populations may be important for protection of infants against TB and its complications. In adults, higher frequencies of CD14+CD16+ and lower of CD14++CD16+ monocyte populations were found in controls compared to LTBI or active TB patients (122). CD14dim monocyte responses have also been associated with protection against TB in individuals exposed to this disease (131), suggesting that contribution of innate immune responses to protection against TB may need to be more extensively studied.

## 1.3 Heterologous effects of BCG

### *1.3.1 BCG and all-cause mortality*

Not very long after beginning to use BCG as an anti-TB vaccine, it was observed that individuals administered this vaccine are less likely to die of diseases other than TB (132). These effects are termed non-specific, heterologous or secondary effects and include reduction of all-cause mortality, acquisition or severity of infectious diseases, occurrence of non-communicable diseases, such as cancers or allergies, and can also influence innate or adaptive immune responses to stimuli not associated with these vaccines or infectious agents for which these vaccines were designed. Although the heterologous effects of BCG were reported as early as the 1930s (132), the interest in this phenomenon dissipated and only rekindled in the early 2000s when observational studies in West Africa suggested that all-cause mortality among infants that had been administered this vaccine was 30-50% lower than that of infants that did not receive this vaccine (133–135). In addition, these and other studies suggested that not only BCG, but diphtheria-tetanus-pertussis (DTP), measles vaccine, OPV or other childhood vaccines may also have effects on all-cause infant mortality (133–136). The reduction in all-cause mortality is also reported in some BCG revaccination trials (137), although the evidence is somewhat conflicting as other studies report no effect or associations with elevated risk of mortality in the vaccinated group, possibly due to interplay with other vaccines or nutritional supplementation (138).

The early studies from the 2000s investigating this effect of BCG were mostly observational and included relatively short follow-up periods – up to 2 years of age, often receiving criticism for potential biases, such as healthier, normal-weight infants receiving their BCG vaccine earlier than preterm and/or low-birth-weight (LBW) infants who would be at a higher risk of life-threatening infections and whose vaccination would be delayed, or infants from the lower socioeconomic background lacking access to health care services (139,140). It has also been proposed that the observed reduction in all-cause mortality in BCG-vaccinated infants may be due to undiagnosed mycobacterial infections (141). Indeed, a trial that found a beneficial effect of BCG immunisation on rates of LBW infant survival because of lower incidence in neonatal sepsis, respiratory infections or fever did not examine the aetiologies for these clinical manifestations (142), although a study in Guinea-Bissau suggested that BCG may reduce all-cause infant mortality both among those exposed or unexposed to TB (143). It should be noted that despite the limitations of such studies, the patterns in findings are frequently consistent, and although many of these findings come from studies conducted in West Africa, investigations conducted elsewhere often report lower incidence of all-cause mortality in BCG-vaccinated infants as well.

Among the earliest observations that associated BCG immunisation with a reduction in all-cause mortality in children was a study conducted in Sweden in the 1930s (132), although there are some historical trials from the United States, Canada or more recent cohort studies from Papua New Guinea (136,144). A recent re-analysis of this historical dataset from Sweden confirmed the association of BCG and lower risk of death due to infectious diseases other than TB in neonatal and post-neonatal period, although it also indicated the possibility of biases in the design of the original historical study contributing to the results observed (145). A reduction in all-cause mortality was also discovered in BCG-immunised children at 1-5 years of age from Uganda, with a 74% reduction in mortality risk (146). These findings have also been replicated in southern India, where higher survival rates were found in BCG-vaccinated infants for up to 6 months of age compared to infants that did not receive this vaccine (147). The beneficial

modulation of all-cause mortality in infants exerted by BCG is also confirmed by larger meta-analyses, some of these predicting 6-72% reduction based on the data from clinical trials or 2-95% as informed by observational studies (136,144,148). Overall, although the number of clinical trials to investigate the non-specific effects of BCG has increased, these estimates are particularly difficult to generate due to differences in study designs, childhood immunisation schedules and large numbers of participants required to detect the differences. Also, although the more recent findings that report beneficial effects of BCG immunisation on all-cause infant mortality still come from cohort studies or randomised trials conducted in settings with high infectious disease burden. The pioneering investigation from the 1930s Sweden also reflects a different public health context, raising a question on how broadly in terms of geography can the influence of BCG on all-cause mortality be investigated.

It should be noted that the evidence on when this non-specific effect of BCG on all-cause mortality manifests in the vaccinated individuals and whether it is universal, is complex even in settings where the burden of infectious diseases is high. Currently available information suggests that in settings where all-cause infant mortality is high, early BCG may be beneficial to neonates or very young infants, especially those with LBW, however, the trends observed vary. While randomised trials in Guinea Bissau implicated that early BCG vaccination may be beneficial to prevent infection-related neonatal deaths of LBW infants (142,149), similar trials in India found no association of the BCG vaccine and LBW infant survival throughout the neonatal period (150). It has been suggested that differences in immunisation schedules, resulting in different vaccine combinations, or use of different BCG strains may have contributed to differences observed (150). Indeed, a study in Guinea Bissau found that immunising LBW infants at birth instead of delaying the BCG vaccine according to the recommended policies may improve infant survival over neonatal and early post-neonatal period (151). The reanalysis of the historical Swedish BCG immunisation study found a reduction in deaths associated with non-tubercular infections in BCG-vaccinated children up to 4 years of age, with this effect manifesting most strongly in the neonatal period (~62%

reduction) or in infants at 1 to 12 months of age (~44% reduction) (145), also suggesting benefits of administering BCG early. In this re-analysis, the strength of the effect was also associated with the period of follow-up, the beneficial effect of BCG on risk of death more pronounced among children followed-up in 1927-1929 than in 1930-1931, implicating possible biases in the design of the original study or contribution of other, unknown effects (145). There is now some evidence that sex may contribute to variation or lack of overall effect observed in some studies. For instance, some investigations suggested that LBW males may benefit from BCG vaccination more early on in the neonatal period than female LBW infants (152) or that season during which BCG is administered could affect the extent of its effects on all-cause mortality (153).

Although it is possible that the beneficial effect of BCG on all-cause mortality may manifest predominantly in neonates or very young infants at risk of severe infections, some studies suggest that reduction of all-cause mortality among BCG-vaccinated individuals can extend to adulthood and may not be limited to settings with high infectious disease burden. An observational study in the United Kingdom associated positive BCG vaccination status with lower all-cause mortality in individuals with TB, the association manifesting up to 10 years since immunisation with this vaccine (154). There are other studies that indicate that BCG-associated reduction in all-cause mortality can extend to adulthood. A cohort study in Denmark found lower risk of death among the BCG-immunised individuals for up to 20 years since vaccination; also, when stratified by age group, the effect was mainly noticeable in the group of individuals aged 20-30 years of age (155). Although this is an encouraging finding and implicates that BCG-associated effects on overall survival may be lasting and not restricted to settings with high infectious disease burden, the extent of this effect and its implications on public health are not yet clear.

### *1.3.2 BCG and protection against non-mycobacterial infectious diseases*

Apart from the possible benefits on overall infant survival, BCG vaccination has been associated with lower rates or risk of mortality due to non-mycobacterial infections in individuals that received this vaccine. Also, because the effects of BCG on all-cause infant mortality can be difficult to investigate in settings with low infant mortality and/or infectious disease burden, some studies examine the effects of BCG on hospitalisation or infection rates among the vaccinated and non-vaccinated infants. In such a set-up, hospitalisation usually indicates a severe case of infectious disease. However, although the findings from all-cause mortality studies suggests that early administration of the BCG vaccine might also reduce the likelihood of hospitalisations, that is not always the case in hospitalisation studies. For instance, a cohort study in Greenland found no difference in hospitalisation rates among the BCG-vaccinated and non-vaccinated infants up to 35 months of age (156). Although this study included data from time periods when different childhood immunisation schedules were applied, randomised BCG immunisation trials show similar patterns, with no difference in hospitalisation rates observed for BCG-vaccinated and non-vaccinated infants up to 15 months of age in Denmark, overall or for infection (157,158). In this setting, parent-reported infection rate was also similar for the infants who received BCG and those who did not (159). Finally, no difference in hospitalisation rates among infants that received early BCG vaccine and infants who received BCG as per recommended immunisation schedule were found in Guinea-Bissau either, although those hospitalised infants that received this vaccine early, had lower case-fatality rate due to neonatal sepsis (160). Again, the latter observation was made in high infectious disease burden setting.

Interestingly, some studies that found association between BCG immunisation and lower risk of death during neonatal or post-neonatal period, also refer to broadly specific aetiologies: respiratory infections, fever and meningitis (142,145,160). Importantly, similar observations were also made elsewhere. For instance, a study in Spain comparing hospitalisation rates in children up to 14 years of age in two regions with different BCG vaccination policies found

lower rates of hospitalisation due to non-mycobacterial respiratory infections and sepsis among the BCG-vaccinated children (161). Of interest, for sepsis, this protective effect differed by area in the selected regions of Spain, suggesting a possible involvement of other modulating factors, with age or time since vaccination being a possibility. While protective effect of BCG against respiratory infections in this study intensified with age, protective effects against sepsis manifested most strongly in children under 1 year of age, similar to observations from infant mortality studies (142,145,161). Lower incidence of acute lower respiratory tract infections in BCG-vaccinated children under 5 yrs of age was also found in a meta-analysis of data from 33 countries, estimated at 17-37% (162). In this analysis, the associations of BCG-dependent protection were found for pneumonia, early BCG vaccination (within 3 months after birth) associated most strongly with protective effects, with waning observed with infant age or time since vaccination (162). However, in a randomised controlled trial in Australia, no significant effects of BCG vaccination on incidence of lower respiratory tract infections were found in infants over the first year of life (163).

Although it has been proposed that associations with lower risk of respiratory infections, meningitis or sepsis may reflect undiagnosed mycobacterial infections (141), there is increasing evidence that BCG immunisation may be associated with lower incidence of non-tubercular infections. Such an observation has been recently made in Uganda, with infants who received this vaccine at birth having 29% lower risk of non-mycobacterial infections for up to 6 weeks of age than infants in the delayed immunisation group (164). An analysis of Demographic Health Survey data from 13 Sub-Saharan African countries associated positive BCG vaccination status with lower risk of malaria in children under 5 years of age (165). Another study in Guinea-Bissau observed a possible association between seasonality and BCG-associated reduction in all-cause neonate mortality, suggesting that the association may reflect a reduction in risk of death due to malaria, as the reduction in risk of death in BCG-vaccinated infants was stronger when BCG was administered in November-January, overlapping with season of high malaria transmission (153). In this study, however, the

reduction of risk of death due to malaria was not significant, despite the trend for lower risk of malaria-associated deaths among infants that received their BCG vaccine early compared to those that received the vaccine delayed (153). Previous investigations conducted in this population also found an association between BCG scar and lower risk of death due to malaria in BCG-vaccinated infants (166). Although implications of differences in the risk of mortality in children with or without BCG scar are not quite clear, it is possible that BCG may prime immune systems of the vaccinated individuals differently, contributing to non-specific protection in some individuals. The evidence on this is conflicting as although BCG-vaccinated human adults had higher frequencies of activated NK and γδ T-cells after controlled infection with *Plasmodium falciparum* and their monocytes expressed lower levels of co-stimulatory molecule CD86 after anti-malarial treatment, individuals that were BCG-vaccinated had more severe symptoms of malaria than unvaccinated participants of the trial (167). A cohort study in Denmark also found no effect of BCG vaccination on risk of mortality due to infectious diseases (155). While this can be interpreted as contradiction of the observations from infant BCG immunisation studies in Sub-Saharan Africa, it is possible that the heterologous effects of BCG may manifest differently in infants or young children and in adults because of their differentially primed immune systems (37,38). And some studies suggest that BCG may still have beneficial heterologous effects on infections in BCG-vaccinated adults. For instance, a study in Uganda associated BCG vaccination with lower incidence of helminth infections in HIV-1-positive individuals with BCG scar (168). Overall, this suggests that BCG may have a beneficial effect on acquisition or severity of infectious diseases; however, the extent of this effect may depend on setting, infectious disease burden and the type of infection.

### *1.3.3 BCG and its effects on non-communicable diseases*

Apart from its effects on all-cause mortality and non-mycobacterial infectious diseases, BCG immunisation may also influence a range of non-communicable diseases. Among the confirmed and the best explored are the anti-tumour effects of BCG, where this vaccine is

used as an approved, licenced treatment against non-muscle invasive bladder cancer (169), working through mechanisms similar to those reported in trained immunity studies (170,171). There is also evidence that BCG may improve the survival of patients with acute myelogenous leukaemia (172), and that it might reduce the risk of acute lymphocytic leukaemia in children (173), although other studies report no effect of BCG on non-bladder cancers (155,174). One study on BCG immunotherapy in bladder cancer patients also suggested that individuals who receive this therapy may have a lower risk of developing Alzheimer's disease (175), with a similar possible effect on risk of neurological disorders observed in a BCG immunisation cohort in Denmark (155). It is also possible BCG might have a beneficial effect on cardiovascular diseases (155), and some studies in mice suggest that BCG may modulate plasma cholesterol in animals given this vaccine, thus regulating the formation of atherosclerotic lesions (176).

This is perhaps not surprising as BCG immunisation was associated not only with changes in the innate immune responses in the vaccinated individuals but the cellular pathways regulating these responses have been shown to involve components that also regulate cellular metabolism (177). It has been suggested that because of its abilities to modulate the metabolic pathways, especially those of glucose metabolism, BCG may be exploited for treatment of such conditions as type 1 diabetes (178,179). At the moment, such studies remain largely explorative, although a retrospective cohort study in Canada investigated incidence of diabetes in BCG-vaccinated children in the first year of life. However, it found no association between BCG immunisation and the risk of diabetes (180,181).

Because the reported heterologous effects of BCG include a broad range of phenomena, from all-cause mortality to interactions between cell metabolism and innate immune responses, and because some infections in infancy can have lasting consequences to childhood development, some explorative studies examined its effects on infant growth. A retrospective cross-sectional study that examined the influence of this vaccine on stunting in Sub-Saharan Africa, a randomised trial in Guinea-Bissau that investigated the impact of BCG on LBW infant growth over the first year of life and the Danish BCG Study found no effects of this vaccine on infant

growth (182–184). The latter study also explored the effect of BCG on psychomotor development in BCG-vaccinated infants in Denmark but found no effects (185). No effects of BCG on the thymic size were found in BCG-vaccinated infants, either (186).

Because BCG is a potent Th1-like response inducer and it stimulates a wide range of inflammatory cytokines, one of the areas of interest in heterologous effects of BCG studies is its possible effects on allergies or atopies. Several randomised trials in Denmark and Australia investigated the effects of BCG immunisation on the development of atopic or allergic reactions in BCG-vaccinated infants. A possible beneficial effect of BCG vaccination on clinically diagnosed atopic dermatitis was found in infants with atopic predisposition in Denmark over 13 months of follow-up (187). Randomised trial in Australia, however, suggested a possible beneficial effect of BCG immunisation on eczema in infants, especially in those at high risk of eczema, over 12 months of follow-up (188). It should be noted that these are skin conditions and BCG is used as an intradermal vaccine. However, its beneficial effects on mortality and acquisition of infectious diseases in the vaccinated infants and adults suggest it may act more broadly. Despite this, the Danish BCG Study found no effect on recurrent wheezing over 13 months of follow-up among the BCG-vaccinated infants (189), and although a cohort study in Canada detected a possible impact of BCG on the incidence of asthma in the vaccinated individuals, the effect was weak and influenced by confounding factors (180). Together, these findings suggested that although BCG may subtly influence innate immune or metabolic responses in the vaccinated individuals, the scope of effects mediated by these responses may be limited to modulation of inflammatory reactions.

## 1.4 BCG and heterologous immune responses in vaccinated individuals

### *1.4.1 Trained immunity*

Of interest, the heterologous effects of BCG vaccination manifest most strongly in those children who receive their BCG vaccine within the first few months since birth, in the time frame when their adaptive immune responses undergo extensive development (37,38,136).

This time frame and broad scope of non-specific effects observed suggests the involvement of the innate immune system. Indeed, some studies detected the influence of BCG on innate immune responses to non-mycobacterial agents and suggested that they could contribute to both the heterologous effects of BCG and antimycobacterial immunity. This phenomenon was named trained immunity (or innate immune memory) and is defined as the property of innate immune cells, once primed by a stimulus, to respond to secondary (or heterologous) stimulation with the same or unrelated agents more strongly and rapidly. First identified in NK cells (190), innate immune memory is now thought to be a property shared with other innate immune cell types, including monocytes (191), innate lymphoid cells (192) or microglia (193–196). The range of agents that have been demonstrated to induce trained immunity *in vitro* or trained-like responses *in vivo* is quite broad (*Tables S1.1; S1.2)*. These agents include, although are not limited to, live microorganisms or vaccines, such as BCG, MTBVAC, *Candida albicans* or *P. falciparum* (170,191,197–202). Purified microbial components, for instance, fungal β-glucan, muramyl dipeptide (MDP) or plasmodial hemozoin have also been shown to enhance secondary monocyte responses to whole cell microorganisms or pattern recognition receptor (PRR) agonists (200,202,203). Cellular or nutritional metabolites – oxidised lipoprotein (oxLDL), lipoprotein A and fumaric or mevalonic acids – have also been shown to enhance secondary cytokine or surface receptor responses in monocytes or macrophages (204–208). Finally, a host component - IL-1β has also been shown to induce trained immunity *in vitro* (209).

Although many of the listed stimuli enhance innate responses to heterologous stimulation, some agents have been associated with the induction of an opposing effect, resulting in lower cytokine or other secondary responses. For instance, modified *Vaccinia* Ankara virus (MVA) or synthetic PRR agonists used to simulate viral infections, such as as polyinosinic:polycytidylic acid (poly I:C) or resiquimod (R848) have been shown to suppress monocytic IL-6 and TNFα responses to Toll-like receptor (TLR) agonists in a dose-dependent manner (201,203). A similar effect has been shown for the bacterial components flagellin or

LPS (203,210). Vitamin A has also been shown to reprogram innate cytokine responses to secondary stimulation with TLR agonists or microorganisms, downregulating both the inflammatory and regulatory cytokine production and also to inhibit the effects of training monocytes with BCG. Although such imprinting of innate immune cells, especially by LPS, is defined as tolerance in the context of trained immunity and can contribute to immunological underresponsiveness in such conditions as sepsis (210–212), it also suggests that trained immunity is a subtle phenomenon, especially as whole, live organisms or vaccines may contain multiple components that determine the outcome of training.

The work on the long-term effects of BCG on the innate immune cells mostly reports potentiating effects on monocytes or NK cells. These effects include expansion of CD14+ monocyte population upon adult human vaccination with BCG and changes in peripheral blood monocyte surface receptor expression, such as an increase in the expression of the monocyte activation marker, CD11b, or context-dependent upregulation or downregulation of TLR4, a receptor for LPS (191,213,214) (*Table S1.1)*. Similar changes happen in human peripheral blood monocyte cultures trained *in vitro* with β-glucan, a cell wall component from yeast, or oxLDL, some studies reporting elevated expression of genes encoding these molecules and a range of scavenger receptors (200,203,204). In addition, human peripheral blood monocyte *in vitro* training with BCG model suggested that TLR10, an anti-inflammatory TLR, is also downregulated after monocyte exposure to BCG (215). These findings, however, may not pertain to cells populations of monocytic or macrophage lineage, especially tissue-resident macrophages as a recent analysis found a downregulation of CD11b and HLA-DR surface expression in alveolar macrophages at 2 weeks and 3 months post immunisation (216).

These changes in surface receptor expression are accompanied by a characteristic pattern in cytokine profiles induced upon secondary stimulation of PBMCs, adherent monocytes or NK cells with TLR agonists or whole cell microorganisms (*Table S1.2*). Most studies on innate immune responses in BCG-vaccinated individuals or mice, or in *in vitro* trained monocyte cultures report enhanced production of inflammatory cytokines, especially TNFα, IL-1β and

IL-6 (170,177,191,198,205,209,217–220). Upon stimulation with secondary innate stimuli or unrelated microorganisms, these cytokines are also produced at higher levels in cell cultures trained with other agents – β-glucan, MTBVAC, *Vaccinia* or metabolites (170,198,200,201,203–208,221), so these inflammatory cytokines have been established as markers of trained immune responses. However, there maybe other downstream mediators of trained innate immune responses. More recent studies that employ broad range cytokine detection methods, such as multiplex bead arrays (MBAs), suggest that responses of multiple inflammatory cytokines or chemokines can be modulated by innate immune cell training with BCG or other inducers of trained immunity (222). Broad and, to some extent, microorganism-specific cytokine profiles have also been induced by secondary non-mycobacterial stimuli in human BCG immunisation studies (131,223), although it is also possible that adaptive immune cells, such as T-cells, may also contribute to production of these cytokines, either directly or through regulation of innate immune responses. Determining which parts of the cytokine responses in human BCG immunisation studies are due to the innate immune cell populations and to which of the innate populations can be complicated, especially where mixed cell populations, such as PBMCs or whole blood are used, although purified TLR or other PRR agonists are usually used to trigger secondary innate responses.

Changes in the expression of genes encoding surface receptors or soluble immune mediators have been associated with metabolic alterations occurring in parallel. Monocytes trained with BCG or other agents *in vitro* or *in vivo* shift from oxidative phosphorylation to glycolysis and glutaminolysis, accompanied by higher rates of lactate production (177,224). In addition, epigenetic changes happen in the promoter or the enhancer regions of genes encoding surface receptors, cytokines or metabolic pathway components upon *in vitro* or *in vivo* training, with the accumulation of transcription-permissive (H3K4me3 or H3K27ac) and reduction of transcription-inhibiting epigenetic marks (H3K9me3) (177,191,198,202,204,207–210,217,224–227). Apart from changes in histone methylation or acetylation patterns, regulation by long non-coding RNAs (lncRNAs) or microRNAs (miRNAs) can also contribute

to changes in chromatin structure and so, transcription of genes encoding immunological mediators in trained innate cells (228,229). Finally, BCG can also influence DNA methylation patterns in the vaccinated individuals (230). Although changes to these patterns were associated with the ability of monocyte-derived macrophages to inhibit mycobacterial growth *in vitro*, there were differences in both the DNA methylation patterns and the extent to which mycobacterial growth was inhibited (230), suggesting that individuals that receive this vaccine respond to it epigenetically differentially and that this may influence their innate immune responses to *M. tuberculosis* or other agents.

It should be noted that interplay between metabolic and epigenetic regulation of trained immunity is particularly complex. Although it would be expected that epigenetic modifications would happen first to enhance the transcription of genes regulating metabolic pathways or immune responses, metabolic components involved in pathways associated with innate immune training frequently interact with enzymes that on their own accord regulate histone methylation or acetylation patterns (207,208,225,231). For instance, modulation of glucose metabolism, e.g. by taking metformin, has been shown to affect cytokine responses to heterologous stimuli in BCG-vaccinated individuals (177), suggesting complex metabolic and epigenetic regulation loops of trained immune responses. It is also possible that BCG may also regulate trained immune responses through other mechanisms, for instance, post-translational modifications of proteins. BCG has been shown to modulate glycosylation patterns in THP-1 cells and human monocyte-derived macrophages (232); however, it is not known whether this effect is lasting and what consequences it may have on the innate immune responses, so further investigations would be needed to test this hypothesis.

It should be noted that epigenetic modifications, especially those of histones or those mediated by lncRNAs, are reversible, so one of the intriguing questions regarding trained immunity is how long for the effects of trained immunity remain. Because the interest in trained immunity is relatively new, few studies have explored the duration of trained immune responses. Since most of the *in vitro* work regarding trained immunity has been done on monocytes, a short-

lived cell type, most assays explore the effects of interest within 3-6 days post training (*Tables S1.1; S1.2)*, although one study reported detectable enhancement of secondary TNFα responses 2 weeks after monocyte training with β-glucan (200). This has been confirmed in human BCG immunisation studies, with changes in surface receptor expression or secondary cytokine production detectable for a period of 2 weeks to several months post immunisation, or, in some cases, 1 year post vaccination with BCG (191,197,213,216,223,233). It has been thought that, considering the flexibility of epigenetic mechanisms that regulate trained immunity induced by BCG or other agents, these effects are likely to be short-term. However, there is some evidence from historical BCG vaccination studies, suggesting that some effects of BCG on monocyte, macrophage or NK cell responses can manifest years after the original vaccination, influencing transcription of PRR encoding genes in monocyte-derived dendritic cells (DCs) or macrophages (234), TLR4 surface expression after macrophage stimulation with *Mycobacterium leprae* (214) or cytokine-producing NK cell frequencies and associated mycobacterial growth inhibition (235). While this suggests long-term impact of BCG on innate immune responses, because of difficulties differentiating trained immunity and heterologous adaptive immune responses or the contribution of stimulus-specific T-cell subsets on innate responses, in some cases it can be difficult to determine whether these effects are mediated by the same mechanisms, so further studies would be needed to determine the long-term influence of BCG on innate immune responses.

### *1.4.2 BCG and innate immune responses in infants*

Despite difficulties in studying and interpreting the impact of heterologous effects of the BCG vaccine, the concept of non-specific, secondary effects of this vaccine has become more widely accepted. It is therefore of interest to know which immunological mechanisms mediate these effects, especially in infants or neonates, who may benefit most from receiving this vaccine early, particularly, in areas with high infectious disease burden. It is possible that trained immunity may be one of these mechanisms. Although studied mostly in adult human

peripheral blood monocyte *in vitro* systems, trained immunity can be also induced in monocytes from infants (219,222) (*Table S1.3*). However, because obtaining and handling blood samples from infants or neonates for their immune response studies *in vitro* can be more difficult than from adults, especially because of small sample volumes available, such analyses have been limited. Most information on which immunological components may mediate the heterologous effects of the BCG vaccine comes from infant BCG immunisation studies and investigation of their cytokine profiles (*Table S1.3*). Because *in vitro* trained immunity investigation systems in the adults study relatively narrow cytokine profiles, because the immune system in neonates or infants is differentially primed, and because in infant studies innate cytokine profiles are frequently investigated in diverse time points, using whole blood systems and multiplex assays, cytokine profiles induced by secondary stimulation in infants are often broader and more diverse than those detected in the adult BCG vaccination studies (*Table S1.3*). Although some studies report an increase in previously reported trained immunity markers IL-1β, IL-6 and TNFα in samples from BCG-vaccinated children (68,236,237), others demonstrate context-dependent modulation of chemokines (CXCL9/MIG, CCL2/MCP-1), regulatory cytokines (IL-10, IL-1RA) or growth factors (EGF, PDGF-AB/BB, G-CSF, GM-CSF) (68,128,223,233,238–240). It should be noted that the cytokine combinations induced are often relatively specific to secondary stimuli or the timing of observation (*Table S1.3*). In some settings, the season of BCG administration has also been associated with the extent of innate cytokine responses to heterologous stimulation (153). Together, this shows large diversity in heterologous cytokine responses in BCG-vaccinated infants, probably reflecting the development of the innate immune system in infants and variation in downstream heterologous effects of BCG, such as all-cause mortality or acquisition of infectious diseases.

Regarding the influence of BCG on long-term surface marker expression on the innate immune cells in BCG-vaccinated infants, few studies investigate them, perhaps because of difficulties distinguishing the direct effects of BCG on the innate immune cells and those where

other cell types, e.g. T-cells, can act as intermediaries. A study in the UK showed that BCG vaccination in infants was associated with elevated surface CD11b, mannose receptor CD206 or NK cell activation marker CD69 expression upon cell stimulation with *M. tuberculosis* lysate (223). The expression of other markers did not differ for the vaccinated and control infants, with the exception of CD69 in cultures stimulated with Pam3Cys (223). This suggested that BCG may also modulate the expression of surface molecules on monocytes or NK cells in infants, so contributing to their responses to mycobacterial or unrelated infectious agents. It still remains to confirm, however, whether these changes in BCG-vaccinated infants contribute to their protection against TB or unrelated infections.

### *1.4.3 BCG and its impact on the innate immune cell populations*

Although the early studies on heterologous effects of BCG focused mostly on cells of the monocytic/macrophage lineage (*Tables S1.1, S1.2*), it has become increasingly clear that this vaccine influences other cell populations as well, and not only in terms of their cytokine production or PRR expression (*Table S1.4*). In addition to the findings that BCG immunisation results in an expansion of CD14+ and/or CD11b+ monocyte and macrophage populations at 1-2 weeks post immunisation in mice or human adults (191,241), other studies report broader changes occurring in haematopoietic compartment of BCG-vaccinated animals or humans (218,227). It has been shown that bone marrow (BM) of BCG-intravenously-vaccinated mice contains higher total numbers and frequencies of multipotent progenitor cells (MPPs), especially those of myeloid lineage (MPP3) (227). These changes were associated with development of epigenetically reprogrammed BM-derived macrophages (BMDMs) in BCG-vaccinated mice (227). In humans, BCG, although given intradermally, can also change gene expression patterns in the BM of the vaccinated individuals, inducing a neutrophil-associated gene signature for up to 3 months post immunisation (218). Indeed, in many murine or human BCG vaccination studies, a temporary elevation in circulating neutrophil levels has been detected, however, it usually peaks at 1-4 d post immunisation and does not extend to longer

periods (218,220,242). Although this transient increase in neutrophils may contribute to enhanced protection against infectious diseases short-term, it is not clear what the implications are for infections contracted later on in life or for TB, where necrosis of *M. tuberculosis*-infected neutrophils has been associated with limited control of its growth in macrophages (88).

Apart from monocytes or neutrophils, BCG vaccination has also been shown to influence the percentage of CD69+ or granzyme+ NK cell populations in BCG-vaccinated adults challenged with *P. falciparum* (167). Other studies suggest that BCG influences NK cell responsiveness to stimulation with pathogens or PRR agonists but does not necessarily alter NK cell numbers or frequencies on its own. A study in The Netherlands found no differences in percentages of $CD56_{dim}$CD16+ NK cell populations in BCG-vaccinated donors at 2 weeks and 3 months post-immunisation compared to pre-vaccination samples (197). No differences in NK cell populations were found in BCG-immunised compared to unvaccinated mice, either (243). BCG or *M. tuberculosis* may induce functional changes, however. Memory-like NK cells have been identified in TB patients and they have been shown to make IFNγ or IL-22 when stimulated with BCG (244,245), although their contribution to the control of TB is not fully clear. Cytokine producing NK cells from historically BCG-vaccinated human adults have been associated with improved control of mycobacterial growth *ex vivo*, in the presence or absence of antimycobacterial drugs rifampicin and isoniazid (235,246), although a murine BCG-immunisation model showed no difference in the contribution of NK cells to the ability of macrophages to inhibit mycobacterial growth in BCG-vaccinated and control mice despite IFNγ+ NK cell population expanded by PPD stimulation (243). Further investigations are therefore needed to assess the influence of the BCG vaccine on NK cell populations and their responses to *M. tuberculosis* or other pathogens.

## 1.5 Innate immune responses in BCG immunisation studies and protection against infectious diseases

### *1.5.1 BCG and innate immune protection against infectious diseases in mouse studies*

Although the number of murine or human BCG immunisation studies that report trained-like cytokine signatures has been growing, the number of studies that mechanistically link innate responses with heterologous protection against infectious diseases specifically is relatively small. Most of the evidence associating BCG-enhanced innate immune responses and protection against infections comes from murine studies (*Table S1.5*). In the 1970s, it was demonstrated that BCG-vaccinated mice treated with cyclophosphamide, an immunosuppressive drug, and challenged with *S. aureus* 4 days later, have lower mortality than the unvaccinated mice (247). A more recent study using a strain of mice with severe combined immunodeficiency (SCID) found an improved survival of BCG-vaccinated mice challenged with a lethal dose of *C. albicans* and lower loads of this microorganism in the kidneys compared to the control, unvaccinated mice (191). Because of the impaired lymphocytic cell populations in SCID mice, this strongly suggested that the heterologous protection of BCG-vaccinated animals was mediated by the innate immune system (191). In a murine polymicrobial sepsis model, the temporary increase in neutrophil counts has been associated with improved survival of BCG-vaccinated immunocompetent murine pups and lower microbial burden in their spleen, blood or lungs (242). Neutrophil or BMDM responses have also been associated with protection of BCG-vaccinated mice against *M. tuberculosis* challenge, reducing mycobacterial burden in the lungs or spleen (227,241). BMDMs from vaccinated mice were shown to be protective in models parabiosis or adoptive transfer of CD3+ T-cell-depleted BM or BMDMs from BCG-immunised to immunodeficient animals, suggesting that trained immunity or otherwise BCG-enhanced innate immune responses contribute to protection against TB, at least in murine models (227). It is possible, however, that these broadly protective effects of BCG may vary depending on the pathogen type to an extent as the BCG vaccine was found to have no influence over survival, weight or lung pathology of mice infected with influenza A/Anhui/1/2013 (H7N9) (248) despite contributing to protection in fungal or bacterial infection models in mice (*Table S1.5*).

### *1.5.2 BCG and innate immune protection against infectious diseases in human studies*

Although a number of studies report heterologous effects of BCG in epidemiological or public health settings, or changes in immune responses to heterologous stimuli, few studies explore both. Probably the most informative investigations so far have been a recent randomised controlled BCG immunisation trial by Prentice et al. (164), being the first study that associated the epigenetic regulation induced by BCG, risk of non-tuberculous infections and serious disease risk in infants; and a BCG and yellow fever vaccine (YFV) immunisation study by Arts et al. (209), linking BCG-induced epigenetic modifications, trained immunity responses and control of YFV replication in the blood of vaccinated individuals.

The trial by Prentice et al. showed that infants that received BCG immunisation at birth not only had lower risk of physician-diagnosed non-tuberculous infection for the first 6 weeks of life until the control group received the BCG vaccine, but this effect was also accompanied by differential histone methylation patterns at the promoters of genes encoding markers previously associated with trained immunity (significant effect for *TNFA*) (164). Of interest, in this trial, although the accumulation both the permissive (H3K4me3) and the inhibitory (H3K9me3) epigenetic marks increased at the promoter of *TNFA* from birth to 6 weeks of age, in BCG-vaccinated infants this increase was lower, with the same trend observed for *IL-1B* (164). It is not fully clear what the biological implications of this observation are as accumulation of both the permissive and inhibitory marks was affected but BCG vaccination was associated with lower risk of non-tubercular infections in LBW infants in this trial (164), suggesting that perhaps there is more flexibility for further epigenetic modifications at the promoters of genes encoding inflammatory cytokines and therefore responses to secondary infections in BCG-vaccinated infants. In addition, this effect was sex-differential, with lower accumulation of the permissive H3K4me3 mark in BCG-vaccinated males (significant for *TNFA,* trends for *IL-6* and *IL-1B*) and the accumulation of inhibitory H3K9me3 mark suppressed in BCG-vaccinated females (significant for *IL-1B*, trend for *IL-6*). BCG-vaccinated

males were at lower risk of serious illness for 6 weeks follow-up (164). Previous findings also suggested that BCG-vaccinated LBW male neonates may benefit from non-specific effects of BCG more than females, especially over the first couple of weeks of life (149), and some studies suggest that innate or heterologous cytokine responses differ in BCG-vaccinated male and female infants (238).

The BCG and YFV immunisation study by Arts et al. included a group of healthy adult volunteers that were vaccinated with BCG and a group of controls that did not receive this vaccine. 28 days later, both groups received YFV, and their blood was sampled at 3 and 5 days, 1 week and 3 months after YFV was administered (209). BCG immunisation was associated with increased accumulation of H3K27ac permissive epigenetic mark at the promoters or enhancers of genes encoding components of chemokine signalling pathways, Fc receptor signalling or transendothelial migration (209). Also, BCG not only enhanced the production of secondary stimuli-induced trained immunity markers (IL-1β, TNFα, IL-6) compared to baseline but also compared to individuals that did not receive BCG. While that would have been expected based on the evidence from previous work on trained immunity, the BCG-vaccinated group of volunteers had lower YFV replication at 5 days post YFV immunisation, and this reduction was associated with enhanced secondary IL-1β responses at 28 d post BCG immunisation (209). Together, this suggested that trained immunity may contribute to control of viral particle replication and, possibly, the outcome of viral infections.

This has been more extensively tested during the recent COVID-19 pandemic. In the initial phases of the pandemic, vaccines specific to SARS-CoV-2 were not yet available, so several trials aiming to test whether the heterologous immune responses induced by BCG could contribute to protection against COVID-19 were set up. One such trial was run in the Netherlands. In this placebo-controlled trial, no differences were found in rates of SARS-CoV-2 infections, associated deaths, admissions to intensive care units, hospital stay or self-reported respiratory tract infections over 12 months of follow-up despite trained-like cytokine responses to influenza H1N1 in the BCG-vaccinated individuals (249). In addition, when

stimulated with SARS-CoV-2 Wuhan Hu-1 strain, PBMCs from the BCG-vaccinated individuals only produced higher levels of IL-6, with a trend for reduced TNFα compared to the controls (249). In another, retrospective cohort study, three cohorts of healthy Dutch patients who had or had not received the BCG vaccine in the 5 years prior to the observation were followed up for 2 months in 2020. This study measured COVID-19 diagnosis or associated hospital admission cases in the vaccinated and unvaccinated cases and found no differences for either of these outcomes, although BCG-vaccinated individuals reported less cases of sickness and fatigue (250). This study also analysed markers associated with trained immunity in the BCG-vaccinated participants. Although it found a trend for lower rates of self-reported sickness in those BCG-vaccinated individuals who produced higher levels of IL-6 upon stimulation with *M. tuberculosis* and lower rates of self-reported symptoms among those who produced more IL-6 in response to *S. aureus* at 3 months post immunisation, the rates of self-reported symptoms were only significantly lower for BCG-vaccinated IL-6 responders to heterologous *S. aureus* when symptoms were grouped together (250). It is possible that the heterologous effects of BCG on infectious diseases may vary depending on infectious agent. In a controlled human malaria challenge, BCG vaccination was associated not only with increased frequencies of activated, cytotoxic NK cells and improved control of *P. falciparum* levels, but also with more severe malarial symptoms (167). Together, this suggested that although BCG can modulate human protection from infections through innate immune responses, this phenomenon warrants further investigation.

### 1.6 Aims and objectives of this study

Although there is evidence that trained immunity can contribute to both the protection against *M. tuberculosis* and the heterologous effects of BCG, there is a lack of well-defined innate immune markers associated both with trained immunity and protection against TB or other infectious diseases. Such markers could be of interest in the development of novel, more efficacious TB vaccines, considering that in TB vaccine trials, only Th1 or Th17-polarised

immune profiles are usually investigated despite correlates of protection being not established for TB.

The hypothesis driving this study was that a broad range of components, induced or modulated by innate immune training – *in vitro* or through BCG vaccination, including, but not limited to the previously reported markers of trained immunity (TNFα, IL-1β and IL-6), contribute to BCG-vaccinated infant or adult protection against TB. The aim of this study was to address the gaps mentioned above, test the behaviour of the markers already identified and screen for novel trained immunity markers with a protective role in mycobacterial or other infections, focusing on the role of innate immune responses in protection against TB.

The study had the following objectives: 1) using samples from healthy donors, to independently investigate and exploit the system of primary human monocyte *in vitro* training with BCG for screening of molecules and novel markers associated innate immune protection against TB or other infectious diseases, including pathogen recognition receptors reported to play a role in mycobacterial recognition and internalisation, antigen presenting molecules or innate cytokines including those previously not associated with trained immunity; 2) explore in PBMCs from 2 BCG-vaccinated infant cohorts these trained immunity associated surface marker and cytokine profiles, the expression of innate cytokines and innate immune response mediating genes associated with protection against TB, and the ability of infant PBMCs to inhibit mycobacterial growth; and 3) to test and compare whole blood cytokine profiles induced by BCG and novel TB candidate vaccines, focusing on the innate compartment, characterise these signatures, identifying their unique features and exploring the potential of these vaccines to induce broad-range innate or adaptive responses (beyond those of previously reported trained immunity markers or IFNγ, Th1, Th17 signatures) that could also be induced *in vivo* and be exploited for design of more efficacious TB vaccines.

# **Chapter 2:** Training of primary human monocytes with BCG *in vitro*

## 2.1 Introduction

Although for many years the general consensus was that immunological memory can only manifest in the adaptive immune compartment, more recently, an alternative type of immunological memory, known as innate immune memory, has been described. Also known as trained immunity, this phenomenon manifests as the ability of innate immune cells, such as monocytes, neutrophils or NK cells once exposed to immunological agents to respond more strongly to subsequent challenges even if the original stimulus is gone.

Multiple agents have been shown to induce trained immunity and include live microorganisms (170,177,191,200), fungal cell wall component β-glucan (200,203,205,224), pattern recognition receptor (PRR) agonists (203), lipoproteins (204–206) or metabolites (208,225). Of these, BCG and β-glucan have been studied the most extensively. Previous studies have shown that exposure to these agents causes the innate immune cells to undergo epigenetic changes at the promoters or the enhancers of genes encoding inflammatory cytokines, surface markers or enzymes regulating glucose metabolism, leading to a metabolic shift to aerobic glycolysis and enhanced expression of surface receptors or cytokines upon exposure to secondary stimuli (170,177,191,200,207,208,224,225). These changes have been both demonstrated *in vitro* or *in vivo* in mouse models or in BCG-vaccinated humans and may last from 2 weeks to 3 months, or, in some cases, up to 1 year post immunisation and may contribute to the protection of BCG-vaccinated humans or animals from infections (177,191,197,209,213,218,223,227).

Many of the *in vitro* innate immune training assays have been successfully exploited as tools to screen for novel markers associated with BCG-dependent trained innate immunity and often utilise adherent monocytes (170,177,191,205). While adherent monocyte cultures have the advantage of including the classical, non-classical and intermediate monocyte populations, human monocyte counts and frequencies vary across individuals. Classical (CD14+CD16-) monocytes make up the largest population and they have been previously shown to undergo changes in BCG-vaccinated individuals; the frequencies of CD14+CD45+ cells increased at 2

weeks post BCG vaccination and the surface expression of TLR4 and CD11b increased at 3 months post immunisation (191). These changes have also been associated with higher expression of IL-1β and TNFα encoding genes in response to stimulation with *M. tuberculosis, S. aureus* or *C. albicans* (191). Both of these cytokines are produced by human monocytes and are considered to play a protective role against TB, and monocytes themselves serve as blood-derived precursors for macrophages (84). In addition, a study of mice given BCG intravenously, associated improved protection of the vaccinated mice against *M. tuberculosis* challenge with enhanced myelopoiesis in the bone marrow (BM) and inflammatory responses of bone marrow-derived macrophages (BMDMs) (227), suggesting that trained monocytes can be used as a screening tool for markers associated both with trained immunity and protection against TB.

In this work, a previously reported innate immune training assay (205) was investigated and optimised using CD14+ monocytes, β-glucan and BCG, using the latter as the primary training agent.

## 2.2 Methods

### *2.2.1 Isolation of PBMCs and CD14+ monocytes*

Venous blood samples (50 ml) from anonymous, healthy, BCG-vaccinated adult volunteers were collected via the LSHTM Blood Donation system (LSHTM Ethics Reference #14576). A written consent was obtained in each case prior to obtaining the sample and any of the study subjects was free to withdraw from the study at any time. Each sample was mixed with equivalent volume of HBSS at 37$^{o}$C (Life Technologies, UK), split into three aliquots and carefully overlaid over 15 ml Histopaque®-1077 (Sigma-Aldrich, UK). The samples were then spun at 800 x g for 20 min with deceleration off. PBMCs were carefully collected with a pastette, transferred to a fresh 50 ml centrifuge tube and then washed three times at 400 x g for 10 min, with aliquots of PBMCs from the same donors pooled after the first wash. The cells were then resuspended in 20 ml HBSS, stained with Trypan Blue stain, 0.4% (ThermoFisher Scientific, USA) and counted with an automated cell counter (Countess II; ThermoFisher Scientific, USA).

CD14+ monocytes were isolated using human CD14 microbeads (Miltenyi Biotec, Germany) following the manufacturer's instructions. Briefly, PBMCs were centrifuged at 300 x g for 10 min, resuspended in 80 µl MACS buffer at 4$^{o}$C (PBS, 0.5% BSA (Sigma-Aldrich, USA), 2 mM sodium EDTA (Sigma-Aldrich)) and labelled with 20 µl beads per 1 x $10^{7}$ PBMCs. After incubation on ice for 15 min, the cells were washed with MACS buffer at 300 x g for 10 min, resuspended in 500 µl MACS buffer and filtered through the MS MACS columns (Miltenyi Biotec, Germany). The columns were washed three times with 500 µl and CD14+ monocytes eluted in 1 ml MACS buffer. They were then stained with Trypan Blue stain and counted.

### *2.2.2 Purity estimation*

Aliquots of cryo-preserved PBMCs from healthy UK donors were thawed rapidly at 37$^{o}$ C with constant, gentle swirling and then transferred to centrifuge tubes containing pre-heated RPMI

supplemented with 10% FCS. The cells were then washed by centrifuging at 500 x g for 5 min. After resuspending cells in 5 ml RPMI with 10% heat-inactivated pooled human AB serum (HI ABS; Sigma-Aldrich, USA), benzonase (final concentration 10 U/ml) was added to each tube. PBMCs were then incubated at 37$^{o}$C for 2 hours, centrifuged for 5 min at 500 x g and carefully resuspended in 1 ml RPMI with 10% HI ABS. PBMCs were counted and then CD14+ monocytes were isolated by MACS as described above.

Monocytes (2.0 x $10^5$ cells/tube) were then added to polypropylene FACS tubes containing RPMI with 10% HI ABS. After this, the cells were washed with PBS followed by centrifugation at 500 x g for 5 min, stained with pre-titrated LIVE/DEAD Fixable Aqua Dead Cell Stain viability dye (Life Technologies, USA) and incubated at room temperature (RT) for 15 min. This was followed by blocking of surface receptors with 10 µl polyclonal Anti-human Fc Receptor Binding Inhibitor (eBioscience, USA) and a further 15 min incubation at 4$^{o}$C, followed by surface staining with 5 µl αCD14-V450 (MΦP9; BD, USA) or 5 µl αHLA-DR-PerCP-Cy5.5 (L243; BioLegend), or 5 µl FACS buffer in 100 µl final staining volume at for 4$^{o}$C for 30 min. This was followed by a wash with FACS buffer (PBS, 0.1% BSA (Sigma-Aldrich, USA), 0.01% sodium azide (Sigma-Aldrich)). Monocytes were then permeabilized with Cytofix/Cytoperm buffer (BD, USA) at 4$^{o}$C for 20 min, washed with Perm/Wash buffer (BD, USA) at 750 x g for 5 min and 100 µl FACS buffer was added to each tube. The cells were incubated at RT for 30 min, washed and fixed overnight with 1% paraformaldehyde (Sigma-Aldrich, Germany). The following day the cells were filtered and transferred to polystyrene FACS tubes for surface receptor expression and intracellular cytokine measurement on LSR II (BD, USA).

Data (100,000 total events) was acquired using FACSDiva (BD Biosciences, USA) and analysed post-acquisition using FlowJo software (version 10; BD Biosciences, USA). Antibody-stained beads (BD Biosciences, USA) were used for compensation and unstained controls were used to set the gates.

### *2.2.3 Optimisation of BCG MOI for monocyte training in vitro*

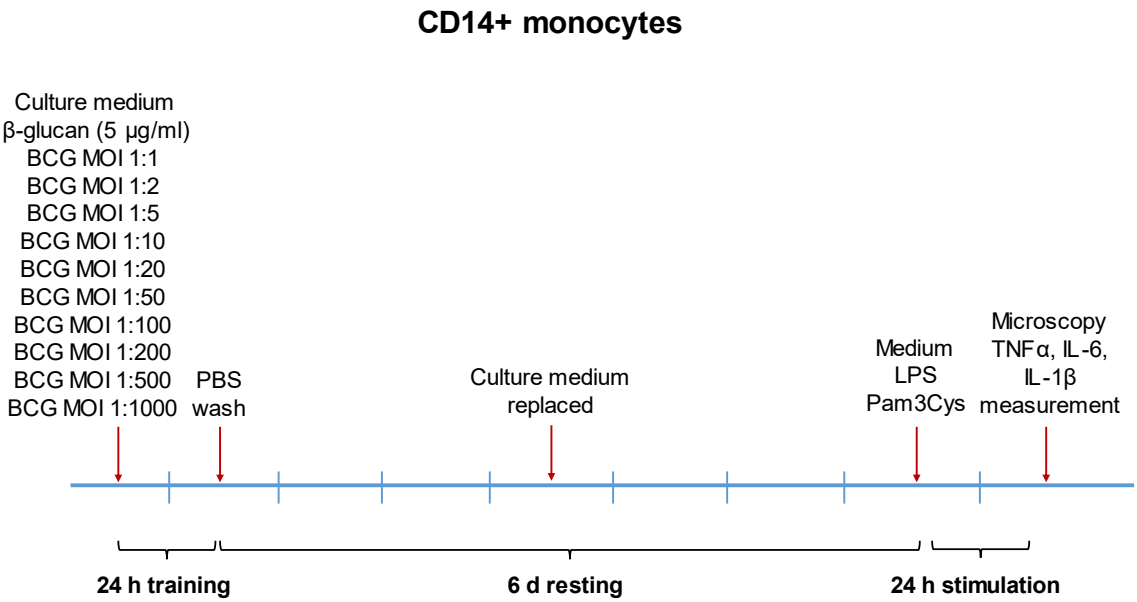



**Figure 2.1.** *Model of monocyte in vitro training with BCG*. Adapted from *Bekkering et al., CVI, 2016.* In this model, monocytes were trained for 24 h with β-glucan or BCG or left untrained. They were then washed and rested for 6 days with cell culture medium replacement on day 3. The cells were then re-stimulated for 24 h with LPS, Pam3Cys or left unstimulated. Cytokine measurements or cell culture images were taken at time points indicated as described in the appropriate Methods sections.

The CD14+ monocyte training procedure (*Figure 2.1)* was adapted from a previously published protocol (205). For optimisation of multiplicity of infection (MOI; expressed as ratio of BCG CFUs per number of monocytes in the well), CD14+ monocytes were diluted to 1 x $10^6$ cells/ml with RPMI 1640 medium (Life Technologies, UK) supplemented with 10% heat-inactivated pooled human AB serum (Sigma-Aldrich, USA) and 1% L-glutamine (Sigma-Aldrich, UK) and plated in duplicates at 1 x $10^5$ or 3 x $10^5$ cells/well on 96- or 48-well plates respectively. After incubation at 37°C, 5% $CO_2$ for 1 hour, monocyte culture medium was replaced with fresh medium alone, 5 μg/ml β-glucan or medium containing BCG-Pasteur (Aeras, Rockville, MD, USA) for monocyte training at the following MOIs: (1:1, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, 1:1000; *Table 2.1*). Following further incubation at 37°C, 5%

$CO_2$ for 24 h, the cells were washed with PBS at 37°C to remove stimuli. They were then rested for 6 days with medium replaced on day 3 of resting. On day 6, monocytes were stimulated with medium control, 10 ng/ml lipopolysaccharide from *Escherichia coli* O55:B5 (LPS; Sigma-Aldrich; UK) or 10 µg/ml N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (Pam3Cys; Invivogen, USA) for 24 h. Supernatants from trained or control monocyte cultures were then collected and stored at -80°C until further analysis.

**Table 2.1.** *Numbers of BCG CFUs per well used to train monocytes at the indicated MOIs*

| **MOI** | **BCG CFU / well** (96-well plate) | **BCG CFU / well** (48-well plate) |
|---|---|---|
| **1:1** | 100,000 | 300,000 |
| **1:2** | 50,000 | 150,000 |
| **1:5** | 20,000 | 60,000 |
| **1:10** | 10,000 | 30,000 |
| **1:20** | 5,000 | 15,000 |
| **1:50** | 2,000 | 6,000 |
| **1:100** | 1,000 | 3,000 |
| **1:200** | 500 | 1500 |
| **1:500** | 200 | 600 |
| **1:1000** | 100 | 300 |

### *2.2.4 Imaging of BCG-trained monocytes*

To visualise the morphological changes occurring in CD14+ monocytes upon training with BCG, bright-field images of monocytes trained with RPMI or BCG at MOI 1:1, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100 or 1:1000 were taken at the indicated time points at magnification = x20

using Nikon Ti-E inverted microscope (Nikon, Japan). NIS elements 4.6 software (Nikon, Japan) was used to extract the images.

### *2.2.5 Cytokine production by BCG-trained monocyte cultures*

Cytokine production by trained monocyte cultures in response to secondary stimulation with LPS or Pam3Cys was tested with Human IL-6 ELISA MAX Standard, Human TNF ELISA MAX Standard and Human IL-1β ELISA MAX Deluxe kits (BioLegend, USA) as per manufacturer's instructions. Briefly, 96-well plates were coated overnight at 4$^{o}$C with anti-IL-6, anti-TNF or anti-IL-1β capture antibodies. The plates were then placed on a plate shaker and blocked with PBS containing 1% BSA for 1 h. Thawed supernatants were added in duplicate to each plate and incubated at room temperature for 2 h, followed by the addition of biotinylated detection antibodies. They were then treated with avidin-labelled horseradish peroxidase (Av-HRP), followed by TMB (3,3´,5,5´-tetramethylbenzidine) substrate solution (ThermoFisher, USA; for ELISA MAX Standard Kit tests) or Substrate F solution (for ELISA MAX Deluxe tests; BioLegend, USA) for 15 min. Colour development reactions were stopped with 0.18M or 2N sulphuric acid respectively. The plates were washed 4 times with PBS-0.05% Tween-20 (Sigma-Aldrich; USA) between the steps to remove excess antibodies or sample analytes, and 5 times with 45 s incubation on the bench top between each washing step to remove surplus Av-HRP. The optical density (absorbance) readings at 450 nm were taken with the Spectramax M3 plate reader (Molecular Devices, UK), the duplicate sample values averaged and plotted against the standard concentrations. They were then log-normalised and 4 parameter logistic (4PL) regression was applied to interpolate the cytokine concentrations using GraphPad Prism 9 software (GraphPad Software, USA).

### *2.2.6 Statistical analysis*

To estimate the differences in secondary cytokine responses between β-glucan- or BCG-trained and control cells, Wilcoxon signed-rank matched-pairs test was applied. In BCG dose-optimisation experiments or comparisons of control cell cultures and monocytes trained with different batches of BCG, Friedman test with Dunn's post hoc multiple corrections test was applied, except for those cases where uneven number of data points for different stimulation conditions was available, in which case Wilcoxon signed-rank matched-pairs test was applied to compare each test condition with the control dataset. All datasets were analysed using GraphPad Prism 9 software (GraphPad Software, USA).

## 2.3 Results

### *2.3.1 Monocyte purity*

To standardise numbers of cells used in monocyte *in vitro* training assay, monocytes isolated from PBMCs by MACS were quantified. On average, from 50 ml of peripheral blood, 6.7 x $10^7$ PBMCs (± 2.15 x $10^7$; n = 16) and 9.23 x $10^6$ (± 3.21 x $10^6$; n = 16) monocytes were isolated. Previous work in the Dockrell lab indicated ~90% purity of monocytes isolated by human CD14 microbeads (251). In this work, the purity of CD14+ monocytes was also examined by FACS, checking the expression of CD14 and HLA-DR in CD14+ monocytes and CD14- PBMCs. 95.4% and 99.2% of CD14+ monocytes isolated with magnetic beads were found to express CD14+ or HLA-DR, suggesting a high purity of monocytes isolated by magnetic bead selection (*Figure 2.2*).

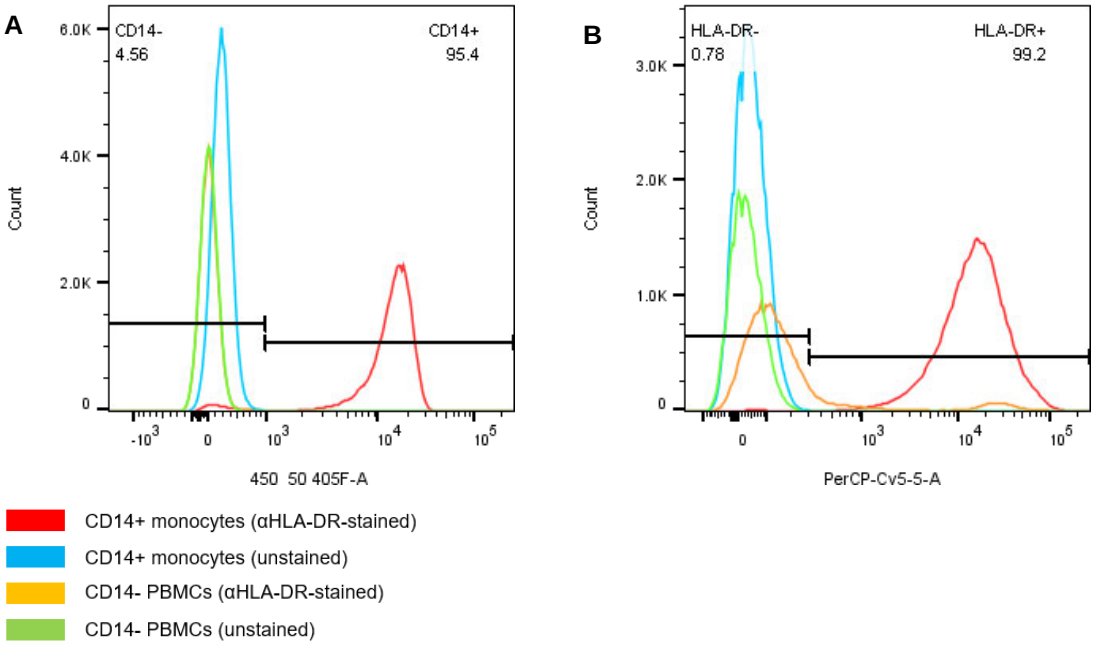



**Figure 2.2.** *CD14 and HLA-DR staining on human CD14+ monocytes isolated by magnetic bead separation*. The histograms show (**A**) frequencies of PBMCs that did (CD14+) or did not (CD14-) stain with anti-CD14-V450 or (**B**) frequencies of PBMCs that did (HLA-DR+) or did not (HLA-DR-) stain with anti-HLA-DR-PerCP-Cy5.5. The histograms also show their staining controls (unstained) and CD14- PBMCs as controls for CD14 or HLA-DR expression. The plots show representative data from two donors in two independent experiments.

### *2.3.2 Assay optimisation to detect differences in TNFα or IL-6 in trained monocyte cultures*

Because of a narrow detectable concentration range in ELISA assays used in this work and a large variation in cytokine concentrations in the supernatants of cell cultures challenged with TLR agonists, a series of experiments to determine the optimal dilution factors allowing the detection of IL-6 or TNFα was conducted. In these experiments, series of two-fold serial dilutions of the supernatants from multiple donors were prepared and the original concentrations of TNFα (*Figure 2.3A*) and IL-6 (*Figure 2.3B)* worked out in the experiments were plotted against the dilution factors. The dilution factors in the linear ranges of the resulting curves that overlapped for most donors were selected to use in the subsequent measurements of TNFα (DF = 2 for untrained LPS-stimulated samples; DF = 20 for BCG-trained and LPS-stimulated samples) and of IL-6 (DF = 50 for untrained and BCG-trained LPS-stimulated samples, except for those samples where IL-6 concentration was above the detection level, in which case DF = 100 was used).

To investigate the inter-experimental variation, an overlay of untransformed (*Figure 2.4A)* and log-transformed (*Figure 2.4B*) standard curves from 6 independent experiments measuring IL-6 in trained monocyte supernatants was generated. The overall alignment of the curves was close but there was also some inter variation between the curves, suggesting this may have contributed to some extent to experimental variability in measurements.

### *2.3.3 Cytokine responses to TLR stimulation by β-glucan trained CD14+ monocyte cultures*

To test the effect of training on monocytes, CD14+ monocytes from human volunteers were trained with BCG and with β-glucan, a fungal polysaccharide, both having been shown in the past to induce trained immunity in human monocytes in previous studies (170,191,200). To train the cells with BCG or β-glucan, the method described by *Bekkering et al.* (205) was applied and the IL-6 or TNFα concentrations after 24 h restimulation with RPMI or TLR agonists were measured (*Figure 2.1*).

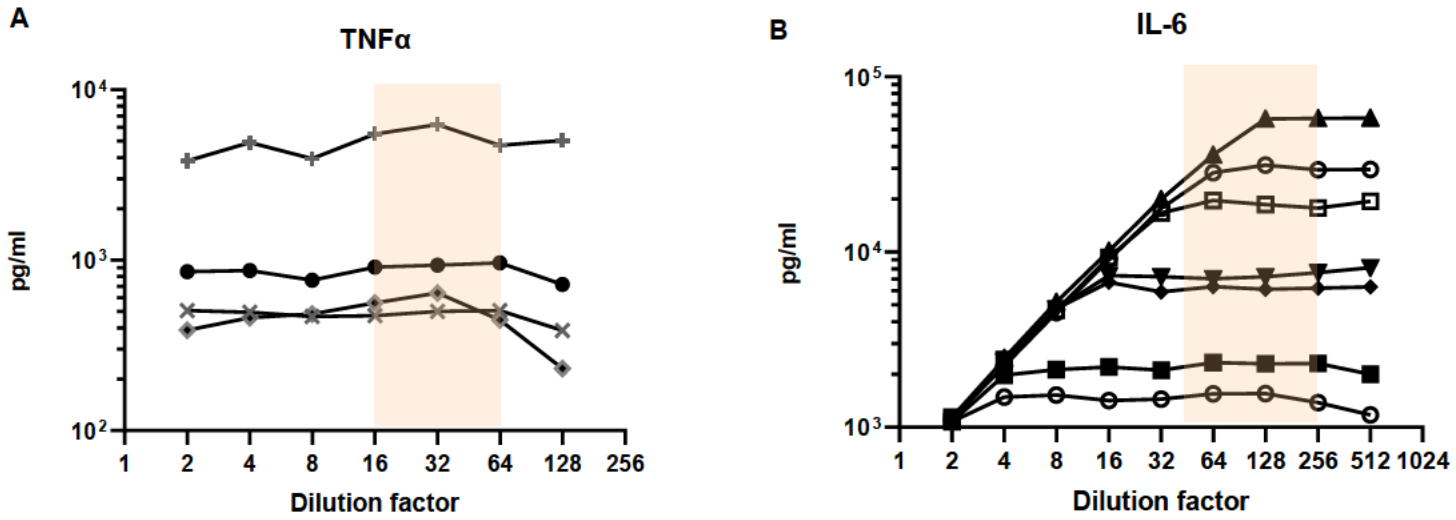



**Figure 2.3.** *Establishing the optimal dilution factors for the optimal TNFα and IL-6 detection in the supernatants from the BCG-trained monocyte cultures*. Supernatants from the CD14+ human monocyte cultures trained with BCG at MOI 1:50 and re-stimulated with LPS (10 ng/ml) as a secondary challenge for 24 h went through a series of two-fold serial dilutions.The concentrations of TNFα **(A)** and IL-6 **(B)** were then measured for each dilution factor, the original cytokine concentrations back-calculated and plotted against the dilution factors. The plots depict the cytokine detection ranges in individual donors, with dilution factor ranges in the linear part of the curves in samples from different donors highlighted in orange. TNFα: n = 4; IL-6: n = 7. BCG MOIs indicated ratios of BCG CFUs to the numbers of cells per well.

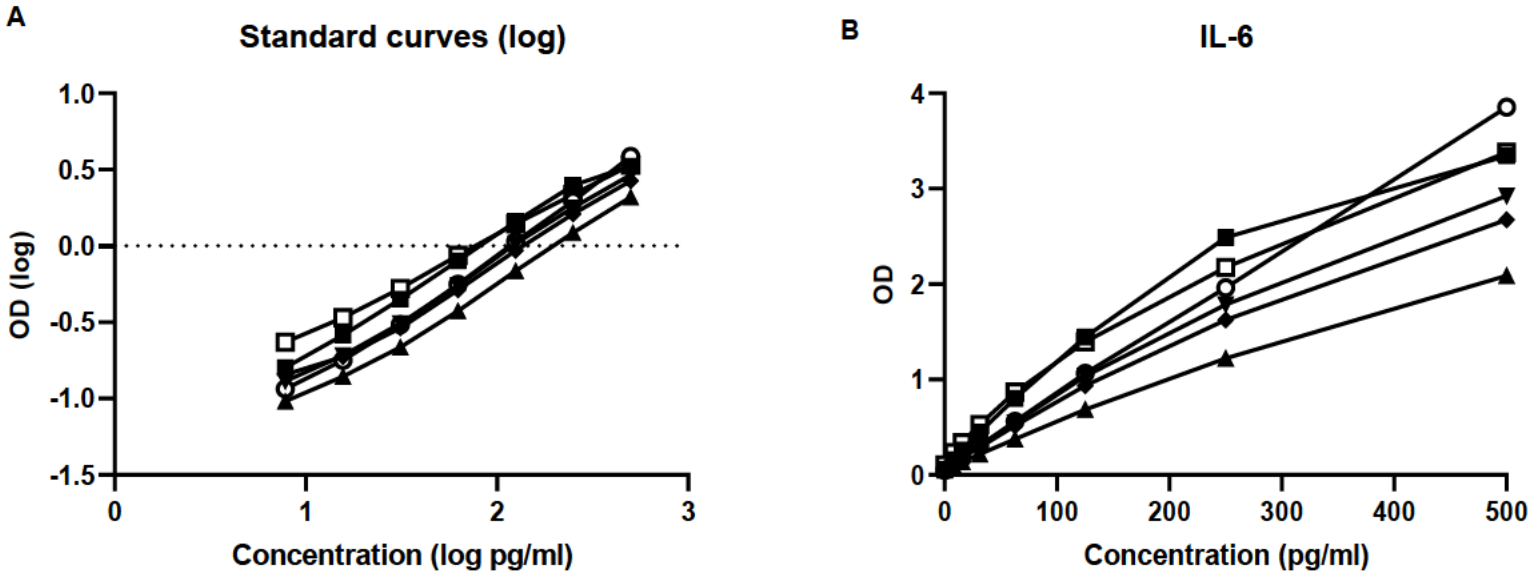



**Figure 2.4.** *The alignment of the standard curves used to estimate the levels of IL-6 in the supernatants from the BCG-trained monocyte cultures*. In this figure, log-transformed (**A**) and untransformed (**B**) standard curves from 6 experiments measuring IL-6 in supernatants from re-stimulated control or trained monocyte cell cultures were overlayed to check for day-to-day variation between the experiments.

Although a strong IL-6 and TNFα response to monocyte stimulation with TLR agonists was detected, no statistically significant differences in concentrations of these cytokines were detected in the supernatants of control or β-glucan trained human CD14+ monocytes cultured in 96-well plates (*Figure 2.5A, B*). To test if increasing the surface area for cell culture improved the responsiveness of human monocytes to training with β-glucan, CD14+ monocytes were also trained in the 48-well plates; however, no statistically significant differences between the trained and control monocyte IL-6 responses to TLR agonists were detected in these cultures (*Figure 2.5 C*).

### *2.3.4 Optimising the dose of BCG used to train human CD14+ monocytes in 96-well culture plates*

To optimise the dose of BCG used to train the cells CD14+ human monocytes were trained in 96-well plates with BCG at varying MOIs, rested for 6 days and challenged with cell culture medium or LPS for 24 h. Poor overall viability of BCG-trained cells was observed by inverted light microscopy in cell cultures treated with BCG; however, a dose-dependent effect of BCG on the survival of monocytes was observed with higher monocyte survival in cultures trained with low doses of BCG (*Figure S2.1*) and this pattern was reflected in IL-6 and TNFα concentrations in supernatants from RPMI- (*Figure 2.6A, C*) or LPS-restimulated cell cultures (*Figure 2.6B, D*). The variation in cytokine production by BCG-trained cells was high and the differences between cytokine levels in control and BCG-trained cell cultures were only significant for IL-6 supernatants from LPS-restimulated monocyte cultures trained with BCG (*Figure 2.6B*). There were no statistically significant differences for TNFα in supernatants from RPMI- or LPS-restimulated cultures trained with BCG compared to the supernatants from the control cultures (*Figure 2.6C, D*). Of interest, the reduction of IL-6 levels in supernatants from BCG-trained cell cultures suggested that BCG did not train the cells, even at the lowest doses.

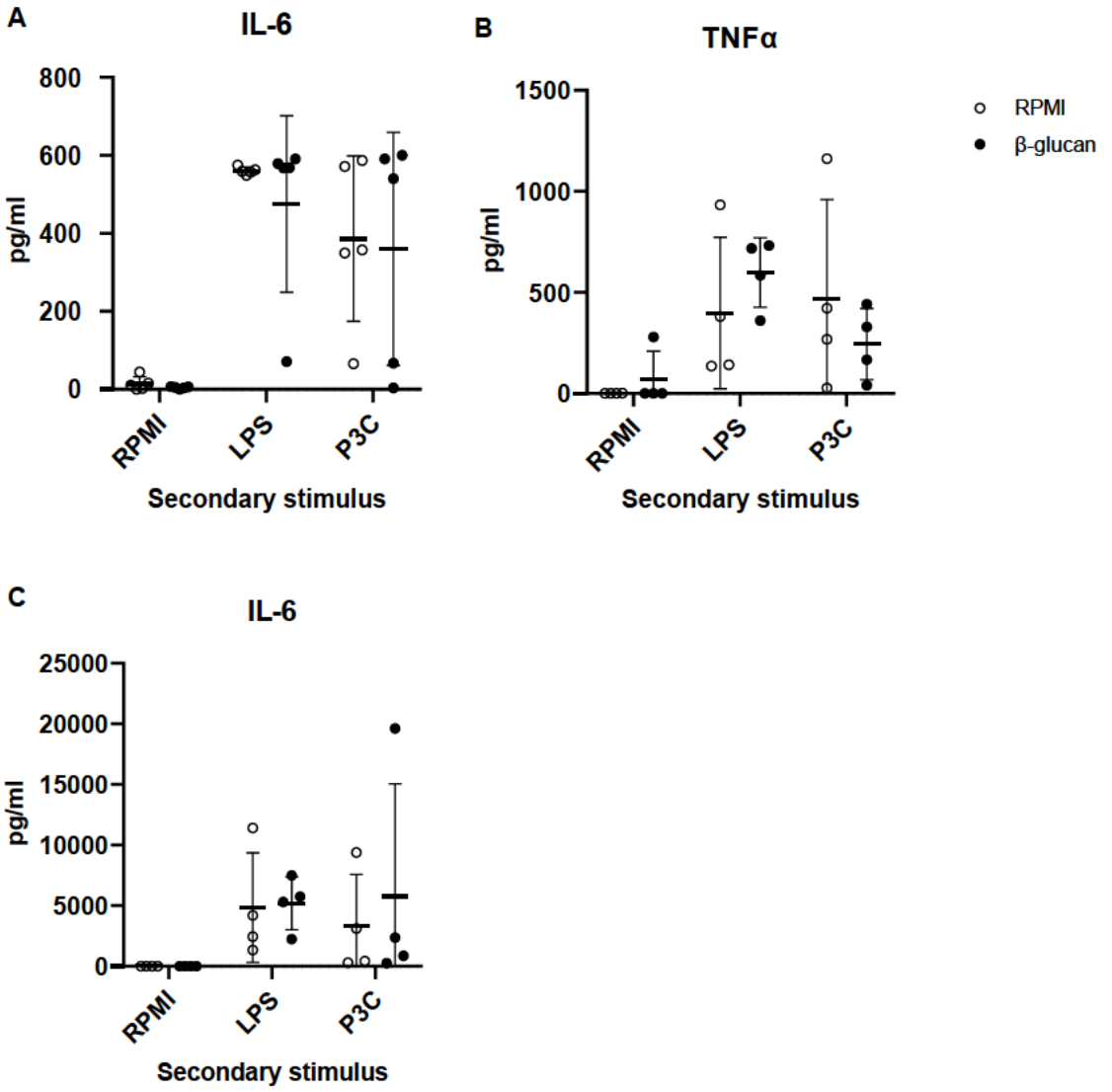



**Figure 2.5.** *The effect β-glucan on CD14+ monocytes*. 100,000 (**A** and **B**) or 300,000 human CD14+ monocytes per well were cultured in the presence or absence of β-glucan for 24 h on 96-well (**A** and **B**) or 48-well plates (**C**) and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then re-stimulated with LPS or Pam3Cys or left unstimulated for 24 h. After 24 h, the concentrations of IL-6 (**A** and **C**) and TNFα (**B**) were measured in the cell culture supernatants. The scatter plots show mean concentrations and their standard deviations. Wilcoxon signed rank matched-pairs test was used to determine the differences. N = 4-5.

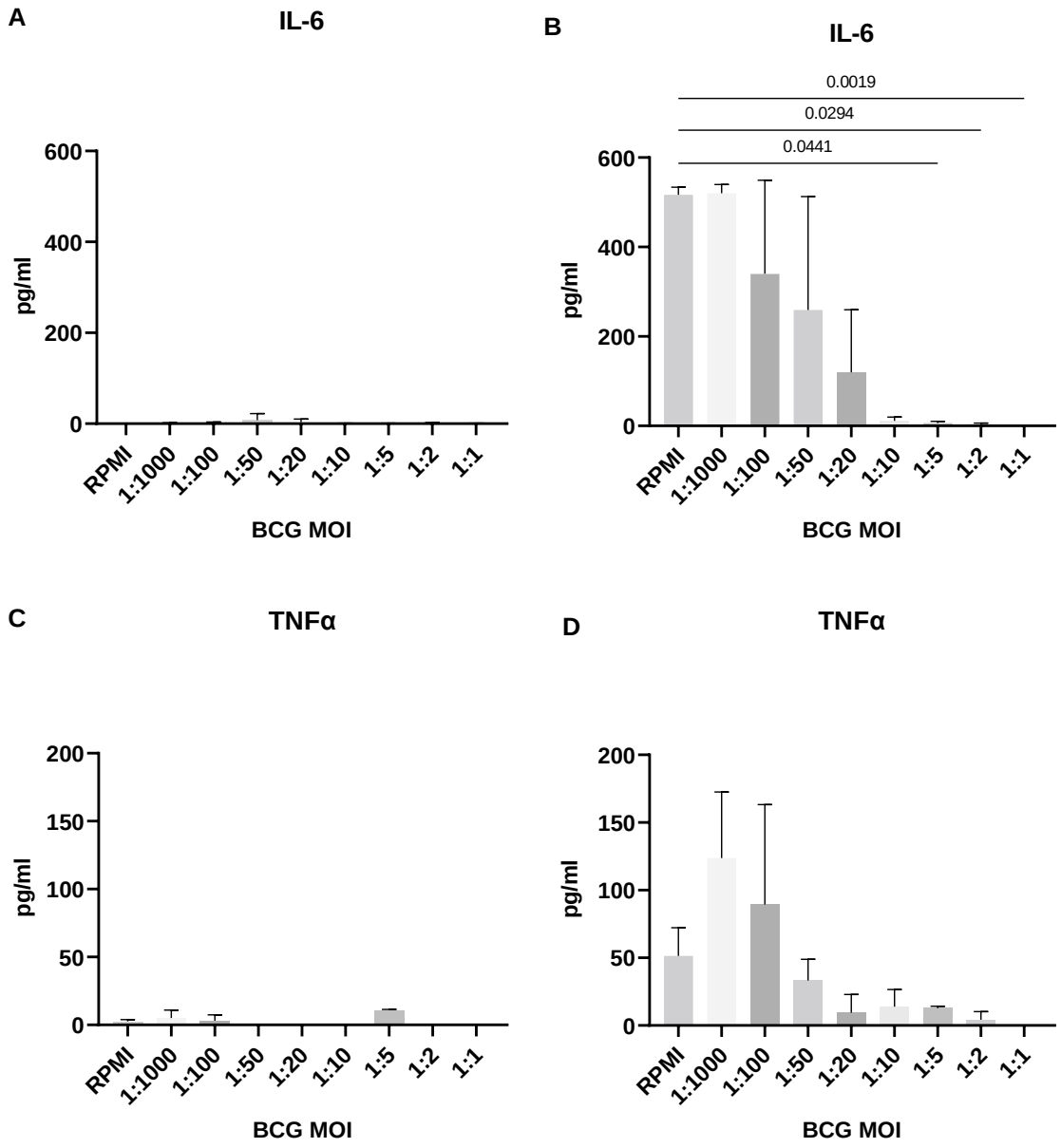
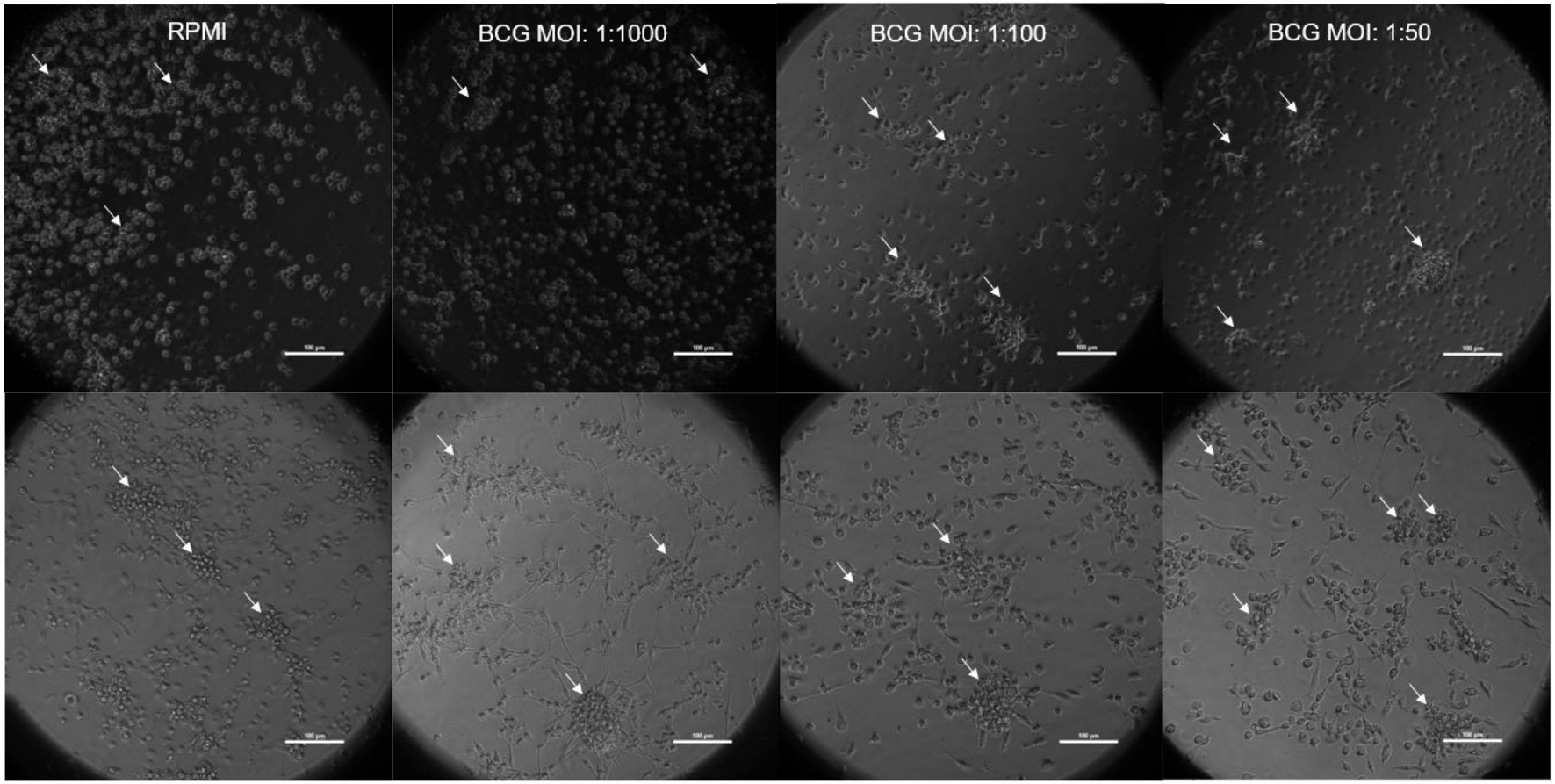



**Figure 2.6.** *BCG dose-dependent cytokine production by CD14+ monocytes*. 100,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at the indicated MOIs for 24 h on 96-well plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then re-stimulated with LPS or left unstimulated for 24 h. After 24 h, the concentrations of IL-6 (**A** and **B**) and TNFα (**C** and **D**) were measured in the cell culture supernatants from unstimulated cells (**A** and **C**) or LPS-treated cells (**B** and **D**). The plots show mean cytokine concentrations and their standard deviations. Friedman test with Dunn‘s post hoc multiple comparison test was used to detect the differences. The numbers on the plot indicate the p values for significant differences between BCG-trained and control monocyte cultures. IL-6: n = 5; TNFα: n = 2. BCG MOIs indicated ratios of BCG CFUs to the numbers of cells per well.

### *2.3.5 Optimising the dose of BCG used to train human CD14+ monocytes in 48-well culture plates*

The absence of the expected training effect despite the modulation of the dose of BCG during the training step of the protocol suggested that the cell numbers or culture conditions in the 96-well plates may have been suboptimal. It was therefore decided to increase the cell numbers and surface area for cell culture, so a follow-up experiment on BCG-dependent training was conducted using 300,000 monocytes per well on 48-well plates. To track the morphological changes happening in BCG-trained monocyte cultures, a series of inverted light microscopy images of monocytes trained with a range of BCG doses were taken throughout the course of the assay: at 24 h post training prior to and after the PBS wash; on day 3 of resting and on day 6 of resting (*Figure 2.7*). Improved cell survival was observed. In addition, BCG-trained cells demonstrated an increased tendency to cluster, especially at higher MOIs (1:100 or 1:50) compared to the control cells, the effect apparent as early as 24 h post training and continuing to the secondary stimulation phase (*Figure 2.7*). It is possible that these clusters reflect activation of monocytes by BCG taken up during the training phase and that they might contribute to enhanced cytokine production upon secondary stimulation reported in previous studies (170,191). Some spontaneous monocyte clustering was observed in the control cultures as well; at the end of monocyte training, monocytes in the control cultures tended to be smaller and more round compared to the larger, spindle-shaped monocytes, reminiscent of M1-type macrophages, observed in BCG-trained cultures during the resting phase (*Figure 2.7*). There was a possible dose-dependent effect of BCG on clustering as samples from cell cultures trained with BCG at MOI 1:50 had more cell clusters compared to the cultures trained with lower doses of BCG or control cell cultures.

Control and trained monocyte cultures at 24 h post training




Control and trained monocyte cultures at day 3 of resting

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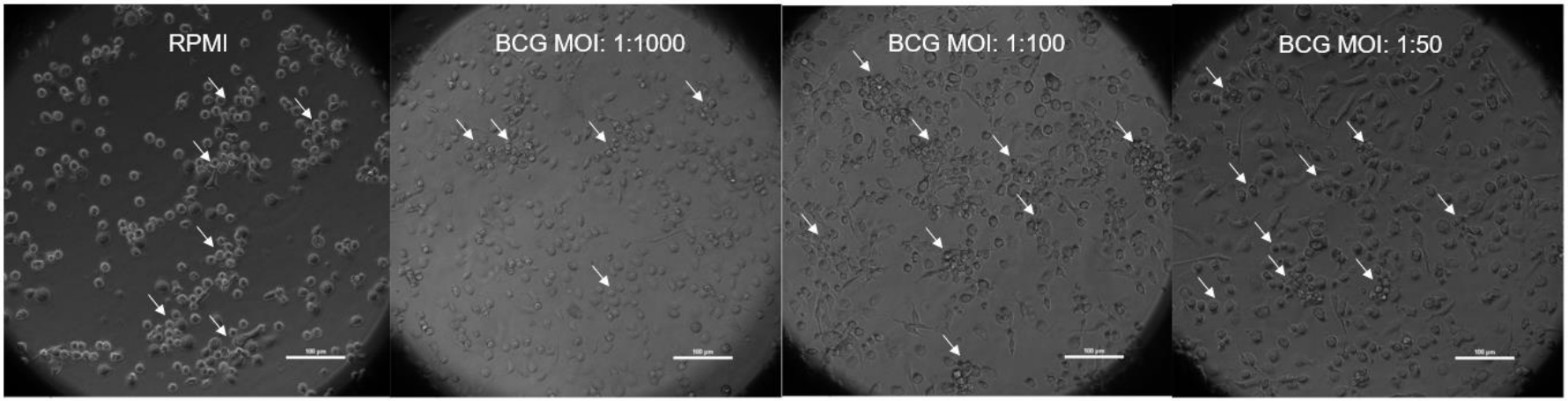



Control and trained monocyte cultures at day 6 of resting,
prior to the addition of LPS

**Figure 2.7.** *Increasing surface area for the cultures of BCG-trained CD14+ monocytes improves their survival*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at the indicated MOIs for 24 h on 48-well plates and rested for 6 d with cell culture medium replaced on day 3 of training. Inverted light microscopy images were taken at 20x magnification at 24 h post-training, on day 3 and 6 of resting. RPMI – training controls. Ratios indicate BCG MOIs used to train the monocytes, and white arrows – BCG-induced monocyte clusters in trained monocyte cultures or spontaneously occurring clusters in control cultures. The images depict morphology of trained monocytes in a culture from a single donor in a representative experiment.

To explore whether monocyte cultures in 48-well plates were trained by BCG and if so, whether the effect was dose-dependent, TNFα concentrations in the supernatants from control and monocyte cultures trained with BCG at MOIs 1:10 – 1:1000 and re-stimulated with cell culture medium or TLR agonists were measured. A BCG-dose-dependent effect on TNFα production was detected irrespective of whether the cells were re-stimulated with cell culture medium or TLR agonists (*Figure 2.8*); however, large interindividual variation in TNFα

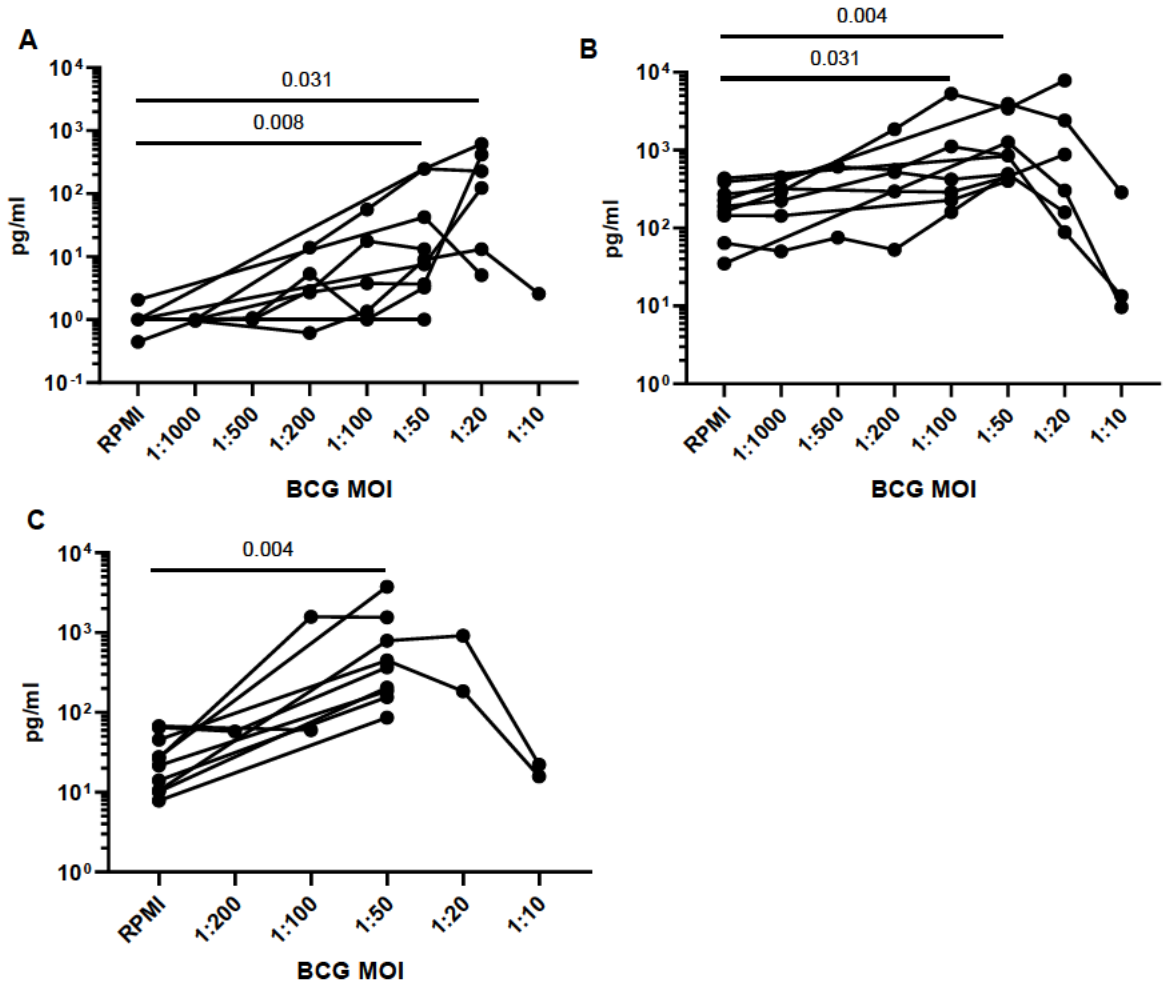



**Figure 2.8.** *The training effect of BCG on TNFα is dose-dependent*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at the indicated MOIs for 24 h on 48-well tissue-culture plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then **A** - left unstimulated or re-stimulated with **B** - LPS or **C** - Pam3Cys for 24 h. After 24 h, the concentrations of TNFα were measured in the supernatants. Data points represent TNFα concentrations in individual donors. Numbers indicate p-values for significant differences. Wilcoxon matched-pairs signed-rank test was used to detect the differences between RPMI- and BCG-trained cells. BCG MOIs indicate ratios of BCG CFUs to the numbers of cells per well. N = 9.

production was observed and only minor overall impact of BCG on secondary responses to TLR agonists was observed for most doses of BCG. There was a statistically significant increase in TNFα production by LPS-challenged monocytes that had been trained with BCG at MOI 1:100 and 1:50 compared to the control cultures, but not for LPS-treated cell cultures trained with higher or lower doses of BCG (*Figure 2.7B*). In addition, BCG-associated increases in TNFα concentrations were found in supernatants from RPMI-only monocyte cultures trained with BCG at MOI 1:50 and 1:20 (*Figure 2.7A*) or Pam3Cys-challenged monocytes trained with BCG at MOI 1:50 (*Figure 2.7C*), suggesting a possible effect of training.

### *2.3.6 Variable effects of BCG-dependent training on monocyte cytokine responses*

Because the experiments investigating the optimal dose of BCG for training of human CD14+ monocytes in 48-well plates suggested that MOI 1:50 was sufficient to induce improved secondary TNFα responses to LPS in human CD14+ monocytes and a similar trend was observed for Pam3Cys, a set of follow-up experiments in 48-well plates was conducted to test the effect of BCG-dependent training on TNFα and other cytokines previously associated with BCG-dependent trained immunity in a larger group of donors (170,191). In this set of experiments, elevated TNFα production in BCG-trained monocytes restimulated with cell culture medium and TLR agonists was observed (*Figure 2.9A*). Enhanced IL-6 production was also found for BCG-trained re-stimulation controls and Pam3Cys-challenged cells (*Figure 2.9B*). However, there was no significant increase in IL-1β production in LPS- or Pam3Cys-challenged BCG-trained monocytes compared to the control cultures (*Figure 2.9C*). in addition, despite the increase in the production of these proinflammatory cytokines in some donors, cytokine levels were decreased in cell cultures from other donors, with the same pattern observed when comparing fold change values for cytokine production in trained and untrained monocyte cultures (*Figure S2.2*). Together, this suggested that although BCG exerted training effect on monocytes, this effect was rather variable.

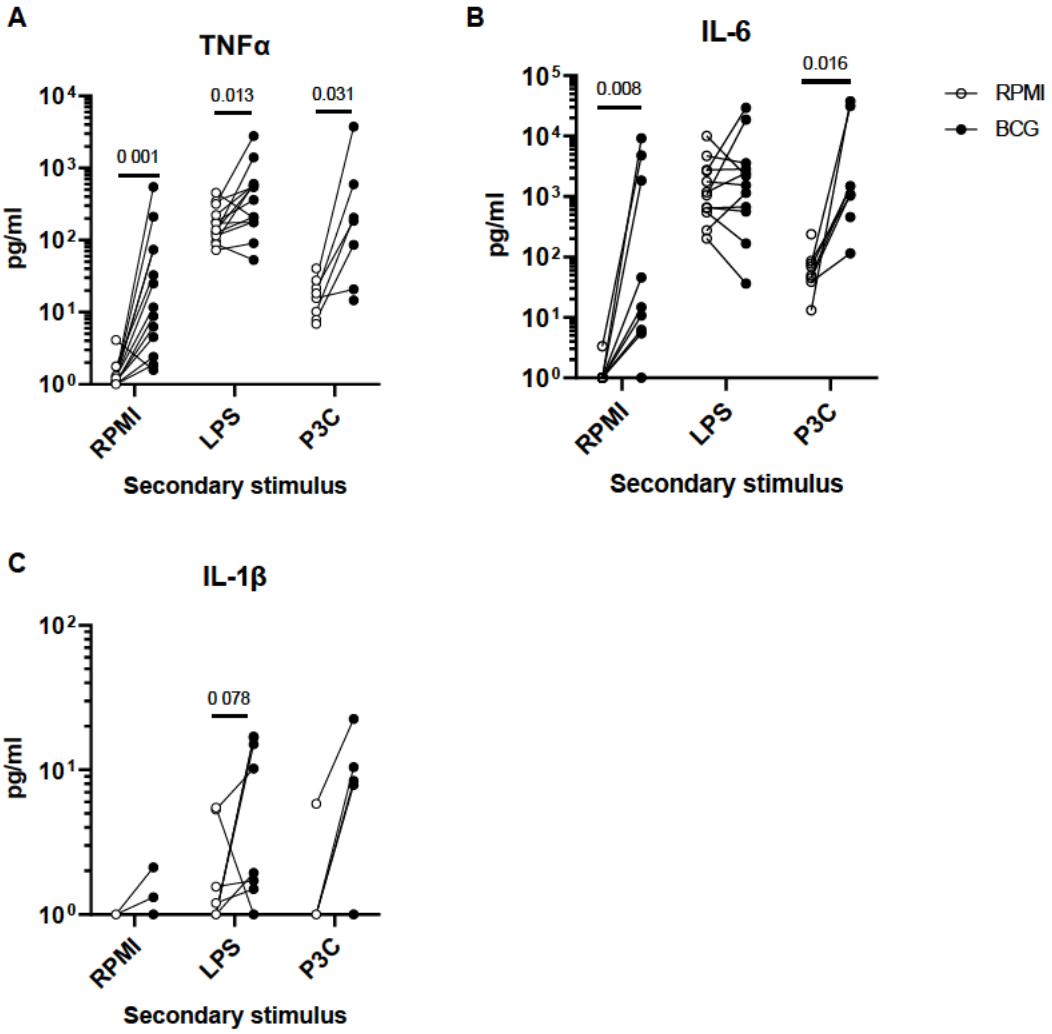



**Figure 2.9.** *The training effect of BCG on human CD14+ monocytes is highly variable*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at MOI 1:50 for 24 h on 48-well tissue-culture plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then re-stimulated with LPS or Pam3Cys or left unstimulated for 24 h. After 24 h of re-stimulation, concentrations of TNFα (**A**; n = 13), IL-6 (**B**; n = 12, and IL-1β (**C**; n = 11) were measured in the supernatants from the control or re-stimulated cells. Data points show cytokine concentrations in individual donors. Numbers indicate p-values for significant differences. Wilcoxon matched-pairs signed-rank test was used to detect the differences between trained and control monocyte responses. BCG MOI indicates ratio of BCG CFUs to the numbers of cells per well.

While the interexperimental variation may have contributed to some extent to the variation observed, there was also a possibility that day-to-day variation in immune responses of

individual donors may have played a role. The TNFα (*Figure 2.10A*) and IL-6 (*Figure 2.10B*) responses of donors tested on two or three separate occasions were therefore compared. Although the responses of both cytokines in supernatants from RPMI-stimulated control and BCG-trained samples were not detectable irrespective of when the cells were trained, TNFα and IL-6 responses to LPS stimulation in control or trained monocyte cultures varied, suggesting that the timing of monocyte training with BCG or other associated variables could contribute to the outcome of training and secondary cytokine responses.

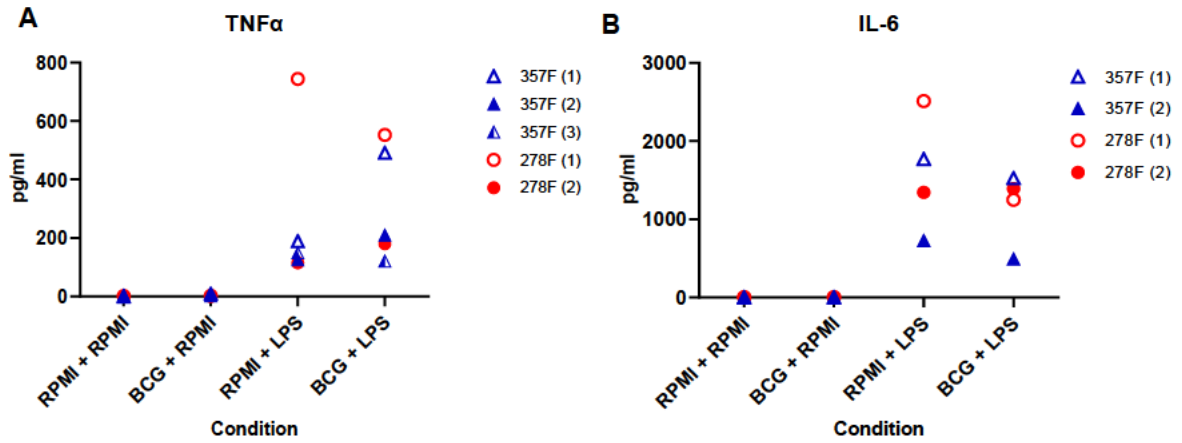



**Figure 2.10.** *Day-to-day variation in individual donor responses*. In this figure, TNFα (**A**) and IL-6 (**B**) secondary responses after the 24 h re-stimulation of control or trained monocyte cultures processed on different days were plotted to address the possibility of day-to-day variation in individual immune responses. N = 2.

### *2.3.7 The effects of training may depend on the batch of BCG*

Apart from inter-individual or day-to-day variation between the experiments or in individual immune responses, other factors, such as the batch – and the associated properties – of BCG can contribute to the outcome of training (252). A set of BCG dose optimisation experiments was conducted using a separately grown batch of BCG (BCG-Pasteur (Aeras) #2) in 48-well plates, using 300,000 monocytes per well. There were no significant differences in terms of TNFα concentrations between the control cells and BCG-trained cells when using this batch irrespective of the MOI of BCG and whether the cells were restimulated with RPMI (*Figure 2.11A*) or LPS (*Figure 2.11B*), although there was a trend for an increase in TNFα levels in supernatants from LPS-challenged cells trained with higher doses of BCG (*Figure 2.11B*).

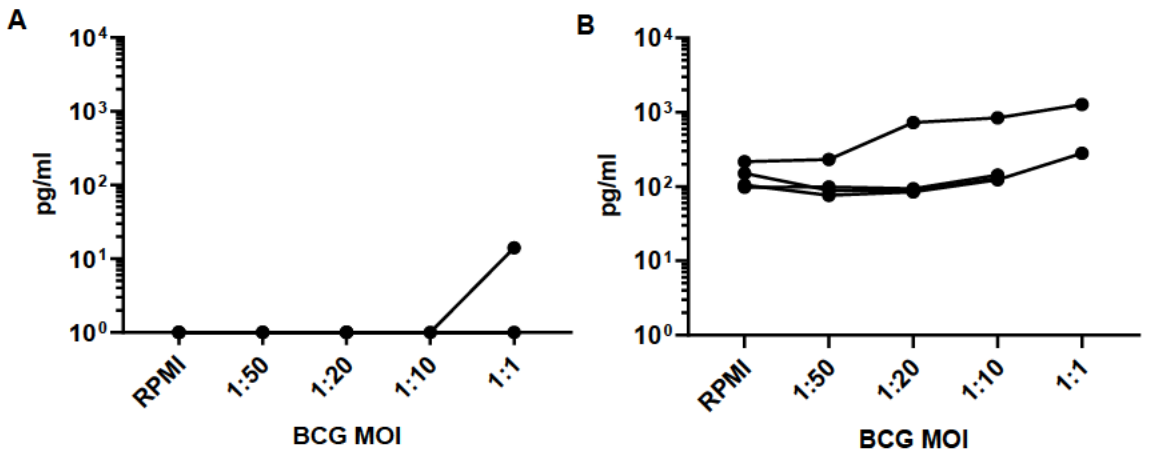



**Figure 2.11.** *The dose-dependent training effect of BCG on TNFα production using BCG-Pasteur (Aeras) batch #2.* 300,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG-Pasteur (Aeras) batch #2 at the indicated MOIs for 24 h on 48-well tissue-culture plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then cultured with RPMI or LPS for 24 h. Concentrations of TNFα in (**A**) - unstimulated and (**B**) – LPS-stimulated cell supernatants were then measured. Data points represent TNFα concentrations in individual donors. Wilcoxon signed-rank matched-pairs test was used to detect the differences between RPMI- and BCG-trained cells. BCG MOIs indicate ratios of BCG CFUs to the numbers of cells per well. N = 4.

Because the previous dose optimisation experiments using the original batch of BCG (BCG-Pasteur (Aeras)) suggested MOI 1:50 as the optimal dose of BCG for human CD14+ monocyte training, monocytes were trained with BCG-Pasteur (Aeras) #2 batch at this MOI and their responses to secondary RPMI or LPS stimulation were compared to those of control monocyte cultures. No differences were found for control or LPS-re-stimulated cells (*Figure 2.12A*), suggesting that BCG-Pasteur (Aeras) #2 batch did not train the cells at MOI 1:50. An additional experiment where CD14+ monocytes from the same individuals were trained in parallel with both batches and challenged with LPS was conducted to test for the differences in monocyte responsiveness to training with these two batches. Although the original batch of BCG induced some TNFα production in RPMI-stimulated cells in contrast to control or BCG-Pasteur (Aeras) #2 trained cells, there were no significant differences in LPS-restimulated samples, possibly,

due to the large variation in TNFα responses to LPS (*Figure 2.12B*). The difference in baseline TNFα production in cells trained with different batches of BCG suggested that some factor, perhaps differences in its viability or growth, contributed to the outcome of training.

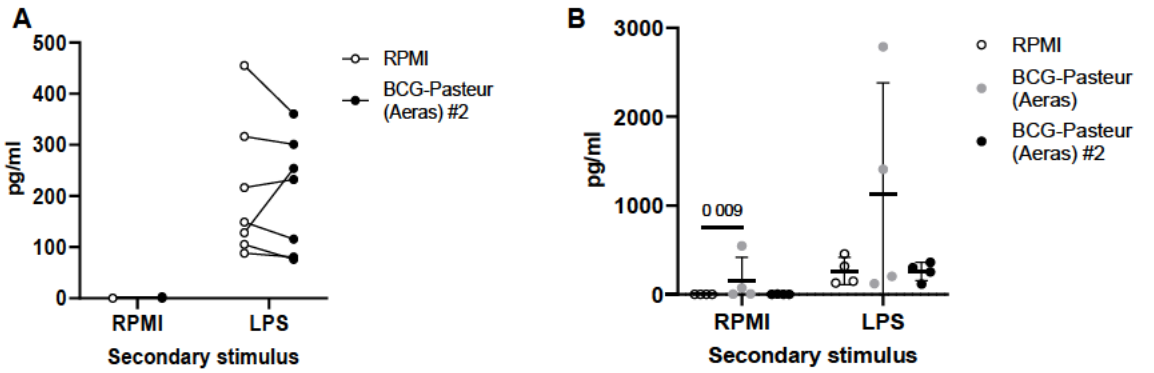



**Figure 2.12.** *The batch of BCG and its effect on TNFα production in trained monocyte cultures*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of **A** – BCG-Pasteur (Aeras) #2 batch at MOI 1:50 (n = 7) or **B** – in the presence of BCG-Pasteur (Aeras) or BCG-Pasteur (Aeras) #2 batches (n = 4) for 24 h on 48-well tissue-culture plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then re-stimulated with LPS or left unstimulated for 24 h. Concentrations of TNFα were then measured. Data points represent TNFα concentrations in individual donors. Numbers indicate p-values for significant differences. Wilcoxon matched-pairs signed-rank test (**A**) or Friedman test with Dunn's post hoc multiple correction test (**B**) were used to detect the differences.

## 2.4 Discussion

### *2.4.1 The effects of primary human monocyte training may be less robust than expected*

This chapter describes a series of experiments performed to optimise a system for measuring innate immune training of human monocytes by BCG. The methods were based on a previously described assay used to train small numbers of human CD14+ monocytes with BCG, aiming to exploit this assay as a tool for screening of novel monocyte markers associated with trained immunity and protection against TB. Two agents previously associated

with trained immunity were used in this work – β-glucan and BCG, with the focus on the latter as the mediator of anti-tuberculosis immunity.

Interestingly, no training effect of β-glucan on human CD14+ monocytes was observed in this study. This was rather surprising as in most *in vitro* training studies β-glucan is reported as a highly potent training agent, able to induce epigenetic or metabolic reprogramming (200,224) and to enhance cytokine responses to secondary stimulation *in vitro*, both with the TLR agonists (200,203) or whole microorganisms (200). In addition, some *in vitro* training studies suggest that β-glucan might be a more potent training agent than BCG or other agents reported to induce trained immunity, enhancing secondary innate cytokine responses to TLR agonists (203–205), and the injection of mice with this fungal component was also shown to enhance myelopoiesis in mice, generation of CD11b+ cells, and production of IL-1β in the BM (253). It is possible that the training effect in this work was missed due to a low number of donors. Inter-individual differences, such as deficiency of dectin-1 – a receptor mediating β-glucan signalling – have been reported to abrogate or modulate innate immune training induced by this fungal compound (200). It is also possible that in this study, the conditions for CD14+ human monocyte cultures in 96-well plates were suboptimal due to frequent cell washes or medium replacement steps as required by the protocol as poor survival of cells was also observed in BCG-trained cell cultures, especially those that received high doses of BCG. Contrary to the literature (170,177,191), no training effect was observed in these cultures. As BCG and the contribution of innate immune responses to anti-tuberculosis effects of this vaccine were the main focus of this study, it was decided to focus on optimising the training conditions for cultures trained with this agent. Culturing CD14+ monocytes in 48-well plates improved the condition of cell cultures irrespective of the training condition.

An interesting clustering phenomenon was observed in BCG-trained monocyte cultures, suggesting that BCG-training may have triggered enhanced integrin production and increased adherence of cells compared to the control, untrained cultures. CD11b, a molecule reported to increase in monocytes in BCG-vaccinated human adults or infants (191,213,223) or in

monocyte cultures trained with plasma lipoprotein A (206) or β-glucan (203) is not only a complement receptor chain, but also an integrin and plays a role in monocyte adhesion (254). Elevation of other adhesion-associated molecules, such as CD11c or CD62L has also been reported in individuals with elevated plasma lipoprotein A levels (206), suggesting that other agents that have been reported to induce trained immunity *in vitro*, including BCG, may also enhance the expression of these molecules. Although this hypothesis was not examined, enhanced monocyte adhesion in BCG-trained cultures was indeed observed in this study. Overall, this suggests that these clusters may involve trained, activated monocytes and that the number of such clusters in BCG-trained monocyte cultures may reflect their responsiveness to secondary stimulation with TLR agonists or whole cell microorganisms. This hypothesis would also be supported by the presence of elongated, M1-like spindle-shaped monocytes observed in BCG-trained monocyte cultures.

To test whether the training effect of BCG indeed manifested in these cell cultures, TNFα responses were measured in control and BCG-trained cultures. Although, as expected, increasing BCG MOIs was associated with elevated TNFα production both in the presence or absence of TLR stimulation, this effect only manifested to an extent and the highest doses of BCG had an inhibitory effect rather than that of training. In addition, elevation of TNFα seemed to correlate with the number of clusters observed in BCG-trained monocyte cultures. TNFα has been previously associated with the maintenance of granulomas in mycobacterial infections in zebrafish models of mycobacterial infection (255), although other cytokines could play a role, too. A relatively recent human study on human individuals exposed to but uninfected with *M. tuberculosis* suggested a protective role for CXCL9, -10 and -11 production by CD14dim monocytes and associated them with trained immunity as these cytokines have been also been elevated in cell culture supernatants from BCG-vaccinated individuals along with IL-1β, IL-6 and TNFα – cytokines associated with trained immunity (131). CD14dim monocytes may also be captured by CD14 magnetic bead isolation; however, the numbers or role of such monocytes in trained monocyte cultures was not tested in this work.

### *2.4.2 High inter-individual variation may contribute to the lack of training observed in some cases*

Interestingly, the increases in TNFα associated with BCG training of monocytes were only statistically significant for some of the conditions as large variations in the concentration of this cytokine was observed in cell cultures from different individuals. This remained true even when CD14+ monocytes were trained with BCG in a larger number of donors at MOI determined as optimal. While monocyte cultures from some of the donors seemed to be responsive to BCG training and their TNFα, IL-6 or IL-1β responses to secondary stimulation with LPS or P3C or baseline production of these cytokines were higher compared to those in the control cultures, secondary cytokine responses to TLR stimulation in BCG-trained monocytes of others were similar or even lower than that of the RPMI controls. Although reasons for variation other than inter-experimental variability in cytokine measurements or day-to-day differences in control or BCG-trained monocyte secondary responses to RPMI and LPS were not addressed in this study, other factors may have contributed to variation in cytokine responses in trained monocyte cultures from different individuals. For instance, a recent study suggested that circadian rhythms can influence the outcome of innate immune training, morning vaccination with BCG possibly enhancing cell responsiveness to training (256). Because in this work the access to the blood samples depended on the availability of donors participating in this study, all samples were trained late in the afternoon and it was not possible to ascertain whether training monocytes at a different time of day would improve their responsiveness to BCG training or reduce variation in cytokine responses secondary stimulation.

Some of the variation in monocyte responsiveness to BCG-dependent training might be encoded by genetic factors. For instance, deficiency of the intracellular pathogen recognition receptor NOD2 has been previously shown to abrogate the enhancing effect of BCG-dependent training on TNFα responses to LPS (191). Apart from genetic deficiencies, single nucleotide polymorphisms (SNPs) in both the autophagy and glycolysis related genes have been shown to affect the ability of human monocytes to be trained with BCG (170,177). It

should be noted that in general, the human population is highly genetically diverse and that human innate immune responses to BCG vaccine are highly influenced by genetic variation – SNPs in cytokines or their receptors, extracellular or intracellular PAMP sensors, or cellular metabolism have all been shown to contribute to variation in direct or secondary innate immune responses to BCG or in its non-specific effects (NSEs) (257).

Although genetic components can significantly contribute to inter-individual variation observed in BCG-trained monocyte responses, there are yet other factors that may have played a role. It has been speculated for a while that some of the NSEs of BCG observed in the vaccinated individuals might be sex-differential and some studies indeed suggest that subtle differences in male and female immune responses to this vaccine may be present (258,259). Of interest, however, when the effect of estradiol and dihydrotestosterone on human monocyte responses to BCG training was examined *in vitro*, no effect of these hormones on monocyte responsiveness to training was observed (260). In this study, the possible effect of sex on individual responses to BCG training or secondary stimulation was not addressed, so it was not possible to confirm whether sex contributed to variation in secondary responses to TLR agonists in the control and BCG-trained cultures.

### *2.4.3 Other factors that may contribute to the variation observed*

It should also be noted that individuals whose monocytes were trained in this study had been BCG-vaccinated in the past. Previous BCG vaccination was associated with enhanced IFNγ production (261), and positive BCG vaccination status has been associated with downregulation of TLR4 upon stimulation with *M. leprae* in human macrophages (214). Of interest, TNFα and IL-1β responses to LPS in BCG-trained monocyte cultures, when compared to the control cultures, despite the general increasing trend, showed quite large variation, decreasing in BCG-trained monocyte culture supernatants in some cases. Historical BCG vaccination has also been associated with enhanced mycobacterial growth inhibition in NK cell cytokine dependent manner (235,246). Although this study did not investigate innate

immune cell responses other than those of control and BCG-trained human CD14+ monocytes, together, this evidence suggests that BCG vaccination could have a lasting impact on the innate immune responses and that there is a possibility that the positive BCG status may have affected the ability of CD14+ monocytes to train.

Some studies have also suggested that variables associated with BCG itself, such as its viability or its ability to grow may affect innate immune responses, with slow-growing batches of BCG enhancing IL-1β or IL-10 production in human monocytes (252). To address the possibility that the responsiveness of human CD14+ monocytes to BCG-dependent training may be affected by the batch of BCG, the cells were also trained with a batch of BCG grown separately from the original BCG-Pasteur (Aeras) strain used in the previous *in vitro* training experiments. In this study, the change in BCG batch abrogated the ability of BCG to train the cells, although direct comparison of the effect of each batch on monocyte secondary responses to LPS suggested that this effect might be masked by the variability in secondary cytokine responses to TLR agonists. The previously reported comparison of the ability of slow and normal growing strains of BCG to train human monocytes also suggested that subtle batch-associated differences in monocyte responsiveness to BCG training may exist (252). This is perhaps not surprising as other studies also suggested that BCG vaccines vary in their viability, ability to grow and the ability to induce innate cytokines, possibly affecting downstream responses to BCG (262).

### *2.4.4 Similarities and differences with other studies on innate immune training*

Although this work was based on monocyte *in vitro* training with BCG model described by *Bekkering et al.* (205), there were some differences in the protocols used to train the cells with BCG in this and the previously reported work that may have contributed to a less consistent effect of training in this work. These differences are summarised in *Table 2.2*.

**Table 2.2.** *Differences in the protocols reported by Bekkering et al. and associated studies and the protocol used in this study*

| | ***Bekkering et al. and related studies*** | **This study** |
|---|---|---|
| **Monocyte isolation** | Percoll gradient, followed by monocyte adherence | Human CD14 magnetic beads |
| **Serum** | Serum, source or inactivation status unknown | Commercial human AB serum, heat-inactivation |
| **BCG quantification** | BCG concentration in terms of µg/ml | BCG CFU to monocyte ratio (MOI); CFU/well |
| **Data presentation** | Fold change in cytokine concentration in stimulated cell cultures compared to unstimulated controls | Cytokine concentrations |

As one of the aims of this optimisation work was to standardise cell numbers in trained monocyte cultures and avoid possible involvement of residual populations of other cell lineages, human CD14+ monocytes were purified by positive selection, using magnetic beads. Previously reported assays on monocyte *in vitro* training with BCG, however, frequently isolate monocytes by PBMC adherence (170,191,217,219) or by Percoll gradient (177,205,215). Isolation with Percoll yields monocyte-enriched cell suspensions (75 ± 20% purity); however, these suspensions can still contain T- or NK cells (263). The work described in *Chapter 3* showed that BCG survives and replicates in trained monocyte cultures and so, throughout the 8 days of culture, could potentially activate residual NK or T-cells through direct stimulation, BCG-derived peptide presentation by monocytes or through bystander cytokine production. Such activated cells could contribute to increases in cytokine concentrations upon secondary stimulation. In this work, purity of monocytes isolated by human CD14 microbeads was estimated as ≥95%, suggesting that the possible contributions from carryover non-monocytic cell populations would have been lower than in cultures of monocytes isolated by other methods, an important consideration for a work where samples from BCG-vaccinated donors

were used to investigate BCG-dependent training. Also, the role of other monocyte populations should be considered. While CD14+ monocytes used in this study are the most abundant, and *in vitro* training studies using positively selected CD14+ monocytes showed that they could be trained just as the adherent monocyte cultures or change surface marker expression upon vaccination (191,200,209,264), non-classical, CD14dim monocytes have also been associated with trained immunity and protection against TB (205). It is possible that different monocyte subsets also differ in their ability to train or that contribution of some monocyte populations may be more important to cytokine responses to heterologous stimulation than others.

In this, and also, in the previous studies on the innate immune training, monocyte cultures were supplemented with human serum (170,177,191,205,215,217). While most of these studies did not specify the source of serum, a recently published detailed description of the *in vitro* training protocol reported use of pooled in-house serum (265). In this study, commercially available pooled AB serum from male donors was used, and it was heat-inactivated in the lab to reduce the possible influence of complement responses. Serum sources, especially, if sera from different species or individuals are used to culture cells *in vitro*, have been shown to influence cell differentiation, surface marker expression or cytokine production in multiple cell culture models (266–270); however, variation in biochemical composition across different batches of sera from the same species can also contribute to differences in cultured cell responses (271), suggesting that the outcome of training could also be affected. Heat-inactivation of human serum has also been shown to reduce monocyte reactivity to LPS (268), suggesting that differences in culture conditions in this and previous *in vitro* training studies may have contributed to differences observed in BCG-trained monocyte secondary responses, for instance, the low IL-1β responses observed in this study.

Another important difference that may have contributed to a less robust effect of innate immune training observed in this study was the dosing of BCG. The innate immune training studies conducted in the Netherlands quantified BCG in terms of micrograms per millilitre,

ranging from 1 to 10 µg/ml (170,177,191,205,215,217). *In vitro* mycobacterial immunology studies, however, usually quantify the dose of BCG as multiplicity of infection (MOI) – the ratio of mycobacterial colony forming units (CFUs) to monocytes or macrophages in cell culture. While a carefully pre-titrated dose of BCG in any units may be able to induce innate immune training, it would be expected that in adherent cell cultures monocyte numbers would vary across individuals, depending on monocyte proportions and the ability of cells to adhere to the culture plates. BCG-Danish from Statens Serum Institut in Denmark was used to train cells *in vitro* in previous studies conducted in the Netherlands (170,177,191,205,215,217,265), with a standard vial of this vaccine containing 2-8 x $10^5$ CFU / 0.1 ml dose (272). While in 200 µl cell cultures containing 100,000 cells/well this would equate to MOI ranges of 1:37 to 1:9, higher than the optimal MOIs determined in this work, in adherent PBMC cultures, numbers of monocytes would vary as well, likely to modify the effective MOIs. In addition, without knowing how much viable BCG is present in the reconstituted vaccine, the effective MOIs would probably be affected even further. This is quite a broad range and implies that irrespective of the concentration selected, the amount of BCG used to train the cells may have varied in these earlier studies. To overcome these issues, in this work, in addition to standardised cell numbers, BCG was quantified in CFUs. Despite this, variation was still a significant issue in this assay, suggesting that other factors may have contributed to differences observed in this and other in vitro training studies.

Finally, there were also some differences in the presentation of data obtained from studies on human monocyte *in vitro* training with BCG. While the previously conducted studies frequently report differences in BCG-trained monocyte cytokine responses to secondary challenges in terms of fold change from baseline (170,177,191,205,215,217,265), this study compared raw differences between the control or BCG-trained unstimulated and re-stimulated monocytes. While the former approach can help emphasise the differences in test conditions, it can also inflate some differences. For instance, when cytokine concentrations in this study were converted to fold changes, large increases in TNFα or IL-6 production were observed in trained

unstimulated monocyte cultures (400- or 9000-fold for some donors), whereas the influence of BCG training on secondary responses to LPS, which is a highly potent stimulus on its own, was modest (10-fold for TNFα; *Figure S2.2*). Although it is difficult to compare the parameters measured in different studies because of differences in experimental set-up, mean TNFα or IL-6 fold-changes in secondary responses to LPS observed in this study (~3-fold) were broadly similar to those observed before, and responsiveness to Pam3Cys was higher in this study (30-fold change for TNFα and 360-fold for IL-6) (205). In addition, in this study, large variation in donor secondary responses was observed, with decreased cytokine responses to secondary stimulation in some cases. Together, all these differences in the study populations and the methods used to train human monocytes with BCG *in vitro* may have contributed to the lesser extent of training observed in this study.

### *2.4.5 Summary*

The goal of the work described in this chapter was to establish and optimise the method for human monocyte *in vitro* training with BCG. Using CD14+ monocytes isolated through magnetic bead selection allowed standardising the cell numbers used in the assay and determination of the specific quantity of BCG needed to train human monocytes *in vitro* in terms of MOIs. However, this study did not replicate the previously published findings (205). Although some effect on TNFα or IL-6 responses to secondary stimulation was observed in BCG-trained cultures, this effect was inconsistent. Even more so, no effect at all was observed after the batch of BCG used in monocyte training experiments was changed despite the same dose of BCG being used in these experiments, and the same protocol being used to grow the BCG. No single factor that determined the variability or lack of effect observed in this study was established, although some factors that may have contributed were addressed in this work, including experimental variation. It is possible that prior BCG vaccination may have influenced the effect of training, but it was not possible to address this hypothesis with the majority of the donors available having had the BCG vaccine in the past. Overall, this work

suggested that assays used to study trained immunity *in vitro* should be carefully optimised if intended to use as a screening tool for novel markers associated with trained immunity or protection against mycobacterial infections. Finally, because the change in BCG batch resulted in no effect on monocyte secondary responses to LPS, it was decided to investigate if factors such as viability of BCG contributed to the variability or the lack of training effect observed in this work,

# **Chapter 3:** Viability of BCG and its effects on innate immune training

## 3.1 Introduction

The BCG optimisation studies described in *Chapter 2* showed that BCG-trained monocyte cultures produced IL-6 or TNFα – markers of innate immune training – in the absence of stimulation with TLR agonists and, in addition, did so in a manner dependent on the dose of BCG used to train the cells. Low doses of BCG used during the training steps of the assay resulted in IL-6 or TNFα concentrations similar to those in the supernatants of the control cell cultures and were barely detectable in the presence of cell culture medium alone. In contrast, high IL-6 or TNFα concentrations were detected in supernatants of some cultures trained with high doses of BCG, suggesting that the amount of viable, replicating bacteria can be important for the extent of responses to secondary stimulation in BCG-trained monocytes. In addition, variation in the ability of different BCG batches to train the cells using the same MOI was observed, suggesting possible differences in percentage viability of the bacteria in these batches, which were standardised based on CFU. Other researchers have experienced differences in the ability to train human monocytes when the strain of BCG used was changed, for example from BCG-Danish to BCG-Russia (email communication with Dr Mihai Netea, Dr Jorge Dominguez-Andres and Dr Siroon Bekkering, Radboud University). Previous studies demonstrated that even licensed, quality-controlled BCG vaccines, among other properties, often vary in terms of viable BCG CFUs and do not always reach the expected growth ranges indicated on the information sheets provided by the manufacturers (262). This variation can sometimes affect the immune responses, both in *in vitro* assays and *in vivo* upon BCG vaccination.

The number of BCG CFUs has been shown to correlate with the levels of innate cytokines in supernatants from BCG-stimulated whole blood cultures of newborns and adults (262). Some of these cytokines, such as G-GSF, GM-CSF, PDGF-AB/BB, IL-1β or TNFα or IFNγ have been associated with trained immunity, non-specific effects of BCG or protection against TB (170,177,191,209,223,227,242), suggesting that variation in viable CFU amounts in BCG vaccines might affect this immunological phenomena. Live BCG has been shown to enhance

the production of trained immunity associated cytokines in THP-1 derived macrophages compared to heat-killed BCG (contains denatured antigens) (273). In cattle, alveolar macrophages stimulated with live as opposed to heat-killed BCG were shown to express higher levels of genes encoding such chemokines as CCL2, -3, -4, and -8, and also, CXCL8 and -10 (274), the latter chemokine associated with trained immunity and protection against TB in exposed but uninfected human individuals (131).

Cellular responses can also be modulated by the viability of BCG or other mycobacteria. Higher viability of THP-1 monocytes was detected upon infection with heat- or streptomycin-killed *M. tuberculosis* compared to live *M. tuberculosis* strains (275). Higher rates of phagocytosis of viable *M. tuberculosis* were detected in Raji cells compared to dead mycobacteria (276), and the acidification of phagosomes in macrophages was partially prevented by live BCG (277), while live *M. tuberculosis* was shown to inhibit phagosomal maturation in THP-1 cells (278). Live mycobacteria can also affect the ability of innate cells to present antigens to T-cells even if the expression of MHC encoding genes remains unaffected (279). Expansion of lymphocyte populations can also be affected by mycobacterial viability. Live *M. tuberculosis* has been shown to expand γδ+ T-cells *in vitro* while heat-killed *M. tuberculosis* expanded conventional αβ+ T-cells (280,281), and live BCG has been shown to stimulate deer lymphocyte proliferation more potently than heat-killed BCG (282).

Some studies also show differences in immune responses to viable and dead mycobacteria *in vivo*. For instance, mice vaccinated with heat-killed attenuated *M. tuberculosis* have poorer survival rates upon challenge with virulent *M. tuberculosis* compared to mice that were vaccinated with live attenuated strain (283). In another model, instillation of murine bladders with viable BCG led to the induction of Th1 type cytokine gene expression, while instillations with dead BCG enhanced the expression of genes encoding Th2-associated cytokines (284). Similarly, PBMCs from deer vaccinated with heat-killed BCG had higher IL-4 mRNA expression compared to PBMCs from deer that received a live vaccine (285).

Heterologous effects of BCG can also be affected by the viability of this organism. While live BCG has been shown to induce heterologous cytokine responses in vaccinated individuals and modulate yellow fever vaccine particle load in blood in an experimental viral infection model (191,197,209,213), immunisation of humans or animals with dead mycobacteria has been less effective at inducing non-specific responses. Vaccination of newborn piglets with an inactivated *M. paratuberculosis* did not enhance IL-6 responses to TLR agonists (286), and in another study, vaccination of human individuals with irradiation-killed BCG was shown to enhance the heterologous production of IFNγ and IL-22 but not of markers previously associated with trained innate immunity (217). In addition, irradiation-killed BCG was less potent at inducing trained immunity *in vitro*, requiring higher dose to train human monocytes (217).

This evidence suggests that mycobacterial viability can modulate a broad range of immunological mechanisms in diverse hosts and may extend not only to direct protective effects of BCG but also its heterologous effects. Although previous studies suggested that dead BCG may also reprogram monocytes epigenetically, the mechanisms mediating the reprogramming and the extent of its downstream effects are not fully understood. It is possible that live, replicating BCG may induce it, complement it or, instead, provide parallel signals enhancing responses to secondary stimuli, such as pathogens or vaccines. To address the hypothesis that trained immunity effects may in fact result from synergistic signals from viable, replicating units of BCG inside the cells that went through training and that the viability of BCG at different stages of the training phase of the assay played an important role on the outcomes of training, human CD14+ monocytes were cultured and/or trained in the presence or absence of an antimycobacterial drug streptomycin, or with irradiation-killed BCG.

## 3.2 Methods

### *3.2.1 Isolation of PBMCs and CD14+ monocytes*

The methods used to isolate PBMCs and CD14+ monocytes from adult volunteer peripheral blood samples were described in *Chapter 2*, *Section 2.2 Methods*. Sample use was approved by LSHTM Ethics Committee (LSHTM Ethics Reference #14576).

### *3.2.2 Examination of mycobacterial viability and its effects on monocyte training*

The method used to train human CD14+ monocytes with BCG was described in *Chapter 2*, *Section 2.2 Methods*, with the amendments used to investigate how the viability of BCG affects the outcome of training described below.

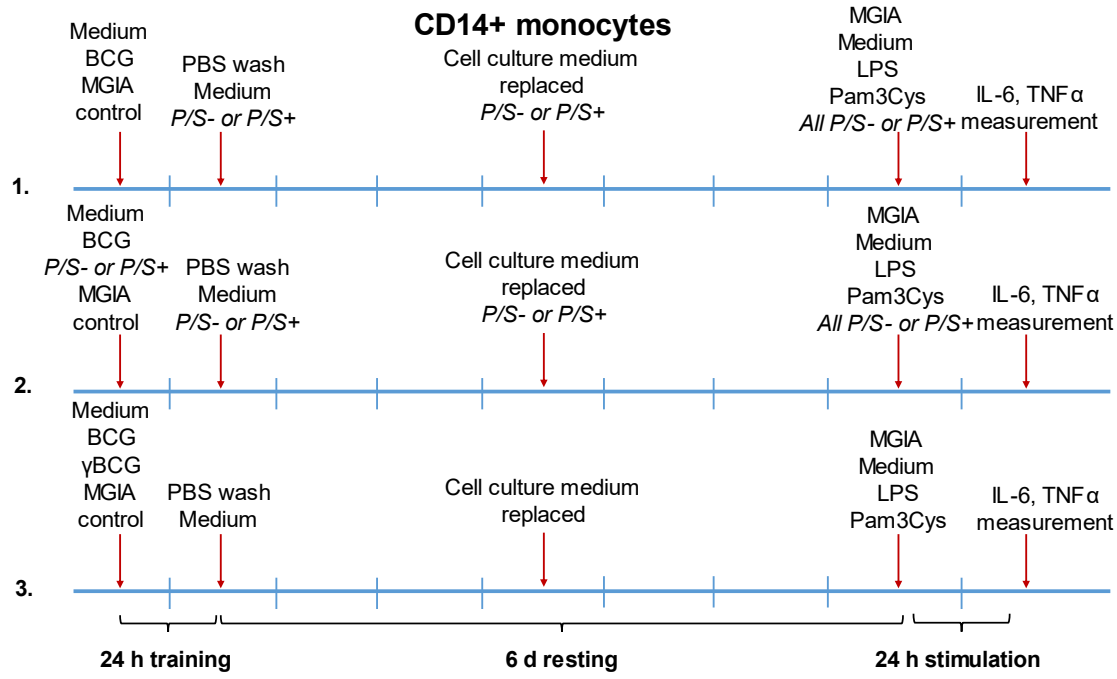



**Figure 3.1.** *The outline of three experimental designs used to train human CD14+ monocytes with live or dead BCG*. In design 1, monocytes were trained for 24 h with live BCG or left untrained. They were then washed and rested for 6 days in the presence or absence of streptomycin with cell culture medium replacement on day 3. The cells were then re-stimulated for 24 h with LPS or Pam3Cys in the presence or absence of streptomycin or left unstimulated. In design 2, monocytes were trained with live BCG for 24 h in the presence or absence of streptomycin, washed and then cultured as cells in design 1. In design 3, monocytes were trained with live or irradiation-killed BCG for 24 h, washed and rested for 6 days with medium replacement on day 3. They were then re-stimulated with LPS, Pam3Cys or were left unstimulated. No streptomycin was used in design 3. Cytokine measurements were taken or MGIA cultures started at the indicated time points.

To test the possible influence of mycobacterial viability on the outcome of monocyte training, three experimental designs were applied (*Figure 3.1*). In the first, 3 x $10^5$ CD14+ monocytes/well in 48-well NTC plates were cultured in duplicate with medium alone or trained with BCG at MOI 1:50 (6,000 CFUs/well) for 24 h. After the PBS washing step, monocytes were rested in RPMI supplemented with 10% HI ABS, 1% L-glutamine and 1% penicillin/streptomycin (P/S; Sigma-Aldrich) or no antibiotics for 6 days, with medium replaced on day 3 of resting. Streptomycin has antimycobacterial properties and can inhibit the growth of BCG (287). In the second, monocytes were trained with BCG in the presence or the absence of 1% penicillin/streptomycin, and then cultured in the presence or absence of antibiotics for 6 days with the cell culture medium changed on day 3. In the third, live or irradiation-killed (γBCG) was used to train and culture monocytes in the absence of antibiotics throughout the duration of the assay. To ensure that the γBCG used for training was indeed killed, it was irradiated with γ-rays for 16 h and cultured on 7H10 agar (BD, USA) supplemented with OADC (BD, USA) for 21 days at 37ºC. A parallel agar culture of live BCG used for monocyte training was set up as a positive control for BCG growth. No growth of colonies was observed on the plates containing γBCG, confirming it was successfully killed by irradiation.

On day 6 of resting, 500 µl of sterile, tissue culture grade water (Sigma-Aldrich, UK) was added to each well to lyse the monocytes. The plates were vortexed three times for 30 s with 5 min breaks in between and monocyte lysates transferred to the MGIT tubes (BD, USA) supplemented with PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) and OADC (oleic acid, albumin, dextrose and catalase) enrichment broth (BD, USA) to reduce the risk of MGIT tube contamination with other bacterial species. The screw-cap tubes were vortexed and transferred to a Bactec MGIT960 incubator for culture at 37ºC until the end of a 42-day protocol.

To control for BCG growth in MGIT tubes and quantify the viable mycobacteria in the samples, 6,000 BCG CFU and a series of ten-fold standard dilutions (1 x $10^5$ to 1 x $10^0$ CFU in 500 µl water) from the same BCG aliquot used to train the cells were resuspended on the day of

monocyte training and prepared in duplicates for MGIA as described above. In addition, to validate the number of BCG CFUs in the MGIA standards, 50 µl of each standard dilution were seeded onto 7H10 agar (BD, USA) plates supplemented with OADC (BD, USA) and incubated at 37$^{o}$C for 21 days. The number of colonies on the plate was then counted for each standard dilution and used to calculate the concentration of BCG within each MGIA standard dilution

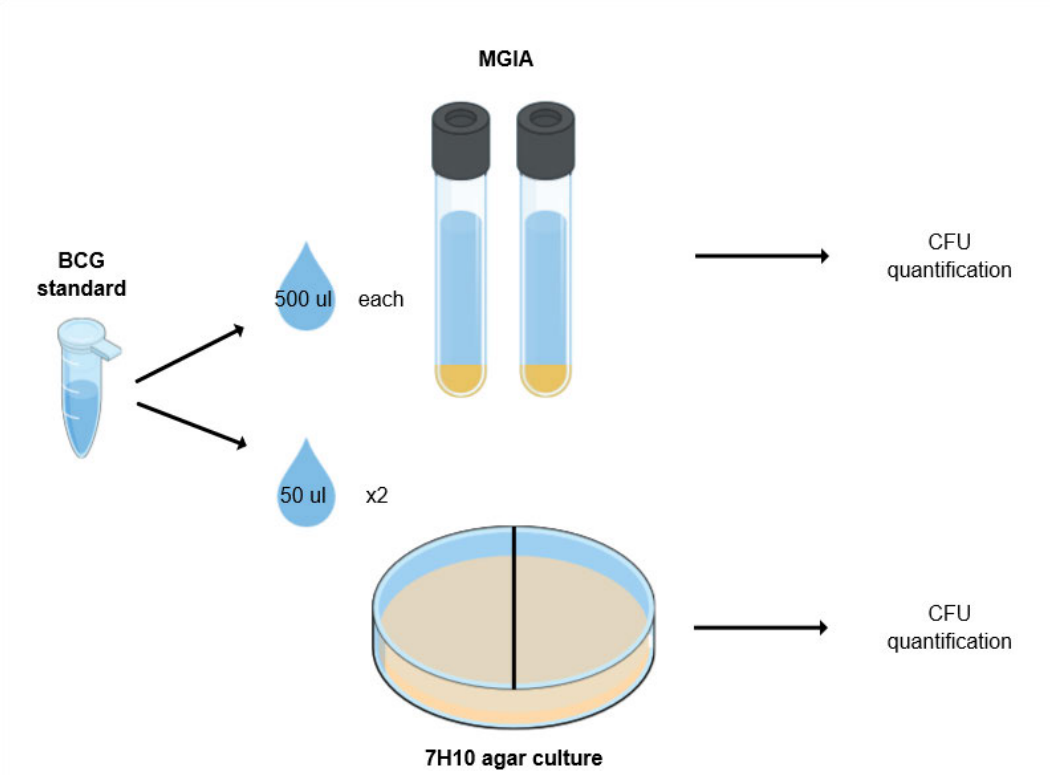



**Figure 3.2.** *The outline of BCG culture experiments used to quantify the number of BCG CFUs in mycobacterial growth inhibition assays or 7H10 agar plates*.

(*Figures 3.2, S3.1*). While the numbers of BCG CFUs detected for the standards containing the highest BCG CFU numbers were too numerous to quantify, the quantifiable concentrations of BCG CFUs were similar, although somewhat higher than the expected standard concentrations (*Table S3.1*).

Trained or control monocyte cultures in replicate plates were stimulated with 10 ng/ml LPS (Invivogen, USA) or 10 µg/ml Pam3Cys (Invivogen, USA) in the presence or the absence of P/S for 24 h, and their supernatants were collected and stored at -70 $^{o}$C until further analysis.

### *3.2.3 Cytokine production by monocyte cultures trained with live or dead BCG*

Cytokine production by trained monocyte cultures in response to secondary stimulation with LPS or Pam3Cys was tested with Human IL-6 ELISA MAX Standard and Human TNF ELISA MAX Standard (BioLegend, USA) kits as described in *Chapter 2*, *Section 2.2 Methods*.

### *3.2.4 Imaging of live or dead BCG trained monocytes*

To visualise the morphological changes occurring in CD14+ monocytes upon training with BCG, bright-field images were obtained as described in *Chapter 2*, *Section 2.2 Methods*.

To measure the uptake of a fluorescent dsRed-BCG strain (BCG-Pasteur, kindly donated by Dr Sam Willcox, LSHTM), human CD14+ monocytes were trained with live or γ-irradiated BCG as described above. After the resting period, the cell culture medium was replaced with ice-cold Dulbecco's PBS A, and the monocytes were then detached by incubating cell culture plates on ice for 30 min and pipetting. The monocytes were then seeded onto the µ-slides (Ibidi, Germany) at 100,000 cells per well and re-stimulated with dsRed-BCG at MOI 10:1 for 3 h. The cells were then washed with PBS and their images were taken at magnification = x20 using Nikon Ti-E inverted microscope (Nikon, Japan). NIS elements 4.6 software (Nikon, Japan) was used to extract the images.

### *3.2.5 Statistical analysis*

To estimate the differences in TTP values in lysates from BCG-trained or control monocytes cultures or the differences in cytokine concentrations in cell cultures treated in the presence or absence of streptomycin Wilcoxon matched-pairs signed rank test was used. To determine if there was a correlation between IL-6 or TNFα concentrations in trained monocyte supernatants and the CFU values in lysates from the corresponding trained monocyte cultures, Pearson r values were calculated. To test if there was a difference in IL-6 or TNFα production by control monocytes or monocyte cultures trained with live or irradiation-killed

BCG, the Friedman test with Dunn's post hoc multiple comparison test was used. All datasets were analysed using GraphPad Prism 9 software (GraphPad Software, USA).

## 3.3 Results

### *3.3.1 The effect of streptomycin on BCG-trained monocyte responses*

To address the possibility that some of the effects of the innate immune training may be affected by viable units of BCG persisting inside BCG-trained cultures and so implicating a synergistic effect rather than just that of training, human CD14+monocytes were left untrained or were trained with BCG and then cultured in the presence or absence of streptomycin throughout the duration of resting and restimulation of cells with LPS. Inverted light microscopy images of these cell cultures showed no difference in terms of cell morphology or density for those monocytes that were trained with BCG and then cultured in the presence or absence of streptomycin (*Figure 3.3C, D*).

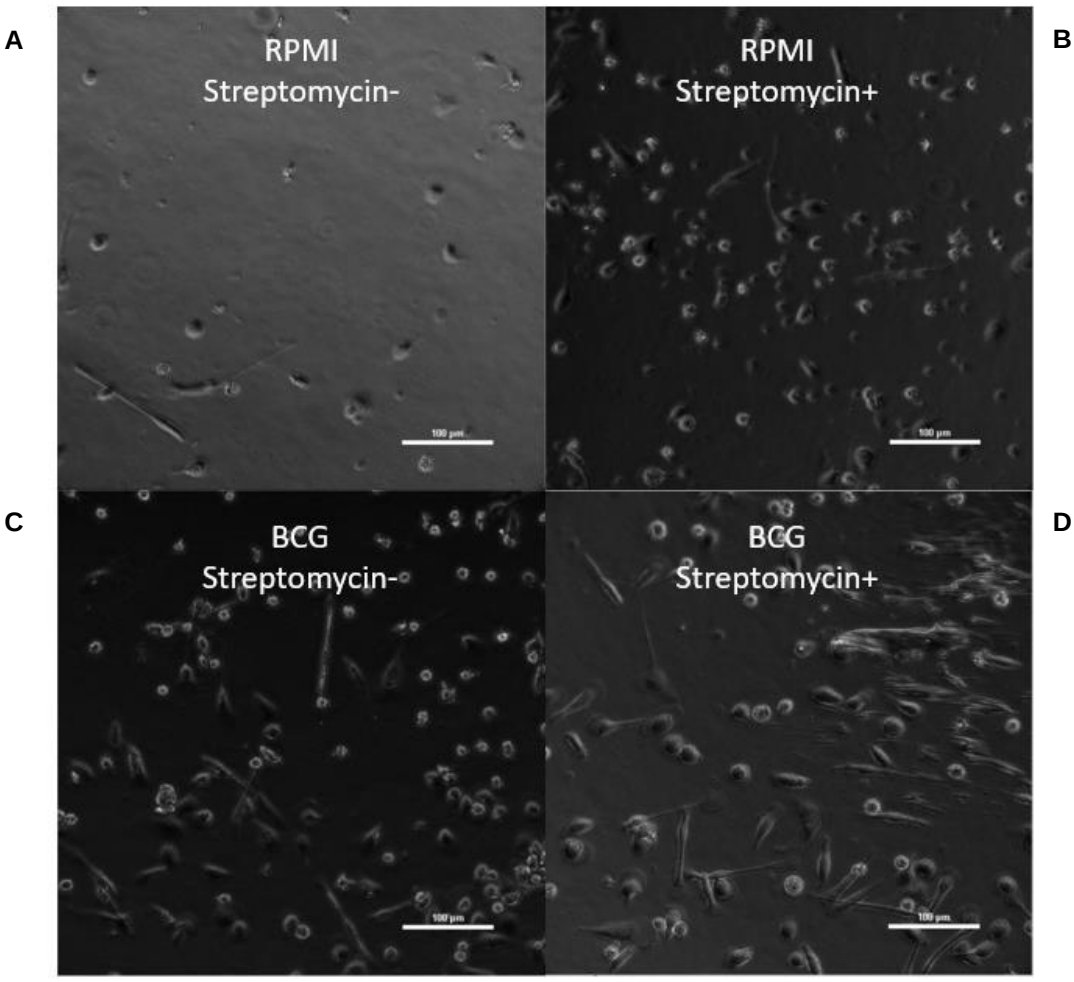



*Figure legend and description in the next page*

**Figure 3.3.** *The effect of streptomycin on morphology and density of BCG-trained CD14+ monocytes*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at MOI 1:50 for 24 h on 48-well non-treated plates and rested for 3 d. Inverted light microscopy images were taken at 20x magnification on day 6 post-training, prior to the secondary re-stimulation of cells. **A:** training controls cultured in the absence of streptomycin, **B**: training controls cultured in the presence of streptomycin, **C:** trained monocyte cultures in the absence of streptomycin, **D:** trained monocyte cultures in the presence of streptomycin. The images depict morphology of trained monocytes from a single donor in a representative experiment. Streptomycin- or Streptomycin+ indicate the presence or absence of this antimicrobial agent in the respective cell cultures.

There was a significant increase in the time it took for BCG to grow in MGIT tubes as shown by time-to-positivity (TTP) values in lysates from those cell cultures that received streptomycin compared to lysates from the cultures that did not receive this antibiotic (mean TTP: 242.40 h and 116.35 h respectively, p = 0.002; *Figure 3.4A*). Additional estimates were also made to quantify the growth of BCG in trained monocytes in cells cultured in the presence or the absence of antibiotics. The number of BCG CFUs increased significantly throughout the duration of trained monocyte cultures as higher numbers of CFUs were found in MGIT tubes containing lysates from monocytes cultured in the absence of streptomycin than in tubes containing the inoculi alone (14,361 CFU vs 6,000 CFU respectively, p = 0.008). The growth of BCG in human CD14+ monocytes was significantly slowed down in the presence of this antibiotic compared to both the monocyte cultures in the absence of antibiotics and BCG controls alone (335 CFU, p = 0.008; *Figure 3.4B*). Together, this data suggested that although streptomycin did not fully kill BCG in trained monocyte cultures, it significantly slowed the growth of BCG inside CD14+ human monocytes.

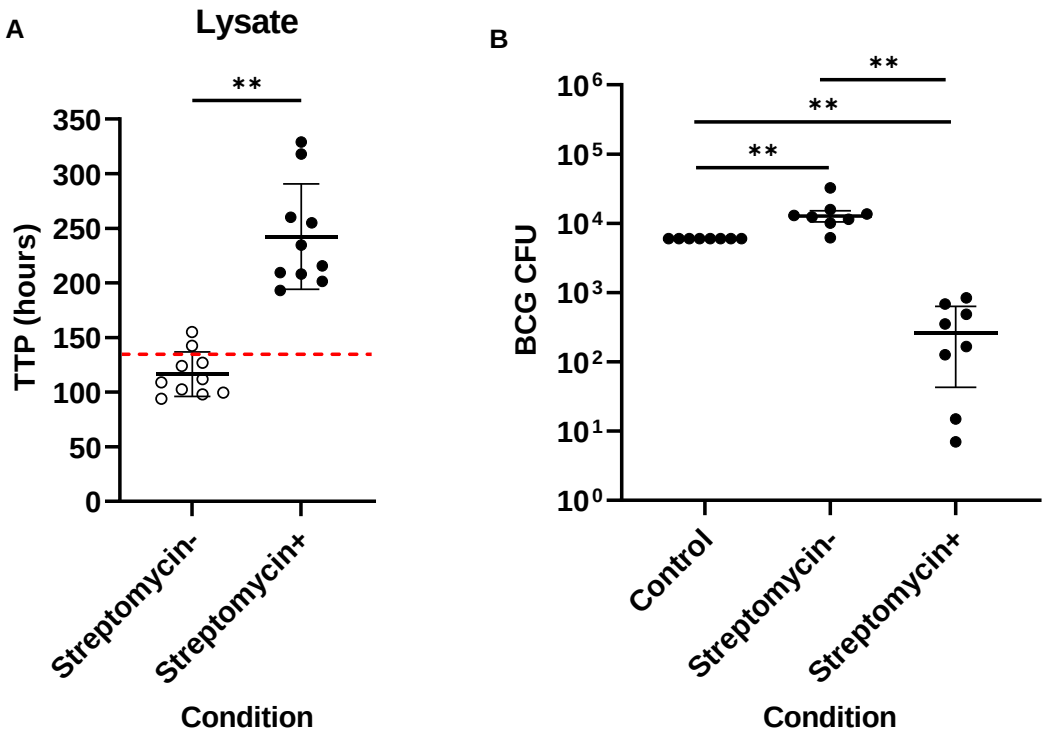



**Figure 3.4.** *Adding streptomycin to BCG-trained monocyte cultures slows the growth of BCG but does not kill it completely*. 300,000 human CD14+ monocytes per well were trained in duplicate with BCG at MOI 1:50 for 24 h on 48-well non-treated plates, washed with PBS and then streptomycin was added to half of the cell cultures. Monocytes were then rested for 6 d, after which the cells were then lysed and their lysates were cultured in BACTEC MGIT960 system for 42 days. **A** shows the TTP values for the lysates extracted from cell cultures treated with streptomycin or control cultures; **B** shows the amount of BCG CFUs in lysates of monocytes BCG-trained monocytes cultured in the presence or the absence of streptomycin or in MGIT tubes containing BCG growth control. Wilcoxon matched-pairs signed-rank test was used to detect the differences in BCG growth. N = 8-10.

To explore the possible role of mycobacterial viability on downstream effects of BCG-dependent training, cytokine concentrations in supernatants of BCG-trained human CD14+ monocytes cultured in the presence or absence of streptomycin during the resting and re-stimulation phases of the assay were also measured. No statistically significant effects of streptomycin were found on the production of IL-6 (*Figure 3.5A*) or TNFα (*Figure 3.5B*).

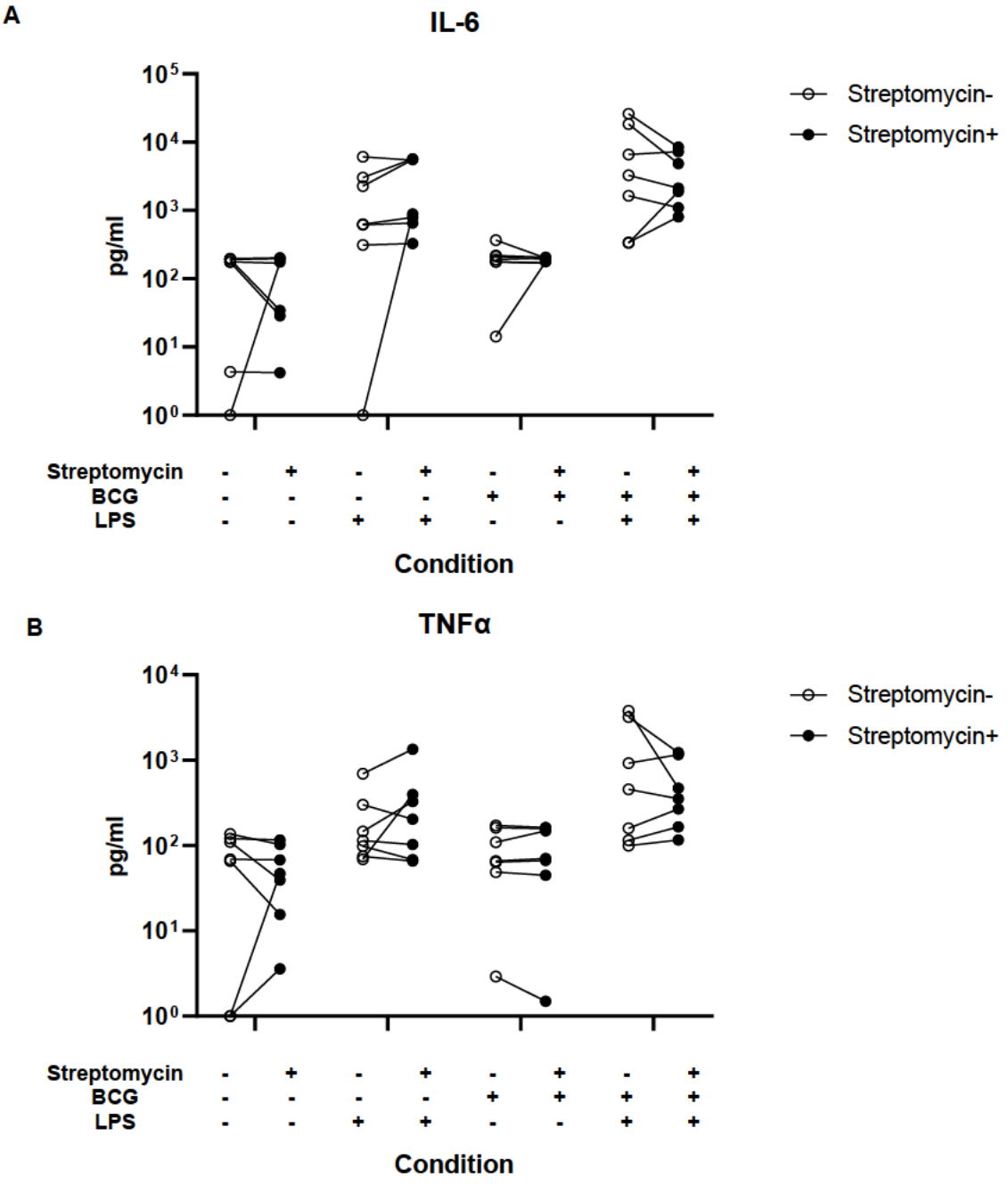



**Figure 3.5.** *The effect of adding streptomycin to BCG-trained cultures after the training step on cytokine production*. 300,000 human CD14+ monocytes per well were trained in duplicate with BCG at MOI 1:50 for 24 h on 48-well non-treated plates, washed with PBS and then streptomycin was added to half of the cell cultures. Monocytes were then rested for 6 d and re-stimulated with RPMI or LPS. The plots show the concentrations of **A** - IL-6; **B** - TNFα. Wilcoxon matched-pairs signed rank test was used to detect the differences in cytokine production in control and streptomycin-treated monocyte culture supernatants. N = 7.

To investigate whether the number of viable BCG in lysates from CD14+ monocytes cultured in the presence or the absence of antibiotics correlated with the IL-6 or TNF concentrations in RPMI- or LPS-stimulated trained monocyte culture supernatants and if so, whether this correlation was linear, the concentrations of these cytokines from the supernatants of the corresponding monocyte cultures were plotted against the number of CFUs detected in lysates

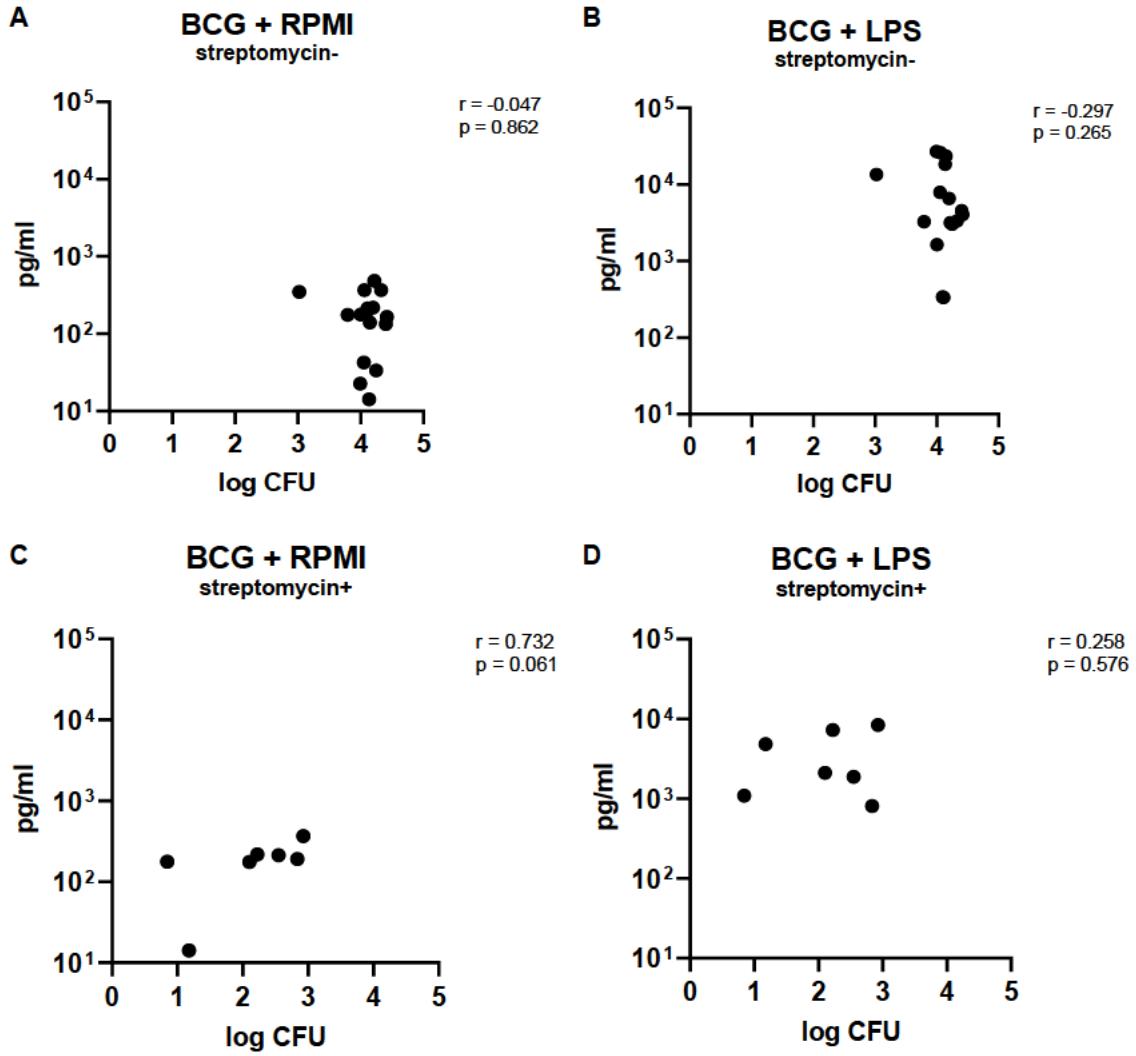



**Figure 3.6.** *Correlation between IL-6 production and the amount of BCG in trained monocyte cultures*. The concentrations of IL-6 in BCG-trained monocyte cultures were plotted against log BCG CFU values obtained in trained monocyte lysate analyses and Pearson r values were calculated to determine if there was a linear correlation between the concentration of IL-6 and BCG CFUs in lysates. The figures show correlations for: **A** – RPMI-treated cultures in the absence of streptomycin, **B** – LPS-treated cultures in the absence of streptomycin, **C** - RPMI-treated cultures in the presence of streptomycin, **D** – LPS-treated cultures in the presence of streptomycin. All data points are shown as the mean of two replicates. N = 7-15.

from trained monocyte cultured in the presence or absence of streptomycin. In this experiment, there was a trend for elevated IL-6 production in those cell cultures trained with BCG that contained higher numbers of BCG CFUs in their lysates; however, this trend was only observed for streptomycin-treated, RPMI-stimulated cell cultures (*Figure 3.6C*). A similar effect was observed in RPMI-restimulated, BCG-trained CD14+ monocyte culture

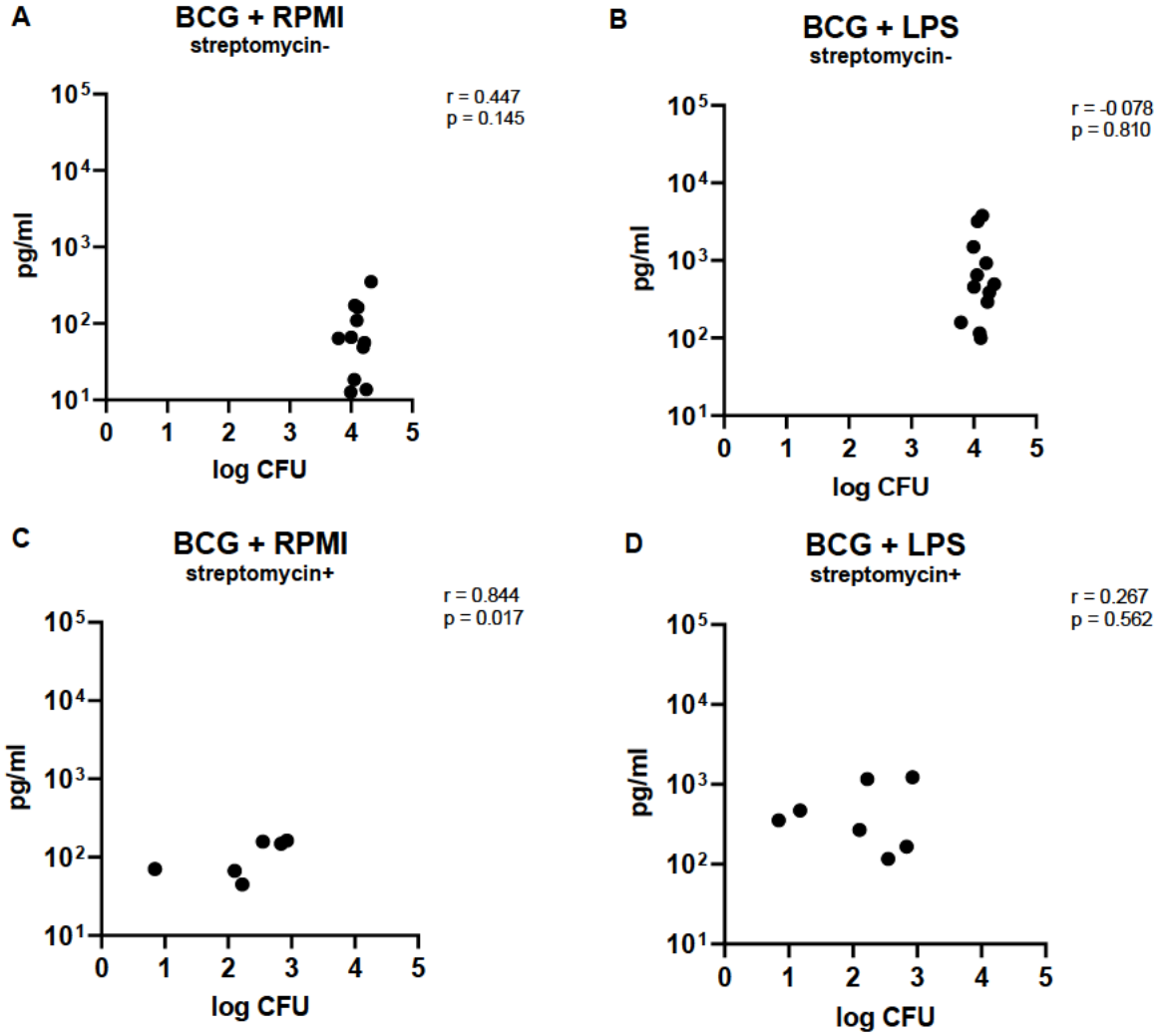



**Figure 3.7.** *Correlation between TNFα production and the amount of BCG in trained monocyte cultures*. The concentrations of TNFα in BCG-trained monocyte cultures were plotted against log BCG CFU values obtained in trained monocyte lysate analyses and Pearson r values were calculated to determine if there was a linear correlation between the concentration of IL-6 and BCG CFUs in lysates. The figures show correlations for: **A** – RPMI-treated cultures in the absence of streptomycin, **B** – LPS-treated cultures in the absence of streptomycin, **C** - RPMI-treated cultures in the presence of streptomycin, **D** – LPS-treated cultures in the presence of streptomycin. All data points are shown as the mean of two replicates. N = 7-12.

supernatants where the concentrations of TNFα correlated with the number of CFUs in lysates from BCG-trained monocytes cultured in the presence of streptomycin (r = 0.844, p = 0.017; *Figure 3.7C*). The correlations between the concentrations of IL-6 (*Figure 3.6*) or TNFα (*Figure 3.7*) and the number of BCG CFUs in lysates from other trained monocyte cultures were not statistically significant, irrespective of whether cells were cultured in the presence of streptomycin or restimulated with LPS.

Together, this suggests that viability of BCG may contribute to the secondary responses in cells trained with BCG; however, this effect is likely to be small and masked by the responses to such potent stimuli as LPS.

### *3.3.2 The effect of killing BCG during the training step*

Because the previous experiment suggested incomplete killing of BCG post-training (*Figure 3.4A and B*) and because streptomycin added after the training step would not have affected BCG during training, human CD14+ monocytes were trained in the presence or absence of streptomycin and lysed on day 6 of resting or re-stimulated with RPMI or LPS for 24 hours to test IL-6 or TNFα production. Again, the TTP conversion rates were statistically significantly higher for lysates from monocyte cultures trained with BCG in the presence of streptomycin compared to lysates from monocytes trained and cultured in the absence of this antimicrobial (mean TTP values 562.2 h and 110.1 h respectively, p = 0.002; *Figure 3.8A*). Similar to the previous experiments, a faster growth of BCG was observed in lysates from monocytes cultured in the absence of streptomycin compared to BCG growth controls, also cultured in the absence of streptomycin (mean BCG CFU values 17,488 and 6,000 respectively, p = 0.006; *Figure 3.8B*). Of interest, even though the TTP signals were positive for lysates from streptomycin-treated trained monocyte cultures, it was not possible to extrapolate the number of BCG CFUs in lysates from these cultures (*Figure 3.8B*). To be able to compare the differences with growth by BCG controls, though, the datapoints from streptomycin-positive cultures were assigned an arbitrary value of 1 CFU. The expected difference between BCG

controls or streptomycin-negative and streptomycin-treated monocyte culture lysates was statistically significant (p = 0.002; *Figure 3.8B*).

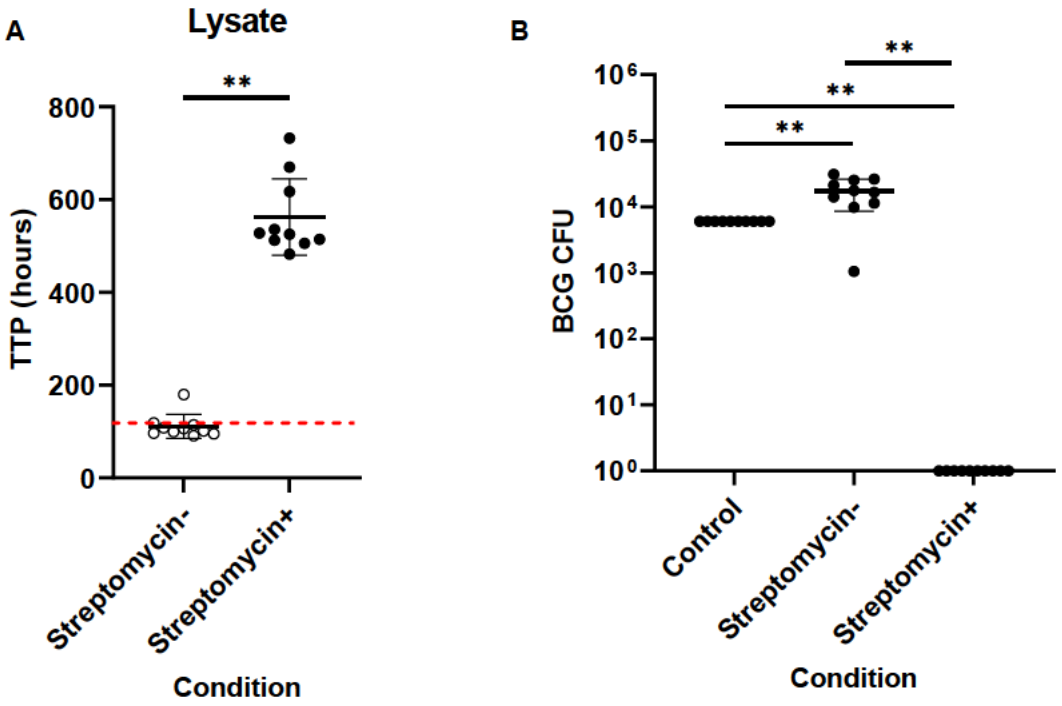



**Figure 3.8.** *The effect of streptomycin on BCG growth in monocytes when added together with BCG*. 300,000 human CD14+ monocytes per well were trained in duplicate with BCG at MOI 1:50 for 24 h on 48-well non-treated plates in the presence or absence of streptomycin. Monocytes were then washed, rested for 6 d in the presence or absence of streptomycin, after which the cells were then lysed and their lysates were cultured in BACTEC MGIT960 system for 42 days. **A** shows the TTP values for the lysates extracted from cell cultures treated with streptomycin or control cultures; **B** shows the amount of BCG CFUs in lysates of monocytes BCG-trained monocytes cultured in the presence or the absence of streptomycin or in MGIT tubes containing BCG growth control. Wilcoxon matched-pairs signed-rank test was used to detect the differences in BCG growth. N =10.

Cytokine concentrations were also measured in supernatants from monocyte cultures trained in the presence or absence of streptomycin. A significant reduction in IL-6 concentration in BCG-trained and control or LPS-challenged cells treated with streptomycin was found (p = 0.049 and p = 0.002 respectively, *Figure 3.9A*). Production of TNFα was also tested in supernatants from control or BCG-trained monocyte cultures in the presence or absence of

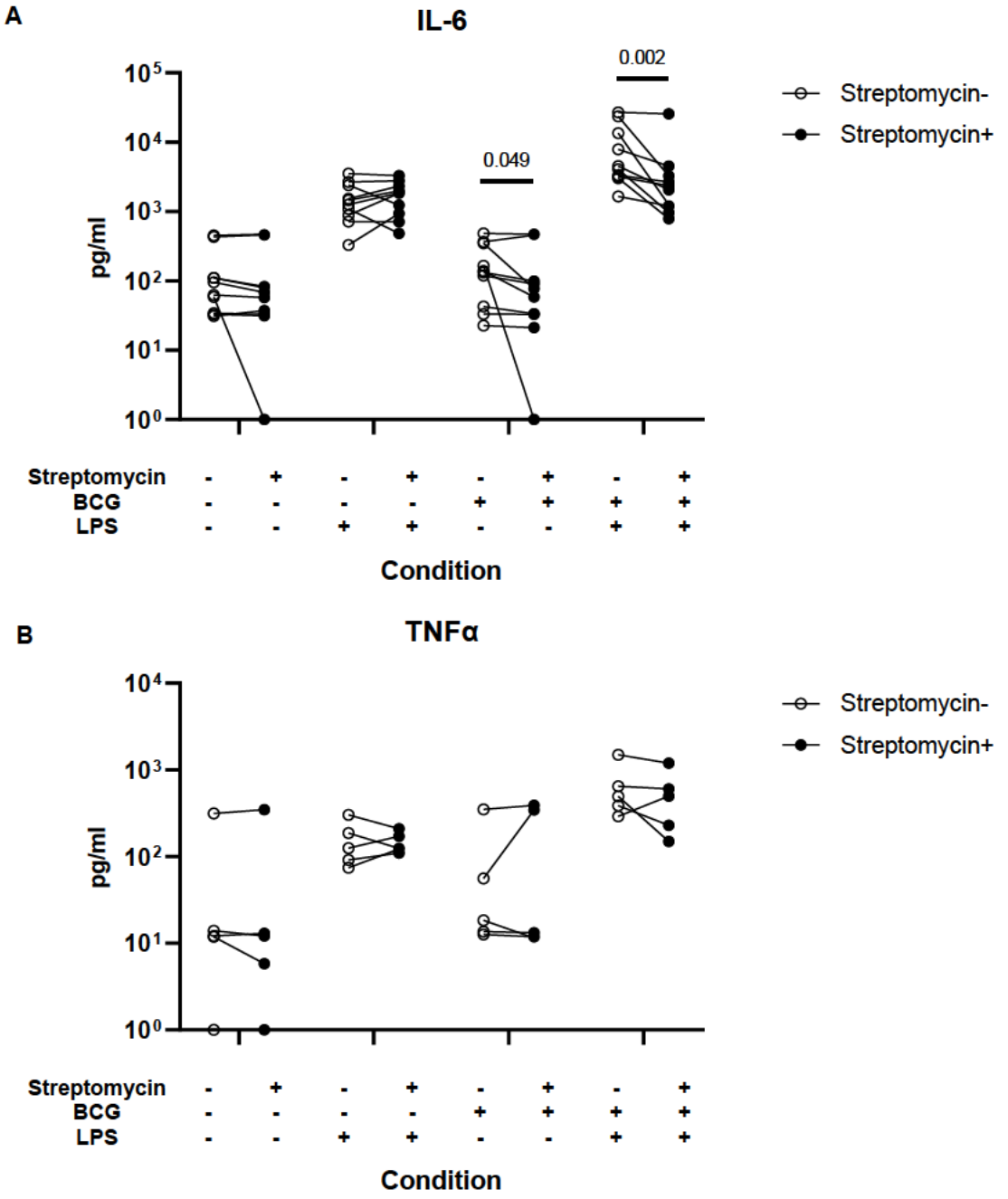



**Figure 3.9.** *The effect of adding streptomycin to BCG-trained monocyte cultures during the training step on cytokine production*. 300,000 human CD14+ monocytes per well were trained in duplicate with BCG at MOI 1:50 in the presence or absence of streptomycin for 24 h on 48-well plates, washed with PBS and then cultured in the presence or absence of streptomycin. Monocytes were then rested for 6 d and re-stimulated with RPMI or LPS. The plots show the concentrations of **A** - IL-6; **B** - TNFα. Wilcoxon matched-pairs signed rank test was used to detect the differences in cytokine production in control and streptomycin-treated monocyte culture supernatants. All data points are shown as the mean of two replicates. IL-6: n = 10, TNFα: n = 5.

streptomycin, however, no concentration differences were detected for this cytokine in

supernatants from cells trained and cultured in the presence or absence of this antibiotic (*Figure 3.9B*). Because it was not possible to extrapolate the amount of CFUs in streptomycin-treated monocyte cultures, the correlation datasets of IL-6 and TNFα concentrations were only generated for monocyte cultures trained and cultured in the absence of streptomycin and analysed together with the data from the previous mycobacterial growth and cytokine production in control and LPS-stimulated trained monocyte cultures (*Figure 3.6, 3.7*).

### *3.3.3 The effect of training human CD14+ monocytes with live or irradiation-killed BCG*

Because the positive TTP signal detected in cultures of monocytes trained with BCG in the presence of streptomycin suggested the presence of some remaining viable BCG, irradiation-killed BCG (γBCG) was used in parallel to live BCG to train CD14+ monocytes. To confirm whether killing BCG by irradiation was successful, lysates from monocyte cultures trained with live or irradiation-killed BCG were cultured in MGIT tubes. In this experiment, the rate of growth of BCG controls or live BCG from trained monocyte lysates was similar to that observed in the previous experiments (mean TTP: 138.7 h and 102.3 h respectively). No growth of BCG was observed in 18 out of 20 lysate replicates from monocytes trained with γBCG, except for lysate samples from two donors (one replicate each, TTP values 349 h and 434 h, equivalent to 1-2 CFU). No growth of γBCG was observed on 7H10 agar plates. Thus, 16 h irradiation was sufficient to kill 99.83% of the BCG.

Next, a direct comparison of cytokine production in response to secondary stimulation of monocytes trained with live or irradiation-killed BCG was made (*Figure 3.10*). Although the production of IL-6 (*Figure 3.10A*) or TNFα (*Figure 3.10B*) did not differ for unstimulated and LPS-re-stimulated control and γBCG trained cells, an increase in IL-6 concentration was observed in supernatants from unstimulated monocyte cultures trained with live BCG compared with control or γBCG-trained cells ($p = 0.005$; *Figure 3.10A*). Similarly, TNFα concentration was also higher in supernatants from monocyte cultures trained with live BCG compared to the control or γBCG trained cells, both in unstimulated or LPS-challenged

cultures (p = 0.003; *Figure 3.10B*). This suggested that viability of BCG may indeed affect the outcome of human monocyte training and their responses to secondary challenges, irradiation-killed BCG having little if any training effect compared to live BCG.

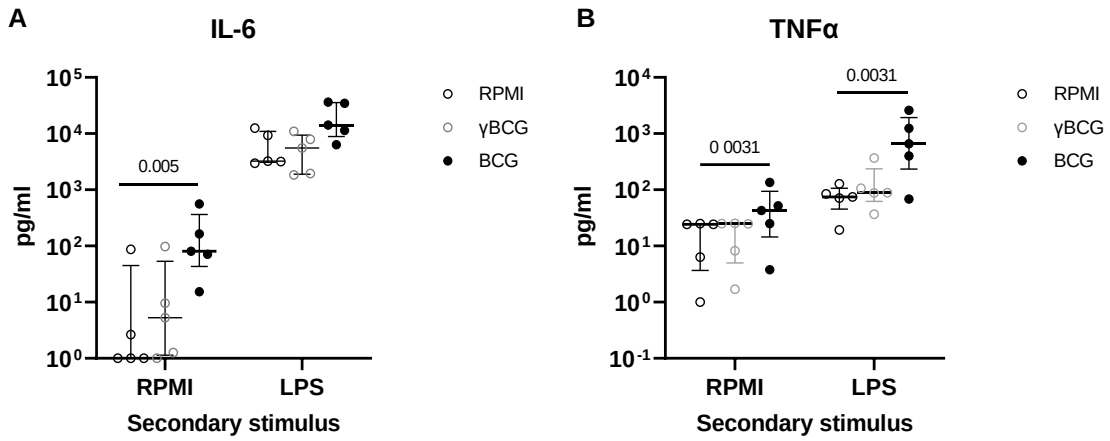



**Figure 3.10.** *Cytokine production by cells trained with RPMI, dead or live BCG*. 300,000 human CD14+ monocytes per well were trained with RPMI, γ-irradiated and live BCG at MOI 1:50, washed with PBS and rested for 6 days. The cells were then restimulated with RPMI and LPS for 24 h and cytokine concentrations in their supernatants were measured by ELISA. The plot shows median concentrations and interquartile ranges of **A** - IL-6 and **B** – TNFα. Friedman test with Dunn's post hoc multiple comparison test was used to detect the differences. N = 5.

To address whether viability of BCG during monocyte training could affect functions other than cytokine production, the association of a fluorescent strain of BCG (dsRed-BCG) with control and live- or dead-BCG trained monocytes at MOI 10:1 was imaged in an experiment using cells from two donors. Although the association of dsRed-BCG and monocytes was observed in all training conditions, it was not possible to determine if dsRed-BCG showed greater binding to or uptake by monocytes trained with BCG(*Figure 3.11*). This method also did not distinguish adherence of BCG and phagocytosis, so further investigations would be required to answer how uptake of microorganisms or phagocytic properties are affected in trained monocytes.

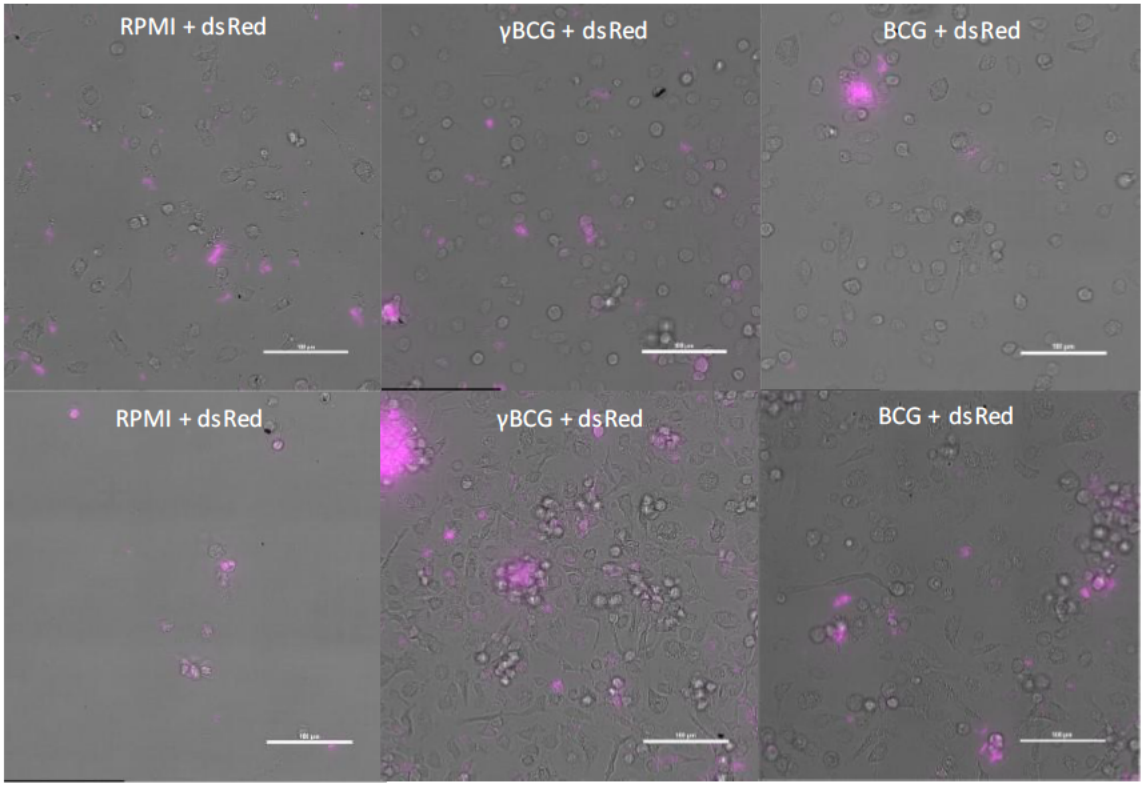



**Figure 3.11.** *The association of fluorescent BCG with control and trained monocytes*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of live or gamma-irradiated BCG at MOI 1:50 for 24 h on 48-well non-treated plates and rested for 6 d with medium replacement on day 3 of resting. The cells were then detached, seeded onto the µ-slides at 100,000 cells per well and re-stimulated with dsRed-BCG at MOI 10:1 for 3 h. Inverted light microscopy images were then taken at 20x magnification. RPMI + ds-Red – training control re-stimulated with dsRed-BCG, γBCG + ds-Red – cells trained with irradiation-killed BCG and challenged with dsRed-BCG, BCG + dsRed – cells trained with live BCG and challenged with dsRed-BCG. The images depict the association of dsRed-BCG with control and trained monocytes from two donors after 3 hour stimulation with this fluorescent BCG strain and the morphology of control and trained cells.

## 3.4 Discussion

### *3.4.1 The effect of adding streptomycin to trained monocyte cultures*

To investigate how the viability of BCG may affect the outcome of human CD14+ monocyte training, and whether there was any synergistic effect of viable BCG on the outcome of monocyte training, control and BCG-trained monocytes were cultured in the presence or absence of streptomycin, a potent antimycobacterial antibiotic. As an aminoglycoside, upon entry into the *M. tuberculosis* cells, streptomycin binds 30S subunits of mycobacterial ribosomes and inhibits their protein synthesis, leading to the bactericidal effects on *M. tuberculosis* cells (288); however, it can also inhibit the growth of BCG (287). In this study, using streptomycin- or irradiation-killed BCG to train monocytes was associated with reduced cytokine production upon heterologous stimulation with TLR agonists but not when monocytes were cultured in the presence of this antibiotic after the training step.

Overall, it was expected that no viable BCG CFUs would be detected in unstimulated or LPS-stimulated BCG-trained monocytes cultured in the presence of this antibiotic as concentrations as low as 0.25 µg/ml have been shown to inhibit the growth of BCG *in vitro* on agar (287), while in this study, a larger final concentration of streptomycin (1%, or 50 µg/ml) was used when culturing BCG-trained monocytes. Contrary to the expectation, a positive TTP signal was detected in the MGIA tubes containing lysates from BCG-trained monocytes cultured in the presence of this antibiotic during the resting and restimulation stages, even though the number of viable CFU detected in these cultures was significantly lower compared to the BCG controls or lysates from BCG-trained monocytes cultured in the absence of streptomycin (*Figure 3.4B*), suggesting an incomplete killing of BCG by this drug. It was possible that streptomycin was not able to kill the BCG CFUs that were internalised by human monocytes during the 24 hours of training, although the experiments on *M. tuberculosis* infected murine macrophages from as early as the 1960s demonstrated the effect of streptomycin against both intracellular and extracellular mycobacteria (289). Other studies on antimycobacterial properties of streptomycin also confirm that addition of streptomycin after human or murine

macrophage infection with *M. tuberculosis* or other mycobacterial species inhibits their growth but does not kill them completely, even at high doses, suggesting a limited effect of streptomycin on the intracellular mycobacterial populations (290–293).

The difference in the quantities of viable BCG detected in lysates from trained monocytes cultured in the presence or absence of streptomycin suggested that if the viability of BCG contributed to secondary responses in BCG-trained cells, there would be corresponding differences expected in cytokine concentrations upon secondary stimulation with TLR agonists. However, the lack of difference in IL-6 or TNFα concentrations in supernatants from BCG-trained unstimulated or LPS-stimulated monocytes cultured in the presence or absence of streptomycin suggested that there was no effect of viability of BCG on human CD14+ cytokine responses to secondary challenges, at least in the cell cultures that received streptomycin after the training step. In agreement with this, the light microscopy images of monocyte cultures trained with BCG and then cultured in the presence or absence of streptomycin also showed no differences to the morphology of trained monocytes, suggesting no effect of streptomycin on the outcome of training. The correlation plots of the concentrations of IL-6 or TNFα and the number of viable BCG in lysates from the corresponding trained monocyte cultures also suggested that there was no correlation between cytokine responses to secondary stimulation and viability of BCG, except for lysates from BCG-trained unstimulated monocytes cultured in the presence of streptomycin, where a correlation between cytokine production and viability of BCG was observed. Overall, this showed that reducing the viability of BCG influenced the quantity of cytokine detected; however, this effect was masked when a strong secondary stimulus for cytokine production, such as LPS, was added. One possible explanation could be that the same number of viable BCG was used to train the cells in both comparison groups and that epigenetic and metabolic changes induced by BCG during the training step would be independent of any further internal stimulation with BCG. However, this does not explain the correlation between viability of BCG CFUs and the concentrations of cytokines in unstimulated trained monocytes cultured with streptomycin. It

is also possible that the overall correlation between the numbers of viable BCG inside the monocytes and cytokine production is not very strong, especially, if different cellular signalling pathways or positive feedback loops are engaged.

### *3.4.2 The effect of applying streptomycin during the training step*

Because the *in vitro* training work (described in *Chapter 2*) suggested that, at least, morphological changes happen to trained monocyte cultures immediately during the training step, it was possible that treating already-trained monocyte cultures with streptomycin after this step did not affect the downstream effects of training. To address how streptomycin would affect human CD14+ monocyte training with BCG, cells were trained in the presence or absence of streptomycin and then rested in the presence or absence of this antibiotic. Although it was expected that streptomycin at 1% (50 µg/ml) final concentration would kill BCG (287), and it was not possible to quantify the number of viable BCG in lysates of monocytes trained and cultured in the presence of streptomycin, positive TTP signals in the MGIT tubes containing lysates from these cell cultures were detected, suggesting that some BCG CFUs were present in these lysates. As 1) the BACTEC™ MGIT™ 960 Mycobacteria Culture System uses fluorometric detection of oxygen consumption by metabolically active bacilli (294); 2) BCG standards including those as low as 1 BCG CFU were used to quantify the numbers of viable BCG CFUs and the TTP signals from those were detected in most measurements; 3) a combination of antibiotics against a range of microorganisms – PANTA – was used in these MGIA experiments; and 4) MGIT tubes where a positive TTP signal was detected had visually detectable colonies with appearance consistent to those of BCG, it was unlikely that the TTP signal was false positive (e.g. due to contamination).

In the case of *M. tuberculosis*, resistance to streptomycin can develop due to the mutations occurring in mycobacterial genes encoding protein or rRNA components of ribosomes, and tolerance to streptomycin can also develop whenever metabolically inactive populations of persisting mycobacteria appear (288). Persister responses and the activity of genes involved

in dormancy regulation and streptomycin resistance in *M. tuberculosis* and other mycobacteria have been previously associated with improved intracellular survival (295). In this work, however, BCG-trained monocytes were only cultured in the presence of streptomycin for 8 days, a period likely not long enough for resistance to develop, especially, as some studies suggest that BCG is inherently less resistant to this drug compared to *M. tuberculosis*, at least *in vitro* (296,297). In the work described in this thesis, the growth of BCG was inhibited by streptomycin. The lowest BCG standards (1 CFU) took ~400 h for a positive TTP signal while lysates from monocytes trained in the presence of streptomycin took 562.2 h on average and were not possible to quantify, suggesting that incomplete killing of BCG was not the only factor contributing to positive signal in these lysates. Slow-growing strains of BCG, although not studied in the context of streptomycin resistance or BCG persistence, have been shown to enhance IL-1β and IL-10 production upon direct stimulation of human monocytes (252). While this suggested that addition of streptomycin to the cells together with mycobacteria or slow growth rates of mycobacteria can affect monocyte or macrophage responses, their training capacity was similar to that of normal-growing strains (252).

To test if the addition of streptomycin during the training step had any effect on cytokine responses to secondary stimulation in this work, concentrations of IL-6 and TNFα were measured in the supernatants from LPS-stimulated human CD14+ monocyte cultures trained with BCG in the presence or absence of this antibiotic or their controls. Although no statistically significant differences were found for TNFα, the addition of streptomycin during the training step resulted in significantly reduced production of IL-6 in LPS-re-stimulated or control monocyte supernatants, suggesting that reducing the viability of BCG during the training step and during monocyte re-stimulation with secondary challenges was important. This effect was stronger than in cell cultures where streptomycin was added after the training step, possibly, due to the antibiotic exerting its antimycobacterial effects prior to BCG being internalised. It also suggested reducing viability of BCG resulted in incomplete training of cells or reducing a possible synergistic effect of BCG CFUs inside trained monocytes. The latter was possible as streptomycin has been previously shown to reduce the percentages of both the TNFα and IL-

10 producing cell frequencies and the rates of cell death in B10R macrophage cultures infected with *M. tuberculosis* (298).

### *3.4.3 The effect of training monocytes with irradiation-killed BCG*

To address whether it was the viability of BCG during the training or the viability of BCG during the restimulation period that mattered for enhancing cytokine responses, human CD14+ monocytes were trained with live or γBCG and their IL-6 or TNFα production in response to challenge with RPMI or LPS was measured. Previous work suggested that, similarly to live BCG, γBCG was also able to reprogram monocytes epigenetically, enhancing accumulation of permissive H3K4me3 marks at the promoters of cytokine encoding genes *in vitro* (217). In contrast to the previous work, in the work described in this chapter only live BCG induced innate immune training compared to the control cells or monocyte cultures trained with γBCG, and this effect was consistent for both IL-6 and TNFα irrespective of whether the cells were unstimulated or stimulated with LPS. It is possible that the dose of γBCG used in this study (MOI 1:50) was insufficient to train monocytes as the previously conducted study found that increasing the dose of γBCG could help achieve the effect of training (217); however, it was not possible to test this because of limited numbers of blood samples available during the COVID-19 pandemic. Unfortunately, it was therefore also not possible to confirm whether it was mycobacterial viability during training or internal synergistic signals from viable bacilli inside trained monocytes that contribute to enhanced IL-6 and TNFα production observed in monocytes trained with live BCG, although it could be argued that internal synergistic signals were likely to contribute as the same MOIs were used for both live and irradiation killed BCG to train the cells and γBCG had been previously shown to induce epigenetic changes (217). As the previous studies also suggested that mycobacterial viability can affect the phagocytic properties of cells (276), this study also attempted to address whether BCG dependent training would improve the phagocytic abilities of monocytes; however, due to extensive clumping of the fluorescent BCG strain, it was not possible to confirm whether BCG-dependent training

improved the phagocytic properties of monocytes and if so, whether that was associated with viability of BCG during training.

### *3.4.4 Summary*

To conclude, this study showed that in monocytes trained with BCG *in vitro*, BCG remained viable not only throughout the training phase, but also throughout the resting and secondary stimulation phases, suggesting a possible synergistic effect that could contribute to the reported enhancement of cytokine production in assays where BCG is used as an agent to train monocyte cultures. This study attempted to address the contribution of this possible synergistic effect and determine at which stage the viability of BCG was the most important to heterologous cytokine responses by killing BCG at different stages of the innate immune training assay. Although complete killing was only achieved for irradiation-killed BCG and this completely abrogated the effect of training, the overall effect of viability of BCG on cytokine responses to LPS was modest and likely to be masked by secondary innate immune responses, especially in those cases where BCG was only partially killed. However, this work suggested that using live BCG to train the cells or during vaccination may induce stronger heterologous cytokine responses.

# **Chapter 4:** Innate immune responses in BCG-vaccinated infants from the UK and South Africa

## 4.1 Introduction

To date, the majority of BCG vaccination studies in infants have focused on adaptive immune responses to this vaccine and its ability to induce long term anti-mycobacterial responses associated with protection against TB (e.g. Th1 or Th17 responses, polyfunctional CD4+ T-cell responses or the ability of infant PBMCs to inhibit mycobacterial growth) (86,299,300). Interestingly, although BCG-vaccinated children have lower risk of developing severe forms of childhood TB, such as TB meningitis or disseminated TB compared to those that did not receive this vaccine (28,29), in the endemic settings, some of the vaccinated infants can still develop TB despite the presence of polyfunctional BCG-specific T-cell responses (113). This suggests that other immunological mechanisms may be involved in mediating BCG-dependent partial protection from TB.

Since the early 2000s, there has been evidence emerging that apart from its antimycobacterial effects, the BCG vaccine exerts heterologous (or non-specific) effects (NSEs) on vaccinated individuals (301). As discussed in *Chapter 1,* these effects range from the reduction in all-cause mortality (133,135,136,144,146,147,155) to lower risk of respiratory or other infections, sepsis (142,161,162,164,165,168,209) or lymphoma (302) in individuals that receive this vaccine. Although it is not fully clear which phenomenon mediates the heterologous effects of BCG, the broad scope of these effects and improved innate immune resistance of BCG vaccinated mice to infectious challenges suggests that the innate immune system might be involved (191,227), although contribution of other mechanisms, such as heterologous T- or B-cell responses may also be possible (301).

As described in *Chapter* 1, although most studies on trained immunity induced by BCG or the heterologous immune responses to this vaccine have been so far conducted *in vitro* or, if *in vivo*, in human adults or animal models of BCG vaccination, there is increasing evidence that BCG can induce similar responses in infants given this vaccine, although the effects observed vary across studies (301). While multiple BCG immunisation studies suggest that there is a beneficial effect on overall infant survival, the confidence intervals for the estimates on the

efficacy of BCG on reducing all-cause infant mortality are broad and may be subject to study design or biases in dataset selection (136,144). Most beneficial effects on all-cause infant mortality have been observed in settings with high infant mortality (133,135,147,164), however, other NSEs, such as reduction on acquisition of infectious diseases in children have been detected across broader settings, including West or Sub-Saharan Africa (142,164,165), or Spain (161), although it is also possible this may be related to the burden of infectious diseases – a recent study in Denmark found no difference on the risk of hospitalization due to infectious diseases in infants who received the BCG vaccine and the control infants (159).

There are some differences in immunological findings from infant BCG vaccination studies carried out in geographically diverse settings as well (301). The MIS BAIR infant BCG vaccination study in Australia found lower IL-1RA, IL-6, IL-10, MIP-1α, MIP-1β and MCP-1 responses to whole blood stimulation with TLR agonists or lower MCP-1 responses to non-mycobacterial microorganisms at 7 days post birth (238), reduced IFNγ responses to heterologous microorganisms and TLR agonists at 7 months of age (233) and higher neutrophil counts (218) in BCG-vaccinated infants compared to unvaccinated controls. Elevated production of EGF, IL-6 and PDGF-AB/BB in response to whole blood stimulation with Pam3Cys or non-mycobacterial microorganisms was found in BCG-vaccinated infants from the United Kingdom at 4 months post vaccination (223). Enhanced production of IL-6, TNFα and IFNγ was also found in whole blood cultures from low-birth-weight (LBW) infants in Guinea-Bissau upon stimulation with PMA or Pam3Cys at 4 weeks post BCG randomisation (236). In another study from South Africa, BCG-vaccinated infant whole blood cultures produced higher levels of IL-8, MCP-1, MIP-1β, EGF and IFN-α2 (239). In Uganda, a reduction in histone methylation levels was found in the promoter of the TNFα encoding gene in BCG-vaccinated infants at 6 weeks of age, suggesting production of this cytokine could be affected upon secondary stimulation (164). In contrast to these studies, in Denmark, no differences were found in whole blood cytokine production in response to stimulation with non-mycobacterial microorganisms at 4 days post BCG randomisation or at 3 and 13 months of

age (237). No differences in frequencies of neutrophil or other leukocyte populations were detected in infants that received BCG and control infants at these time points either, although BCG vaccination was associated with a transient reduction or increase of monocytes depending on whether the infants were vaccinated within the first day of birth or at 2 days of age or later (303).

Multiple factors can contribute to differences in human responses to vaccines. These include age at BCG vaccination, or the measurement of their immune responses, sex of the vaccinees, nutrition, infections, environmental factors and many others (304). It is also possible that population of origin – due to genetic or other factors - could contribute to some differences found in these studies. There is evidence suggesting that *M. tuberculosis*-specific immune responses may differ in vaccinated individuals from different countries; for instance, in a study comparing anti-tuberculosis responses in BCG-vaccinated UK and Malawian infants, almost all UK infants had detectable IFNγ responses to PPD stimulation at 3 and 12 months of age while only 53% and 48% of Malawian infants produced IFNγ in response to PPD (305). In addition, differences were observed not only for IFNγ but for cytokines produced by the innate immune cell populations as well: IL-6, IL-12p40, MIP-1α, IP-10 were produced at higher levels in PPD-stimulated whole blood samples from the UK infants while IFNα2, IL-1α, IL-1RA, IL-10, MCP-3, MDC, PDGF-AA, PDGF-AA/BB, GM-CSF or G-CSF elevated in PPD-treated whole blood cultures from the Malawian infants at 3 months post BCG vaccination (306). Differences in the production patterns of innate cytokines (IL-1α, IL-6, TNFβ, TGFα, IL-12p70, RANTES and IP-10) were also found in a study comparing Malawian and the Gambian infant whole blood responses to PPD (307). Of interest, for some of these cytokines this difference remained significant even when season of birth was adjusted for (307). Together, these differences suggest that innate immune responses to the BCG vaccine, mycobacterial components or other stimuli could also vary across different populations and contribute to differences in NSEs of BCG or its mediated protection against TB.

The work described in this chapter was inspired by the hypothesis that innate immune responses to mycobacteria and heterologous stimuli may differ in BCG-vaccinated infants from different populations and that these differences could contribute to BCG-immunised infant protection against TB and other infectious diseases. To address this hypothesis and potential issues associated with variation, however, a comparative study of innate immune responses of BCG-vaccinated infants from different settings would need to be made, with the participants recruited using the same study protocol and the same strain of BCG. Although it was not possible to perform such a comparative study as part of this thesis, samples were available from two small BCG vaccination cohorts from the UK and South Africa and were exploited to investigate the expression of surface markers and intracellular cytokines in supernatants associated with trained monocyte phenotype in PBMCs in 10-week-old BCG-vaccinated infants from the UK and South Africa. In addition, this study also examined the profiles of cytokines associated with trained immunity, heterologous effects of BCG or protection against TB and the expression of genes associated with protection against TB in PBMCs from these two groups stimulated with BCG or TLR agonists. To explore if the responses observed contributed to protection against mycobacterial infections, this study compared the ability of PBMCs from the UK and South African infants to inhibit the growth of BCG. Finally, to address whether sex of the infants contributed to their antimycobacterial or heterologous innate immune responses, differences in surface marker expression, cytokine profiles, expression of genes associated with protection against TB, and the ability to inhibit mycobacterial growth was examined in male and female UK infants.

## 4.2 Methods

### *4.2.1 Study participants*

Samples were available from 10 week old infants who received the BCG vaccine at birth or shortly after in the UK and South Africa.

The BCG-vaccinated UK infant cohort has been described before (308). In this study arm, infants offered the BCG vaccine as per UK National BCG immunisation programme were enrolled at North Middlesex Hospital, London, United Kingdom in the UgUK Infant BCG Study upon their parents' or guardians' voluntary informed consent. In this cohort, samples were collected from healthy 10-week-old-infants vaccinated with the InterVax BCG vaccine (strain SL222 Sofia, (309)). Samples from infants born after a complicated delivery, those born with low birth weight (≤2500 g) or from twins were excluded from this study. This study received approval from NRES Committee London – Camden & Islington (#15/LO/0048) and LSHTM Ethics Committee (reference #8720 and #14576) and the cohort of infants.

The BCG vaccination study conducted by the South African Tuberculosis Vaccine initiative has been described before (113,310). Briefly, in South Africa, samples were obtained from 10-week-old infants vaccinated with BCG (strain Tokyo 172) at birth and enrolled in a BCG vaccination study at the SATVI vaccine trial site in the Worcester area near Cape Town upon their parents' or guardians' voluntary consent. Infants that received their BCG vaccine later than 24 hours since birth, had a mother living with HIV, symptoms of an acute or chronic disease at the time of enrolment, anaemia, perinatal complications or had been in contact with individuals with TB disease in their household, were excluded from this study. Infants included in this study were followed up for two years and only samples from those infants that did not develop TB were used in the comparison of the UK and South African infant innate immune responses. The BCG vaccination study conducted by SATVI was approved by the University of Cape Town Research Ethics committee. Sample use for the investigation of innate immune responses in the UK and South African samples was approved by the LSHTM Ethics Committee (reference #8720-1 and #14576-2).

The PBMC samples and associated clinical data on the South African infant cohort were collected by SATVI as part of studies comparing immune responses to intradermal and percutaneous BCG and funded by Bill and Melinda Gates foundation. Blood samples and associated clinical data from the UK cohort were collected at North Middlesex Hospital by the research nurse Mrs Rose Blitz as part of the UgUK infant BCG vaccination study (TBVAC, funded by the EU).

### *4.2.2 Sample processing*

A sample of 2-5 ml peripheral venous blood was collected at North Middlesex Hospital by a trained phlebotomist from each participant in the UK infant group. The blood was then delivered to LSHTM (mean time from bleeding till sample receipt: 2 h 29 min; ±34 min) and processed immediately. Briefly, the samples were diluted with pre-warmed Hank's Balanced Salt Solution (HBSS; Life Technologies, UK) and carefully overlaid over 3 ml Histopaque®-1077 (Sigma-Aldrich, UK). Then, the samples were centrifuged at 800 x g for 20 min with brake off. PBMC layers were then collected and washed three times with HBSS by spinning at 400 x g for 5 min. Then the pellets were resuspended in 1 ml Roswell Park Memorial Institute 1640 medium (RPMI) with 20% FCS and small aliquots were stained with Trypan Blue stain, 0.4% (ThermoFisher Scientific, USA) and counted with an automated cell counter (Countess II; ThermoFisher Scientific, USA). The large aliquots were cooled on ice for 15 min and then 1 ml of FCS (Life Technologies, UK) with 20% DMSO (Sigma-Aldrich, UK) was added dropwise to the cell suspension. The cells were then transferred to cryovials in duplicate and stored at -70$^{o}$C overnight. After this, the cells were transferred to liquid nitrogen for long term storage until further use.

Cryovials with frozen PBMCs from South African infants were kindly donated by Dr Thomas Scriba from University of Cape Town and were stored in liquid nitrogen until further use.

### *4.2.3 Cell processing for functional analyses*

Aliquots of PBMCs from both the UK and South African infant groups were thawed rapidly at 37$^{o}$C with constant, gentle swirling and then transferred to centrifuge tubes containing pre-heated RPMI supplemented with 10% foetal calf serum (FCS). The cells were then washed by spinning at 500 x g for 5 min. After resuspending cells in 5 ml RPMI with 10% heat-inactivated pooled AB serum (HI ABS) (Sigma-Aldrich, USA), benzonase (Novagen; final concentration 10 U/ml), a nuclease to prevent cell clumping, was added to each tube. Infant PBMCs were then incubated at 37$^{o}$C for 2 hours, centrifuged for 5 min at 500 x g and carefully resuspended in 1 ml RPMI with 10% HI ABS. PBMCs were counted and diluted to 2.0 x $10^{6}$ cells/ml for further analyses.

### *4.2.4 FACS analysis*

Infant PBMCs (2.0 x $10^{5}$ cells/tube) were added to polypropylene FACS tubes containing RPMI with 10% HI ABS, 20 ng/ml ultrapure *E. coli* O55:B5 lipopolysaccharide (LPS; Invivogen, USA), 20 µg/ml N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine (Pam3Cys; Invivogen, USA) or 10,000 BCG CFU. PBMCs were incubated at 37$^{o}$C, 5% $CO_2$ for 2 h, after which brefeldin A (Sigma-Aldrich) was added at the final concentration of 3 µg/ml and the cells were further incubated for another 4 hours.

After this, the cells were washed with PBS followed by centrifugation at 500 x g for 5 min, stained with pre-titrated LIVE/DEAD Fixable Aqua Dead Cell Stain viability dye (Life Technologies, USA) and incubated at room temperature (RT) for 15 min. This was followed by blocking of surface receptors with 10 µl polyclonal Anti-human Fc Receptor Binding Inhibitor (eBioscience, USA) and further 15 min incubation at 4$^{o}$C, followed by surface staining (*Table S4.1*) with 2.5 µl αCD14-V450 (MΦP9; BD, USA), 2.5 µl αCD16-AF700 (3G8; BD, USA), 2.5 µl αHLA-DR-PerCP-Cy5.5 (L243; BioLegend), 2.5 µl αTLR4-PE (HTA125; BioLegend), 1.25 µl αCD11b-BV711 (ICRF44; BioLegend) in 50 µl final staining volume at for 4$^{o}$C for 30 min. This was followed by a wash with FACS buffer (PBS, 0.1% BSA (Sigma-

Aldrich, USA), 0.01% sodium azide (Sigma-Aldrich, Germany)). For intracellular staining, PBMCs were permeabilized with Cytofix/Cytoperm buffer (BD, USA) at 4°C for 20 min, washed with Perm/Wash buffer (BD, USA) at 750 x g for 5 min. Antibodies for intracellular staining were then added to each tube: 2.5 µl αIL-6-FITC (B-E8; eBioscience, USA) and 2.5 µl αTNFα-APC (Mab11; BioLegend) in 50 µl final staining volume (*Table S4.1*). The cells were incubated at RT for 30 min, washed and fixed overnight with 1% paraformaldehyde (Sigma-Aldrich, Germany). The following day the cells were filtered and transferred to polystyrene FACS tubes for surface receptor expression and intracellular cytokine measurement on LSR II (BD, USA).

Data (100,000 total events) was acquired using FACSDiva (BD Biosciences, USA) and analysed post-acquisition using FlowJo software (version 10; BD Biosciences, USA). Antibody-stained beads (BD Biosciences, USA) were used for compensation and Fluorescence Minus One (FMO) cellular controls were used to set the gates.

### *4.2.5 PBMC stimulation for qRT-PCR and multiplex bead array analysis*

Infant PBMCs (2.0 x $10^5$ cells/well) were loaded in duplicates to 96-well plates containing RPMI with 10% HI ABS, 40 ng/ml ultrapure *E. coli* O55:B5 lipopolysaccharide (LPS; Invivogen, USA), 40 µg/ml Pam3Cys (Invivogen, USA) or 5,000 BCG CFU. PBMCs were then cultured for 24 h at 37°C, 5% $CO_2$ in 200 µl final volume of cell culture medium or medium containing each stimulus. After this, PBMCs were centrifuged at 400 x g for 5 min. Cell supernatants were then collected in microcentrifuge tubes, and PBMCs were pooled and lysed in 200 µl total RLT buffer volume (QIAgen, Germany) for each stimulation condition. Both PBMC supernatants and cell lysates were frozen and stored at -70°C until further analysis.

### *4.2.6 Multiplex bead array (MBA) analysis*

The workflow of two experiments investigating the cytokine profiles associated with trained immunity or antimycobacterial responses is outlined in Figure 4.1.

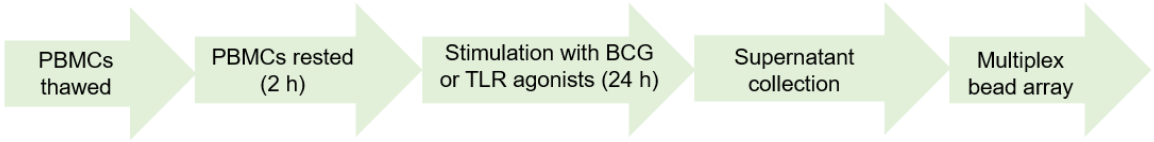



**Figure 4.1.** *The workflow of analysis of cytokine responses to BCG and TLR agonists using infant PBMC samples from the UK and South African cohorts*.

Supernatants from infant PBMCs stimulated with cell culture medium or TLR agonists were thawed and analysed using multiplex bead array using the human cytokine/chemokine Milliplex MAP Human Cytokine/chemokine Magnetic Bead Panel (Merck-Millipore, USA). The kit covered the following analytes: IL-1α, IL-1β, IL-1RA, epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-AB/BB, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6, TNFα, CCL2 (MCP-1), CXCL10 (IP-10), and IL-12p40 (*Tables S4.2*). Supernatants were processed as per the manufacturer's instructions.

Briefly, one day before the experiment, microbeads for each cytokine of interest were warmed to room temperature (RT), sonicated for 30 s and vortexed for 1 min. Then 60 µl of each type of microbeads were added to a bottle and diluted with a Bead Diluent to a final 3.0 ml volume. The beads were then mixed and stored at 4$^{o}$C until further use. The wash buffer was then prepared using 10X concentrate diluted with deionized water and stored at 4$^{o}$C until further use. On the day of the experiment, the cell culture supernatants were thawed, the beads and the wash buffer warmed to RT. Quality controls and a series of five-fold dilutions of human cytokine standards were prepared immediately before running the assay.

The plate was gently washed and dried before adding 25 µl of cytokine standards and quality controls (in duplicate) to the appropriate wells, followed by 25 µl assay buffer added to all the wells. The samples were then loaded to the appropriate wells in single 25 µl replicates. This was followed by adding 25 µl vortexed microbeads to each well on the plate, which was then sealed and incubated at RT for 2 hours on a plate shaker. The plate was then washed twice with 200 µl wash buffer before adding 25 µl detection antibodies to each well. This was

followed by 1 hour incubation on a plate shaker and another 30 min incubation with 25 µl streptavidin-phycoerythrin per well. The plate was then washed two more times and 150 µl PBS was then added to each well. The beads were then resuspended by vortexing the plate for 5 min.

Data were acquired using the Magpix system (Luminex, USA) and xPONENT software (Luminex, USA) and analysed using GraphPad Prism 9 software (GraphPad Software, USA). During the analysis of cytokine production in infant PBMC samples, in those cases where cytokine concentration values exceeded those of the top cytokine standard, they were assigned an arbitrary value of 10,000 pg/ml, and if they were lower than the lowest cytokine standard (3.2 pg/ml), they were assigned an arbitrary value of 1.6 pg/ml.

### *4.2.7 Literature search for genes of interest and primers for the qPCR analysis*

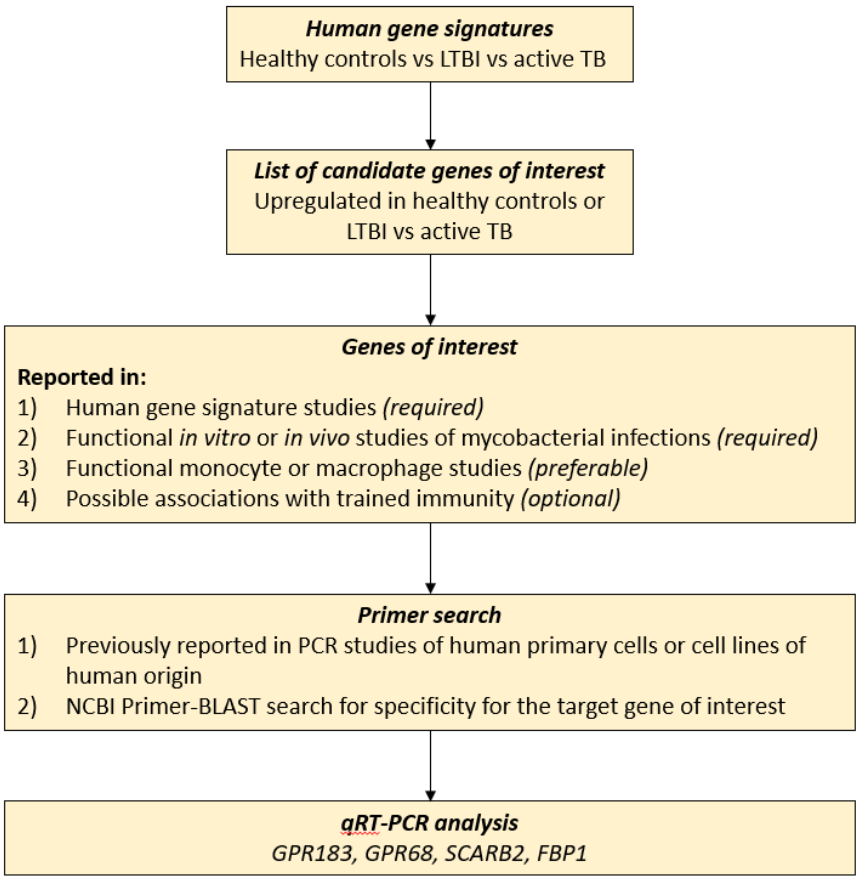



**Figure 4.2.** *Workflow of gene of interest and primer selection for qRT-PCR experiments.*

A literature search for human gene signature studies in healthy individuals, LTBI and active TB patients was performed in the NCBI PubMed database. A list of candidate genes of interest, including those that were expressed at higher levels in healthy controls or LTBI patients compared to individuals with active TB was made. A subsequent search was then run in PubMed using the symbols of genes of interest and keywords “TB” or “tuberculosis” or “mycobacterium” or “mycobacterial infection” or “BCG”. This was a required criterion for the selection of gene of interest for further analysis. Additional searches using the same symbols for genes of interest and keywords “monocyte” or “macrophage” were run to investigate if the genes of interest or their downstream products were expressed by cells of monocytic lineage.

The genes of interest were also searched for associations with trained innate immunity; however, as this phenomenon has been only relatively recently characterised and its role in the protection against TB is unclear, this criterion was only additional in selecting genes of interest for the qRT-PCR analysis. A literature search was then run for the primer pairs reported in previous qRT-PCR analyses of these genes (*Figure 4.2)*.

**Table 4.1**. *Primers used in amplification of cDNA from infant PBMCs*

| Symbol | Name of target gene | 5'→ 3' primer sequence | Direction | Reference |
|---|---|---|---|---|
| **FBP1** | Fructose-bisphosphatase 1 | GATTGCCTTGTGTCCGTTG | Forward | (311,312) |
| | | TGCCATACAGTGCGTAGCC | Reverse | |
| **GPR68** | G-protein coupled receptor 68 | CAACTCCTCGATGAGCTGTACCA | Forward | (313) |
| | | AGGTAGCCGAAGTAGAGGGACA | Reverse | |
| **GPR183** | G-protein coupled receptor 183 | TGCACCCTCTACGCTACAACAAG | Forward | (314) |
| | | ATGCCCCAAGCAGAATCCAG | Reverse | |
| **SCARB2** | Scavenger receptor class B member 2 | GTACTGAGGCATTTGACTCCT | Forward | (315) |
| | | AGTTCCCTGTAGGTGTATGGC | Reverse | |
| **HuP0*** | Human large ribosomal protein P0* | GCTTCCTGGAGGGTGTCC | Forward | (316) |
| | | GGACTCGTTTGTACCCGTTG | Reverse | |

* In NCBI Gene database, gene symbol of this gene is reported as RPLP0 and the name of the target gene is "ribosomal protein lateral stalk subunit P0" (317).

Only those primer pairs that had been previously used in PCR studies of primary human cells or cell lines of human origin were considered for the selection. A search was then run on NCBI Primer-BLAST tool to check the specificity of the selected primer pairs for the genes of interest. Primer pairs were then selected for four genes of interest which were selected for further analysis: *GPR183 (G protein-coupled receptor 183)*, *GPR68 (G protein-coupled receptor 68)*, *SCARB2 (scavenger receptor class B member 2)* and *FBP1 (fructose 1,6-bisphosphatase 1); Figure 4.2; Table 4.1.*

### *4.2.8 qRT-PCR analysis*

RNA from infant PBMCs was extracted using RNeasy RNA extraction kit (QIAgen, Germany) as per manufacturer's instructions. Briefly, frozen cell lysates were thawed on ice, diluted with

an equal volume of 70% molecular grade ethanol (Fisher Scientific, UK), vortexed and transferred to RNeasy spin columns in 2 ml centrifuge tubes. The lysates were then centrifuged at 12,000 x g for 15 s, supernatants poured off and the tubes blotted against absorbent tissue. This was followed by two 350 µl washes with RW1 buffer and two 500 µl RPE washes, with the tubes and columns spun at 12,000 x g for 2 min during the second wash. The columns were then transferred to fresh 2 ml centrifuge tubes and spun at 12,000 x g for 1 min and then transferred again to 1.5 ml microcentrifuge tubes. Then 25 µl of nuclease-free water (Ambion, USA) was added to each column directly onto the membrane and the tubes were incubated for 1 min at RT. The tubes were then spun at 14,000 x g for 1 min to elute RNA. Concentration of RNA was measured by a NanoDrop DS-11 FX spectrophotometer (DeNovix, USA) and the samples were frozen and stored at -70$^{o}$C until analysis by qRT-PCR.

On the day of qRT-PCR analysis, RNA samples were thawed on ice and 50 ng of RNA from each sample was transferred to 0.2 ml microcentrifuge tubes (DeNovix, USA). Genomic DNA was then digested with ezDNAse (ThermoFisher Scientific, Lithuania) on ice for 2 minutes and then 10 µl master mix containing primers, reverse transcriptase and nucleotides (SuperScript IV VILO Master Mix; ThermoFisher Scientific, Lithuania) was added to each reaction tube. Tubes containing 20 µl reaction were then transferred to the BIO-RAD T100 thermal cycler (Bio-Rad Laboratories, Inc.) and the following protocol was run: 37$^{o}$C for 2 min, 4$^{o}$C for 10 min, 25$^{o}$C for 10 min (primer annealing), 50$^{o}$C for 10 min (reverse transcription) and 85$^{o}$C for 5 min (enzyme inactivation), 4$^{o}$C until further processing. The resulting complementary DNA (cDNA) was then stored on ice until loaded into plates for qRT-PCR experiments.

Primers used to amplify cDNA from infant PBMC samples were purchased from Sigma-Aldrich (United Kingdom) and are described in *Table 4.1*. Lyophilized primers were reconstituted in nuclease free water to reach a stock concentration of 100 µl and stored at -20$^{o}$C until further use. Twenty microliter qRT-PCR reactions were then set up in triplicate in 96-well PCR plates (4titude, UK) using 15 µl reaction mix containing 10 µl Fast SYBR$^{TM}$ Green Master Mix (Applied

Biosystems, Lithuania), 1 µl of each forward and reverse primers and 3 µl nuclease-free water and 5 µl of 1:5 diluted cDNA for each stimulation condition. The qRT-PCR reaction was run on an ABI Fast7500 system, using the protocol described in *Table 4.2.* Data from qRT-PCR experiments was acquired using Applied Biosystems 7500 Fast Real-time PCR software (version 2.3; ThermoFisher Scientific, USA).

**Table 4.2.** *ABI 7500 Fast protocol used to amplify cDNA from the infant cells*

| Stage | Temperature | Duration | Number of cycles |
|---|---|---|---|
| **Holding** | 50°C | 2 min | |
| | 95°C | 2 min | |
| **Cycling** | 95°C | 15 s | 40 |
| | 60°C | 1 min | |
| **Melting curve** | 95°C | 15 s | |
| | 60°C | 1 min | |
| | 95°C | 30 s | |
| | 60°C | 15 s | |

### *4.2.9 Mycobacterial growth inhibition (MGIA) analysis*

The experimental workflow outlining the mycobacterial growth inhibition experiments is shown in *Figure 4.3.*


**Figure 4.3.** *The workflow of mycobacterial growth inhibition experiments using infant PBMC samples from the UK and South African cohorts.*

The methods used to analyse the ability of infant PBMCs to inhibit mycobacterial growth have been described before (114). One million infant PBMCs were added in duplicate to 2 ml sterile screw-cap tubes containing 500 colony forming units (CFUs) of BCG-Pasteur (Aeras, USA).

A series of 10-fold dilution of BCG standards (2 x $10^5$ to 2 x $10^0$ CFU/ml in sterile water) and control BCG inoculi containing 1000 CFU/ml in sterile water were prepared from the BCG stock solution (6 x $10^5$ CFU/ml in sterile water) and loaded in duplicate to mycobacterial growth inhibition tubes (MGIT; BD, USA; 500 µl/tube) containing growth supplement and reconstituted PANTA™ Antibiotic Mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; BD, USA) to reduce the risk of MGIT tube contamination with other bacterial species and OADC (oleic acid, albumin, dextrose and catalase) enrichment broth (BD, USA), vortexed and cultured in Bactec MGIT960 system (BD, USA) for 42 days at 37ºC.

The screw-cap tubes containing PBMCs and BCG were rotated for 96 h. After this, the tubes were centrifuged for 10 min at 12,000 rpm. Supernatants were taken off by careful pipetting and the cells were lysed by adding 500 µl tissue-culture grade sterile water (Sigma-Aldrich, UK), vortexing the cells for 30 s three times with 5 min breaks between each pulse. The cell lysates were then transferred to the MGIT tubes containing growth supplement and PANTA antibiotic mixture and OADC and transferred to BACTEC MGIT960 incubator for culture until a positive signal was obtained or the end of a 42-day protocol.

### *4.2.10 Statistical analyses*

A power size calculation was made to determine the number of samples to be used in this study using data from pilot FACS experiments using StataSE 16 software (StataCorp LLC, USA). It was estimated that to detect a 12.21% difference in frequencies of TNFα+IL-6+ CD14++CD16- cells at 90% power and 5% statistical significance threshold, 23 samples would be required in each – UK and South African – sample group. However, as the UK and South African cohorts were not recruited as a part of a single study, there were a number of

differences between them (discussed in section 4.3.1), which meant that a direct comparison between the two cohorts was not valid.

To determine if there were differences in the clinical characteristics of the UK and South African infants, the unpaired t-test was applied to compare the mean maternal age, mean gestation duration, mean age at BCG vaccination and mean age at blood sampling. Fisher's exact test was used to investigate the differences in the proportions of male and female infants or infants that received intradermal or percutaneous vaccination. To investigate 1) the per cent contribution of stimulus and donor variation to total variation in the UK and South African infant responses to BCG and TLR agonists and 2) the per cent contribution of sex, stimulus and donor variation to the total variation in the UK male and female infant innate responses to BCG and TLR agonists, two-way ANOVA was used in the FACS, qRT-PCR and MBA analyses. This test was followed by Šidák's multiple comparison test used for pairwise comparisons between UK male and female infant responses and for the comparisons of BCG- or TLR agonist- stimulated PBMC responses with baseline surface receptor, intracellular cytokine or gene expression or baseline cytokine concentrations. Mann-Whitney U-test was used in MGIA analyses for pairwise comparisons of the ability of PBMCs from the UK male and female infants or the UK infants vaccinated within the first day of birth or later to inhibit the growth of BCG.

All datasets were analysed using GraphPad Prism 9 software (GraphPad Software, USA).

## 4.3 Results

### *4.3.1 Study participants*

**Table 4.3.** *Characteristics of the UK and South African (SA) infants whose PBMC samples were investigated in this study*

| **Characteristics** | **UK** (n = 21) | **SA** (n = 27) | *P-value* |
|---|---|---|---|
| Mean maternal age (years) | **35.94** | **27.38** | ***<0.0001*** |
| Mean birth weight (kg) | 3.47 | 3.19 | *n. s.* |
| Mean gestation (weeks)* | 39.35 | 39.8 | *n. s.* |
| Caesarian delivery, no. (%) | NA | 4 (14.81) | - |
| Vaginal delivery, no. (%) | NA | 22 (81.48) | - |
| Unknown, no. (%) | NA | 1 (3.70) | - |
| Male, no. (%) | 13 (61.90) | 12 (44.44) | *n. s.* |
| Mean age at BCG vaccination (days) | **2.33** | **0.30** | ***0.0006*** |
| Intradermal BCG, no. (%) | **21 (100)** | **19 (70.37)** | ***0.0064*** |
| Black African, no. (%) | 4 (19.05) | NA | - |
| Other Black, no. (%) | 1 (4.76) | NA | - |
| White British, no. (%) | 9 (42.86) | NA | - |
| Other White, no. (%) | 6 (28.57) | NA | - |
| Asian (Pakistani), no. (%) | 1 (4.76) | NA | - |
| South African, Black, no. (%) | NA | 8 (30.77) | - |
| South African, Mixed, no. (%) | NA | 17 (65.38) | - |
| Unknown, no. (%) | NA | 1 (3.70) | |
| Mean age at blood sampling (weeks) | **14.50** | **9.82** | ***<0.0001*** |
| Mother exposed to TB** | 5 (25) | NA | - |
| Infant exposed to TB | NA | 0 (0) | - |

* Gestation data was not available for 2 SA infants

** The status of TB exposure was unknown in 1 of the mothers of infants from the UK cohort

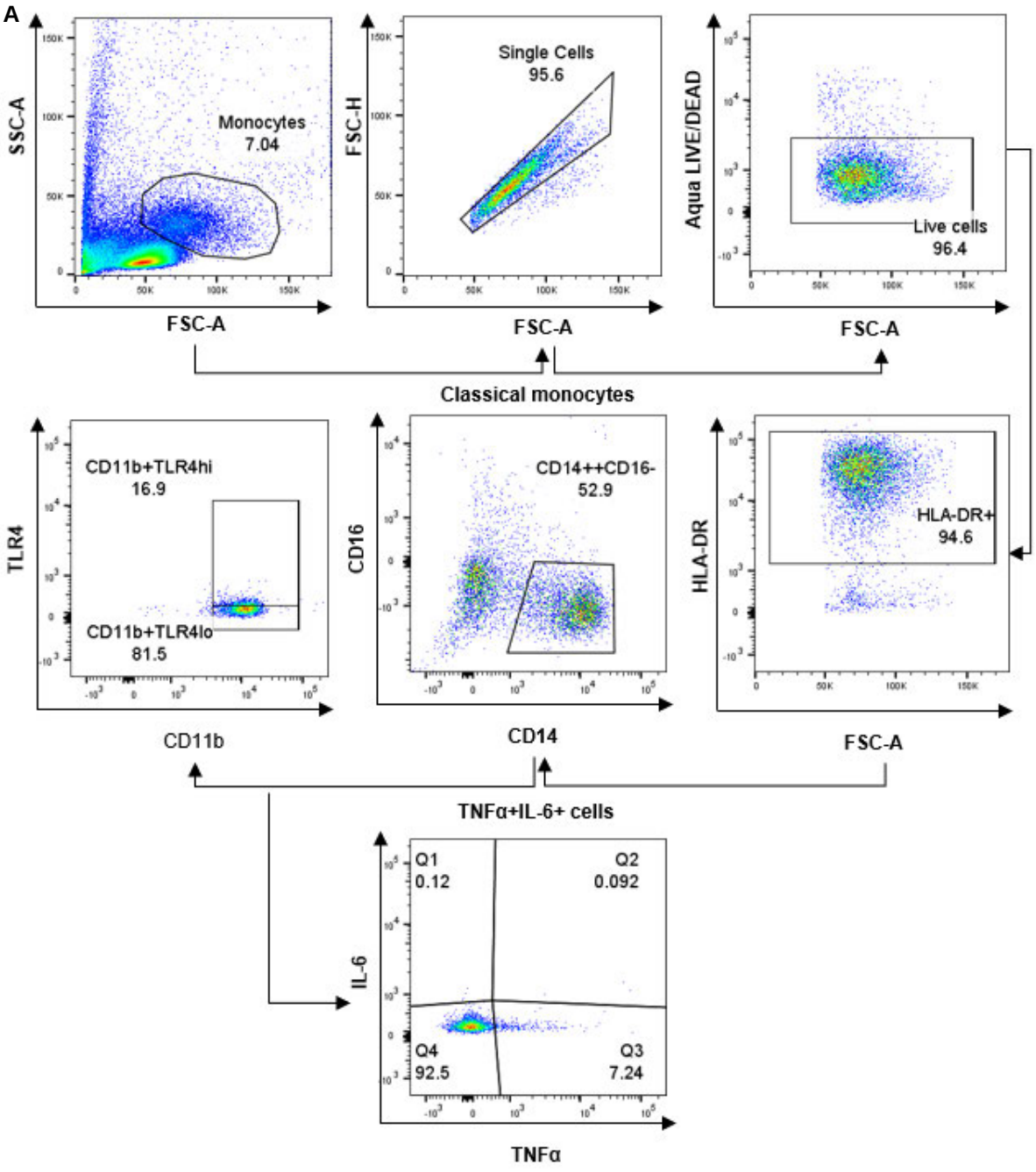
Unpaired t-test was applied to compare mean age of mothers of the infants, mean gestation duration, mean age at BCG vaccinarion and mean age at blood sampling. Fisher's exact test was applied to

compare the proportions of male and female infants and the proportions of infants that received the BCG vaccine via different routes (intradermal vs percutaneous).

In this chapter, responses of PBMCs to stimulation with BCG and Toll-like receptor (TLR) agonists were examined in 21 infants from the North Middlesex Hospital recruited for the UgUK Infant BCG Study in the UK and 27 infants recruited for the previous SATVI BCG vaccination study in South Africa. *Table 4.3* shows the characteristics of study participants recruited in the UK and South Africa. Infants in both cohorts had similar birth weight, were delivered at term (except for one UK infant) and came from varied ethnic backgrounds. Although the study population of the UK infants included more males (61.90%) than females, and the South African – more females (55.56%), this difference was not statistically significant. However, infants in the UK cohort were older than those in the South African cohort at blood sample collection (mean age 14.50 weeks vs. 9.82 weeks respectively), with only 61.90% of the UK infants having received their BCG vaccine immediately after birth, as recommended by the WHO (31), with mean age at BCG vaccination being 2.38 days (± 2.73 days). The mean maternal age at delivery was also different, with 35.94 years (± 4.95 years) in the UK cohort and 27.38 years (± 5.48 years) in the South African cohort. Finally, the South African infant cohort included 8 samples from percutaneously vaccinated infants. Although previous work by SATVI showed no differences in the efficacy of intradermal vs percutaneous BCG immunisation against TB (310), route of immunisation has been shown to influence TNFα production and IFNγ to IL-4 ratios in BCG-vaccinated infants (318). The 8 percutaneously vaccinated South African infants were therefore excluded from downstream analyses and, because of the differences in the cohorts, the investigation was focused on exploring the innate immune responses of BCG-vaccinated UK and South African infants in parallel without comparing them directly.

### *4.3.2 Surface receptor and intracellular cytokine expression by PBMCs from UK and South African infants*

Previous studies showed that the heterologous effects of BCG vary across studies. To investigate the heterologous responses to BCG and other stimuli in the UK and South African infants, a FACS panel was designed to explore monocyte surface markers and intracellular cytokines previously associated with trained immunity. The gating strategy for the detection of monocytes with the phenotype consistent with that previously reported in the innate immune training studies *in Chapter 2* is outlined in *Figure 4.4*. This figure also shows a representative example of the expression patterns of surface molecules (CD11b and TLR4) and intracellular cytokine production (IL-6 and TNFα) by infant CD14++CD16- monocytes in the absence (*Figure 4.4A)* or presence of BCG stimulation *(Figure 4.4B)*. Although this study did not have control groups of PBMC samples from infants that did not receive the BCG vaccine in the cohorts and so was not powered to assess the potential of BCG to train monocytes in infants that received this vaccine, the assay allowed distinction and quantification of surface receptor or cytokine responses by the innate immune cells to *in vitro* stimulation with the BCG vaccine or TLR agonists (*Figure 4.5).*

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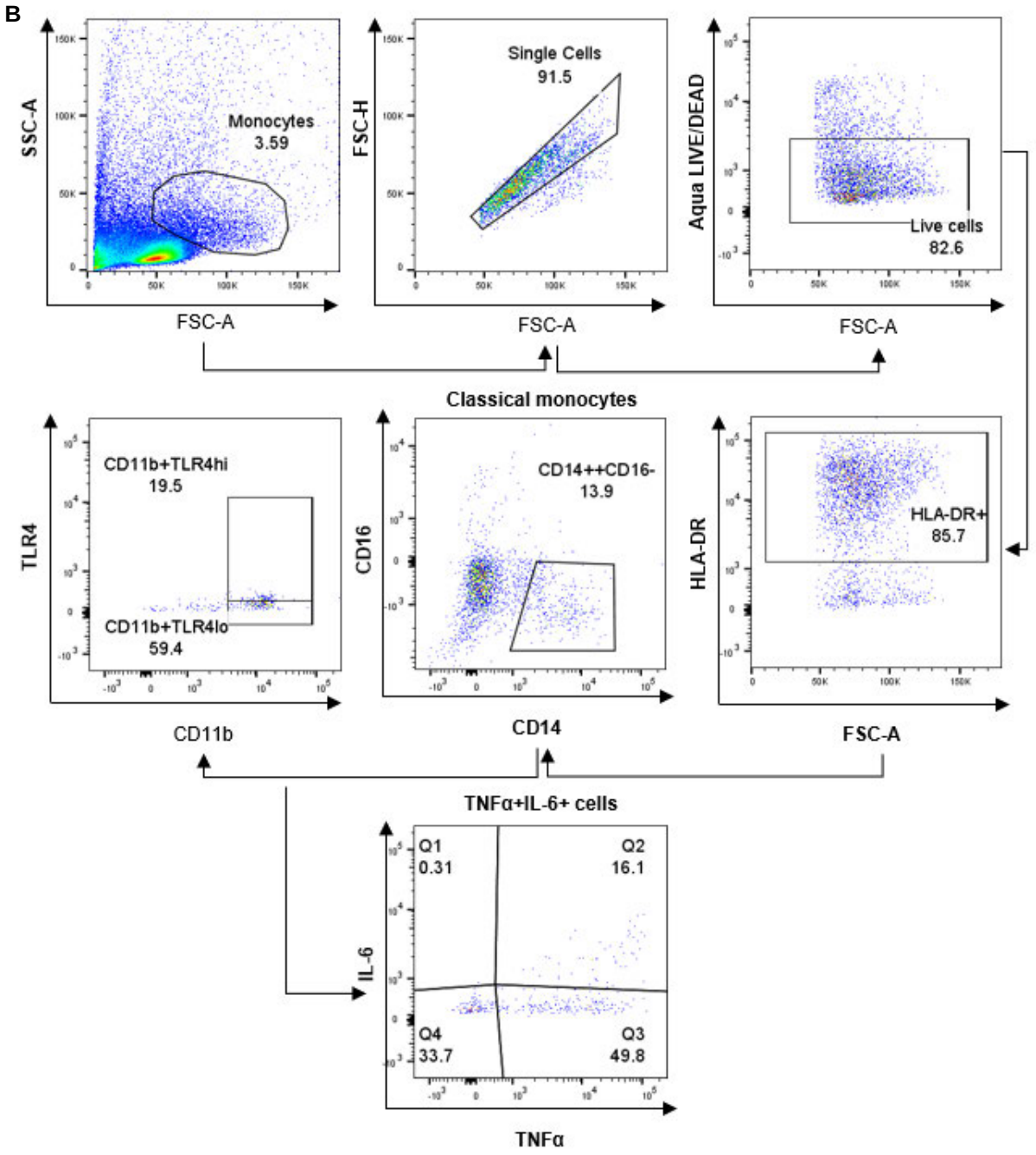



**Figure 4.4.** *Gating strategy used to detect monocytes with phenotype consistent with that of monocytes trained with BCG in in vitro or human vaccination studies.* **A** shows the gating strategy for unstimulated infant PBMCs and **B** – the same gating strategy in BCG-stimulated infant PBMC samples. The same gating strategy was also used to analyse the expression of CD11b, TLR4, IL-6 and TNFα in PBMC samples stimulated with LPS or Pam3Cys. The diagrams depict data from a single donor in a representative experiment.

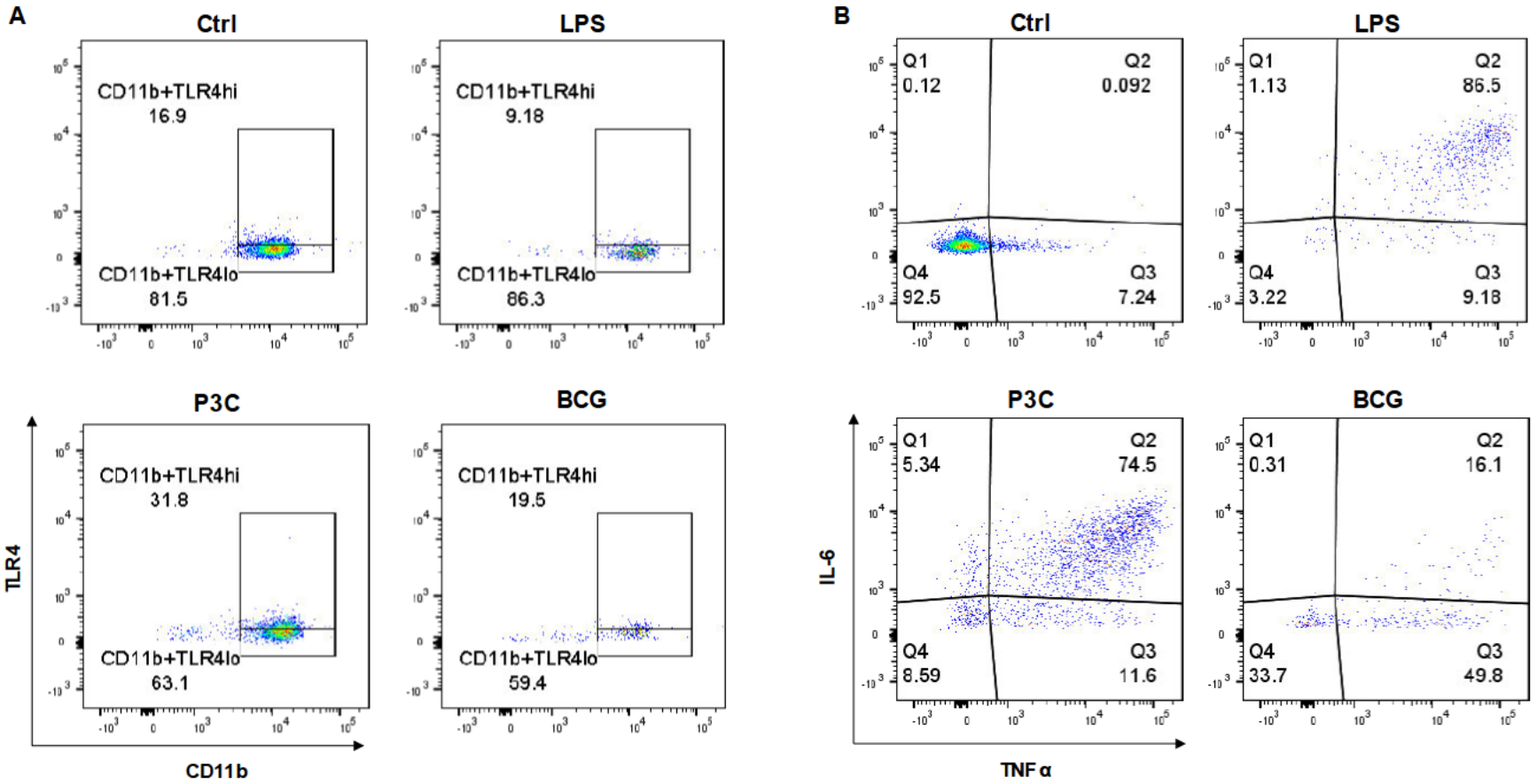



**Figure 4.5.** *Differences in infant HLA-DR+ CD14++CD16- PBMC responses to BCG and TLR agonists.* **A** shows surface expression of CD11b and TLR4 and **B** - production of intracellular IL-6 and TNFα in unstimulated infant PBMCs (ctrl) or cells given LPS, Pam3Cys or BCG treatment. The diagrams depict data from a single donor from a representative experiment.

To investigate if cytokine production or surface receptor expression was altered in monocytes following PBMC stimulation with BCG or TLR agonists in both cohorts, frequencies of CD14++CD16- cells (classical monocytes) expressing CD11b, TLR4, IL-6 or TNFα and mean fluorescence intensity (MFI) values for these molecules were examined using samples from the UK (n = 19) and South African (n = 18) infants.

There were clear differences in responses of CD14++CD16- monocyte subpopulations to individual stimuli in both cohorts (*Figure 4.6, Table S4.3)*. For instance, after the 6 hours of PBMC stimulation with BCG or TLR agonists, BCG or Pam3Cys increased the proportion of TNFα+IL-6- frequency of CD14++CD16- monocytes more strongly than LPS in both the UK and South African samples compared to unstimulated samples (*Figure 4.6A*, *Table S4.4;* respective Šidák multiple comparisons test p-values of LPS vs BCG and LPS vs P3C: $p < 0.0001$ and p 0.0033 in the UK cohort, and $p < 0.0001$ for both in the SA cohort). LPS was the most potent of the three stimuli in expanding TNFα+IL-6+ responses in CD14++CD16- monocytes compared to baseline, followed by Pam3Cys (*Figure 4.6C, Table S4.4*; both $p < 0.0001$ in the UK and SA cohorts). The latter TLR agonist induced IL-6 responses by CD14++CD16- monocytes most strongly (*Figure 4.6B, Table S4.4*; $p < 0.0001$ in both the UK and SA cohorts) and increased the frequency of CD11b+TLR4hi CD14++CD16- monocyte population compared to unstimulated cells (*Figure 4.6D, Table S4.4*; $p < 0.0001$ in both the UK and SA cohorts). In contrast, the latter was reduced in cells treated with LPS compared to unstimulated cells in the UK but not the SA cohort (*Figure 4.6D, Table S4.4;* $p = 0.0282$ vs $p = 0.0834$ respectively).

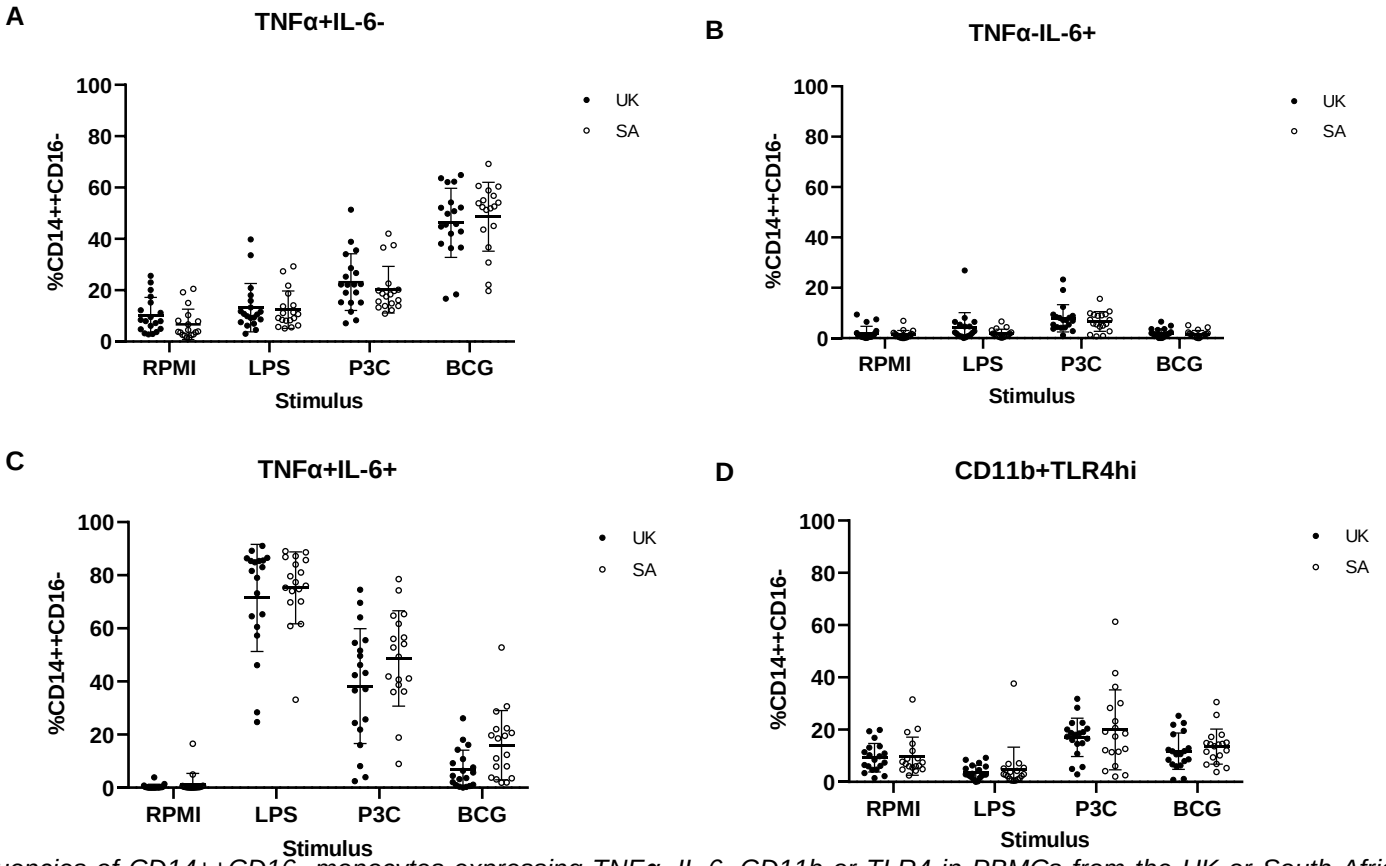



**Figure 4.6.** *Frequencies of CD14++CD16- monocytes expressing TNFα, IL-6, CD11b or TLR4 in PBMCs from the UK or South African infants.* The diagrams show frequencies of cells expressing TNFα (**A**), IL-6 (**B**), both TNFα and IL-6 (**C**) and co-expressing CD11b and TLR4 (**D**). Two-way ANOVA was applied to investigate the contribution of stimulus or donor to total variation. Šidák's multiple comparison test was applied for differences in PBMCs from both cohorts stimulated with different stimuli. For clarity, the p-values referring to the effects of stimuli are listed in *Table S4.4*. UK: n = 19; SA: n = 18.

To test if there were also differences in protein expression per cell rather than changes in frequencies of cytokine-producing CD14++CD16- monocyte populations upon stimulation with BCG and TLR agonists, differences in MFI values for these molecules of interest were examined in PBMCs from the UK and South African infants. As expected, stimulation with TLR agonists increased the MFI values for both IL-6 and TNFα compared to the unstimulated cells in both the UK and South African cohorts (*Figure 4.7A, B; Table S4.4*). LPS induced TNFα more strongly than P3C or BCG in both the UK and SA cohorts while P3C and BCG stimulated the production of this cytokine comparably in both cohorts (*Figure 4.7A; Table S4.4*). It was also the most potent stimulus for IL-6, while BCG stimulated IL-6 to a lesser extent compared to LPS and P3C in both infant cohorts ($p < 0.0001$ respectively in the UK and SA infant samples; *Figure 4.7B; Table S4.4*). Although the expression of CD11b in CD14++CD16- monocytes was mostly stable upon stimulation, LPS enhanced its expression in the UK infants ($p = 0.0004$; *Figure 4.7C; Table S4.4*). LPS also reduced surface expression of TLR4 in CD14++CD16- monocytes compared to unstimulated or P3C or BCG stimulated cells from both the UK and South African infants ($p < 0.0001$ in both cohorts; *Figure 4.7D; Table S4.4*).

Apart from the stimulus, the second largest factor contributing to variation was variation among the donors (two-way ANOVA per cent estimates of total variation: 11.69%, $p = 0.0033$ for TNFα+IL-6-, 41.46%, $p < 0.0001$ for TNFα-IL-6+, 8.87%, $p < 0.0001$ for TNFα+IL-6+ and 42.18%, $p < 0.0001$ for CD11b+TLR4hi CD14++CD16- monocyte frequencies, *Table S4.3*). Data from the UK infant dataset were therefore broken down by sex. No statistically significant differences in TNFα, IL-6, CD11b or TLR4 expressing CD14++CD16- monocyte frequencies were found in unstimulated or stimulated female and male infant PBMC samples (*Figure 4.8; Table S4.5*). No differences in the MFI values for these cytokines or surface markers were found in the male vs female infant CD14++CD16- monocytes, except for IL-6 in unstimulated samples (higher in females, Šidák multiple comparison test, $p = 0.0475$; *Figure 4.9B; Table S4.5*) and also a trend for higher IL-6 MFI values in stimulated CD14++CD16- monocytes.

This suggested that factors other than the sex of infants contributed to the variation among the samples.

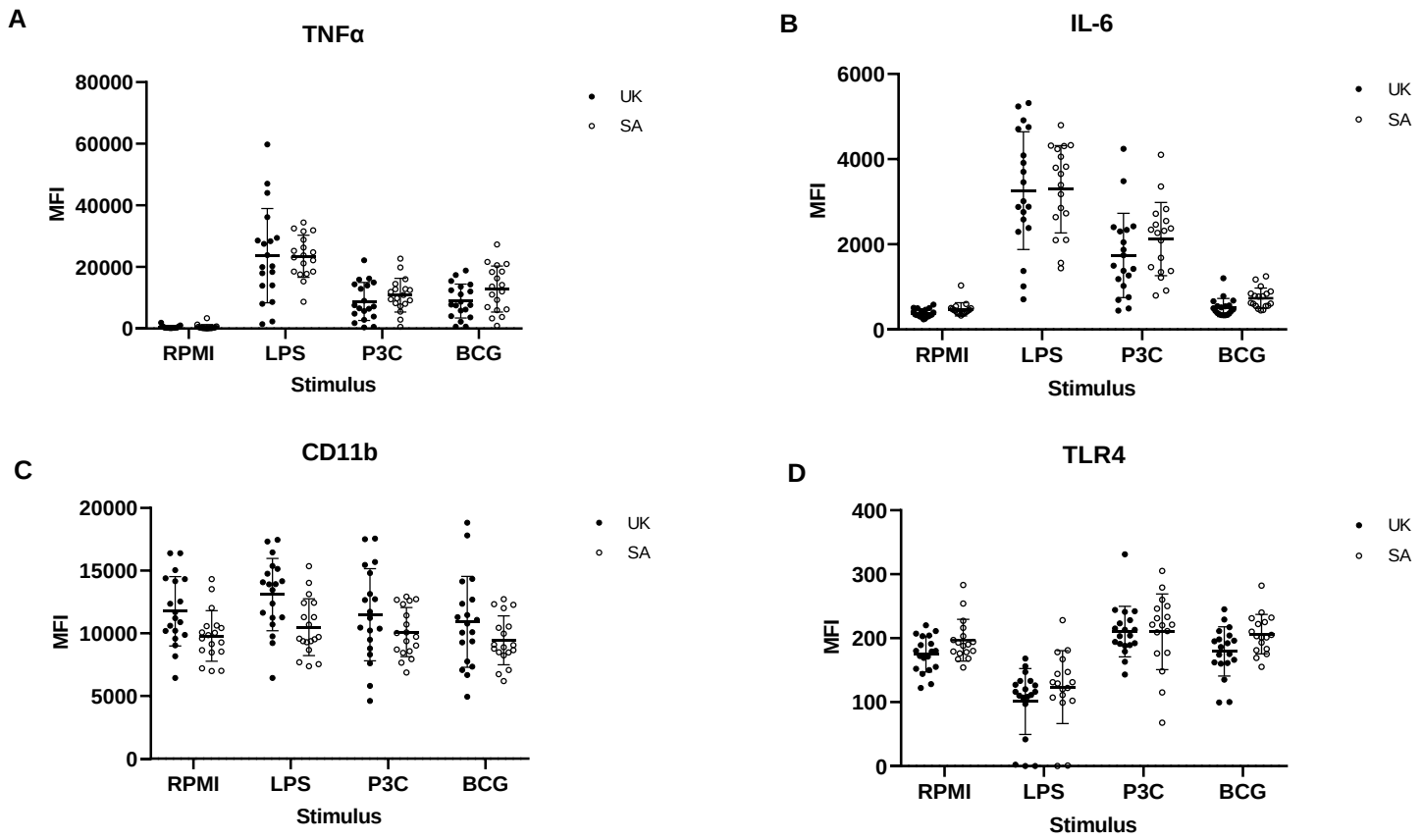



**Figure 4.7.** *MFI values of intracellular cytokines and surface expression markers expressed by CD14++CD16- monocytes.* The diagrams show MFI values for TNFα **(A)**, IL-6 **(B)**, CD11b **(C)** and TLR4 **(D)**. Two-way ANOVA was applied to investigate the contribution of stimulus or donor to total variation. Šidák's multiple comparison test was applied to detect differences PBMCs from both cohorts stimulated with different stimuli.. For clarity, the p-values referring to the effects of stimuli are listed in *Table S4.4*. UK: n = 19; SA: n = 18, with the exception of TLR4 where the readings of the MFI values from 1 SA donor were affected by voltage settings.

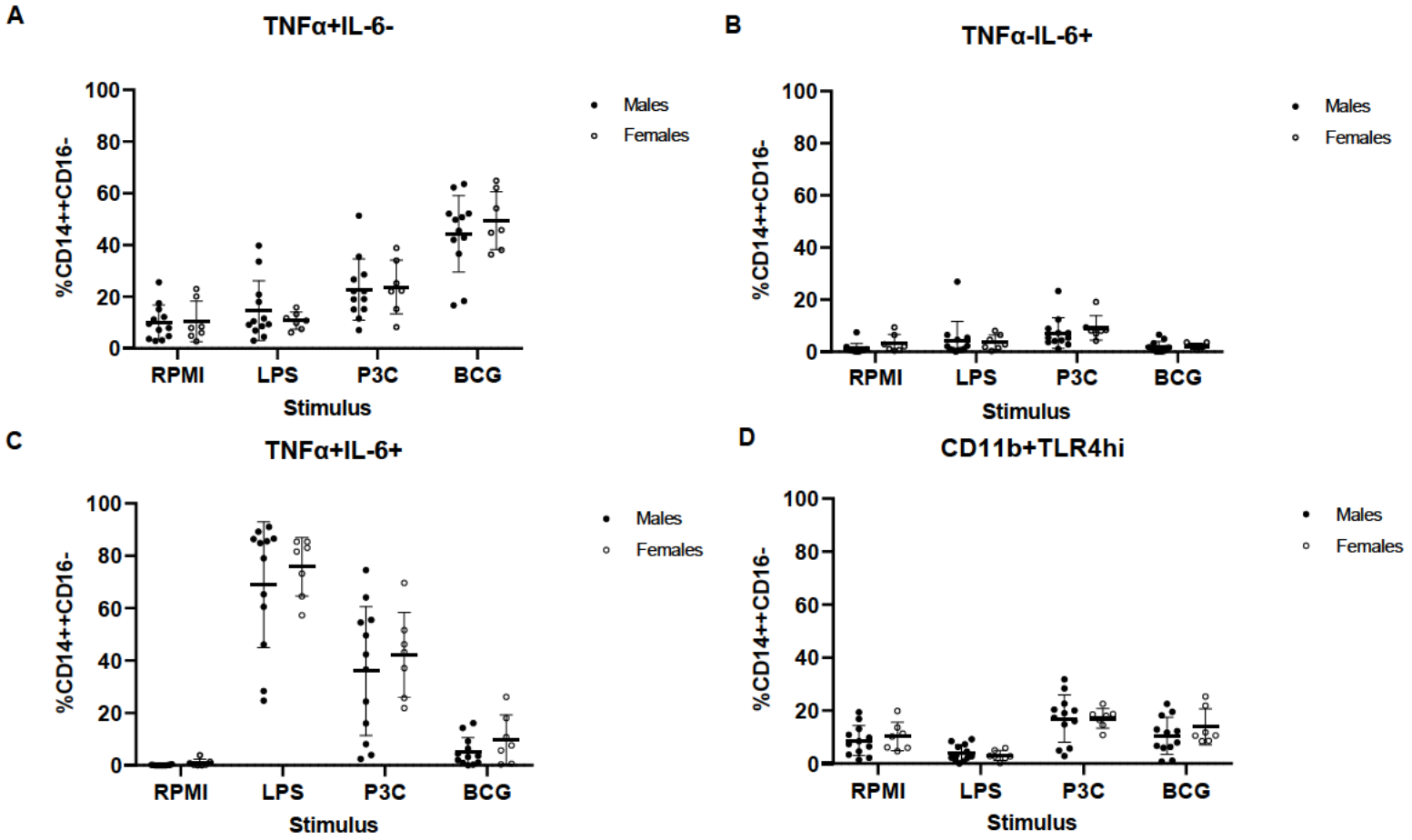



**Figure 4.8.** *Frequencies of CD14++CD16- monocytes expressing TNFα, IL-6, CD11b or TLR4 in PBMCs from male or female UK infants.* The diagrams show frequencies of cells expressing TNFα (**A**), IL-6 (**B**), both TNFα and IL-6 (**C**) and co-expressing CD11b and TLR4 (**D**). Two-way ANOVA was applied to investigate the contribution of sex, stimulus or donor to total variation. Šidák's multiple comparison test was applied for differences in male and female infant responses. Males: n = 12; females: n = 7.

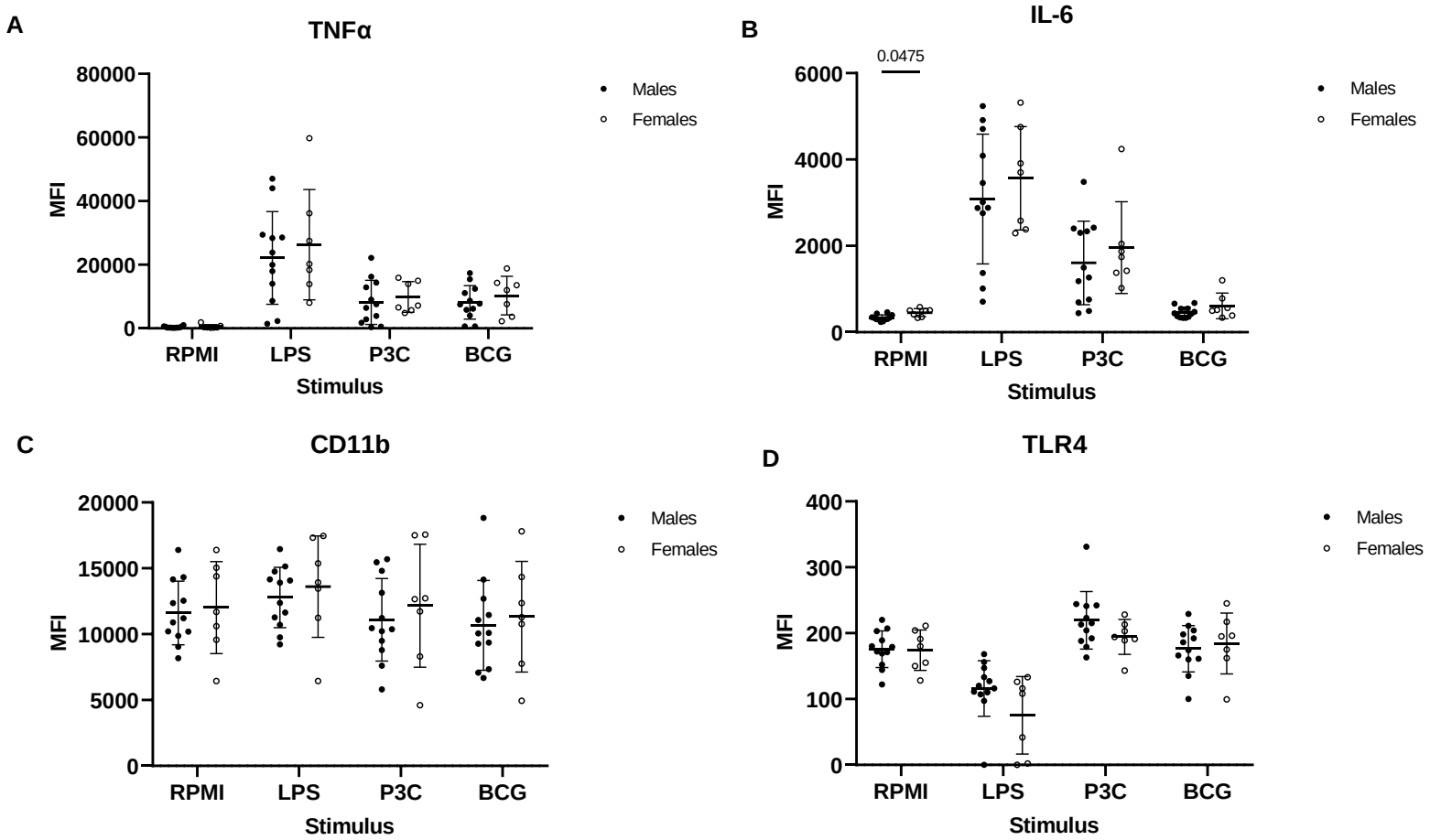
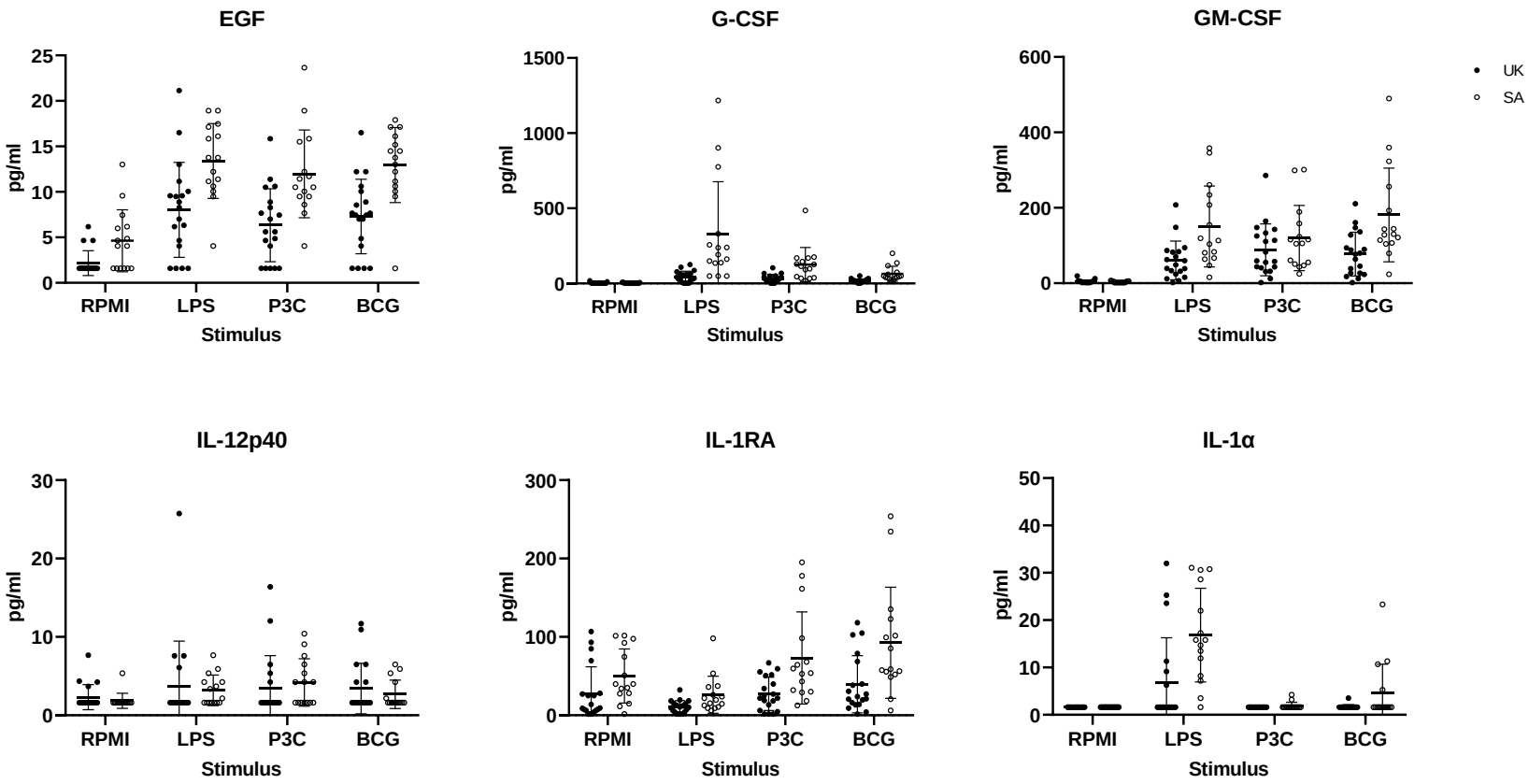



**Figure 4.9.** *MFI values of intracellular cytokines and surface expression markers expressed by CD14++CD16- monocytes in male and female UK infants.* The diagrams show MFI values for TNFα (**A**), IL-6 (**B**), CD11b (**C**) and TLR4 (**D**). Two-way ANOVA was applied to investigate the contribution of sex, stimulus or donor to total variation. Šidák's multiple comparison test was applied for differences in male and female infant responses. Males: n = 12; females: n = 7.

### *4.3.3 Production of cytokines potentially associated with protection against TB by PBMC samples from the UK and South African infants*

In addition to surface marker expression and intracellular cytokine measurements, production of innate cytokines were also analysed in supernatants from PBMC cultures from the UK and South African infants stimulated for 24 h. Multiplex bead array analysis of 12 innate immune cytokines associated with trained innate immunity or protective effects against TB showed that BCG and TLR agonists stimulated the production of 7 of these cytokines: EGF, G-CSF, GM-CSF, IL-1β, IL-6, IP-10 and TNFα both in the UK and South African infant cohorts (*Figure 4.10, Table S4.4*). The production of some cytokines, however, was stimulus specific. For instance, IL-1RA was induced by BCG but not TLR agonists in the UK cohort while LPS stimulated the production of IL-1α in South African cohort (*Figure 4.10, Table S4.4*). In contrast, MCP-1 was induced by P3C in samples from the UK and South African infants. IL-12p40 was not detectable above the baseline irrespective of stimulus in both cohorts (*Figure 4.10, Table S4.4*). Overall, LPS was the most potent stimulus of the innate cytokines, followed by P3C. Of interest, although in the UK cohort IP-10 was induced by all stimuli, P3C was the most potent inducer of this cytokine (*Figure 4.10, Table S4.4*).

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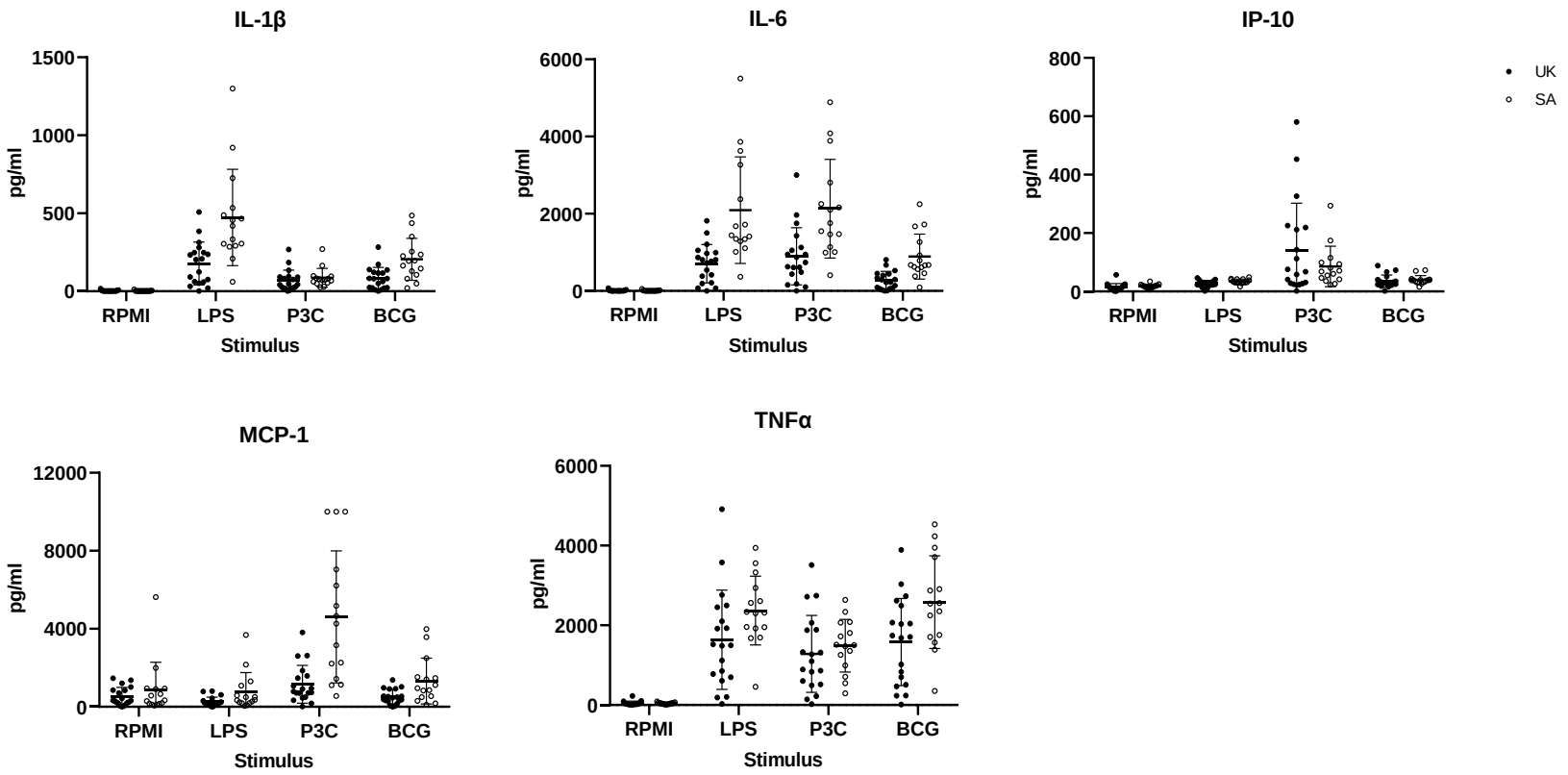
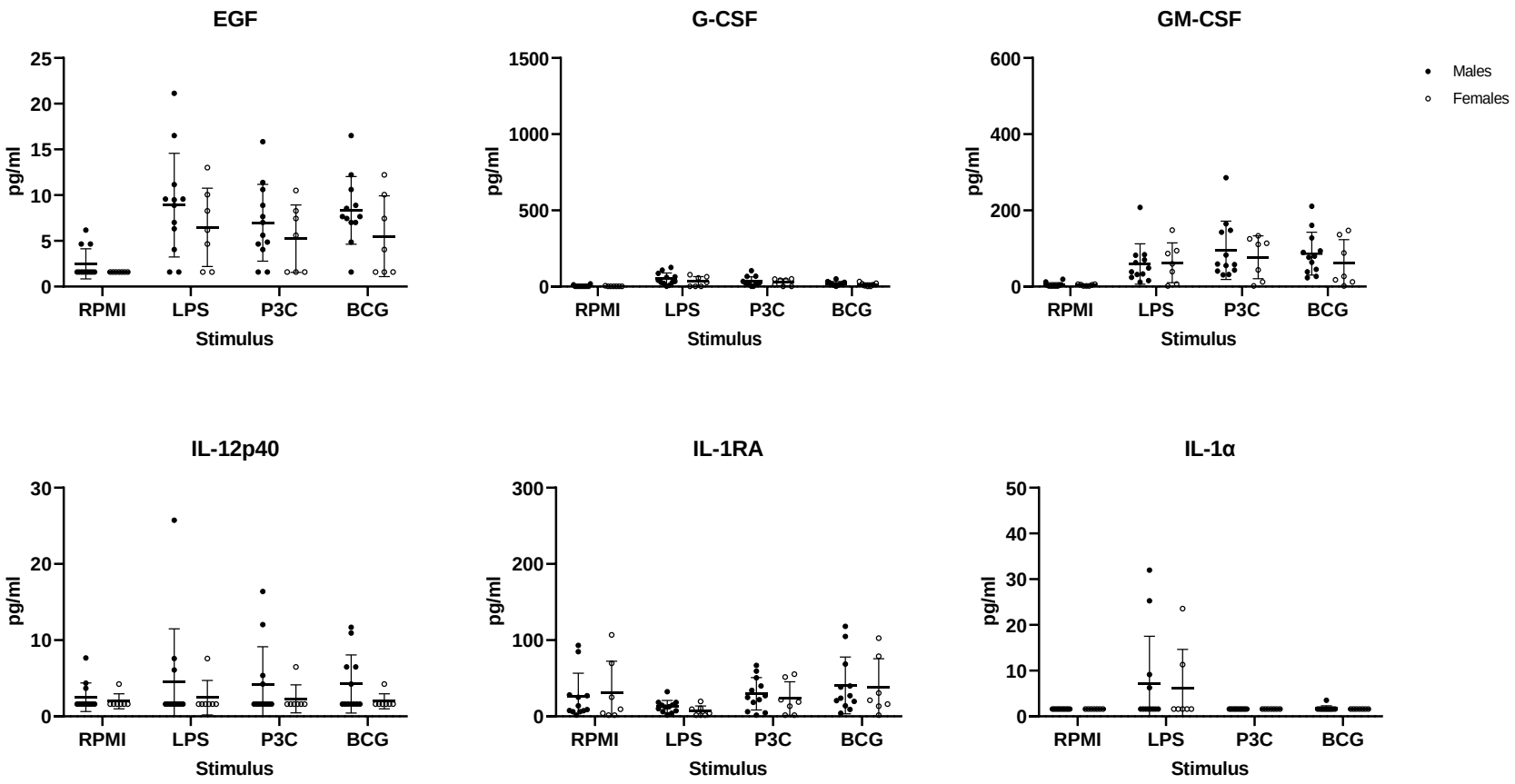



**Figure 4.10.** *Cytokine production by the UK and South African infant PBMCs in response to BCG and innate immune stimuli.* PBMCs from the UK and South African (SA) infants were stimulated with LPS, Pam3Cys or BCG for 24 hours or left unstimulated. PBMC supernatants were analysed by multiplex bead array. The scatter plots depict cytokine concentrations, and bars indicate mean values and standard deviation. Two-way ANOVA was applied to investigate the contribution of stimulus or donor to total variation. Šidák's multiple comparison test was applied for differences in PBMCs from both cohorts stimulated with different stimuli. For clarity, the p-values referring to the effects of stimuli are listed in *Table S4.4*. Data on 11 cytokines out 12 are shown (PDGF-AB/BB was not detectable). UK: n=19, SA: n=15.

Donor variation was one of the major factors contributing to the variation in the expression of surface markers and intracellular cytokines associated with trained immunity as shown by the FACS analysis of HLA-DR+CD14++CD16- infant PBMCs (*Table S4.3*). Contribution of donor variation to variation in the production of cytokines associated with protection against TB or trained immunity was therefore explored in infant PBMC cultures. For most cytokines investigated in this study, donor variation contributed most to the variation in their concentration (EGF, G-CSF, GM-CSF, IL-12p40, IL-1RA, MCP-1 two-way ANOVA - 23.89 to 50.18% variation, $p = 0.0073$ and $p < 0.0001$ respectively; *Table S4.3*) or was the second largest factor contributing to variation in cytokine production levels in infant PBMCs after stimulus (IL-1β, IL-6, TNFα: two-way ANOVA - 20.83 to 33.92% variation, $p = 0.0003$ and $p < 0.0001$; *Table S4.3*).

The cytokine production dataset from the UK infants was therefore broken down by sex. No statistically significant differences between male and female cytokine responses to BCG and TLR agonists were observed for any of the cytokines examined, except for IP-10 (*Figure 4.11; Table S4.5).* This cytokine was produced at higher levels by BCG-stimulated PBMCs in male infants from the UK compared to female infants (Šidák multiple comparison test, $p = 0.0478$; *Table S4.5*). No statistically significant differences were detected in production of other cytokines in cell culture samples from male and female infants (*Figure 4.11)*, although they suggest it might be worth to investigate possible differences in male and female cytokine responses to PBMC stimulation with BCG or TLR agonists in a larger study.

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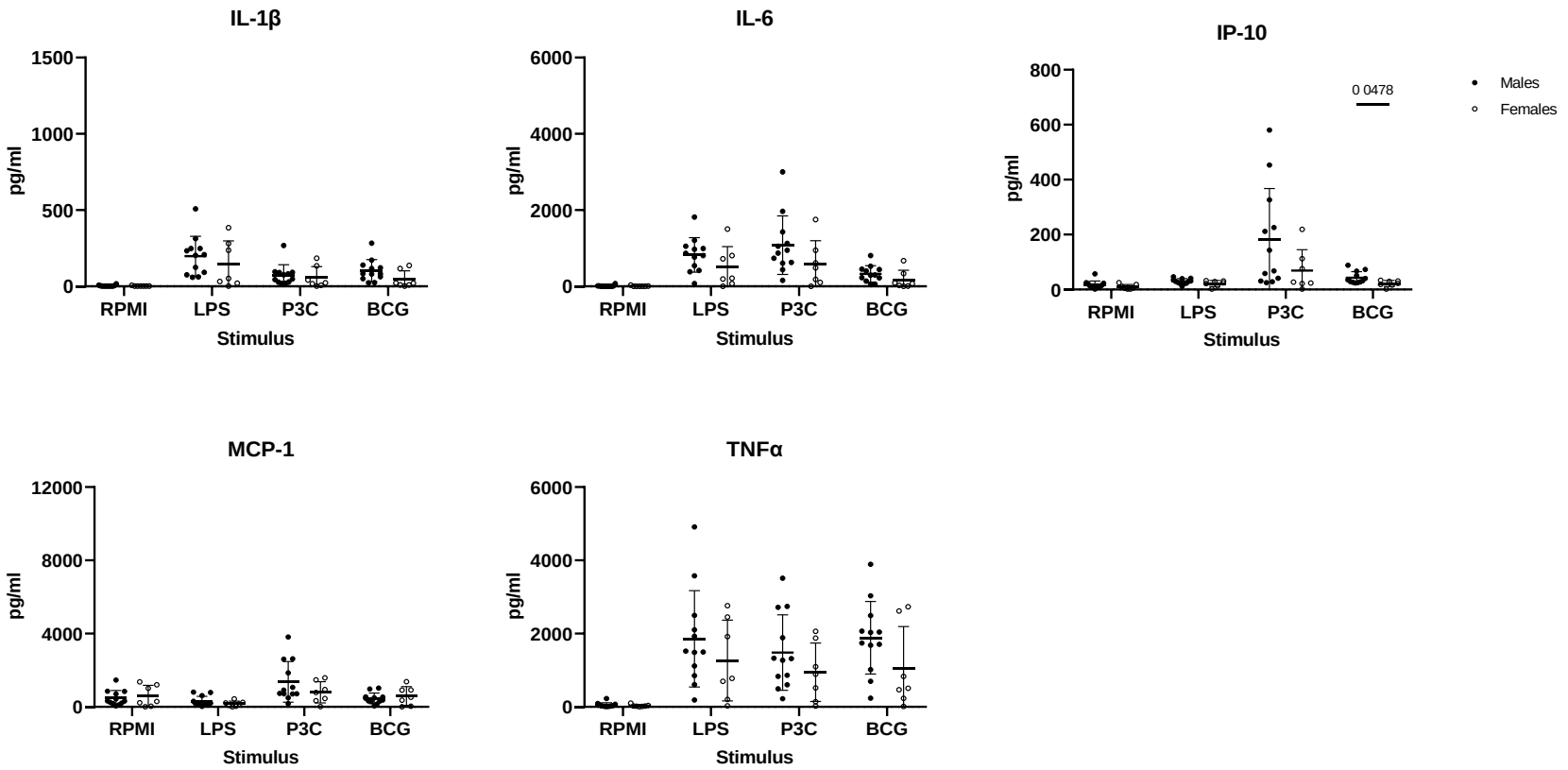



**Figure 4.11.** *Cytokine production by PBMCs from male and female UK infants in response to BCG and innate immune stimuli.* PBMCs from male and female UK infants were stimulated with LPS, Pam3Cys or BCG for 24 hours or left unstimulated. PBMC supernatants were analysed by multiplex bead array. The scatter plots depict cytokine concentrations, and bars indicate mean values and standard deviation. Two-way ANOVA was applied to investigate the contribution of sex, stimulus or donor to total variation. Šidák's multiple comparison test was applied for differences in male and female infant responses. Males: n=12, females: n=7.

### *4.3.4 Expression of genes potentially associated with protection against TB in PBMC samples from the UK and South African infants*

To investigate novel markers that could contribute to infants protection against TB, a literature search on human gene signature studies was conducted to identify genes with a possible role in the protection against TB. The process outlining the strategy used to identify the genes of interest is depicted in *Figure 4.2*. Human gene signature studies identified during the literature search on gene expression in healthy individuals, LTBI and active TB patients are listed in *Table 4.4*. It was expected that *M. tuberculosis* would suppress the expression of genes associated with the protection against TB, so the search for genes of interest was narrowed down to those genes or molecules that were upregulated in healthy controls or individuals with LTBI compared to their expression in individuals with active TB.

Because of the expectation that these novel markers should contribute to the protection against TB in BCG-vaccinated infants, the genes of interest were further investigated for associations with protection against mycobacterial infections (*Figure 4.2)*. Additional searches for the association of these genes with monocyte or macrophage responses and trained immunity and searches for the qRT-PCR primer pairs previously reported to amplify these genes in studies of primary human cells or human cell lines revealed 4 genes of interest selected for further analyses. The genes and their associations with responses to mycobacterial infections, their involvement in innate immune responses or metabolic pathways regulating trained innate immunity are outlined in *Table 4.5*.

**Table 4.4.** *Studies exploring gene expression differences in individuals with LTBI, active TB or healthy controls*

| Study | Cohort or comparison group | No. of active TB patients | No. of individuals with LTBI | No. of healthy controls | Reference |
|---|---|---|---|---|---|
| **Transcriptional Profiling of Human Peripheral Blood Mononuclear Cells Identifies Diagnostic Biomarkers That Distinguish Active and Latent Tuberculosis** | Biomarker identification cohort | 28 | 25 | 31 | (319) |
| | Biomarker validation cohort | 51 | 44 | 35 | |
| **Genome-wide transcriptional profiling identifies potential signatures in discriminating active tuberculosis from latent infection** | Microarray cohort | 4 | 4 | 4 | (320) |
| | Validation cohort | 66 | 78 | 86 | |
| **A predictive signature gene set for discriminating active from latent tuberculosis in Warao Amerindian children** | Biomarker identification cohort | 9 | 9 | 9 | (321) |
| | Biomarker validation cohort | - | 20 | 16 | |
| | Children with radiologically confirmed pneumonia | - | 6 | 12 | |
| **Messenger RNA Expression of IL-8, FOXP3, and IL-12β Differentiates Latent Tuberculosis Infection from Disease** | Healthy controls | 30 | 24 | 10 | (322) |
| **Transcriptomic Biomarkers for Tuberculosis: Evaluation of DOCK9.** | Pilot investigation cohort | 6 | 16 | 12 | (323) |

| | | | | | |
|---|---|---|---|---|---|
| **EPHA4, and NPC2 mRNA Expression in Peripheral Blood** | | | | | |
| **Gene expression profiling identifies candidate biomarkers for active and latent tuberculosis** | Microarray cohort | 7 | 7 | 7 | (324) |
| | Validation cohort | 15 | 17 | 15 | |
| **Existing blood transcriptional classifiers accurately discriminate active tuberculosis from latent infection in individuals from south India** | Direct comparison | 28 | 16 | - | (325) |
| **Novel Biomarkers Distinguishing Active Tuberculosis from Latent Infection Identified by Gene Expression Profile of Peripheral Blood Mononuclear Cells** | - | 25 | 36 | | (326) |
| **The Transcriptional Signature of Active Tuberculosis Reflects Symptom Status in Extra-Pulmonary and Pulmonary Tuberculosis** | Healthy controls | | - | 61 | (327) |
| | Pulmonary TB | 45 | - | | |
| | Extrapulmonary TB | 47 | - | | |
| | Sarcoidosis | | - | 49 | |
| **Blood Transcriptional Biomarkers for Active Tuberculosis among Patients in the United States: a Case-Control** | - | 35 | 35 | -* | (328) |

| | | | | | |
|---|---|---|---|---|---|
| **Study with Systematic Cross-Classifier Evaluation** | | | | | |
| **Gene-expression patterns in whole blood identify subjects at risk for recurrent tuberculosis** | - | 6** | 6 | -*** | (329) |

* Data compared with that from 39 pneumonia patients

** Additional 6 samples from recurrent TB patients

*** 6 samples from cured TB patients were used

**Table 4.5.** *Characteristics of genes examined in the study of the UK and South African infant innate immune responses to BCG and TLR agonists*

| Elevated in healthy controls vs TB patients | | | |
|---|---|---|---|
| **Gene candidate** | **Function** | **Study** | **Criterion* met (required, preferable, additional?)** |
| ***GPR183*** | Increased expression in healthy controls vs TB patients | (327) | Required |
| | Reduced in patients with TB and type II diabetes compared to TB patients without diabetes | (330) | Preferable |
| | Silencing of *GPR183* in primary human monocytes correlates with increased growth of BCG in the infected cells and with higher *IFNB1* and *IRF* expression | | |
| | Stimulation of GPR183 on primary human monocytes with 7α,25-dihydroxycholesterol correlates with lower expression of *IFNB1* and *IL-10*, enhanced autophagy and reduced growth of *M. tuberculosis* and BCG | | |
| | C57BL/6 mice deficient of *GPR183* have poorer control of *M. tuberculosis* growth in their lungs compared to WT mice | | |
| | Expression downregulated in blood of C57BL/6 mice upon infection with *M. tuberculosis* | (331) | Preferable |
| | Silencing of *GPR183* in RAW264.7 macrophages reduced *M. tuberculosis* growth at 4 h post-infection | | |
| | Overexpression of *GPR183* in RAW264.7 macrophages enhanced *M. tuberculosis* growth at 48 h post-infection | | |
| | Stimulation of GPR183 of murine macrophages with 7α,25-dihydroxycholesterol slows *M. tuberculosis* growth at 4 h post-infection. | | |
| ***GPR68 / OGR1*** | Increased expression in healthy controls vs TB patients | (327) | Required |

| | | | |
|---|---|---|---|
| | Expression increased by hypoxia and low pH in THP1 and MonoMac6 cells.<br>Expression increased by TNFα.<br>The promoter of *GPR68* has binding sites for HIF-1α, a transcription factor involved in trained immunity. | (332) | Preferable, additional |
| | Expression in MonoMac6 cells induced by TNFα.<br>Contributes to intestinal inflammation in mice. | (333) | Preferable |
| | **Elevated in LTBI vs active TB** | | |
| ***FBP1*** | Increased expression in LTBI vs TB patients | (319) | Required |
| | Regulates glucose metabolism, expression correlates with that of *HIF1α* (regulator of innate training) | (334) | Additional |
| | Expression of *FBP1* is enhanced by the inhibition of histone deacetylases and accumulation of permissive H3K27Ac epigenetic mark. | (335) | Additional |
| | Increased expression of *FBP1* associates with reduced glycolysis in NK cells. | (336) | Additional |
| | **Elevated in healthy controls and in LTBI vs TB patients** | | |
| ***SCARB2/LIMP-2*** | Increased expression in healthy controls and LTBI vs TB patients | (329) | Required |
| | *D. discoideum* that lacks analogue of *SCARB2* has higher susceptibility to infection with *M. marinum* | (337) | Preferable |

*The following criteria were applied when selecting the genes of interest for the qRT-PCR analysis:

- *Required* – upregulated in healthy or LTBI individuals compared to active TB patients in human gene signature studies. Also, reported in functional studies of *in vitro* or *in vivo* mycobacterial infections.

- *Preferable* – reported in functional monocyte or macrophage studies.
- *Additional* – possible associations with trained immunity.

To test the responses to innate stimulation in PBMC samples from the UK and South African infants, their expression was measured in PBMCs incubated with BCG or TLR agonists for 24 hours. All of these genes were responsive to stimulation in the UK and South African infant PBMC cultures, with *GPR183, SCARB2* and *FBP1* downregulated upon stimulation with BCG or TLR agonists and *GPR68* - upregulated; however, the extent of their responses depended on the type of stimulus *(Figure 4.12; Table S4.4)*. For instance, *GPR183* was more strongly upregulated upon P3C stimulation compared to stimulation with LPS or BCG in both the UK and South African cohorts. In contrast, in South African cohort, *FBP1* was less strongly downregulated upon stimulation with BCG compared to TLR agonists.

In this set of experiments, for two of the genes, the strongest factor driving variation in gene expression was the donor variation (two-way ANOVA: 45.97% of total variation for *GPR183*, $p < 0.0001$; 44.74% - for *GPR68,* $p < 0.0001$; *Table S4.3*). For the other two genes, donor was the second largest factor contributing to variation (two-way ANOVA: 9.62% of total variation for *SCARB2*, $p = 0.0004$; and 14.47% - for FBP1, $p = 0.0015$; *Table S4.3*), suggesting that inherent variation among the individuals alone could contribute to variation in their innate immune responses. Of note, the contribution of donor variation was followed by stimulus in the analysis of *GPR183* and *GPR68* expression in the UK and South African infant samples (two-way ANOVA: 18.31% of total variation for *GPR183*; 22.59% - for *GPR68,* both $p < 0.0001$*; Table S4.3*). In the case of *SCARB2* and *FBP1,* the stimulus was the predominant factor driving the variation in the expression of these genes (two-way ANOVA: 77.92%, $p < 0.0001$; and 64.69%, $p < 0.0001$; *Table S4.3*), suggesting that other factors that can potentially modulate the immune responses to BCG.

Considering the strength of donor association with variation in the expression of these genes, the relative expression of *GPR183, GPR68, SCARB2* and *FBP1* in PBMC samples from the UK infants was broken down by sex. Again, there were no statistically significant differences in gene expression in male and female PBMCs *(Figure 4.13; Table S4.5)*. Of note, in the case of *FBP1,* sex contributed to 7.181% variation in *FBP1* expression in the analysis of the

expression of this gene in samples from male and female UK infants (two-way ANOVA: p = 0.0237; *Table S4.3*), suggesting some effect of sex on the expression of this gene.

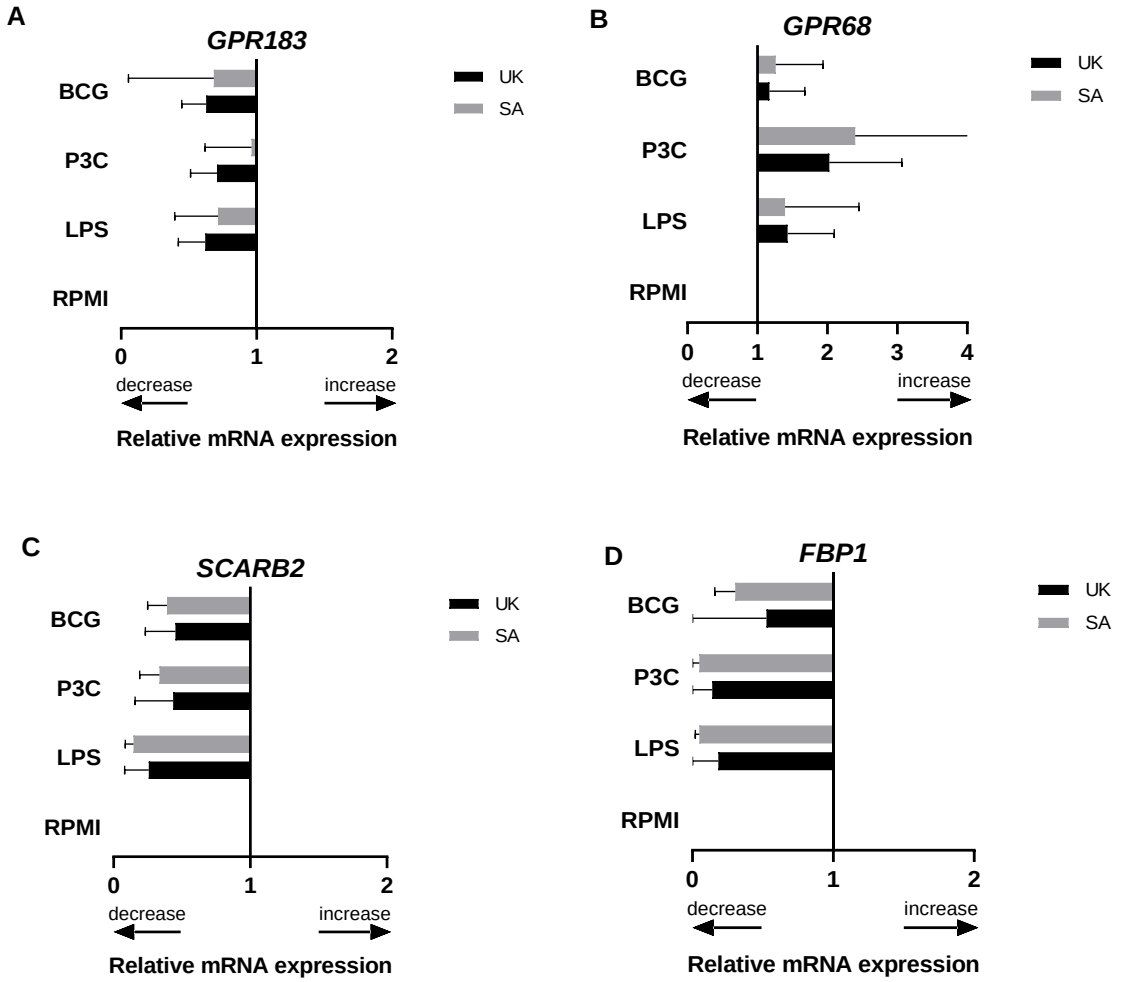


**Figure 4.12.** *Expression of genes potentially associated with protection against TB in PBMCs stimulated with BCG and innate immune stimuli in UK and South African infants.* Relative mRNA expression of **A)** *GPR183*, **B)** *GPR68*, **C)** *SCARB2* and **D)** *FBP1* in response to BCG and innate immune stimuli in PBMCs from the UK and South African (SA) infants are shown as assessed by qRT-PCR. Bars depict means and error bars the standard deviation. In two of the diagrams (C and D), some of the error bars were clipped at 0 as the lowest possible value. Two-way ANOVA was applied to investigate the contribution of stimulus or donor to total variation. Šidák‘s multiple comparison test was applied for differences in PBMCs from both cohorts stimulated with different stimuli. For clarity, the p-values referring to the effects of stimuli are listed in *Table S4.4*.. UK: n = 16; SA: n = 15.

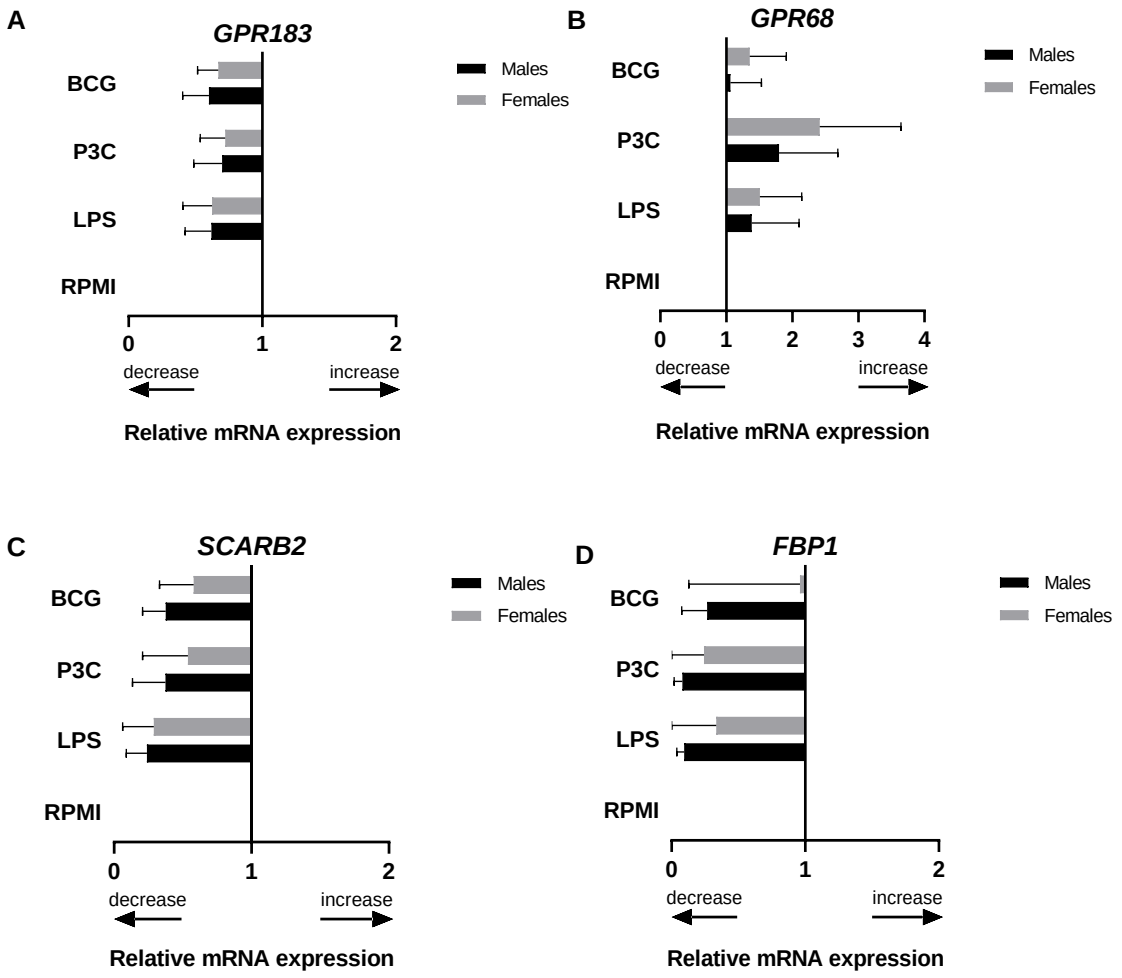



**Figure 4.13.** *Expression of genes potentially associated with protection against TB in response to BCG and innate immune stimuli in UK male and female infants.* Relative mRNA expression of **A** - *GPR183*, **B** - *GPR68*, **C** - *SCARB2* and **D** - *FBP1* in response to BCG and innate immune stimuli in PBMCs from male and female UK infants as assessed by qRT-PCR . Bars depict means and error bars – stardard deviation. In two of the diagrams (C and D), some of the error bars were clipped at 0 as the lowest possible value. Two-way ANOVA was applied to investigate the contribution of population, stimulus or donor to total variation. Šidák‘s multiple comparison test was applied for differences in male and female infant responses. Males: n = 10; females: n = 6.

### *4.3.5 Mycobacterial growth inhibition by the PBMCs from the UK and South African infants*

To explore the capacity of PBMCs of infants from different populations to inhibit mycobacterial growth, mycobacterial growth inhibition by PBMCs from infants from the UK and South African populations was investigated.

The ability of the PBMCs from the UK and South African infants was tested in three independent experiments where the PBMCs from these infants were incubated with 500 BCG CFU, rotated for 96 hours after which recovered BCG was cultured in the BACTEC MGIT960 system. *Figure 4.14.* shows the MGIA standard curves generated during these experiments. Although the growth of the BCG standards was consistent among the experiments, TTP variation was high for the lowest BCG standards (log CFU = 0; *Figure 4.14A)*. To avoid interpolating log CFU values based on BCG standard values from the lower end of the curve, the TTP values for the BCG standards at log CFU = 0 were excluded during the analysis (*Figure 4.14B)* and the TTP values that fell outside the lower range of the line of fit were extrapolated or, where that was not possible, they were assigned an arbitrary value of log CFU = 0 (*Figure 4.15)*.

In some cases, it was necessary to use single replicate samples. The data points from single-replicate samples were aligned against the BCG standard curves from each individual experiment and examined for how strongly they deviated from the curves or distribution of double-replicate samples around the lines of fit from each experiment (*Figure 4.15*). Samples with single or double replicates distributed similarly around the lines of fit, and when the lowest BCG standard values (log CFU = 0) were excluded, data points that fell out of the range of the line of fit were from samples with either double or single replicates (*Figure 4.15 A-C*). Although the log CFU values for some of these data points could not be interpolated or extrapolated, these samples had detectable TTP values (>350 h in experiments 1 and 2 and >270 h in experiment 3) and were assigned arbitrary values of log CFU = 0.

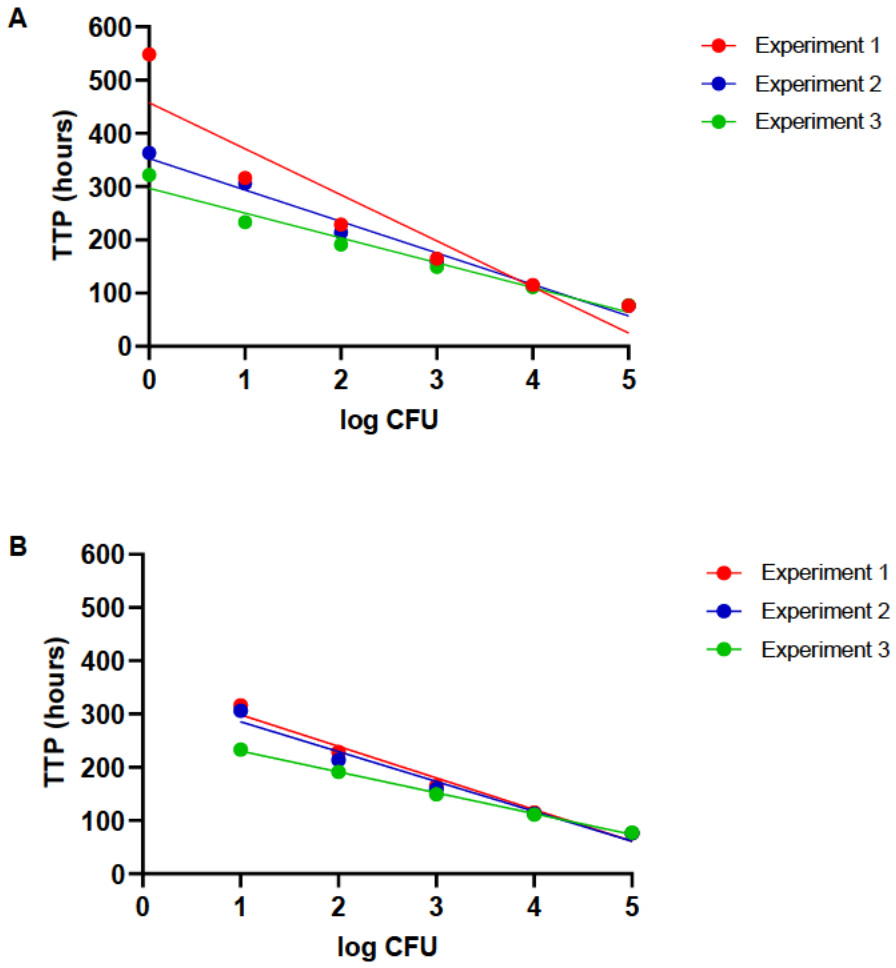



**Figure 4.14.** *MGIA standard curves and their comparison.* In these experiments, series of ten-fold BCG dilutions were prepared as standards for mycobacterial growth inhibition experiments (top standard - 100,000 colony-forming units (CFU), bottom standard – 1 BCG CFU) and loaded in duplicate in MGIT tubes. The Y-axis of the graph depicts time to positivity (TTP; in hours) measured as a proxy indicator of mycobacterial growth; the X-axis shows the log-transformed number of CFUs for each standard. Mean TTP values were used to generate the estimates of the amount of BCG in each sample. The diagrams depict the overlap of standard curves from three independent experiments. **A** shows the overlap of the curves when the TTP values for the lowest BCG standards are included; **B** shows the overlap of the curves when the TTP values for the lowest BCG standards (log CFU = 0) are excluded.

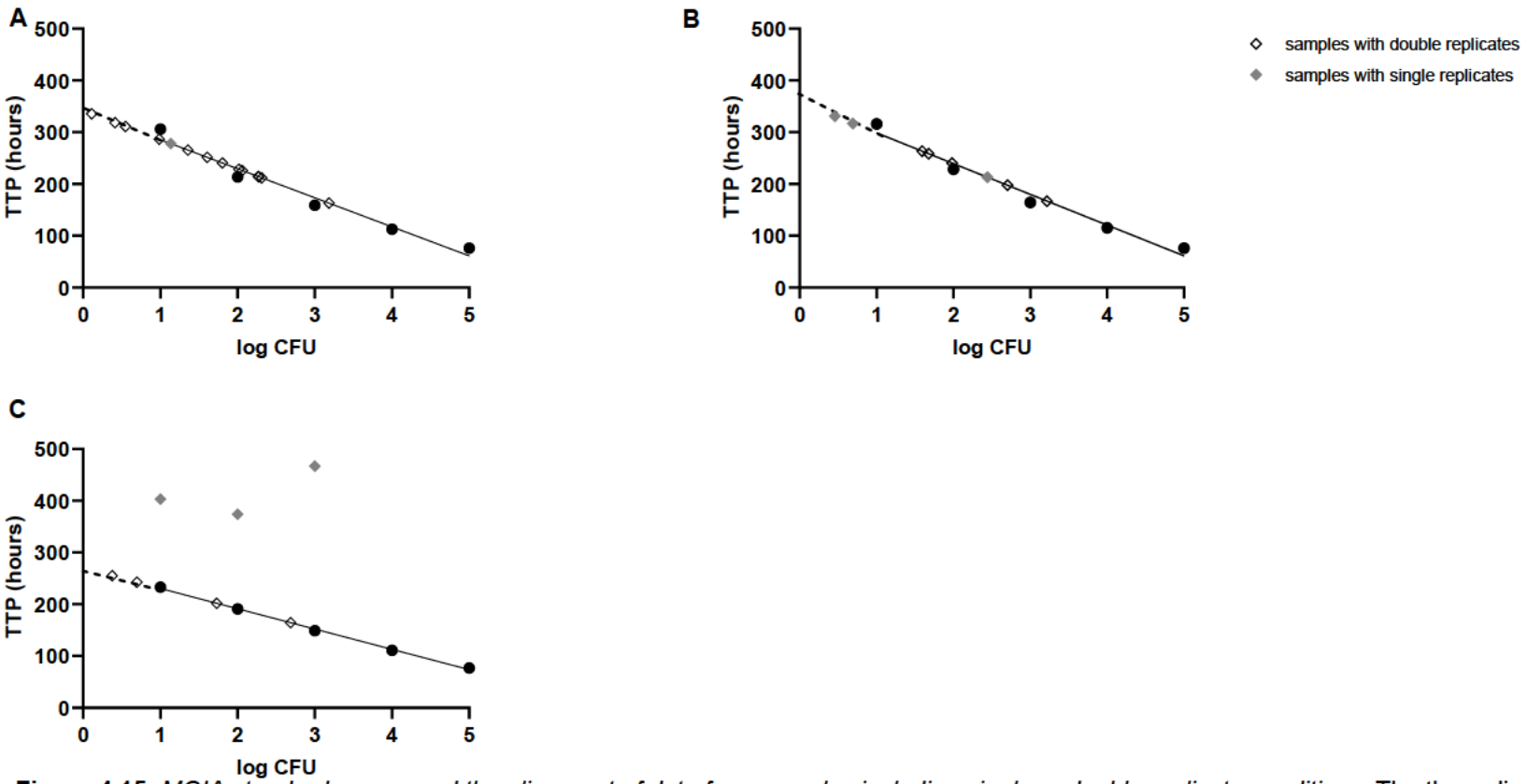



**Figure 4.15.** *MGIA standard curves and the alignment of data from samples including single or double replicate conditions*. The three diagrams show the alignment of data points from samples involving samples with double (open diamonds) or single (closed diamonds) replicates. Each panel (**A, B** or **C**) shows data from an individual experiment. Dotted lines show extrapolation of the lines of fit. The plots show datapoints for those TTP values that could be interpolated (data points on the lines of fit) or extrapolated (data points on the dotted lines), except for the 3 single-replicate samples in **C** that had particularly high TTP values (≥370 h or more), that could not be extrapolated and were aberrantly plotted based on the row numbers by the software.

The ability of PBMCs from the UK and South African populations to inhibit mycobacterial growth was studied using the TTP values and the estimated log CFU values from these three independent experiments of mycobacterial growth inhibition. *Figure 4.16* shows the data from these experiments. As observed in the previous experiments, the variation in the ability of

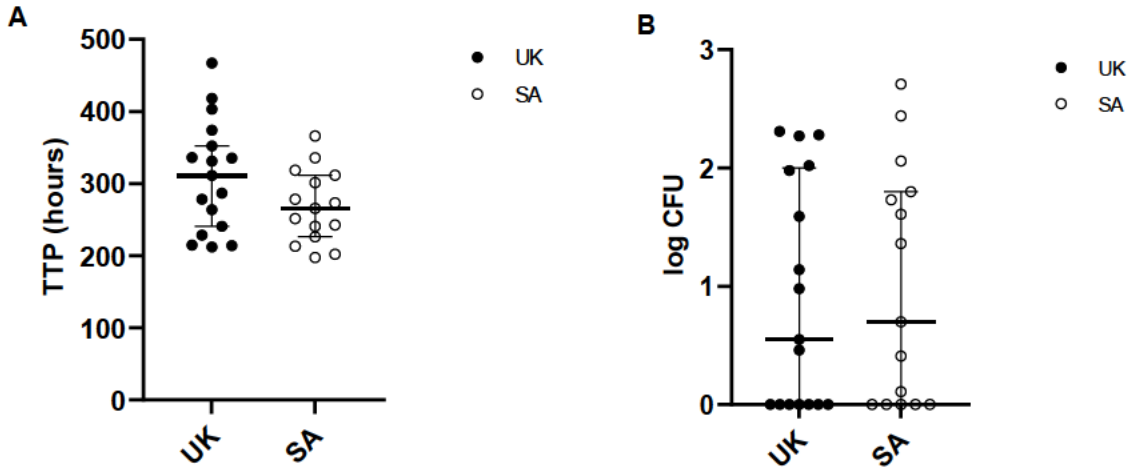



**Figure 4.16.** *Mycobacterial Growth inhibition in the UK and South African PBMC samples.* PBMCs from 10-week-old UK and South African (SA) BCG-vaccinated infants were infected with 500 CFU BCG and rotated for 96 h at 37°C. The samples were then centrifuged, supernatants taken off, the cells lysed and cultured in BACTEC960 MGIA system for 42 days. **A** shows scatter plots of TTP values, and **B** – the estimated log-transformed CFU values for samples obtained from the UK (closed circles) and SA (open circles) infants. The bars indicate the median and interquartile range values. The datasets include samples with data available from single replicates only. The log CFU values could not be extrapolated for 7 UK samples (3 single-replicate samples) and 6 SA samples (1 single- and 5 double-replicate samples). In these cases, an arbitrary log CFU value of 0 was assigned (**B**). UK: n = 17; SA: n = 15.

infant PBMCs to inhibit the growth of BCG as shown by both the TTP values and BCG log CFU estimates was large in both groups, suggesting a role for factors contributing to individual differences among the infants in determining the differences in their ability to inhibit mycobacterial growth.

One of the factors previously reported to contribute to differences in immune responses to BCG vaccine, is the sex of the infant. To examine whether the sex of the infants had any effect on the ability of infant PBMCs to inhibit mycobacterial growth, the MGIA data from the UK infant group was then broken down by sex (*Figure 4.1716)*. In this set of samples, PBMCs from female infants had statistically significantly higher TTP values than PMBCs from male infants *Figure 4.17A*; p = 0.0395). The estimated BCG log CFU values, however, did not differ between male and female UK infants (*Figure 4.17B*; p = 0.1625).

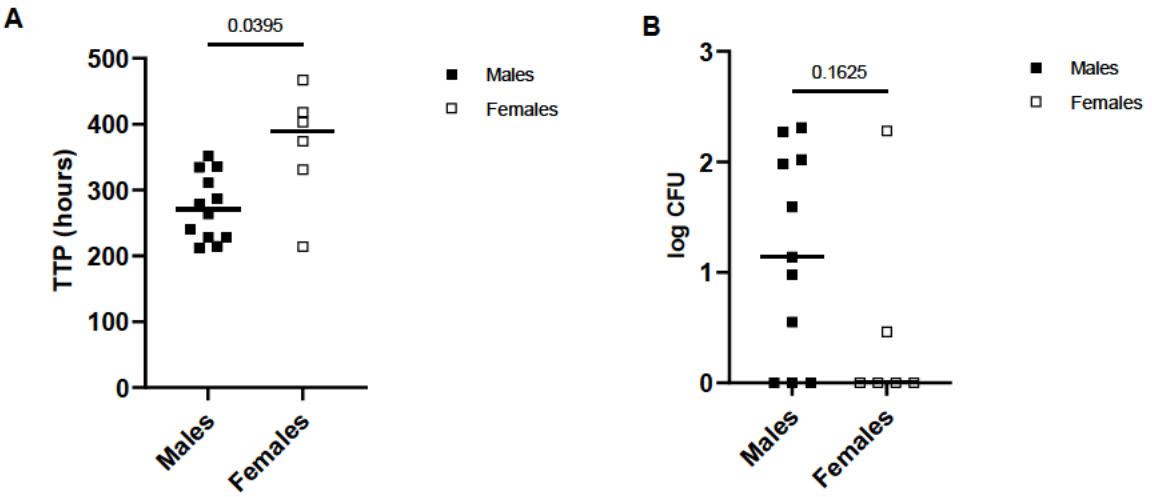



**Figure 4.17.** *Mycobacterial Growth inhibition by PBMCs from UK male and female infants.* PBMCs from 10-week-old UK BCG-vaccinated infants were infected with 500 CFU BCG and rotated for 96 h at 37°C. The samples were then centrifuged, supernatants taken off, the cells lysed and cultured in the BACTEC960 MGIT system for 42 days. **A** shows scatter plots of TTP values, and **B** – the estimated log-transformed CFU values for samples obtained from male (closed squares) and female (open squares) infants. The bars indicate the median values. A Mann-Whitney U-test was performed to compare the datasets. The datasets include samples with data available from single replicates only. The log CFU values could not be extrapolated for 3 male samples (1 single-replicate sample) and 4 female samples (3 single-replicate samples). In these cases, an arbitrary log CFU value of 0 was assigned (**B**). Males: n = 11; females: n = 6.

Because previous studies associated the timing of BCG vaccination with the extent of responses to heterologous stimulation in BCG vaccinated infants (237), the MGIA results from UK BCG vaccinated infants who received their vaccination in the first 24 hrs of life was therefore compared to those who were vaccinated slightly later (*Figure 4.18*); however, no statistically significant differences in the two groups with respect to their TTP values or BCG log CFU estimates were found *(*p=0.4421 and p=0.2594 respectively; *Figure 4.18A, B)*, possibly because of the small dataset.

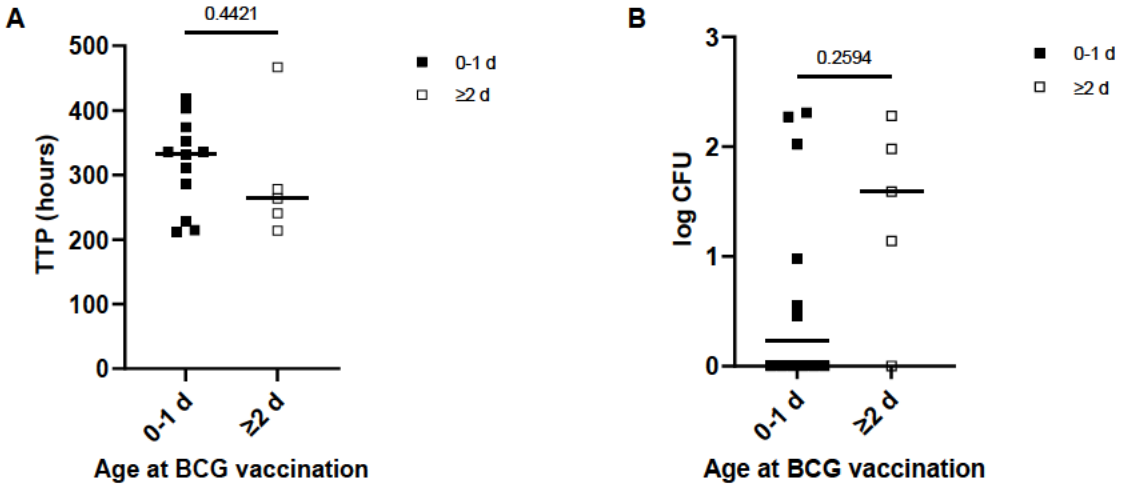



**Figure 4.18.** *Mycobacterial Growth inhibition by PBMCs from the UK infants given BCG at 0-1 or ≥2 days after-birth.* PBMCs from 10-week-old UK BCG-vaccinated infants were infected with 500 CFU BCG and rotated for 96 h at 37°C. The samples were then centrifuged, supernatants taken off, the cells lysed and cultured in BACTEC960 MGIA system for 42 days. **A** shows scatter plots of TTP values, and **B** the estimated log-transformed CFU values for samples obtained from infants who received their vaccine at 0-1 d (closed squares) or ≥2 days post birth (open square). The bars indicate the median values. A Mann-Whitney U-test was performed to compare the datasets. The datasets include samples with data available from single replicate only. The log CFU values could not be extrapolated for six 0-1 d samples (2 single-replicate samples) and in one ≥2 d sample (single-replicate). In these cases, an arbitrary log CFU value of 0 was assigned **(B)**. Samples from infants who received their BCG vaccine at 0-1 d post birth: n = 12; ≥2 days post birth: n = 5.

## 4.4 Discussion

### *4.4.1 BCG and TLR agonists induce differential CD14++CD16- monocyte subpopulations and define the expression pattern of trained immunity associated markers in the UK and South African infants*

Although there is evidence that mycobacteria-specific immune responses in BCG-vaccinated infants can differ across human populations (305,306), few studies have conducted parallel comparisons of such responses. There are even fewer studies that directly compare the innate immune responses to this vaccine in different human populations, although circumstantial evidence from studies on heterologous effects of BCG or infant BCG immunisation studies suggests there might be inherent differences in the innate immune responses to the BCG vaccine or other stimuli (*Tables S4.6; S4.7*). The work described in this chapter was inspired by this hypothesis, however, because it was conducted on samples from two cohorts rather than a single study designed specifically to compare the innate immune responses of BCG-vaccinated infants in the UK and South Africa, it was not possible to directly compare the findings from each study group. Therefore, this study focused on investigating in parallel the expression of surface markers associated with trained immune responses, intracellular and cell culture supernatant cytokines, expression of innate immune genes potentially associated with protection against tuberculosis or trained immunity and the capacity for mycobacterial growth inhibition in PBMC samples from UK and South African infants.

The work described in this chapter observed differential effects of BCG and TLR agonists on the frequencies of TLR4 expressing CD14++CD16- cells and surface expression of TLR4: downregulation of TLR4 in response to infant PBMC stimulation with LPS and an enhancing effect on cells stimulated with Pam3Cys. Importantly, this effect was observed in samples from both cohorts. Differential effects of TLR agonists or β-glucan on the expression of some monocyte surface markers, such as CD11b, CD14, CD25, CD68, or CD86 have been described before and have also been reported to influence monocyte responses to subsequent stimuli; for instance, TLR3-primed monocytes were more prone to apoptosis upon

LPS stimulation (203,338). The downregulatory effect of LPS on TLR4 surface expression observed in this study has also been previously described and associated with CD14-mediated endocytosis (339–341). No effect of BCG stimulation on surface expression of TLR4 or CD11b was observed in the work described in this chapter. This contrasted with previous BCG immunisation studies in adults where lowered or elevated expression of TLR4 was found at 2 weeks or from 3 months to 1 year post BCG vaccination respectively (191,213). Previous studies also detected elevation in surface CD11b was also detected in BCG-vaccinated UK infants at 4 months post immunisation (223). In the work described in this chapter, only samples from BCG-vaccinated infants were used in the analysis, so it was not possible to determine if BCG vaccination influenced the responsiveness of CD14++CD16- monocytes to BCG and TLR agonists. Different monocyte subpopulations have been reported to express varying levels of CD11b (342–344), so if any intermediate monocytes were detected as CD14++CD16- monocytes, that may have influenced the estimation of CD11b+ cell frequencies or surface expression of this molecule. It is also possible that 6 hour PBMC stimulation was too short for any effects of BCG on the expression of TLR4 to manifest in this assay or that the positive BCG vaccination status of the infants recruited for this study may have prevented BCG-dependent modulation of TLR4. Further work will be needed to determine how surface CD11b and TLR4 expression might influence UK or South African infant responses to *M. tuberculosis* or other infectious agents or such cellular functions as monocyte adhesion, complement signalling or recognition and internalisation of microorganisms.

Previous studies on trained immunity and mycobacteria-specific or heterologous effects of BCG suggested that production of some inflammatory innate cytokines, especially, IL-1β, IL-6 and TNFα can be modulated by BCG vaccination (170,177,191,205,209,213,217,219,223,236,238). To investigate the behaviour of cytokines associated with trained immunity and heterologous effects of BCG, intracellular levels of IL-6 and TNFα were measured in the UK and South African infant PBMCs treated with BCG or

TLR agonists. Just as with the expression of TLR4, there was a clear pattern for stimulus-specific responses. While BCG stimulated TNFα production by CD14++CD16- cells, LPS induced production of both TNFα and IL-6, and Pam3Cys induced production of IL-6 more strongly than BCG or LPS, although it was also a potent inducer of TNFα responses. These stimulus-specific differences in TNFα and IL-6 responses were also consistent with previous findings (338).

### *4.4.2 Profiles of cytokines associated with heterologous responses in the UK and South African cohorts*

Apart from the established markers of trained immunity, enhanced or suppressed production of other innate cytokines and chemokines has also been associated with heterologous responses to secondary stimulation in BCG-vaccinated children (*Table S4.6*). The work described in this chapter examined the production of cytokines associated with trained immunity or heterologous responses in BCG-vaccinated infants, including IL-1 family cytokines or growth factors (*Table S4.2*). This work found that apart from IL-12p40 or IL-1α, BCG and TLR agonists stimulated the production of innate cytokines in PBMCs from both infant cohorts, although some of the cytokines tended to prefer specific stimuli: IL-1RA was induced by BCG or LPS, IL-1α – by LPS, while MCP-1 – by Pam3Cys specifically. Overall, BCG and Pam3Cys tended to induce more similar cytokine profiles than LPS and BCG or LPS and Pam3Cys, especially in the UK infant cohort.

Although this study was not able to compare the cytokine profiles in BCG-vaccinated UK and South African infants directly, the production of EGF, G-CSF and IL-6 in South African infant PBMCs cultures and the production of GM-CSF and IL-1β in cultures stimulated with LPS and BCG tended to be higher than that in the samples of the UK infants. This was similar to findings from studies designed to compare the cytokine profiles in BCG-vaccinated infants in the UK and other countries. Higher production of G-CSF, GM-CSF but not IL-6 had also been found

in Malawian infants at 3 months post BCG vaccination compared to the UK infants (306,307). Increased production of IL-1β was found in Uganda compared to the UK in another infant cytokine profile comparison (130).

It is unknown if these subtle differences in the cytokine milieu of BCG-vaccinated infants from different populations could influence their responses to mycobacterial or other infections, although such a possibility exists. Macrophages derived by monocyte stimulation with GM-CSF have been associated with higher levels of CD1a and CD206 and lower levels of CD14, CD163, IL-6, IL-8, and TNFα than macrophages derived through M-CSF or *M. obuense* differentiation (345). In murine models, injection of mice with β-glucan or BCG enhanced myelopoiesis in GM-CSF and IL-1β dependent manner, and, in the case of BCG, could contribute to murine protection against polymicrobial sepsis or *M. tuberculosis* challenge (221,227,242,253). In the work described in this chapter, leukocyte populations other than monocytes in samples from the UK or South African infants were not quantified, so it was not possible to estimate the possible differences in non-monocytic myeloid cell populations or their frequencies in the two infant groups. However, even if possible differences in cytokine profiles reflected possible cellular differences in the infant groups, whether this could be associated with protection against TB, is not quite clear. Variations in monocyte to lymphocyte (ML) ratio and differentially polarised monocyte transcriptional or cytokine responses in BCG-vaccinated South African infants have been associated with susceptibility to TB in the past (346). In addition, *Mtb* has been shown to exploit haematopoietic cell differentiation in mice and manipulate monocyte proliferation in human CD34+ PBMC *in vitro* studies to enhance its spread (347,348). Further work, ideally, in a specially designed, standardised multi-site study would be needed to determine which factors contributed to differences in the UK and South African infant cytokine profiles detected by the MBA analysis.

It should be noted that other, non-monocytic cellular sources of cytokines may have also contributed to cytokine production by PBMCs from the UK and South African infants in the MBA analyses. While the cytokines examined in this study are produced by monocytes

(349,350), only minor effects on IL-6 production were observed in FACS analysis of CD14++CD16- cell population. Apart from monocytes, NK cells have also been associated with trained immunity and have been shown to produce higher levels of IL-1β, IL-6 and TNFα or correlate with higher IL-10 or IL-12p40 responses in BCG-vaccinated individuals upon mycobacterial or heterologous stimulation (197,223). BCG revaccination in infants has also been shown to expand IFNγ-producing NK and NKT-like cell frequencies for up to 1 year post revaccination (351). Other potential contributors to differences in innate cytokine production in BCG or TLR agonist stimulated PBMC cultures could be other innate lymphoid cells (ILCs). ILCs are innate lymphoid cell lineage similar to T-cells; they can respond to a range of stimuli and can also be found in PBMC cultures. It is not known, however, if frequencies of these cells influenced cytokine profiles in the UK and South African infants in this study, however, it would be interesting to study their involvement or potential contribution against TB, whether through cytokines or other mechanisms.

### *4.4.3 UK and South African infant PBMCs show high variation in expression of genes associated with protection against TB and/or trained immunity and inhibition of mycobacterial growth*

Trained immunity and the associated downstream cytokine production or other antimicrobial responses can contribute to the protection against infectious diseases, including TB (191,209,227,242). The work described in this chapter identified 4 innate immune genes with a possible role in protection against TB. Two of these genes – *GPR68* and *FBP1* – have been previously associated with signalling of HIF-1α, a transcription factor regulating metabolic pathways associated with trained immunity and heterologous responses to BCG (224,332,334). *GPR68* contains a promoter binding site for HIF-1α and is upregulated by hypoxia (332) while expression of *FBP1* has been shown to correlate with that of HIF-1α, modulate glycolysis in NK cells and be modulated by epigenetic modifications (334–336), suggesting that both of these genes may play a role in trained immunity or heterologous effects

of BCG. Their responses to BCG and heterologous stimuli were therefore explored in BCG-vaccinated UK and South African infants. All of these genes responded to stimulation with BCG and TLR agonists, possibly, due to high donor-associated variation, with the extent of stimulatory or suppressive effect varying for each of the stimulus. For instance, BCG stimulated GPR68 less potently than Pam3Cys and suppressed SCARB2 less than LPS in the UK and South African cohorts. Further work would be needed to investigate the cellular signalling pathways affected by these genes and their relevance in mycobacterial or heterologous infections in BCG-vaccinated individuals.

The work described in this chapter also investigated the ability of infant PBMCs obtained from the UK and South African infants to inhibit mycobacterial growth. Both infant groups showed large variation, suggesting the importance of host-associated factors to mycobacterial growth inhibition. This was not unexpected as stimulus or donor variation were the major factors associated with surface receptor expression, cytokine production or expression of genes encoding molecules involved in innate immune responses in both cohorts.

### *4.4.4 Male and female infant PBMCs respond to BCG and innate stimuli similarly but females might inhibit mycobacterial growth more strongly*

In the work described in this chapter, donor variation was strongly associated with infant responses to BCG or TLR agonists in this study. Although multiple factors could influence the heterologous effects of BCG (304), multiple studies reported possible sex-differential effects on BCG-vaccinated infant responses to heterologous stimuli or all-cause mortality (152,233,236,238,258,352). In addition, a recent BCG immunisation study in adults suggested that BCG vaccine may differentially influence cytokine responses to *M. tuberculosis* or *S. aureus*, enhancing inflammatory cytokines in females and suppressing them in males (259). The work described in this chapter analysed the expression of monocyte surface markers, production of cytokines, expression of the 4 genes associated with protection against TB and

mycobacterial growth inhibition in UK male and female infants. Overall, male and female infant responses to BCG and TLR agonists were very similar, and only a few minor diferences were detected. Unstimulated female CD14++CD16- monocytes expressed more IL-6 and their PBMCs produced less IP-10 upon stimulaton with BCG. PBMCs from female infants inhibited the growth of BCG more strongly than those from male infants. This was reminiscent of the findings from BCG-vaccinated UK adults where females with positive historical BCG immunisation status tended to produce less IP-10 but inhibit the growth of BCG more strongly (235). It should also be noted that the study described in this chapter was small and further investigations would be needed to validate this observation. Also, it should be noted that while sex-differential effects on responses to heterologous stimulation may be present in BCG-vaccinated infants or adults, they may not necessarily directly translate to differential protection from infectious diseases in males and females. For instance, previous systemic reviews on non-specific effects of BCG in male and female infants found no difference in the effects on all-cause mortality (136,144).

### *4.4.5 Limitations*

It should be also noted that there were some limitations to the analysis described in this chapter. First, the work described in this chapter was not designed as a standardised, multi-center study meant to directly compare the innate responses against mycobacteria and heterologous stimuli. Instead, samples available from two infant BCG vaccination cohorts were used. In addition, the characteristics of the two cohorts showed some substantial differences in terms of infant age at vaccination or bleeding. Also, the sample size used in this study was small and reduced further by the exclusion of data from 8 South African infants that received percutaneous BCG. Overall, this resulted in investigating the markers associated with trained immunity and antimycobacterial responses in parallel in these two cohorts. Ideally, a direct comparison would be preferred, however, for this a special multi-site study using standardised participant recruitment and laboratory protocols would be required.

Second, maternal BCG immunisation status has been previously associated with BCG-vaccinated infant responses to heterologous stimuli or the non-specific effects of this vaccine (159,233,238,353,354). In this work, information on maternal BCG immunisation status was not available for either of the two cohorts. Although generally higher maternal TB incidence would be expected for mothers from South Africa, in this study information on maternal TB status was not available in the UK cohort, although the comparison group included several infants whose mothers had been exposed to TB at some point, Although a recent study in Uganda did not find an effect of latent maternal TB infection on their infant cytokine responses to PPD (355), other studies reported differences in production of some innate cytokines or PPD-specific T-cell responses (130,356). Finally, the infant cohorts examined in the work described in this chapter were vaccinated with different strains of BCG (the possible contribution of different strains of BCG to variation in immunological studies is described in *Chapter 6*).

### *4.4.6 Summary*

The study described in this work investigated markers associated with trained immunity and antimycobacterial responses in two infant cohorts from the UK and South Africa. Although the study was affected by the differences in the characteristics of the two cohorts, including different strains of BCG used for vaccination and the data could not be directly compared, it should be noted, that the results obtained from both cohorts showed highly similar patterns in terms of frequencies of CD14++CD16- monocyte subpopulations, surface marker or intracellular cytokine expression, cytokine profiles, expression of genes associated with trained immunity and antimycobacterial responses and mycobacterial inhibition. In this study, the innate immune responses investigated in both infant cohorts were primarily determined by stimuli and interindividual variation. This study therefore investigated the effect of sex in infant immune responses to BCG and TLR agonists. Although some differences were detected, e.g. in mycobacterial inhibition or IP-10 production upon BCG stimulation, the responses were

overall similar in male and female UK infants. Together, this work suggested that the BCG vaccines used to immunise the two cohorts induced similar mycobacteria-specific or heterologous responses in the vaccinated infants irrespective of the variation expected due to the limitations outlined above or other factors.

# **Chapter 5:** Whole blood-based tool for *in vitro* analyses of innate and adaptive cytokine profiles

## 5.1 Introduction

Since the Bacillus Calmette-Guérin (BCG) vaccine was derived in 1920s, BCG has been the only anti-TB vaccine for human use. Although, as described in *Chapter 1*, this vaccine is effective against severe forms of childhood TB (28,29) and induces long lasting immunity in some populations (26,35,36), its efficacy in preventing pulmonary TB disease in adults varies (12,27,30). For many years, novel TB vaccine candidates, designed to replace or boost BCG, have been created and investigated in animal models or human clinical trials, however, no candidate has been licenced for use in humans yet and many still remain in clinical trials or in the development phase (357).

Most of the clinical trials or anti-TB vaccination studies have traditionally focused on measurements of IFNγ, TNFα, IL-2 and/or IL-17 recall responses in the vaccinated individuals, numbers and frequencies of Th1 or Th17 cells expressing these cytokines, or their ability to expand upon stimulation with mycobacterial antigens (358,359). As described in *Chapter 1,* while associated with mycobacterial growth inhibition *in vitro* in some studies, frequencies of polyfunctional CD4+ T-cells expressing these cytokines were shown not to correlate with the BCG-vaccinated infant protection against TB in others (113,114). It has been proposed that other immunological mechanisms could contribute to protection against TB in individuals vaccinated with BCG or TB vaccine candidates, including NK or B-cells, antimycobacterial antibodies, trained immunity or cytokines other than IFNγ, TNFα, IL-17 or IL-2 and that a broader range of antimycobacterial responses should be investigated in future studies (358–360).

Diluted whole blood assays (WBAs) and multiplex bead arrays (MBA) are an effective tool in detecting subtle differences in broad-spectrum cytokine or chemokine responses. Some of these assays allow simultaneous measurement of as many as 41 analytes or more, and, involving broad concentration detection ranges, they are highly sensitive. Previous work conducted in Professor Dockrell's lab using these assays showed that cytokine and chemokine profiles induced by purified protein derivative (PPD) or *M. tuberculosis* lysate in

BCG-vaccinated infant whole blood cultures extend beyond Th1 or Th17 responses, including chemokines (IL-8, IP-10, MIP-1α, MCP3, MDC), growth factors (G-CSF, GM-CSF, PDGF-AA, PDGF-AB/BB, TGFα, Flt3-L), interferons (IFNα2) or other soluble mediators (128,299,306), suggesting that anti-TB responses involve a broad range of factors. Because of their sensitivity and broad range of analytes, MBAs using diluted blood have also been exploited in studies of UK and Ugandan or Malawian infant cytokine signature comparisons or in studies of influence of maternal immunisation on BCG-vaccinated infant cytokine or chemokine profiles (130,307,353,361). Some infant BCG immunisation studies explored them for analyses of heterologous cytokine responses (223,233,236,238). Finally, apart from cytokine profiling in BCG-vaccinated individuals, MBAs can be applied for screening of novel markers of interest in plasma, serum or cell culture supernatants, not only reducing the need for animal use when run on human samples but also allowing prediction of which immune responses could be expected when tested in human volunteers. This may be of particular interest in the context of TB diagnostics or anti-TB immunisation studies using novel vaccine candidates.

Although BCG is a live attenuated mycobacterium, alternative vaccine design approaches have been applied in attempts to develop an anti-TB vaccine with an improved efficacy for prevention of infection, TB disease, its recurrence or for antitubercular therapy. These designs include *M. tuberculosis* proteins encoded by viral vectors, fusion proteins made up of its antigens, and inactivated or live attenuated mycobacterial species, including *M. tuberculosis* itself (358). Because of the diverse nature of these vaccine candidates, they are likely to interact with the immune system via different immunological pathways, induce distinct cytokine profiles and differentially prime the immune systems of the vaccinated individuals. In addition, recent murine immunisation studies suggested that BCG and other trained immunity inducing agents can contribute to protection against *M. tuberculosis* or other microbial challenges in animals injected with these agents (221,227,242). Although it is not fully clear how BCG or other stimuli epigenetically reprogram myeloid cell progenitors in the bone marrow (BM) of the immunised mice and whether BCG modulates gene expression in the BM in vaccinated human

adults using the same mechanisms, IL-1β and G-CSF have been implicated in reprogramming or regulation of downstream effects on myelogenesis (221,227,242,253). However, previous investigations of BCG-vaccinated infant whole blood responses to mycobacteria and heterologous stimuli suggest that other cytokines can play a role in either inducing or mediating downstream effects of trained immunity and that MBAs can be a useful tool to identify such cytokines and determine the breadth of these responses (223,233,238).

One of the aims of this work was to optimise a system involving a 41-plex MBA, whole blood cultures from healthy UK volunteers and BCG or TB vaccine candidates. Importantly, instead of investigating frequently studied Th1- or Th17-specific cytokine profiles, this work aimed to explore broad whole blood cytokine signatures induced by BCG or TB vaccine candidates. Importantly, this study aimed to characterise cytokine signatures unique to different types of TB vaccine candidates, including viral vectors, subunit vaccines and viable mycobacteria. To do this, cytokine signatures including but not limited to Th1 and Th17 responses were explored for 4 previously reported TB vaccine candidates: *M. tuberculosis* antigen 85A encoded by modified *Vaccinia* Ankara (MVA) viral vector (362), fusion protein 1 (FP1) in spore (363) or nanoparticle (364) formulations and the ΔBCG1419c strain of BCG (365). To address the adjuvanticity potential of the components making up these vaccines, whole blood responses to the components and adjuvants of these vaccines or their combinations, or, where live vaccine candidates themselves were used, their parental strains were tested to investigate their individual contributions to the total cytokine production. Finally, this study also aimed to investigate the potential of vaccines other than BCG or *Vaccinia*-based vaccines to induce trained immunity. To address this and predict whether such vaccines or their components could have heterologous effects *in vitro* or *in vivo*, this study investigated whether FP1 vaccine candidates or their components induced cytokine profiles associated with this mechanism.

## 5.2 Methods

### *5.2.1 Whole blood cultures and supernatant preparation*

Peripheral blood samples were obtained from anonymous adult volunteer donors recruited via the LSHTM blood donation system. A prior written, informed consent was obtained from each donor recruited in this study, with an opportunity to withdraw from the study at any point. Four ml of each blood sample were diluted with 6 ml RPMI 1640 (Life Technologies, UK) supplemented with 1% (2 mM) L-glutamine (Sigma-Aldrich, UK) and pre-warmed to 37$^{o}$C. Blood was then mixed with the cell culture medium by gently inverting the tube a few times and 100 µl of the diluted blood was then loaded onto 96-well U-bottom plates containing 100 µl of twice the final concentration of each TB vaccine candidate, its component and/or adjuvant (or their ten-fold dilutions) and RPMI (with 1% L-glutamine) to reach the final 1 in 5 blood dilution. The plates were then sealed with micropore tape and cultured at 37$^{o}$C, 5 % $CO_2$ for 48 h, after which, the cells were centrifuged at 400 x g for 5 min and their supernatants were collected and stored at -70$^{o}$C until further analysis. The information on the TB vaccine candidates, their composition and the final concentrations used to stimulate whole blood cultures are listed in *Table 5.1*.

**Table 5.1.** *The TB vaccines, their components and concentrations tested.*

| Vaccine / component | Vaccine or control category | Amount / concentration code | Amount / final concentration |
|---|---|---|---|
| **MVA-GFP** | **Viral vector control** | **1** | 1x10$^{7}$ PFU/well |
| | | **2** | 1x10$^{6}$ PFU/well |
| **MVA85A** | **Viral vector** | **1** | 1x10$^{7}$ PFU/well |
| | | **2** | 1x10$^{6}$ PFU/well |
| | | **3** | 1x10$^{5}$ PFU/well |
| **FP1** | **Fusion protein 1** | **1** | 10 µg/ml |
| | | **2** | 1 µg/ml |
| | | **3** | 0.1 µg/ml |
| **Spore-FP1** | ***B. subtilis* spores + fusion protein 1** | **1** | 1x10$^{8}$ units/well |
| | | **2** | 1x10$^{7}$ units/well |
| | | **3** | 1x10$^{6}$ units/well |

| | | | |
|---|---|---|---|
| **Spore-FP1 + Quil A** | ***B. subtilis* spores + fusion protein 1 + adjuvant** | **1** | $1x10^8$ units/well<br>50 µg/ml adjuvant 1 |
| | | **2** | $1x10^7$ units/well<br>5 µg/ml adjuvant 1 |
| | | **3** | $1x10^6$ units/well<br>0.5 µg/ml adjuvant 1 |
| **Spore** | ***B. subtilis* spores** | **1** | $1x10^8$ units/well |
| | | **2** | $1x10^7$ units/well |
| | | **3** | $1x10^6$ units/well |
| **Nano-FP1** | **Nanoparticles + fusion protein 1** | **1** | 0.05% |
| | | **2** | 0.005% |
| | | **3** | 0.0005% |
| **Nano-FP1 + Quil A** | **Nanoparticles + fusion protein 1 + adjuvant** | **1** | 0.05%<br>50 µg/ml adjuvant 1 |
| | | **2** | 0.005%<br>5 µg/ml adjuvant 1 |
| | | **3** | 0.0005%<br>0.5 µg/ml adjuvant 1 |
| **Nano** | **Nanoparticles** | **1** | 0.05% |
| | | **2** | 0.005% |
| | | **3** | 0.0005% |
| **Quil A** | **Adjuvant** | **1** | 50 µg/ml adjuvant 1 |
| | | **2** | 5 µg/ml adjuvant 1 |
| | | **3** | 0.5 µg/ml adjuvant 1 |
| **ΔBCG1419c** | **A mutant strain of BCG (deletion of c-di-GMP phosphodiesterase (PDE) encoding gene)** | **1** | $1x10^5$ CFU/well |
| | | **2** | $0.5x10^5$ CFU/well |
| | | **3** | $0.1x10^5$ CFU/well |
| **BCG WT** | **BCG-Pasteur 1173P2 (wild-type BCG strain)** | **1** | $1x10^5$ CFU/well |
| | | **2** | $0.5x10^5$ CFU/well |
| | | **3** | $0.1x10^5$ CFU/well |
| **LPS** | **Positive control** | **-** | 10 ng/ml |
| **Medium** | **Negative control (cell culture medium)** | **-** | - |

### *5.2.2 Preliminary supernatant screen*

Because the experiments on monocyte training described in *Chapter 2* suggested that some cytokines, such as IL-6 can be produced at high levels and because whole blood containing cell mixtures rather than purified cell populations were used to test the novel TB vaccines, IL-6 levels were first measured in supernatants from these cultures at 1 in 10 dilution. Concentrations of IL-6 were measured with a Human IL-6 ELISA MAX Standard kit (BioLegend, USA) as per manufacturer's instructions. Briefly, 96-well plates were coated overnight at 4$^{o}$C with αIL-6 capture antibodies. The plates were then placed on a plate shaker and blocked with PBS containing 1% BSA. Thawed supernatants were loaded in duplicate onto each plate and incubated at room temperature for 2 h, followed by the addition of biotinylated detection antibodies. They were then treated with avidin-labelled horseradish peroxidase (Av-HRP), followed by TMB (3,3´,5,5´-tetramethylbenzidine) substrate solution (ThermoFisher, USA; for ELISA MAX Standard Kit tests) for 15 min. Colour development reactions were stopped with 0.18M sulphuric acid. The plates were washed 4 times with PBS-0.05% Tween-20 (Sigma-Aldrich; USA) between the steps to remove excess antibodies or sample analytes, and 5 times with 45 s incubation on the bench top between each washing step to remove Av-HRP. The optical density (absorbance) readings were taken with a Spectramax M3 plate reader (Molecular Devices, UK), the duplicate sample values averaged and plotted against the standard concentrations. They were then log-normalised and 4 parameter logistic (4PL) regression was applied to interpolate the cytokine concentrations using GraphPad Prism 9 software (GraphPad Software, USA).

### *5.2.3 Multiplex beads array analysis of supernatants from whole blood cultures stimulated with novel TB vaccine candidates*

The procedures for sample analysis by multiplex bead array were described in *Chapter 4*. Briefly, supernatants from whole blood cultures stimulated with cell culture medium or TB vaccine candidates were thawed and analysed by multiplex bead array using a 41-plex human

cytokine/chemokine Milliplex MAP Human Cytokine/chemokine Magnetic Bead Panel (Merck-Millipore, USA). The kit covered the following analytes: epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), eotaxin (CCL11), transforming growth factor alpha (TGFα), granulocyte colony stimulating factor (G-CSF), Flt-3 ligand (Flt-3L), granulocyte-macrophage colony stimulating factor (GM-CSF), fractalkine (CX3CL1), IFNα2, IFNγ, GRO (CXCL1), IL-10, MCP-3 (CCL7), IL-12p40, MDC (CCL22), IL-12p70, platelet-derived growth factor (PDGF)-AA, IL-13, PDGF-AB/BB, IL-15, soluble CD40 ligand (sCD40L), IL-17A, IL-1RA, IL-1α, IL-9, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), TNFα, TNFβ, and vascular endothelial growth factor (VEGF). Involvement of these cytokines in mycobacteria-specific and heterologous responses in BCG-vaccinated infants are listed in *Table S4.5*., and the reported association of some of these cytokines with antimycobacterial responses in TB, trained immunity and immune response to BCG studies in *Table 5.3*.

Whole blood supernatants were processed as per the manufacturer's instructions.

One day before the experiment, microbeads for each cytokine of interest were warmed to RT, sonicated for 30 s and vortexed for 1 min. Then 60 µl of each type of microbeads were added to a bottle and diluted with Bead Diluent to a final 3.0 ml volume. After this, the beads were mixed and stored at 4$^{o}$C until used the next day. The wash buffer was also prepared on the same day, using 10X concentrate diluted with deionized water. This buffer was also stored at at 4$^{o}$C until further use. On the day of the experiment, the cell culture supernatants were thawed, and the beads and the wash buffer warmed to RT. Quality controls and a series of five-fold dilutions of human cytokine standards were prepared immediately before running the assay.

The assay plate was gently washed and dried by blotting on absorbent paper before adding 25 µl of cytokine standards and quality controls (in duplicate) to the appropriate wells, followed by 25 µl assay buffer added to all the wells. The samples were then loaded to the appropriate wells in single 25 µl replicates. This was followed by adding 25 µl vortexed microbeads to each

well on the plate, which was then sealed and incubated at RT for 2 hours on a plate shaker. The plate was then washed and dried twice with 200 µl wash buffer before adding 25 µl detection antibodies to each well. This was followed by 1 hour incubation on a plate shaker and another 30 min incubation with 25 µl streptavidin-phycoerythrin per well. The plate was then washed two more times, dried, and 150 µl PBS was added to each well. The beads were then resuspended by vortexing the plate for 5 min.

Data were acquired using the Magpix system (Luminex, USA) and xPONENT software (Luminex, USA) and analysed using GraphPad Prism 9 software (GraphPad Software, USA). During the analysis of cytokine production in infant PBMC samples, in those cases where cytokine concentration values exceeded those of the top cytokine standard, they were assigned an arbitrary value of 10,000 pg/ml, and if they were lower than the lowest cytokine standard (3.2 pg/ml), they were assigned an arbitrary value of 1.6 pg/ml.

### *5.2.4 Statistical analyses*

To test the differences in the production of individual cytokines induced by different TB vaccines or their components and compare them to cytokine levels in unstimulated samples, or to test the differences in cytokine levels in response to stimulation with fusion protein, its adjuvant or different vaccine formulations, a Kruskal-Wallis test with Dunn's post-hoc correction was used. To analyse the differences in cytokine levels induced by different doses of TB vaccines or their components, viral vector and its control or ΔBCG1419c and its control, a Wilcoxon matched-pairs signed rank test with Benjamini, Krieger and Yekutieli correction was applied. Principal component analysis (PCA) was conducted to test the similarity of cytokine responses to full formulations of the TB vaccine candidates used in this study. All dataset analyses, including the PCA, were analysed using GraphPad Prism 9 software (GraphPad Software, USA).

## 5.3 Results

### *5.3.1 Preliminary supernatant screens*

One of the aims of the assay optimisation work in this study was to test the effect on whole blood cytokine profiles of different doses of TB vaccine candidates and their components or adjuvants. Because of the high expense of MBAs and the limited amount of the reagents available for screening, to determine which concentrations of TB vaccine candidates, their components or adjuvants should be tested using MBA, a preliminary IL-6 ELISA screen of diluted supernatants from whole blood cultures stimulated with TB vaccine candidates listed in *Table 5.1* was therefore run using samples from two donors. *Table 5.2* and *Figure 5.1* show the concentrations of IL-6 detected in these supernatants for each donor.

**Table 5.2.** *IL-6 ELISA screen of samples stimulated with BCG or TB vaccine candidates or their components used in selection of conditions to be tested by Luminex*.

| **TB vaccine or its component and concentration code** (see *Table 5.1*) | **Donor** | | **Mean IL-6 concentration (pg/ml)** | **Condition selected for Luminex analysis?** | **Reason for excluding the condition** |
|---|---|---|---|---|---|
| | **280** | **284** | | | |
| **MVA-GFP (1)** | 80 | 213 | **147** | Yes | -♣ |
| **MVA-GFP (2)** | 116 | 191 | **154** | Yes | -♣ |
| **MVA85A (1)** | 84 | 194 | **139** | Yes | -♣ |
| **MVA85A (2)** | 119 | 178 | **148** | Yes | -♣ |
| **MVA85A (3)** | 108 | 173 | **140** | No | Similar to baseline |
| **FP1 (1)** | 573 | 555 | **564** | Yes | - |
| **FP1 (2)** | 93 | 185 | **139** | No | Similar to baseline |
| **FP1 (3)** | 83 | 184 | **134** | No | Similar to baseline |
| **Spore-FP1 (1)** | 3 | 1 | **2** | No | Dose toxic to cells |
| **Spore-FP1 (2)** | 5000* | 23 | **2511** | Yes | - |
| **Spore-FP1 (3)** | 5000* | 5000* | **5000** | Yes | - |
| **Spore-FP1 + Quil A (1)** | 481 | 12 | **246** | No | Dose toxic to cells |
| **Spore-FP1 + Quil A (2)** | 1536 | 5000* | **3268** | Yes | - |

| | | | | | |
|---|---|---|---|---|---|
| **Spore-FP1 + Quil A (3)** | 5000* | 5000* | **5000** | Yes | **-** |
| **Spore (1)** | 0 | 3 | **1** | No | Dose toxic to cells |
| **Spore (2)** | 5000* | 5000* | **5000** | Yes | - |
| **Spore (3)** | 5000* | 5000* | **5000** | Yes | - |
| **Nano-FP1 (1)** | 101 | 191 | **146** | Yes | - |
| **Nano-FP1 (2)** | 100 | 206 | **153** | Yes | - |
| **Nano-FP1 (3)** | 97 | 162 | **129** | No | Similar to baseline |
| **Nano-FP1 + Quil A (1)** | 108 | 175 | **142** | Yes | **-♣** |
| **Nano-FP1 + Quil A (2)** | 90 | 188 | **139** | Yes | **-♣** |
| **Nano-FP1 + Quil A (3)** | 81 | 192 | **136** | No | Similar to baseline |
| **Nano (1)** | 96 | 181 | **138** | Yes | **-♣** |
| **Nano (2)** | 94 | 175 | **135** | Yes | **-♣** |
| **Nano (3)** | 96 | 200 | **148** | No | Poor IL-6 response |
| **Quil A (1)** | 5000* | 5000* | **5000*** | Yes | - |
| **Quil A (2)** | 141 | 367 | **254** | Yes | - |
| **Quil A (3)** | 104 | 191 | **148** | No | IL-6 response lower than J1 or J2 |
| **ΔBCG1419c (1)** | 173 | 369 | **271** | Yes | - |
| **ΔBCG1419c (2)** | 153 | 252 | **202** | Yes | - |
| **ΔBCG1419c (3)** | 96 | 203 | **149** | No | IL-6 response lower than K1 or K2 |
| **BCG WT (1)** | 208 | 242 | **225** | Yes | - |
| **BCG WT (2)** | 969 | 264 | **616** | Yes | - |
| **BCG WT (3)** | 105 | 215 | **160** | No | IL-6 response lower than L1 or L2 |
| **LPS** | 5000* | 5000* | **5000*** | Yes | - |
| **Medium**** | 73 | 176 | **124** | Yes | - |

***** Denotes an arbitrary assigned value where OD of samples diluted at factor 1:10 exceeded that of the standards.

** Denotes the mean value of two replicate samples.

♠ Although these vaccines or their components did not stimulate strong IL-6 responses, they were included in Luminex analysis as they might induce greater secretion of other cytokines.

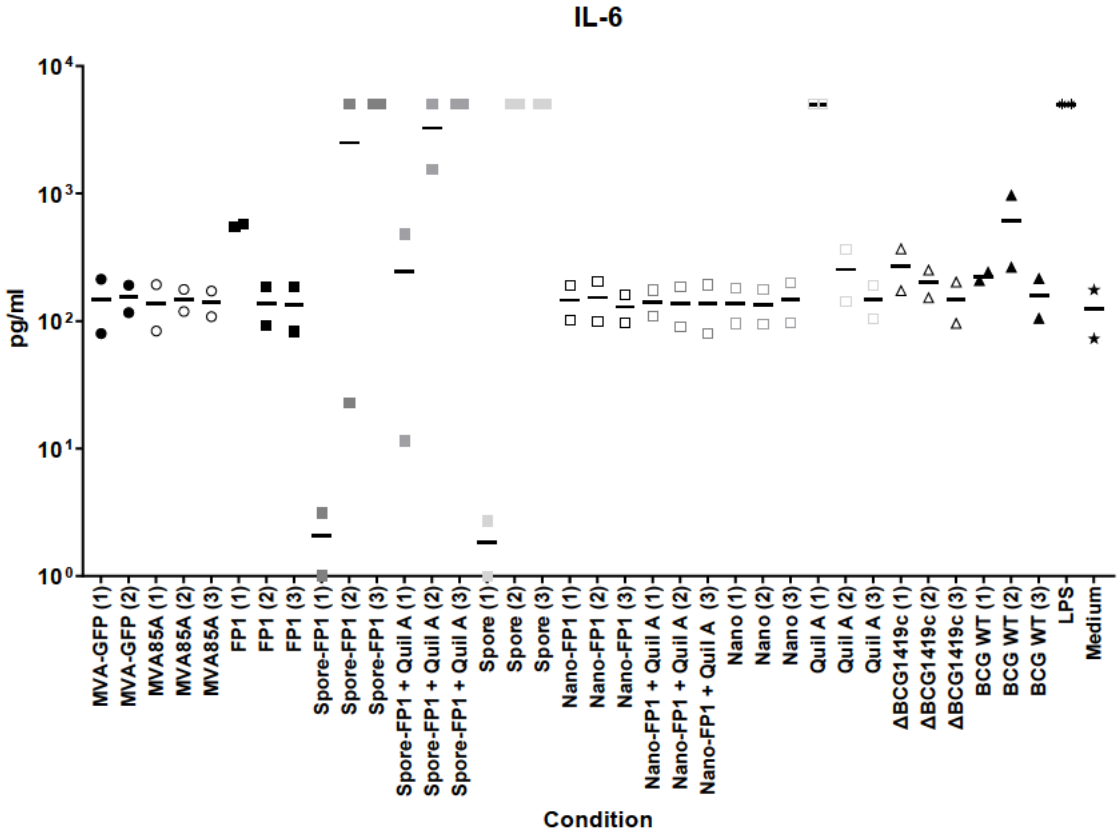



**Figure 5.1.** *IL-6 concentrations in whole blood supernatant samples after 48 h stimulation with BCG or TB vaccine candidates and their components as measured in the IL-6 ELISA.* The bars show mean IL-6 concentrations in supernatants of whole blood cultures treated with different vaccines or their components. Vaccines are labelled, and numbers in the brackets indicate the highest dose of each vaccine or its component, with 1 being the highest dose and 3 being the lowest dose as indicated in *Table 5.1*. N = 2.

As expected, some stimulation conditions induced cytokine responses above the detection limit of the IL-6 ELISA assay used in this work (*Table 5.2*). Because no toxic effect of these vaccine candidates or their components on whole blood cultures was observed and other cytokines produced upon stimulation with these vaccines or their components may fall within a detectable range of the MBA used, it was decided to use these vaccines or their components in cytokine profiling experiments. Some TB vaccine candidates or their formulations (Spore-

FP1, Spore-FP1 + Quil A and Spore) were found to have a toxic effect on whole blood cultures if used to stimulate the cells at high doses ($1x10^8$ units/well of Spore-FP1 in the presence of 50 µg/ml Quil A and $1x10^8$ Spore or Spore-FP1 units/well), reflected both by the appearance of cultures (data not shown) and barely detectable concentrations of IL-6 in their supernatants, possibly due to high activation of cells by high doses of *B. subtilis* spores (*Table 5.2*, *Figure 5.1*). It was therefore decided to exclude the highest concentrations of Spore-FP1, Spore-FP1 + Quil A and Spore formulations (concentration (1)) from the study. Also, some vaccine formulations or their components induced IL-6 responses very similar to those of unstimulated whole blood cultures when added at the lowest doses (MVA85A (3), FP1 (2), FP1 (3), Nano-FP1 (3) or Nano-FP1 + Quil A (3)), and some vaccine candidates or their components induced lL-6 responses barely above the baseline (Nano (3), Quil A (3), ΔBCG1419c (3) or BCG WT (3)). Because of the limited MBA reagents available, it was decided to exclude them from subsequent cytokine profile analyses. Finally, while two of the vaccine candidates did not induce detectable above-the-baseline IL-6 responses (MVA-GFP and MVA85A), it was decided to include this vaccine in downstream analysis as due to the viral nature of the vector used in this vaccine, it may have stimulated production of cytokines other than IL-6.

### *5.3.2 Profiles of IFNγ and the innate cytokines associated with antimycobacterial immunity or heterologous effects of BCG*

Because the BCG vaccine is known to induce antimycobacterial Th1 responses associated with protective antitubercular effects in the vaccinated individuals, the ability of diverse TB vaccine candidates to induce IFNγ responses is studied frequently. Although this study investigated the responses of 41 cytokines and chemokines in whole blood cultures, in this work, 10 innate cytokines previously associated with antimycobacterial immunity, trained immunity or heterologous effects of the BCG vaccine, or previously reported to be differentially expressed in different infant or children populations in this study or others were selected for the analysis (*Table 5.3*).

**Table 5.3.** *Cytokines associated with TB, BCG or trained immunity*

| Cytokine | Reported in studies on | Reported effect | Reference |
|---|---|---|---|
| **MCP-1 / CCL2** | Serum TB markers | ↑ in TB vs HC* in individuals from China | (366) |
| | | ↑ in TB vs IGRA+ contacts from Taiwan | (367) |
| | | ↑ in IGRA -ve vs IGRA +ve contacts from Taiwan | |
| | Plasma TB markers | A trend for ↑ in TB vs HC individuals from India | (368) |
| | Blood TB markers | ↑ in TB vs HC and LTBI individuals from Japan | (369) |
| | Human genetic polymorphisms associated with TB | SNPs associated with susceptibility to LTBI in Thai population | (370) |
| | Heterologous responses to BCG vaccination | ↓ in BCG vaccinated Australian infants at 7 days post vaccination in response to *S. pneumoniae, E. coli, L. monocytogenes, C. albicans*, PEPG, R848 | (238) |
| **IP-10 / CXCL10** | Gene signatures associated with TB | ↑ in TB vs HC individuals from China | (326) |
| | Serum and blood TB markers | ↑ in TB vs LTBI or HC individuals from South Korea | (371) |
| | Plasma TB markers | ↑ in TB vs LTBI in HIV+ and HIV- individuals from Norway | (372) |
| | | ↑ in TB vs HC individuals from India | (368) |
| | | ↑ in TB cases vs household contacts from Ethiopia | (373) |
| | | ↑ in TB cases vs LTBI individuals from China | (374) |
| | Blood TB markers | ↑ in TB or LTBI vs HC individuals from Japan | (369) |
| | Trained immunity | ↑ in recently TB-exposed but uninfected individuals | (131) |

| | | | |
|---|---|---|---|
| | Heterologous responses to BCG vaccination | ↓ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *C. albicans* | (223) |
| | | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate | |
| | | ↓ in BCG vaccinated Australian infants at 7 months post vaccination | (233) |
| | Infant immune responses to BCG vaccination in different populations | ↑ in UK vs Malawian infants at 3 and 12 months post BCG vaccination | (306) |
| | | ↑ in Malawian vs Gambian infants at 3 months post BCG vaccination | (307) |
| | Infant immune responses to BCG vaccination | ↑ in IFNγ responders vs non-responders at 3 yrs post-BCG vaccination | (361) |
| | | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| **MIP-1α / CCL3** | Gene signatures associated with TB | ↑ in HC or LTBI vs TB individuals from UK | (329) |
| | Plasma TB markers | A trend for ↑ in TB vs HC individuals from India | (368) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate, Pam3Cys | (223) |
| | | ↓ in BCG vaccinated Australian infants at 7 days post vaccination in response to PEPG, R848 | (238) |
| | | A trend for ↓ in BCG vaccinated Australian infants at 7 months post vaccination | (233) |
| | | ↓ in BCG vaccinated Australian infants at 7 months post vaccination upon stimulation with *L. monocytogenes* | |

| | | | |
|---|---|---|---|
| | Infant immune responses to BCG vaccination in different populations | ↑ in UK vs Malawian infants at 3 and 12 months post BCG vaccination | (306) |
| | Infant immune responses to BCG vaccination | ↑ in IFNγ responders vs non-responders at 3 yrs post-BCG vaccination | (361) |
| | | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| **EGF** | Plasma TB markers | ↑ in TB cases vs household contacts from Ethiopia | (373) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *E. coli,* Pam3Cys, *C. albicans, S. aureus* | (223) |
| | BCG-vaccinated infant protection from TB | ↑ in TB cases (cluster 1) compared to the controls (cluster 1 and 2)<br>A trend for ↑ in TB cases (cluster 2) compared to the controls (cluster 1 and 2) | (346) |
| **G-CSF / CSF-3** | Serum TB markers | ↑ in TB vs HC in individuals from China | (366) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated mice within first 36 h since BCG vaccination | (242) |
| | Infant immune responses to BCG vaccination in different populations | ↑ in Malawian vs UK infants at 3 months post BCG vaccination | (306) |
| | Infant immune responses to BCG vaccination | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| **GM-CSF / CSF-2** | Serum TB markers | ↑ in TB vs HC in individuals from China | (366) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated mice within first 12 h and at 72 h since BCG vaccination | (242) |

| | | | |
|---|---|---|---|
| | | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate<br>↓ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to LPS and *E. coli* | (223) |
| | Infant immune responses to BCG vaccination | ↑ in IFNγ responders vs non-responders at 3 yrs post-BCG vaccination | (361) |
| | | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| | Infant immune responses to BCG vaccination in different populations | ↑ in Malawian vs UK infants at 3 and 12 months post BCG vaccination | (306) |
| **IL-1α** | Serum TB markers | ↑ in TB vs HC in individuals from China | (366) |
| | TB-associated gene signatures in PBMCs | mRNA ↑ in LTBI vs TB individuals from USA | (322) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate | (223) |
| | Infant immune responses to BCG vaccination | ↑ in IFNγ responders vs non-responders at 3 yrs post-BCG vaccination | (361) |
| | | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| | Infant immune responses to BCG vaccination in different populations | ↑ in Malawian vs UK infants at 3 months post BCG vaccination<br>↑ in UK vs Malawian infants at 12 months post BCG vaccination | (306) |
| | | ↑ in Gambian vs Malawian infants at 3 months post BCG vaccination | (307) |
| **IL-1β** | TB-associated gene signatures | mRNA ↑ in LTBI vs TB individuals from USA | (322) |

| | | | |
|---|---|---|---|
| | Mycobacterial infection model | ↑ in LTBI vs TB macrophages from individuals from Taiwan | (375) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated Guinea Bissau infants at 4 weeks post vaccination in response to PPD, Pam3Cys | (236) |
| | Heterologous responses to BCG vaccination and trained immunity | ↑ in BCG-vaccinated adult individuals from the Netherlands at 3 months post vaccination in response to *M. tuberculosis* and *C. albicans* | (191) |
| | | ↑ in BCG-vaccinated adult individuals from the Netherlands at 1 month post vaccination in response to LPS, *M. tuberculosis* and *C. albicans* | (209) |
| **IL-6** | Serum TB markers | ↑ in TB vs HC individuals from Japan | (366) |
| | | ↑ in TB vs IGRA+ contacts from Taiwan | (367) |
| | Plasma TB markers | ↑ in PTB vs IGRA-positive or IGRA-negative, asymptomatic individuals from Hong Kong | (376) |
| | TB-associated gene signatures | mRNA ↑ in LTBI vs TB individuals from USA | (322) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate, Pam3Cys, *C. albicans, S. aureus* | (223) |
| | | ↑ in BCG vaccinated Australian infants at 7 days post vaccination<br>↓ in BCG vaccinated Australian infants at 7 days post vaccination in response to PEPG and R848 | (238) |
| | | ↑ in BCG vaccinated Australian infants at 7 months post vaccination in response to BCG | (233) |
| | | ↑ in BCG-vaccinated Guinea Bissau infants at 4 weeks post vaccination in response to PMA, PPD, Pam3Cys | (236) |

| | | | |
|---|---|---|---|
| | Heterologous responses to BCG vaccination and trained immunity | ↑ in BCG-vaccinated adult individuals from the Netherlands at 1 month post vaccination in response to LPS, *M. tuberculosis* and *C. albicans* | (209) |
| | BCG-vaccinated infant protection from TB | A trend for ↓ in TB cases (cluster 2) compared to the controls (cluster 1 and 2) | (346) |
| | Infant immune responses to BCG vaccination in different populations | ↑ in UK vs Malawian infants at 3 months post BCG vaccination | (306) |
| | | ↑ in Gambian vs Malawian infants at 3 months post BCG vaccination | (307) |
| | Infant immune responses to BCG vaccination | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| **TNFα** | Serum TB markers | ↑ in TB vs HC individuals from China | (366) |
| | Blood TB markers | ↑ in TB vs HC individuals from Japan | (369) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate | (223) |
| | | ↑ in BCG vaccinated Australian infants at 7 months post vaccination in response to BCG | (233) |
| | | ↑ in BCG-vaccinated Guinea Bissau infants at 4 weeks post vaccination in response to medium, PPD, Pam3Cys | (236) |
| | Heterologous responses to BCG vaccination and trained immunity | ↑ in BCG-vaccinated adult individuals from the Netherlands at 2 weeks and 3 months post vaccination in response to *M. tuberculosis, S. aureus* and *C. albicans*<br>↑ in monocytes trained *in vitro* with BCG in response to LPS, *C. albicans, S. aureus* and *E. coli* | (191) |

| | | | |
|---|---|---|---|
| | | ↑ in BCG-vaccinated adult individuals from the Netherlands at 1 month post vaccination in response to LPS | (209) |
| | Infant immune responses to BCG vaccination | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| **IFNγ** | Plasma TB markers | ↑ in TB vs HC individuals from India | (368) |
| | | ↑ in household contacts vs TB cases from Ethiopia | (373) |
| | Blood TB markers | ↑ in TB vs HC and LTBI individuals from Japan | (369) |
| | TB-associated gene signatures | mRNA ↑ in LTBI vs TB individuals from USA | (322) |
| | | ↑ in TB or LTBI vs HC individuals from China | (326) |
| | Heterologous responses to BCG vaccination | ↑ in BCG-vaccinated UK infants at 4 months post BCG vaccination in response to *M. tuberculosis* lysate | (223) |
| | | ↑ in BCG vaccinated Australian infants at 7 months post vaccination in response to BCG and *M. tuberculosis*<br>↓ in BCG vaccinated Australian infants at 7 months post vaccination in response to *E. coli, H. influenzae,* and LPS<br>↑ % of IFNγ responders to BCG and *M. tuberculosis* in the BCG-vaccinated infant group<br>↓ % of IFNγ responders to *S. aureus, S. pyogenes, E. coli, H. influenzae, L. monocytogenes, C. albicans*, PEPG, Pam3Cys, LPS in the BCG-vaccinated infant group | (233) |
| | | ↑ in BCG-vaccinated Guinea Bissau infants at 4 weeks post vaccination in response to medium, PMA, PPD, Pam3Cys | (236) |

| | | | |
|---|---|---|---|
| | Heterologous responses to BCG vaccination and trained immunity | ↑ in BCG-vaccinated adult individuals from the Netherlands at 2 weeks and 3 months post vaccination in response to *M. tuberculosis* and *S. aureus* | (191) |
| | Infant immune responses to BCG vaccination | ↑ in IFNγ responders vs non-responders at 3 yrs post-BCG vaccination | (361) |
| | | ↑ in BCG-vaccinated vs unvaccinated infants at 3 mos post-BCG vaccination | (128) |
| | Infant immune responses to BCG vaccination in different populations | ↑ in UK vs Malawian infants at 3 and 12 months post BCG vaccination | (306) |
| | | ↑ in Gambian vs Malawian infants at 3 months post BCG vaccination | (307) |

* HC – healthy control

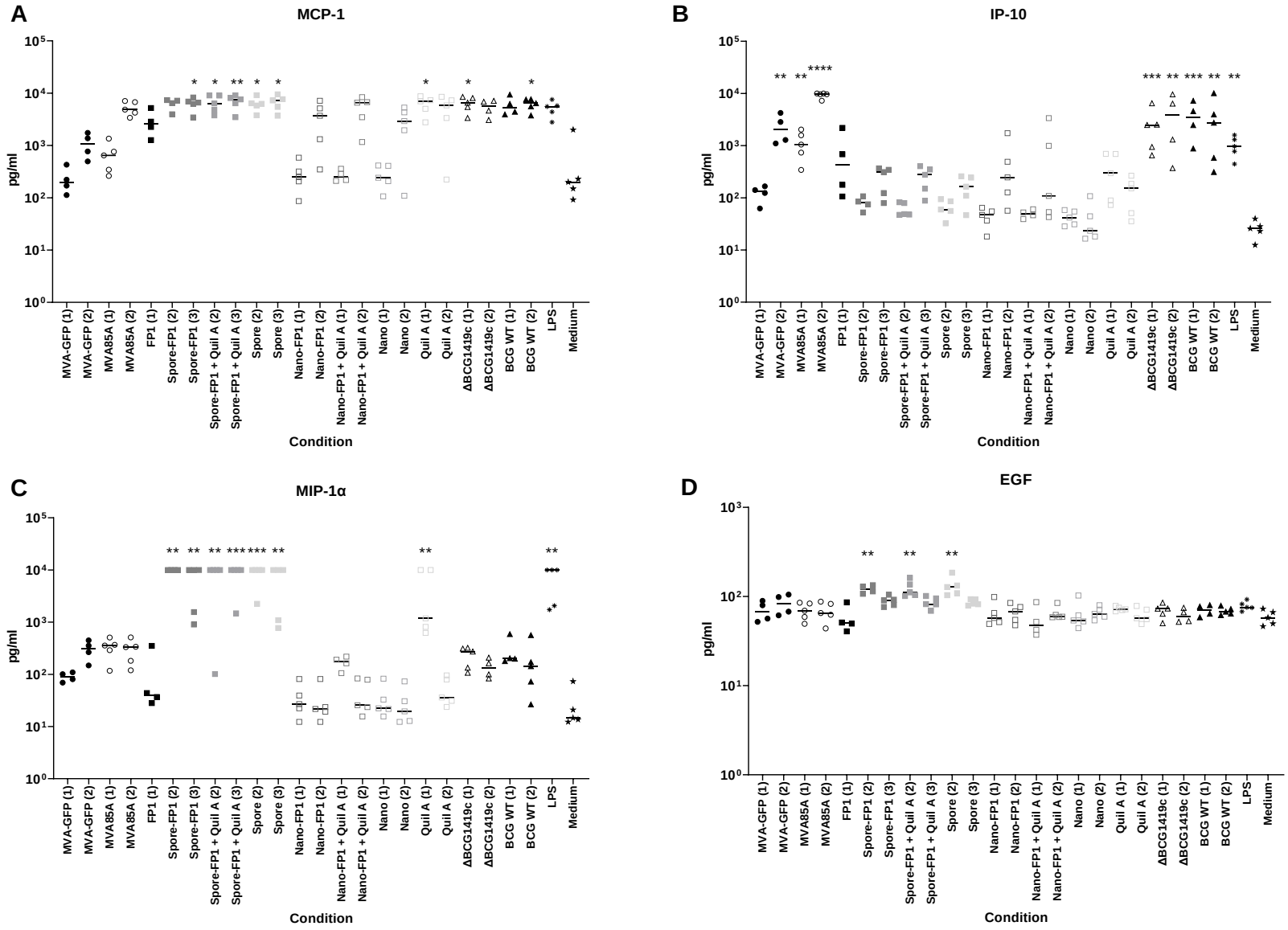
For most of the cytokines or chemokines investigated, production patterns associated with the category of the vaccine candidate or associated controls were observed (*Figure 5.2*). For instance, both the ΔBCG1419c and its control parental BCG WT strain induced high MCP-1 responses ($p = 0.0311$ for ΔBCG1419c (1) and $p = 0.0342$ for BCG WT (2), *Figure 5.2A*) and IP-10 ($p < 0.002$ for all stimulation conditions, *Figure 5.2B*). Elevated IFNγ production was found in cultures stimulated with control BCG (BCG WT (1): $p = 0.0181$, *Figure 5.2K*) and a trend for enhanced production of this cytokine was found in cultures treated with ΔBCG1419c (ΔBCG1419c (1): $p = 0.0732$, *Figure 5.2K*). No statistically significant differences in whole blood cultures stimulated with BCG compared to the unstimulated samples were found for MIP-1α, EGF, G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, and TNFα. Overall, there was no difference in the production of these innate cytokines and IFNγ in cell cultures stimulated with ΔBCG1419c or its parental strain. Regarding the TB vaccine candidate that contained a viral vector, both the vector expressing the mycobacterial protein (MVA85A (1) and MVA85A (2), $p = 0.0049$ and $p < 0.0001$ respectively) and its control (MVA-GFP (2), $p = 0.0017$, *Figure 5.2B*) stimulated production of IP-10, although there was a trend for stronger production of this cytokine in cultures stimulated with lower dose MVA85A. No differences with respect to unstimulated cultures were found for MCP-1, MIP-1α, EGF, G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, TNFα and IFNγ were found for cell cultures stimulated with either MVA-GFP or MVA85A.

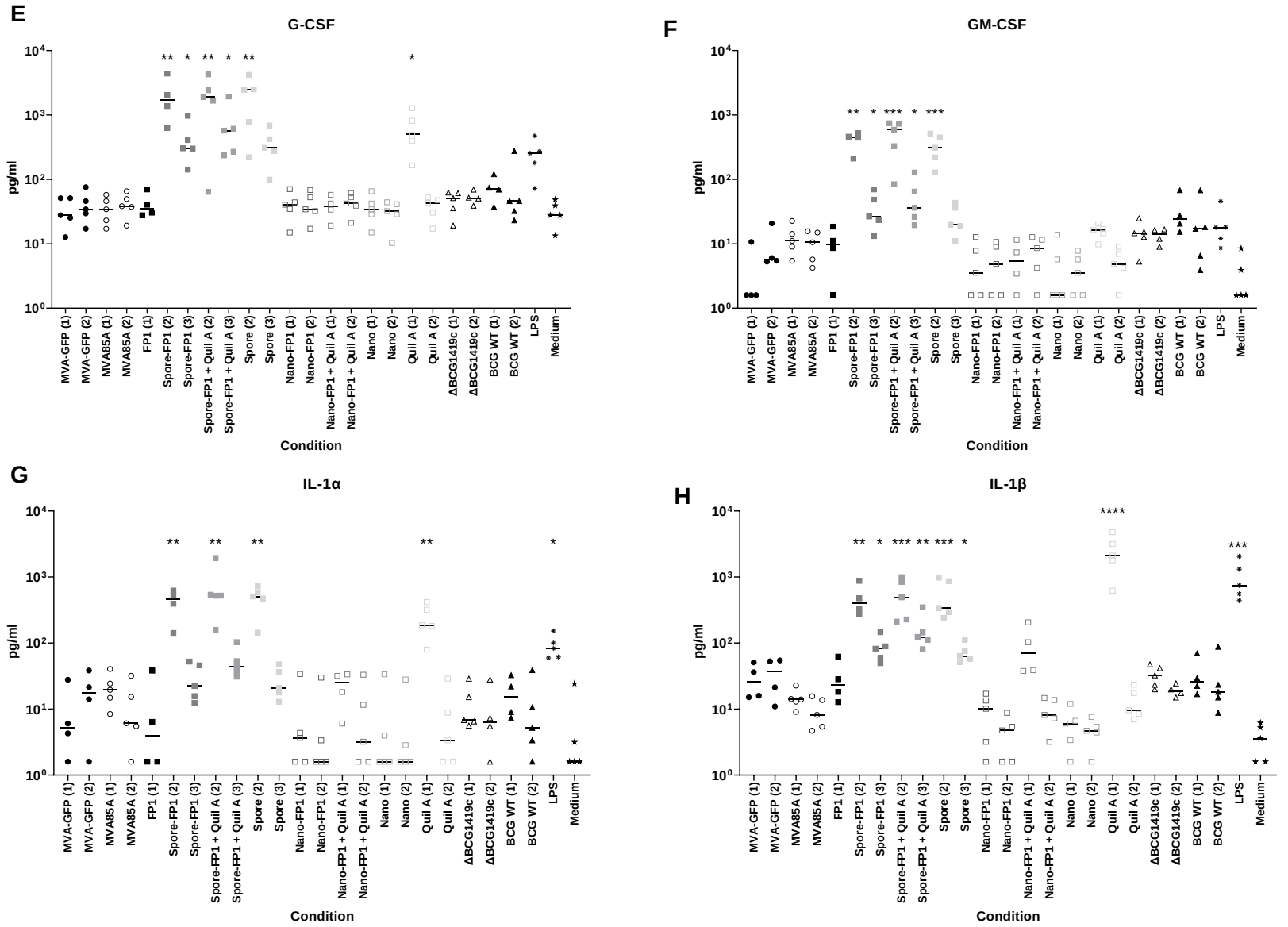
Fusion protein 1 (FP1) based vaccines and their components were investigated next. Although FP1 on its own did not induce a release of MCP-1, MIP-1α, EGF, G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, TNFα or IFNγ there was a significant increase of MCP-1 in whole blood cultures treated with carrier 1 bound FP1, both in the absence of adjuvant (Spore-FP1 (2): $p = 0.051$ and Spore-FP1 (3): $p = 0.0353$, *Figure 5.2A*) and in the presence of adjuvant (Spore-FP1 + Quil A (2): $p = 0.0240$ and Spore-FP1 + Quil A (3): $p = 0.0083$, *Figure 5.2A*). This was possibly an effect of *B. subtilis* spores on their own as they induced the release of MCP1 in the absence of FP1 (Spore (2): $p = 0.0455$ and Spore (3): $p = 0.0131$, *Figure 5.2A*) and the levels of this cytokine in whole blood cultures treated with *B. subtilis* were comparable to those of full Spore-

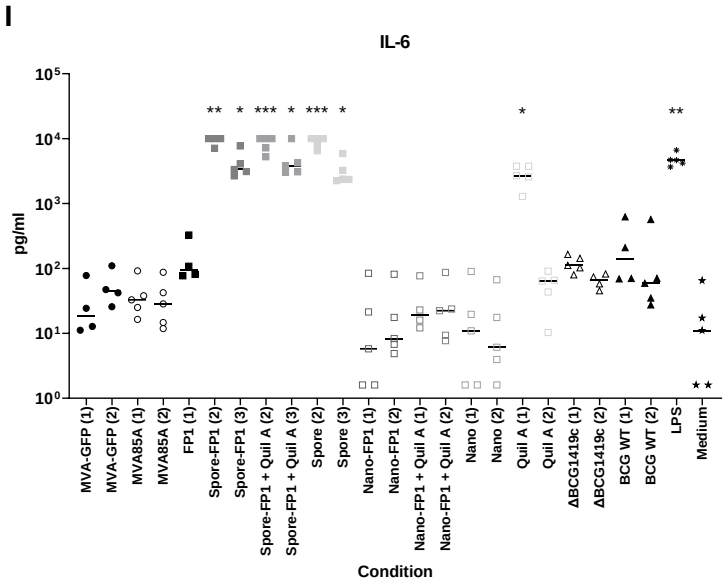
FP1 vaccine formulation. Of interest, this effect was not achieved in whole blood cultures stimulated with nanoparticles, in the presence or absence of FP1 or the adjuvant (*Figure 5.2A*). An similar, statistically significant effect of *B. subtilis* spores but not nanoparticles was observed for MIP-1α, G-CSF, GM-CSF, IL-1β, IL-6, and TNFα (*Figure 5.2C, E, F, H, I, J*). A dose dependent effect of *B. subtilis* spores was found for EGF (Spore-FP1 (2): p = 0.0094, Spore-FP1 + Quil A (2): p = 0.0058, Spore (2): p = 0.0043, *Figure 5.2D)*, IL-1α (Spore-FP1 (2): p = 0.0077, Spore-FP1 + Quil A (2): p = 0.0018, Spore (2): p = 0.0024, *Figure 5.2G*) and IFNγ (Spore-FP1 (2): p = 0.0042, Spore-FP1 + Quil A (2): p = 0.0427, F2: p = 0.0097, *Figure 5.2K*) but nanoparticles alone had no effect on the production of these cytokines. The effect of the adjuvant on its own on the production of these cytokines was also investigated. Significant effects induced by the highest dose of the adjuvant were found on MCP-1, MIP-1α, G-CSF, IL-1α, IL-1β and IL-6 production compared to the unstimulated cultures (*Figure 5.2)*. No effect of FP1 or its carriers – *B. subtilis* spores or the nanoparticles – or the adjuvant was observed for IP-10 in whole blood samples treated with these compounds.

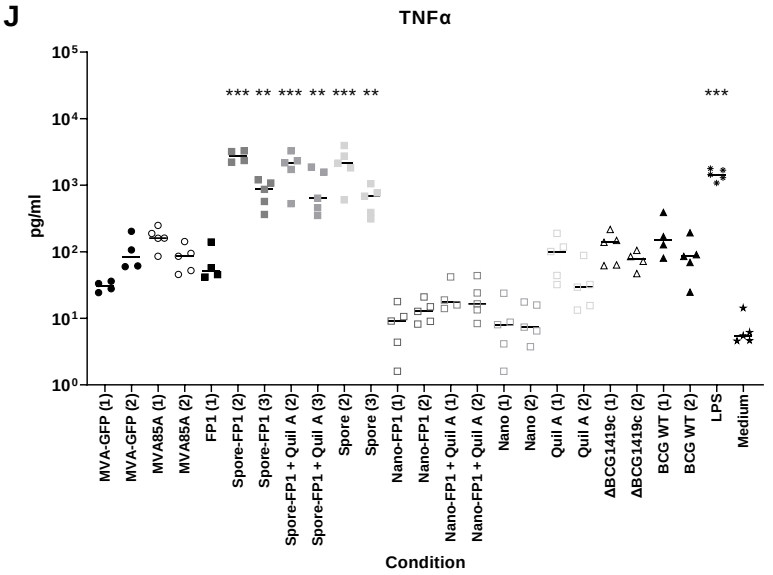
### *5.3.3 The effect of TB vaccine candidates or their components on cytokine profiles*

To test for the differences in cytokine profiles or biosignatures induced by the TB vaccine candidates themselves and their components or control stimuli, concentrations of cytokines screened using this MBA were plotted for MVA85A or its control MVA-GFP (*Figure 5.3A*); FP1, the adjuvant Quil A, *B. subtilis* spores (Spore), Spore-FP1, Spore-FP1 + Quil A (*Figure 5.3B, C*); FP1, Quil A, nanoparticles (Nano), Nano-FP1, Nano-FP1 + Quil A (*Figure 5.3D, E*) and for ΔBCG1419c or its parental strain (BCG WT, *Figure 5.3F*). Overall, there were no statistically significant differences in cytokine responses to the viral vector vaccine (MVA85A) and its control (MVA-GFP, *Figure 5.3A*), suggesting that most of the cytokine response to this vaccine was induced by the vector. A similar observation was made when exploring cytokine responses to ΔBCG1419c and its control, parental strain, where no differences in whole blood cytokine responses to these vaccines was observed (*Figure 5.3F*).


A
MCP-1
pg/ml
Condition
B
IP-10
pg/ml
Condition
C
MIP-1α
pg/ml
Condition
D
EGF
pg/ml
Condition
MVA-GFP (1)
MVA-GFP (2)
MVA85A (1)
MVA85A (2)
FP1 (1)
Spore-FP1 (2)
Spore-FP1 (3)
Spore-FP1 + Quil A (2)
Spore-FP1 + Quil A (3)
Spore (2)
Spore (3)
Nano-FP1 (1)
Nano-FP1 (2)
Nano-FP1 + Quil A (1)
Nano-FP1 + Quil A (2)
Nano (1)
Nano (2)
Quil A (1)
Quil A (2)
ΔBCG1419c (1)
ΔBCG1419c (2)
BCG WT (1)
BCG WT (2)
LPS
Medium


E
G-CSF
pg/ml
Condition
F
GM-CSF
pg/ml
Condition
G
IL-1α
pg/ml
Condition
H
IL-1β
pg/ml
Condition
MVA-GFP (1)
MVA-GFP (2)
MVA85A (1)
MVA85A (2)
FP1 (1)
Spore-FP1 (2)
Spore-FP1 (3)
Spore-FP1 + Quil A (2)
Spore-FP1 + Quil A (3)
Spore (2)
Spore (3)
Nano-FP1 (1)
Nano-FP1 (2)
Nano-FP1 + Quil A (1)
Nano-FP1 + Quil A (2)
Nano (1)
Nano (2)
Quil A (1)
Quil A (2)
ΔBCG1419c (1)
ΔBCG1419c (2)
BCG WT (1)
BCG WT (2)
LPS
Medium



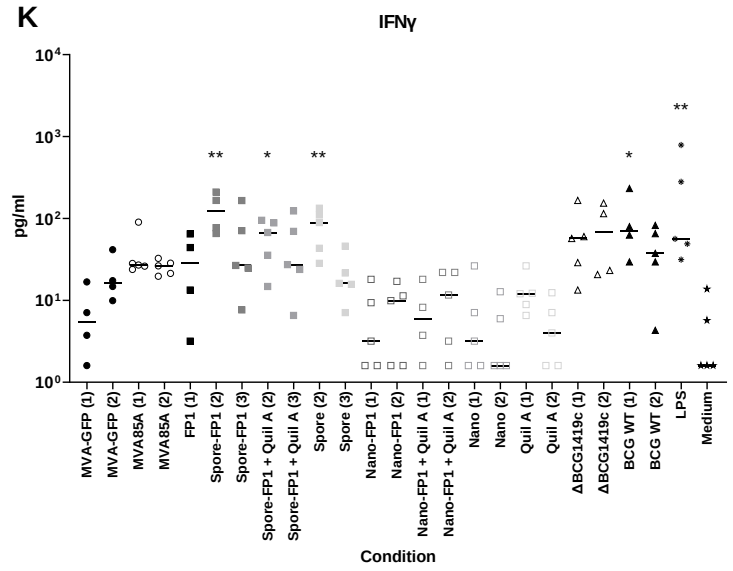
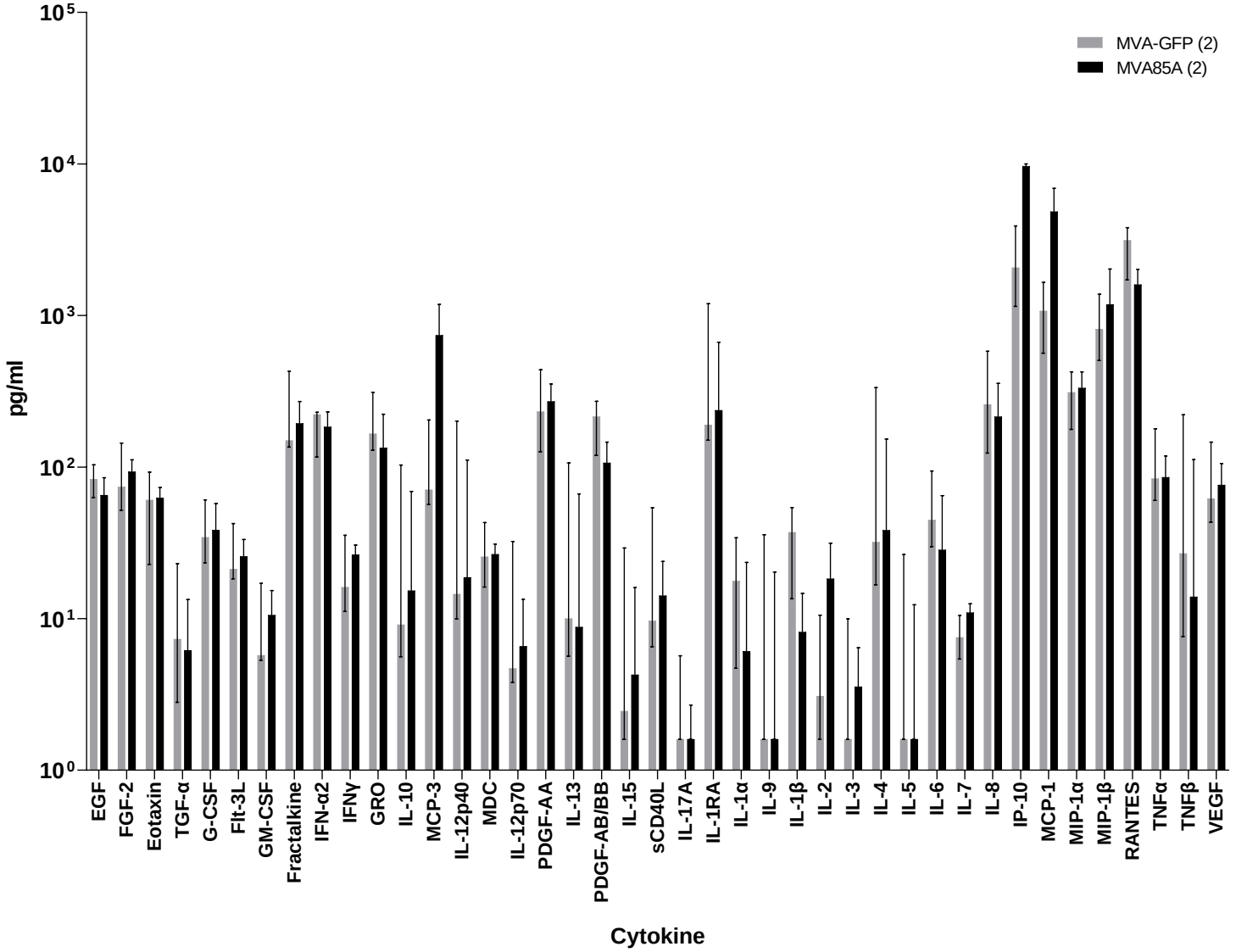



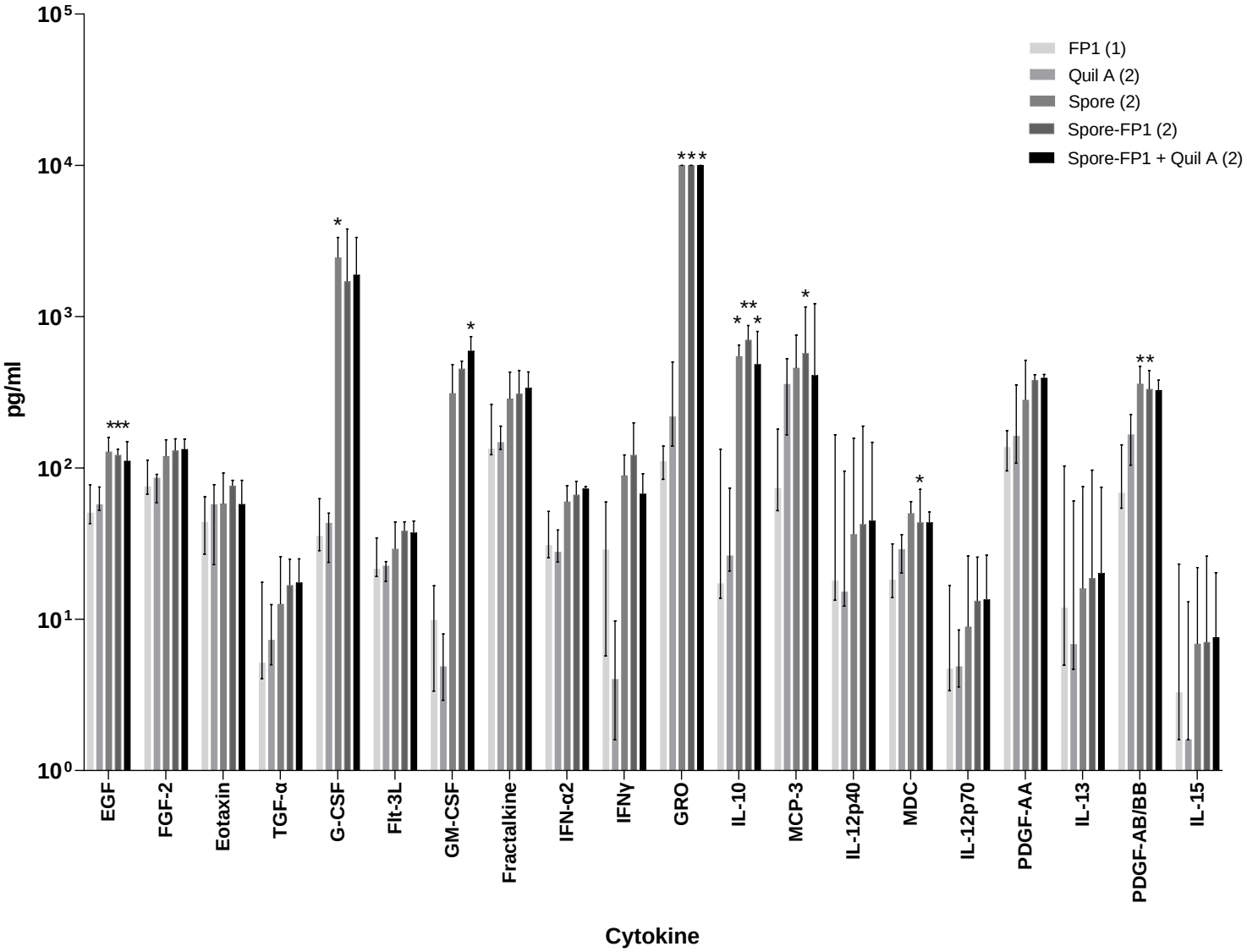

**Figure 5.2**. *Production of individual cytokines by whole blood cultures stimulated with candidate TB vaccines and their components for 48 h as measured by Luminex*. The graphs depict cytokine concentrations, testing the preparations with blood from 5 healthy UK donors, and their median values for MCP-1 (**A**), IP-10 (**B**), MIP-1α (**C**), EGF (**D**), G-CSF (**E**), GM-CSF (**F**), IL-1α (**G**), IL-1β (**H**), IL-6 (**I**), TNFα (**J**), IFNγ (**K**). The scatter plots depict cytokine concentrations and the lines indicate the median values. Kruskal-Wallis with Dunn's post-hoc correction was used to detect the differences to the unstimulated samples. N = 5.

In contrast, the analysis on whole blood cytokine responses to FP1, its adjuvant, *B. subtilis* spores or Spore-FP1 in the presence or absence of the adjuvant showed some differences between FP1 and the different formulations that include this protein (*Figure 5.3B, C)*. Although the adjuvant used in the full formulation of this candidate vaccine (Quil A (2)), when applied on its own, stimulated cytokine production to a similar extent as FP1 *Figure 5.3B, C*), it enhanced the production of GM-CSF (*Figure 5.3B*) and suppressed IP-10 (*Figure 5.3C)* production upon whole blood stimulation with Spore-FP1. Most cytokine responses, however, were driven by *B. subtilis* spores alone (Spore (2)), which was able to induce responses of EGF, GRO, PDGF-AB/BB (*Figure 5.3B*) and IL-8, MIP-1α, RANTES or TNFα (*Figure 5.3C*) at significantly higher levels than FP1 in the absence of this protein or adjuvant. Regarding the nanoparticle formulation of the FP1 vaccine, for most of the cytokines, the high dose of Nano-FP1 induced comparable responses to FP1 (*Figure 5.3D*), except for IP-10 or TNFα, the production of which was significantly lower in whole blood samples treated with nanoparticles or Nano-FP1 formulation compared to FP1 or high dose of adjuvant alone (*Figure 5.3E*), suggesting a possible inhibitory effect of the nanoparticles used in the FP1 vaccine formulation. This inhibitory effect was mostly overcome by the adjuvant Quil A as the full vaccine formulation induced similar responses to FP1 alone, and, in the case of RANTES, exceeded its production compared to cultures stimulated with FP1 (*Figure 5.3E*).

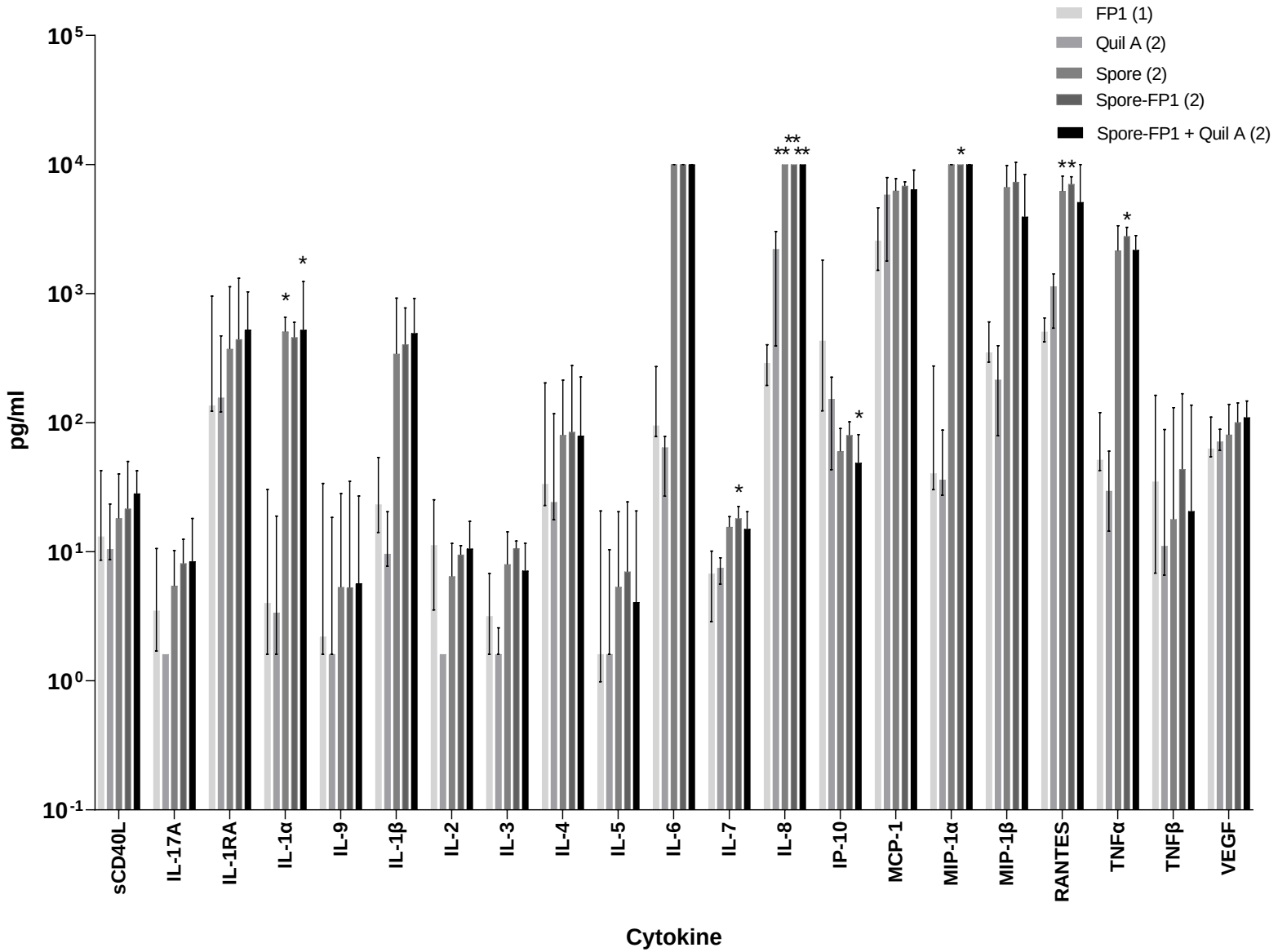
A


MVA-GFP (2)
MVA85A (2)
pg/ml
10^5
10^4
10^3
10^2
10^1
10^0
EGF
FGF-2
Eotaxin
TGF-α
G-CSF
Flt-3L
GM-CSF
Fractalkine
IFN-α2
IFNγ
GRO
IL-10
MCP-3
IL-12p40
MDC
IL-12p70
PDGF-AA
IL-13
PDGF-AB/BB
IL-15
sCD40L
IL-17A
IL-1RA
IL-1α
IL-9
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IP-10
MCP-1
MIP-1α
MIP-1β
RANTES
TNFα
TNFβ
VEGF
Cytokine

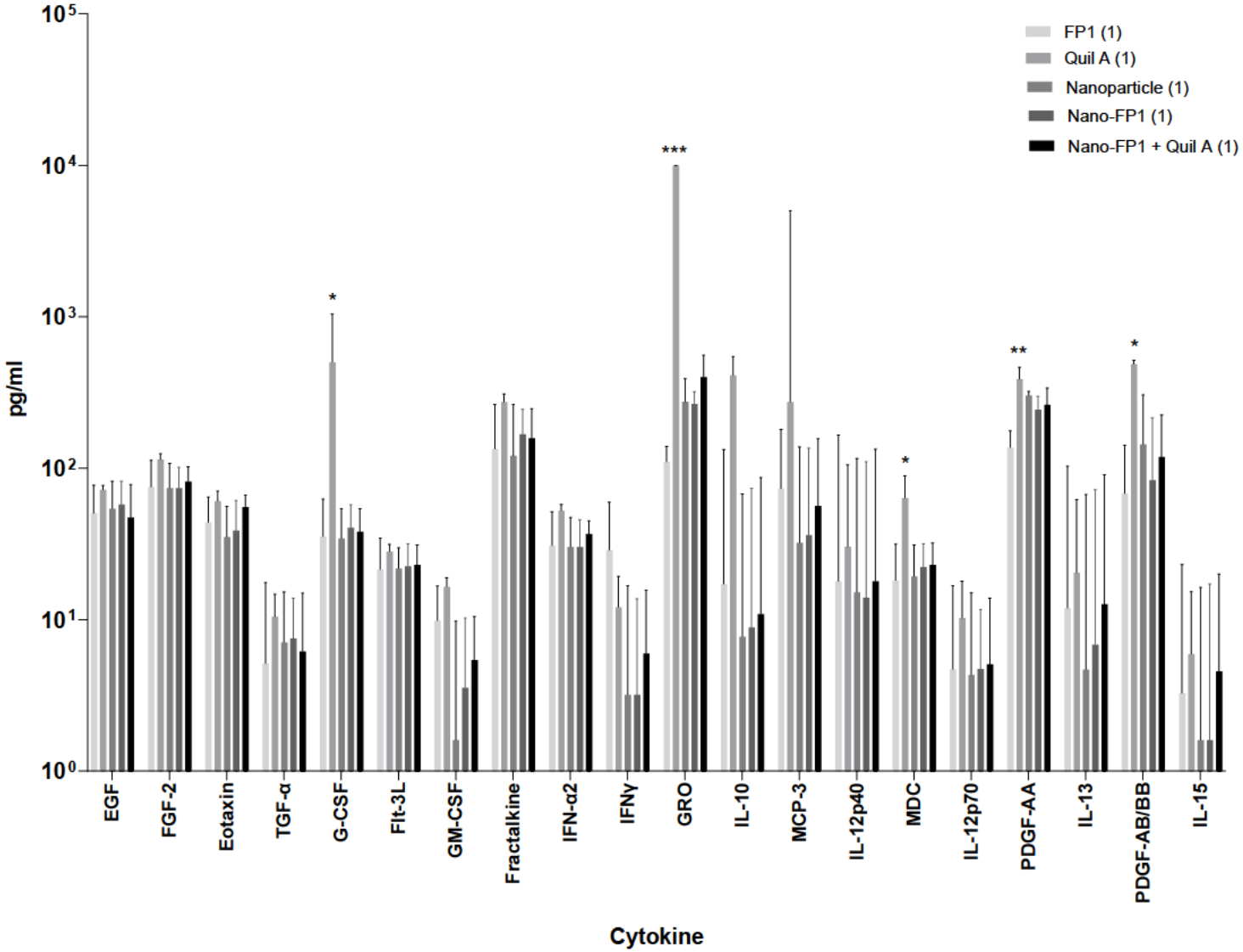
**B**


FP1 (1)
Quil A (2)
Spore (2)
Spore-FP1 (2)
Spore-FP1 + Quil A (2)
pg/ml
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
EGF
FGF-2
Eotaxin
TGF-α
G-CSF
Flt-3L
GM-CSF
Fractalkine
IFN-α2
IFNγ
GRO
IL-10
MCP-3
IL-12p40
MDC
IL-12p70
PDGF-AA
IL-13
PDGF-AB/BB
IL-15
Cytokine

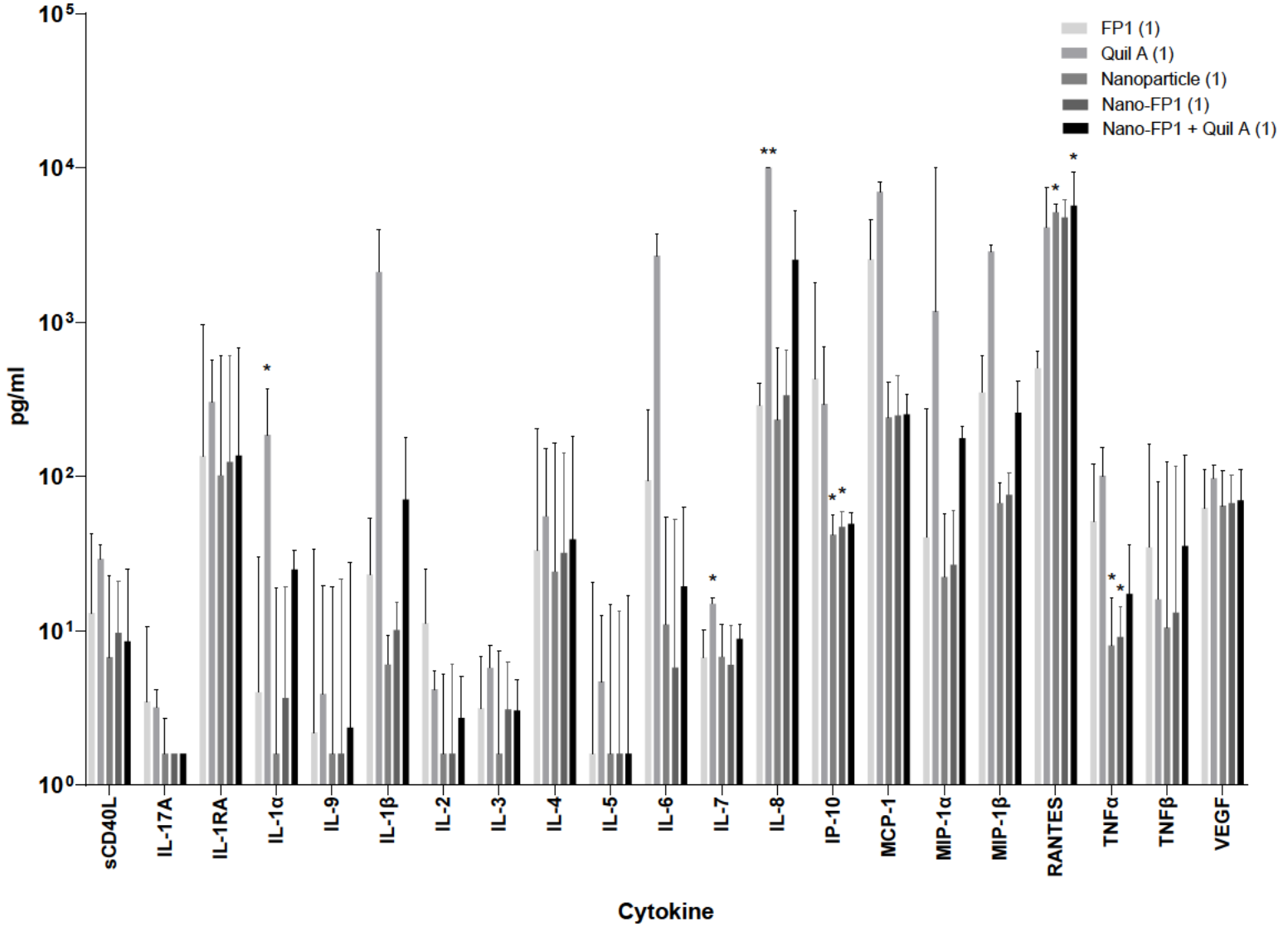
C


FP1 (1)
Quil A (2)
Spore (2)
Spore-FP1 (2)
Spore-FP1 + Quil A (2)
pg/ml
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^{-1}$
sCD40L
IL-17A
IL-1RA
IL-1α
IL-9
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IP-10
MCP-1
MIP-1α
MIP-1β
RANTES
TNFα
TNFβ
VEGF
Cytokine

D


FP1 (1)
Quil A (1)
Nanoparticle (1)
Nano-FP1 (1)
Nano-FP1 + Quil A (1)
pg/ml
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
*
***
*
**
*
EGF
FGF-2
Eotaxin
TGF-α
G-CSF
Flt-3L
GM-CSF
Fractalkine
IFN-α2
IFNγ
GRO
IL-10
MCP-3
IL-12p40
MDC
IL-12p70
PDGF-AA
IL-13
PDGF-AB/BB
IL-15
Cytokine

E


FP1 (1)
Quil A (1)
Nanoparticle (1)
Nano-FP1 (1)
Nano-FP1 + Quil A (1)
pg/ml
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
sCD40L
IL-17A
IL-1RA
IL-1α
IL-9
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IP-10
MCP-1
MIP-1α
MIP-1β
RANTES
TNFα
TNFβ
VEGF
Cytokine

F

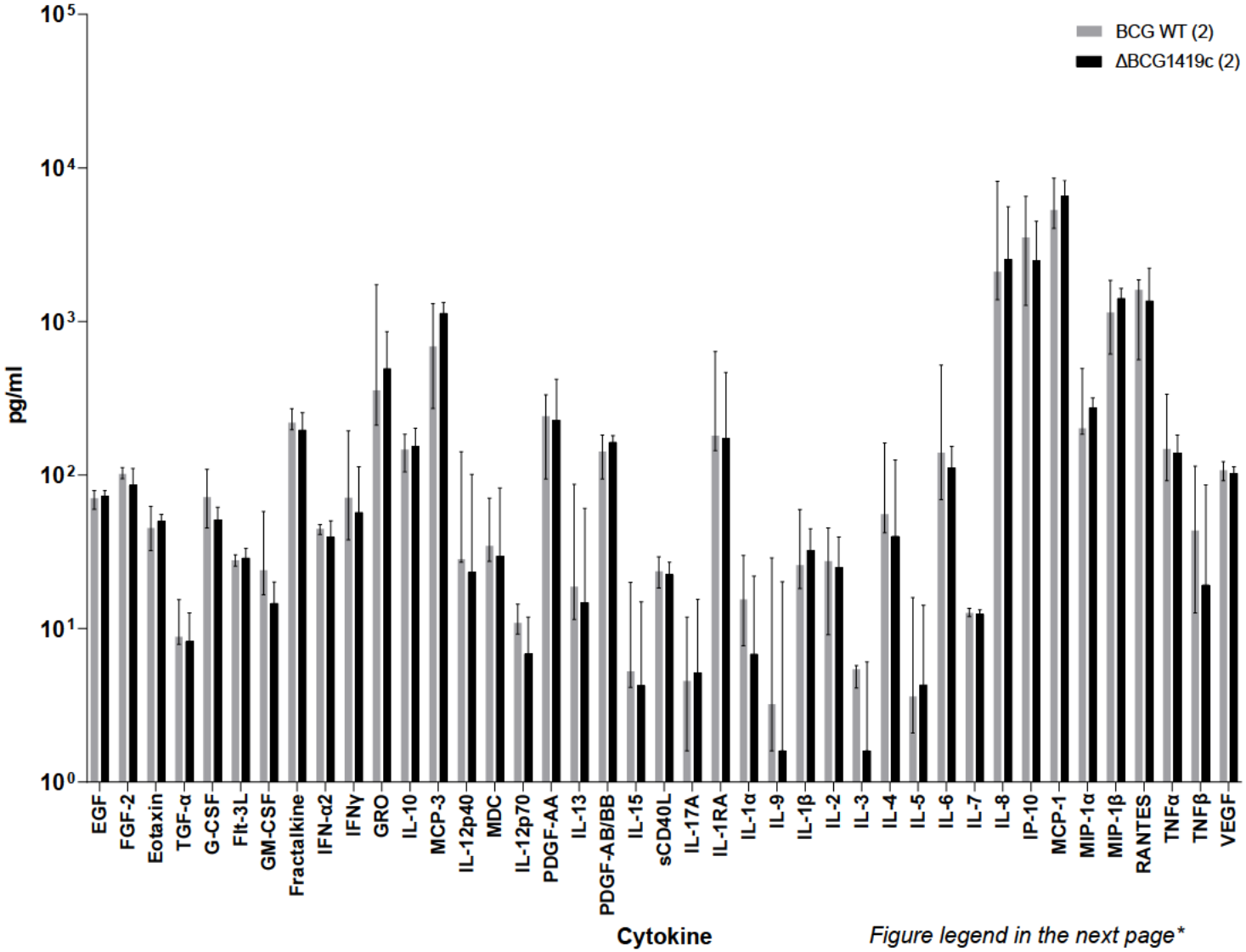



*Figure legend in the next page**

**Figure 5.3**. *Differential cytokine production by whole blood cultures stimulated with candidate TB vaccines for 48 h as measured by Luminex*. The graphs depict median cytokine concentrations and their interquartile ranges in supernatants from whole blood cultures stimulated with: MVA-GFP and MVA85A (**A**); FP1, the adjuvant (Quil A), *B. subtilis* spores (Spore) or the FP1 vaccine in the absence (Spore-FP1) or in the presence of adjuvant Spore-FP1 + Quil A), cytokines EGF-IL-15 (**B**) **or** sCD40L-VEGF (**C**); FP1, the adjuvant (Quil A), nanoparticles (Nanoparticle) or the FP1 vaccine in nanoparticle formulation in the absence (Nano-FP1) or in the presence of adjuvant (Nano-FP1 + Quil A), cytokines EGF-IL-15 (**D**) or sCD40L-VEGF (**E**); and ΔBCG1419c or its parental, wild-type strain (BCG WT) (**F**). Wilcoxon matched-pairs signed-rank test with Benjamini, Krieger and Yekutieli correction was used to detect the differences between MVA85A and MVA-GFP or ΔBCG1419c and BCG WT strain. Kruskal-Wallis test with Dunn's post-hoc correction was used to detect the differences between FP1, its adjuvant, spore or nanoparticle carriers or combinations of FP1 with either type of carrier in the presence or absence of adjuvant. N = 5. Numbers in the brackets indicate cytokine concentrations as described in *Table 5.1.*

### *5.3.4 Differences in whole blood cytokine profiles induced by different TB vaccine candidates*

While overall similar whole blood cytokine profiles were observed for most of the TB vaccine candidates or their components investigated in this study, *B. subtilis* spores on their own, in the presence of FP1 and in the presence or absence of the adjuvant Quil A stimulated the strongest cytokine responses, and the levels of some cytokines varied depending on the type of vaccine or its component, reflecting subtle differences in cytokine profiles induced (*Figure 5.2*). Because this observation resulted from analysis of 11 out of 41 cytokines selected based on their associations with TB, heterologous effects of BCG or trained immunity studies, to further investigate the similarities and differences in cytokine profiles induced by the TB vaccine candidates, a principal component analysis (PCA) was conducted (*Figure 5.4*). Two components – PC1 and PC2 – contributed most to variation in whole blood cytokine production induced by BCG and TB vaccine candidates, contributing to 42.49% and 22.19% respectively, and two major principal component score clusters were observed for BCG and TB vaccine candidates. Both clusters contained scores for almost all the vaccines compared, suggesting close similarity in cytokine profiles induced, except for the adjuvanted Spore-FP1 vaccine, the scores for which were dispersed along both axes (*Figure 5.4*). This was similar to the previous observation – that adjuvanted Spore-FP1 induced stronger cytokine responses than BCG or other TB vaccine candidates. Interestingly, responses to MVA85A and ΔBCG1419c overlapped almost completely in one of the clusters (*Figure 5.4*). Differential clustering of FP1 vaccines was observed, suggesting that the carrier used to deliver the active components of the vaccines to the cells of the immune system, may be particularly important in determining downstream cytokine or other immune responses.

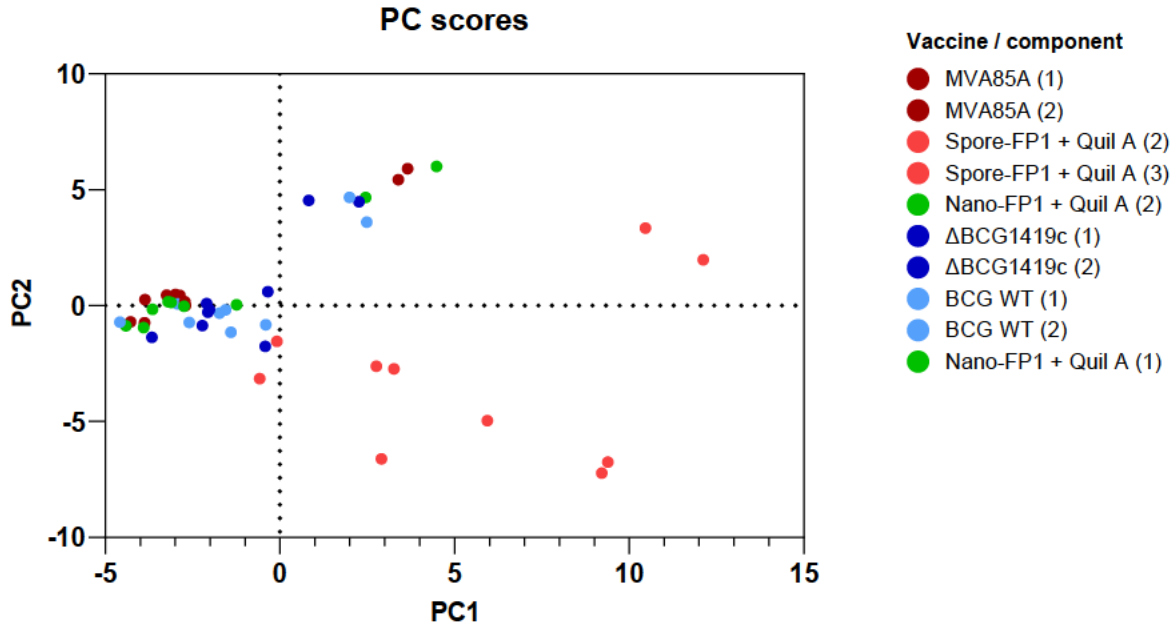
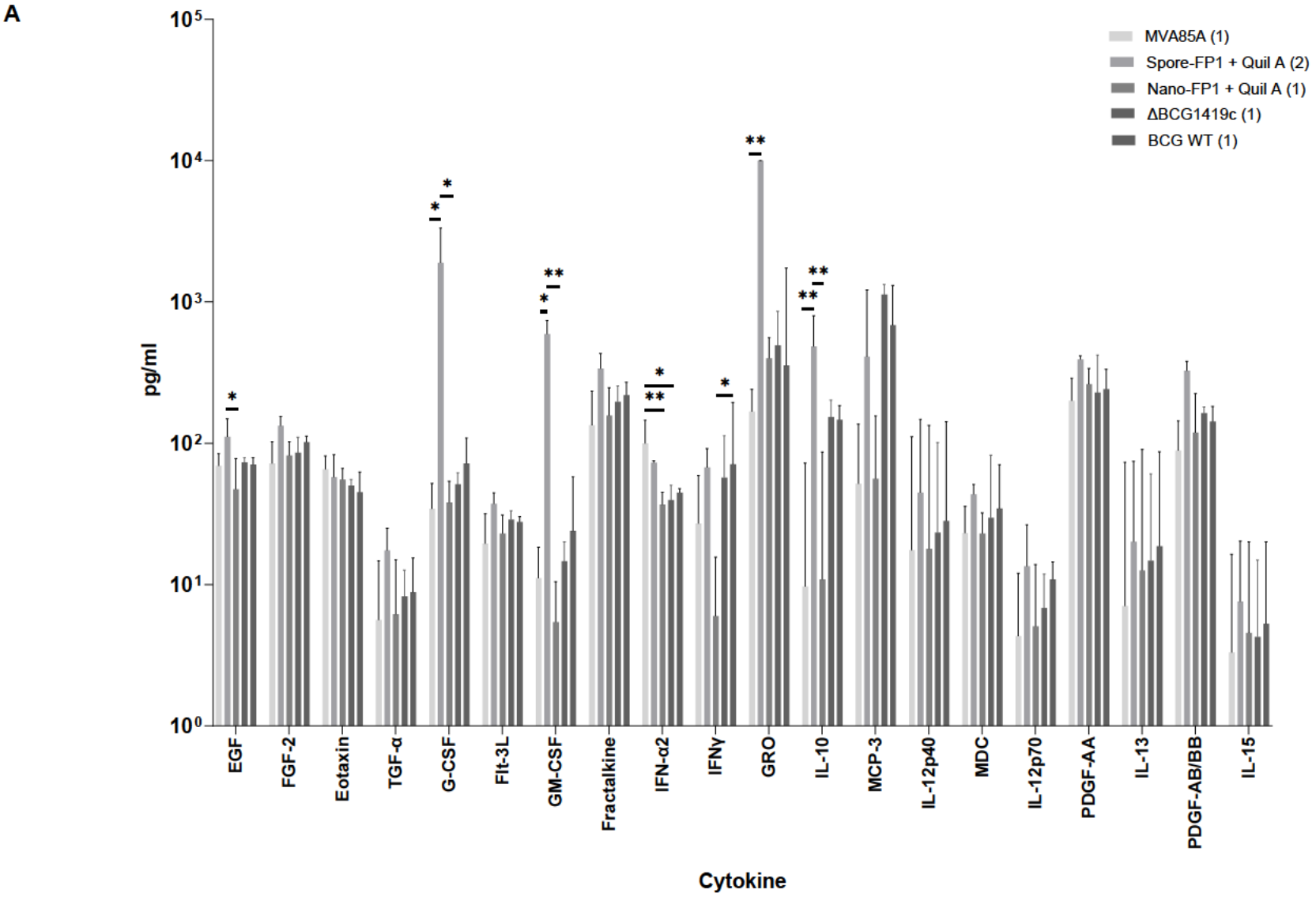



**Figure 5.4.** *Principal component analysis of cytokine responses to BCG and candidate TB vaccines*. The plot shows clustering of results from parental BCG strain (BCG WT) and TB vaccine candidates (MVA85A, FP1 vaccine in spore formulation (Spore-FP1 + Quil A), FP vaccine in nanoparticle formulation (Nano-FP1 + Quil A), ΔBCG1419c and its parental, wild-type strain (BCG WT) based on whole blood cytokine responses at 48 h post-stimulation. Each vaccine preparation was tested at 2 concentrations designated 1 and 2, or 2 and 3 as indicated in *Table 5.1*.

A more detailed breakdown indeed revealed small differences in cytokine profiles induced by BCG and TB vaccine candidates analysed in this study (*Figure 5.5; Table 5.4*). For instance, adjuvanted Spore-FP1 vaccine induced significantly higher levels of EGF, G-CSF, GM-CSF, IL-10, IL-1α, IL-6, MCP-1, MIP-1β and TNFα compared to adjuvanted Nano-FP1 and elevated EGF, G-CSF, GM-CSF, GRO, IL-10, IL-1β, IL-6 and IL-7 compared to MVA85A. MVA85A stimulated IFN-α2 more strongly than adjuvanted Nano-FP1 or ΔBCG1419c, while ΔBCG1419c potentiated IP-10 production more than adjuvated Nano-FP1. In contrast, parental BCG strain induced IFNγ production more strongly than Nano-FP1 + Quil A and more IP-10 than both adjuvanted FP1 vaccines (*Figure 5.5A, B*). There were trends for further differences but they did not reach statistical significance.


A
MVA85A (1)
Spore-FP1 + Quil A (2)
Nano-FP1 + Quil A (1)
ΔBCG1419c (1)
BCG WT (1)
pg/ml
10^5
10^4
10^3
10^2
10^1
10^0
EGF
FGF-2
Eotaxin
TGF-α
G-CSF
Flt-3L
GM-CSF
Fractalkine
IFN-α2
IFNγ
GRO
IL-10
MCP-3
IL-12p40
MDC
IL-12p70
PDGF-AA
IL-13
PDGF-AB/BB
IL-15
Cytokine

B

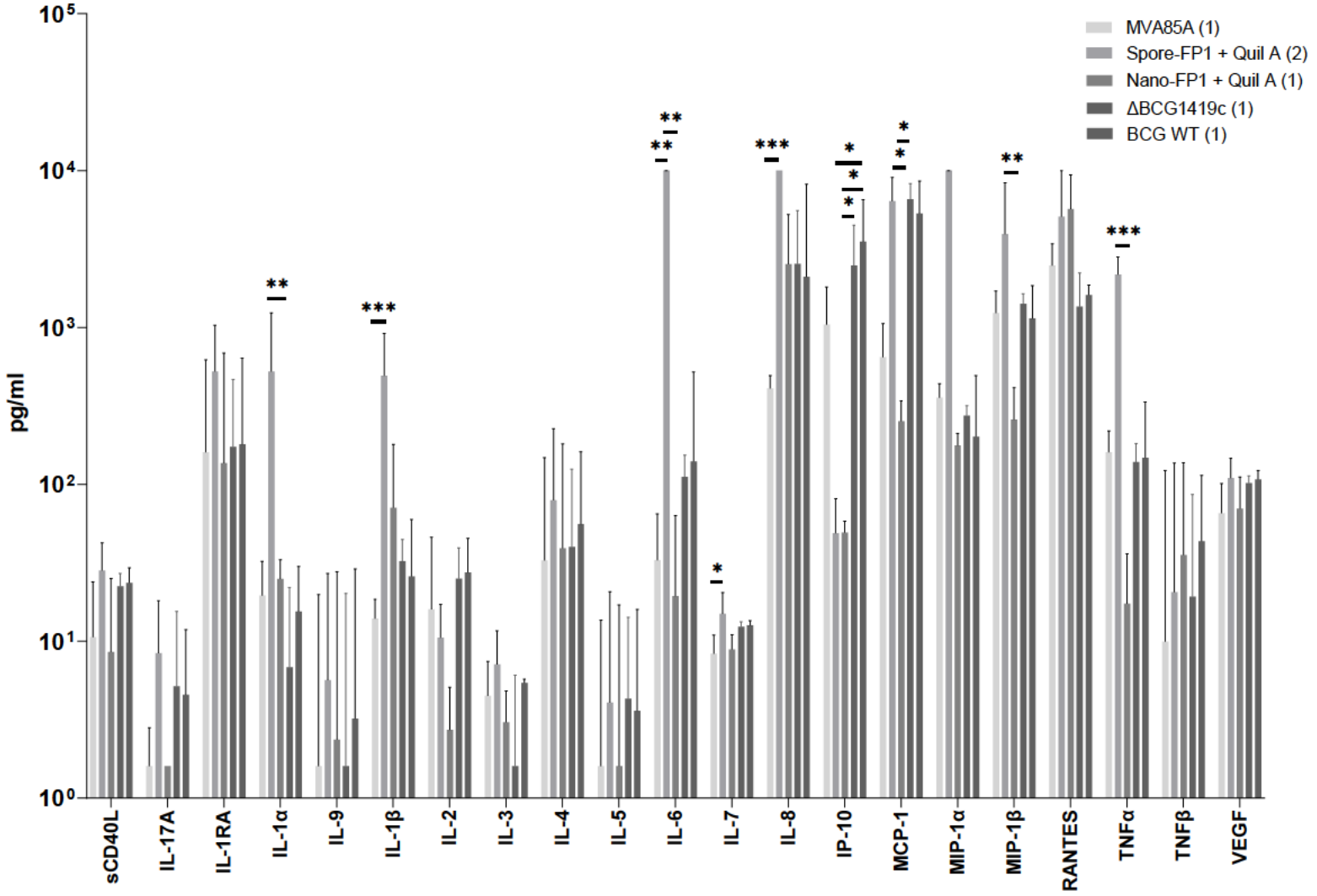



*Figure legend in the next page**

**Figure 5.5.** *Differences in whole blood cytokine responses to BCG and TB vaccine candidates*. The bar plots depict median cytokine (**A** – EGF-IL-15 and **B** – sCD40L-VEGF) concentrations and their interquartile ranges for whole blood samples stimulated with MVA85A, adjuvanted FP-1 vaccine in spore (Spore-FP1 + Quil A) and nanoparticle (Nano-FP1 + Quil A) formulations, ΔBCG1419c and it parental, wild-type strain (BCG WT). Kruskal-Wallis test with Dunn's post-hoc correction was used to detect the differences between MVA85A, Spore-FP1 + Quil A, Nano-FP1 + Quil A, ΔBCG1419c, and BCG WT. N = 5.

**Table 5.4.** *TB vaccine-specific cytokine signatures*

| TB vaccine or candidate | Effect on whole blood culture cytokine production compared to other vaccines |
|---|---|
| **MVA85A** | ↑IFNα2 compared to cultures stimulated with Nano-FP1 + Quil A or ΔBCG1419c |
| **Spore-FP1 + Quil A (2)** | ↑EGF, G-CSF, GM-CSF, IL-10, IL-1α, IL-6, MCP-1, MIP-1β and TNFα compared to Nano-FP1 + Quil A<br>↑EGF, G-CSF, GM-CSF, GRO, IL-10, IL-1β, IL-6 and IL-7 compared to MVA85A |
| **Nano-FP1 + Quil A (1)** | - |
| **ΔBCG1419c (1)** | ↑IP-10 compared to Nano-FP1 + Quil A |
| **BCG WT (1)** | ↑IFNγ compared to Nano-FP1 + Quil A<br>↑IP-10 compared to Spore-FP1 + Quil A or Nano-FP1 + Quil A |

## 5.4 Discussion

### *5.4.1 B. subtilis spores induce strong innate cytokine and chemokine responses and may act as a potent adjuvant*

In this study, whole blood cytokine and chemokine responses to 4 TB vaccine candidates and their components or control compounds were screened using a 41-plex MBA. Because most of these vaccine candidates have been previously explored *in vivo*, in mice or in human clinical

trials, because of the limited availability and high expense of the MBA reagents available for this study and because the previous work in this study suggested high variation in the production of some cytokines, such as IL-6, a preliminary ELISA screen was run to downselect the number of concentrations of TB vaccine candidates or their components to be screened by MBA. Twenty-five stimulation conditions, including the controls, were selected for the cytokine profile analysis by MBA. Although the ranges of concentrations of the vaccine candidates or their component doses tested in this assay were predicted based on the literature on *in vivo* immunisation in animals or human TB vaccine trials, doses of some candidates or their components were too high for the whole blood cell cultures and a lytic effect was observed in some cases, so these doses of vaccines or their components were excluded from further analysis. The lowest concentrations of some of the other vaccine candidates were also excluded from the study as only the higher doses induced IL-6 responses above the baseline.

Although cytokine production by whole blood cultures was measured for all the cytokines and chemokines covered by this array, the ability of the selected TB vaccines or their components to induce 10 innate cytokines and chemokines previously reported in TB marker or BCG immunisation studies along with IFNγ, a correlate of protection against TB, was investigated first. Distinct patterns of cytokine induction were observed for each category of vaccines or their components, formulations including *B. subtilis* spores inducing production of these cytokines most potently, irrespective of the cytokine analysed. Similarly to the observations from the preliminary IL-6 screen, this effect was associated with *B. subtilis* spores as they alone were sufficient to induce strong production of these cytokines. This somewhat differed from previous observations where *B. subtilis* spores, although found to have an additive effect on IFNγ or TNFα induction in human tonsil cells, were not the predominant drivers of their responses (377). Overall strong immunogenicity of *B. subtilis* spores in this study was reminiscent of previous findings in murine immunisation studies where effector memory T-cell expansion and IFNγ, IL-10 or IL-17A production by splenocytes was elevated in Spore-FP1

boosted compared to BCG-only vaccinated mice (363). While previous work suggested that *B. subtilis* spores had limited potency inducing the production of cytokines associated with trained immunity or heterologous effects of BCG, mostly stimulating TNFα by dendritic cells (DCs) (363), in this study, the spores on their own or in the presence of FP1 strongly stimulated innate cytokine production, including IL-1β, IL-6 and TNFα. It should be noted that in both this and previous work, the effect of spores was dose-dependent and that in this study whole blood assays were used instead of purified antigen presenting cell (APC) populations, with longer culture stimulation, possibly contributing to a stronger effect observed in this work.

### *5.4.2 Nanoparticles were associated with poorer innate and adaptive cytokine responses compared to B. subtilis spores or adjuvant Quil A*

Of interest, FP1 vaccine formulations including nanoparticles stimulated the production of IFNγ and the 10 cytokines associated with antitubercular responses, trained immunity or heterologous effects of BCG least strongly and even suppressed the production of some, for instance MIP-1α, G-CSF, IL-1α, IL-1β or TNFα, production of which was potentiated by the adjuvant alone (Quil A (1), Quil A (2)), especially when applied to whole blood cultures at the higher dose. This suppressive effect of nanoparticles was also observed for MCP-1 and IP-10 as reducing the dose of the full or partial formulation of the vaccine containing nanoparticles potentiated the production of these cytokines. Of note, FP1 itself was only mildly immunostimulatory with respect to these cytokines as although there was a slight increase in MCP-1 and IP-10 production compared to unstimulated samples, this was not statistically significant. This was somewhat surprising because the previous work on murine immunisation with the Nano-FP1 vaccine showed that it was more potent than BCG in reduction of the microbial burden in lungs of mice challenged with *M. tuberculosis* both in the presence or absence of BCG priming and enhanced T-cell proliferation or IFNγ, IL-10, IL17 or IL-4 release by splenocytes in Nano-FP1 immunised animals compared to BCG (364). A possible dose-dependent inhibitory effect of Nano-FP1 on production of some cytokines associated with anti-

mycobacterial immunity (MCP-1, IP-10) was observed in this study, however, its contribution to lower immunogenicity of Nano-FP1 in this work is not clear. Previous work suggested that nanoparticles on their own prime macrophages differently from other stimuli, inducing Type I interferons (IFN) and IP-10 rather than Th1-like IL-1β, IL-6 or TNFα responses (364). It is possible that patterns of other cytokine production may have been modulated by Type I IFN signalling, however, in this work, a high dose of adjuvanted Nano-FP1 induced lower levels of IFNα2 than MVA85A and similar concentrations of this cytokine and others compared to the adjuvanted Spore-FP1, ΔBCG1419c or BCG WT, with overall similar cytokine patterns observed.

### *5.4.3 BCG and its derivative ΔBCG1419c induce highly similar cytokine profiles*

Both BCG strains, the ΔBCG1419c and its parental, control strain, induced IFNγ, MCP-1 and IP-10 responses, however, no statistically significant increases in the production of other cytokines associated with antimycobacterial immunity or heterologous effects of BCG were found even where the trends for the elevated cytokine production were present, e.g. for cytokines associated with antimycobacterial or trained immunity, such as IL-1β, IL-6 or TNFα, possibly due to high interindividual variation and small study size, although previous studies showed that ΔBCG1419c was a potent inducer of TNFα and IL-6, even if less capable of stimulating IL-1β compared to the parental strain (378). No significant differences were found in whole blood samples treated with the BCG strains and unstimulated cultures with respect to EGF, G-CSF or GM-CSF. This was mildly surprising as BCG-dependent increase in these cytokines have been previously associated with heterologous effects of BCG. Elevated EGF and GM-CSF production has been previously found in BCG-vaccinated infants at 4 months post BCG vaccination upon stimulation with heterologous stimuli or *M. tuberculosis* lysate respectfully (223), and G-CSF and GM-CSF was shown to increase in BCG-vaccinated murine pups and, in the case of G-CSF, associate with their protection from death by polymicrobial sepsis (242). It is possible that the peak production of these (and other) innate cytokines in

response to BCG may have been missed in this study as elevation of G-CSF and GM-CSF in BCG-vaccinated mice was observed within the first 36 h and GM-CSF – within the first 12 h post BCG immunisation (242), and peak TNFα responses to ΔBCG1419c in murine macrophages – at 24 h post stimulation (378). In this work, cytokine production in whole blood samples was analysed at 48 h of culture with TB, this time point selected as a compromise aiming to detect both the innate and adaptive cytokines. If that was the case in this study, a recommendation could be made for analyses of cytokine profiles at different time points.

### *5.4.4 MVA85A induces IP-10 production but may suppress the production of trained immunity associated cytokines in a dose-dependent manner*

The viral vector vaccine MVA85A used in this study induced a significant increase of IP-10 and there were also trends for increased production of MCP1 and IFNγ, although neither reached statistical significance. There was also a possible inverse dose-dependent effect as the levels of these cytokines were higher in whole blood cultures stimulated with lower doses of these vaccines. No effect of MVA85A or its control MVA-GFP was observed on the production of other cytokines. This was somewhat unexpected as live viral vaccines, such as *Vaccinia,* similarly to BCG, have also been shown to induce trained immunity *in vitro* (201). Of interest, its derivative, MVA, used as a vector for antigen 85A in TB candidate vaccine studies, although also demonstrated to induce trained immunity, had an inverse dose-dependent effect on the ability of human cells to train (201), not unlike the effect on whole blood cytokine responses to direct stimulation observed in this study. Relatively high doses of MVA85A or its control MVA-GFP were used in this study, so it is possible that these vaccines may have had an inhibitory effect on the production of the cytokines examined. It is also possible that they stimulate the production of other cytokines or chemokines that were not analysed in this comparison.

### *5.4.5 Similarities and differences in cytokine profiles induced by BCG and TB vaccine candidates*

Because the preliminary IL-6 screen and analysis of individual innate cytokine responses to TB vaccine candidates and their components suggested that not only the active component of the vaccine candidate but also the vector, the carrier or the adjuvants used in the candidate vaccine formulation can contribute significantly to the innate cytokine responses but in some cases be the major drivers of cytokine responses, the cytokine profiles of candidate vaccines and their counterparts or components were compared directly. Overall, MVA85A and MVA-GFP or the ΔBCG1419c strain and its control, parental strain induced highly similar cytokine profiles, and this similarity was confirmed by the principal component analysis. This was perhaps not surprising as only minimal differences were present in the control strains and the TB vaccine candidates – Ag85A in the case of MVA85A and the deletion of a single gene encoding c-di-GMP PDE in ΔBCG1419c (365) but some differences in downstream responses were reported for the vaccine candidates and their control strains. For instance, ΔBCG1419c was shown to induce lower murine macrophage TNFα and IL-1β responses and elevated IL-6 production compared to its parental strain (378), induce IL-6 more strongly than the parental strain in human lung tissue model (379) or reduce levels of IFNγ, TNFα, IL-6 and IL-10 in lungs of *M. tuberculosis* infected vaccinated mice compared to those that were administered BCG (380). It is not clear why these observations were not replicated in this study, but it is possible that since PDE, knocked out from ΔBCG1419c, is an intracellular enzyme, differences between the parental and modified strains may be less recognised by the innate immune cells upon short-term whole blood culture stimulation. However, as this enzyme regulates biofilm formation, altered morphology of mutant BCG may result in long-term differential recognition of the mutant strain and higher activation of the immune system upon vaccination (380). While the cytokine profiles of ΔBCG1419c and its parental strain were almost identical, there were some trends for higher MCP-3, IP-10 and MCP-1 concentrations in cultures that received

MVA85A, implying a contribution from Ag85A inserted into the MVA vector, however, the size of this effect would need to be investigated in a larger study.

In contrast, different formulations of the FP1 vaccine resulted in differential cytokine expression; with the exception of IP-10, FP1 or the adjuvant induced significantly lower cytokine expression than formulations containing *B. subtilis* spores. Overall strong immunogenicity of *B. subtilis* spores in this study was reminiscent of previous findings in murine immunisation studies where effector memory T-cell expansion and IFNγ, IL-10 or IL-17A production by splenocytes was elevated in Spore-FP1 boosted compared to BCG-only vaccinated mice (363). This again suggested that *B. subtilis* spores themselves were the immunodominant drivers of differences in cytokine responses in cultures treated with formulations containing this carrier, a hypothesis supported by the principal component analysis. Changing the vaccine formulation to the one including nanoparticles instead of *B. subtilis* spores resulted in lower concentrations of cytokines in whole blood cultures even if high concentrations of FP1 or the adjuvant were used in the full formulation. In addition, lower cytokine induction by adjuvanted Nano-FP1 compared to the adjuvant itself suggested a possible suppression of cytokine responses in whole blood cultures by the nanoparticles, partially supported by the trends for lower induction of MCP-1 and IP-10 in samples treated with high dose of Nano-FP1 vaccine or nanoparticles alone. This is supported to an extent by previous findings – although murine immunisation with Nano-FP1 expanded their memory T-cell populations in tissues, direct activation of murine macrophages with nanoparticles was less potent than that using Spore-FP1 (363,364). In addition, consistent with low cytokine responses to nanoparticle formulation observed in this study, instead of activating NF-κB, a master transcription factor of inflammatory cytokines, nanoparticles increased expression of IRF3, a transcription factor associated with Type I IFN responses (364). Of interest, enhanced IP-10 expression in macrophages treated with nanoparticles was observed in the previous work, although no such effect was observed in this study.

More subtle nuances were revealed by the principal component analysis and direct comparison of whole blood cytokine responses to BCG and TB vaccine candidates investigated in this study. Overall, cytokine responses produced by BCG and TB vaccine candidates were similar, except for the adjuvanted Spore-FP1 vaccine, probably due to high cytokine induction by *B. subtilis* spores. The vaccines could be divided into two clusters, however, it was not possible to determine the factors that separated them. Interestingly, although MVA85A and adjuvanted Nano-FP1 or its parental strain are structurally very different vaccines, there was a similarity in cytokine profiles induced by these vaccines, as suggested by the principal component analysis as the data almost completely overlapped in one of the clusters. It is possible that these vaccines prime the immune cells similarly – while the previous work associated Nano-FP1 with Type I IFN pathway activation (364), in this study MVA85A induced the strongest IFNα2 production, although it was higher than that of Nano-FP1.

When analysed more closely, more subtle cytokine associations with each vaccine were found. While BCG vaccines were more potent than FP1 vaccines at IFNγ or IP-10 induction, adjuvanted Spore-FP1 was more likely to induce cytokines associated with heterologous effects of BCG than Nano-FP1 vaccine or MVA85A. Although this is something that should be confirmed by further work, it is possible that *B. subtilis* spores may have heterologous effects similar to BCG, an important consideration as trained immunity was associated with protection against TB and may add to classical T-cell mediated anti-tuberculosis immunity (131,221,227). Also, because it stimulated whole blood cytokine production more potently than other vaccines, it is possible that it may induce a broader range of cellular responses than Nano-FP1 or MVA85A and could be investigated both as a booster and a priming vaccine. It is also likely that ΔBCG1419c may also have heterologous effects, although its previously reported propensity for lower IL-1β induction should be taken into account, especially, considering the role of this cytokine in heterologous effects or antimycobacterial immunity (209,221,227,378). Regarding MVA85A, it is possible that it may prime or boost the immune system differentially,

enhancing Type I IFN responses. Although its vector, MVA has been previously shown to train innate immune cells *in vitro*, it was less potent at doing so compared to *Vaccinia* (201). Although the capacity of MVA85A to train innate immune cells is yet unknown, it is highly likely this vaccine would share the property. Type I IFN signalling, however, has been previously associated with inhibition of IL-1β dependent responses (381,382), so it is possible that further modifications of this vaccine may be needed to enhance its antimycobacterial or potential innate immune training effects. As for adjuvanted Nano-FP1, further investigation would be needed as to why this vaccine induced cytokine productionless potently in this study than in previous reports.

### *5.4.6 Summary*

To conclude, this study showed that MBAs, especially those covering a broad spectrum of analytes can be a useful tool to screen for differences in cytokine signatures induced by different vaccines and can provide useful information on the immune responses or mechanisms induced by these vaccines. Although cytokine profiles induced by BCG and TB vaccine candidates were mostly similar, Spore-FP1 vaccine showed a distinct cytokine signature compared to other vaccines. This difference was likely to be driven by *B. subtilis* spores implicating high adjuvanticity of spores; however, further investigation would be required to determine why that was the case and why nanoparticles stimulated cytokines to a lesser extent, even in the presence of FP1 and adjuvant Quil A. The work described in this chapter also detected subtle differences in otherwise similar cytokine profiles induced by BCG and TB vaccine candidates, suggesting that despite the small scale, the system was still sensitive enough to allow the prediction of some potential immunological properties of these vaccines, even if further work will be required to confirm the validity of these predictions. This study also confirmed the importance of careful selection of carrier platforms and their quantities for subunit vaccines, especially where the carrier is designed to act as an adjuvant.

# **Chapter 6:** Discussion

## 6.1 Trained immunity, heterologous effects of BCG and protection against TB

In this study, innate immune responses to the BCG vaccine and Toll-like receptor agonists were investigated in the context of trained immunity and mechanisms involved in the vaccinated individuals' protection against TB.

It has been speculated for a while that trained immunity may contribute to the protective anti-tuberculosis effects of BCG in vaccinated individuals (383,384). Indeed, there is evidence accumulating from murine and human BCG immunisation studies supporting this hypothesis. In mice, the BCG vaccine can influence the development of macrophages with enhanced antimycobacterial properties in the bone marrow (BM) (227). Models using β-glucan, another trained immunity inducing agent, suggested that downstream IL-1β production can contribute to these changes in the BM and so protect mice against *M. tuberculosis* (221,253). Previously conducted human BCG vaccination and monocyte *in vitro* training studies using BCG consistently indicate increased IL-1β production upon later PBMC, monocyte or NK cell re-stimulation with *M. tuberculosis* or its antigens (170,191,197,213,236,385), suggesting that BCG vaccination may also modulate myelopoiesis in humans via IL-1β and so contribute to their protection against TB or other infectious diseases. Neutrophil-associated gene expression changes were detected in the BM of BCG-vaccinated individuals at 3 months post immunisation (218), and innate cell populations and trained immunity-like responses have been associated with protection against TB in *M. tuberculosis*-exposed but uninfected individuals (131,386). Therefore, it is possible that regulation of innate immune responses through trained immunity or cytokines modulated by this mechanism can play an important role in protection against TB and that *in vitro* systems exploring trained immune responses can provide useful information on factors contributing to protection against TB.

## 6.2 Human CD14+ monocyte *in vitro* training with BCG

### *6.2.1 Overview*

The *in vitro* system in which BCG is used as an agent inducing trained innate responses in human monocytes has been described before (191,205,265). Apart from innate immune training with BCG, this system has also been used to test the training effects of naturally occurring microorganisms or their live attenuated strains (198,200–202), microbial components (203,224) or metabolites (204,207,208,225) on human monocytes, and for the possible associations of this mechanism with the beneficial heterologous effects of BCG in individuals who received this vaccine (164,177,191,209) or the association of monocytes with metabolic and/or inflammatory disorders (206,208,264). Most of the *in vitro* studies on trained immunity induced using BCG have been so far conducted in the Netherlands, using adherent PBMCs or Percoll-isolated monocytes from healthy human adults. As described in *Chapters 2* and *3*, this study independently optimised and tested the *in vitro* training system using BCG and CD14+ monocytes from healthy adult volunteers from the UK, with an aim to use this system as a screening tool for markers associated both with trained immunity and protection from TB. This study confirmed the effects of innate immune training and also showed that this phenomenon can be variable and donor-dependent. In addition, this work also confirmed that the downstream effects of innate immune training depend on viability and quantity of BCG used during the training step and, in the absence of other stimuli, may influence baseline production of innate cytokines.

### *6.2.2 Prior BCG exposure and in vitro training of primary human monocytes*

As described in *Chapter* 2, in contrast to the previous *in vitro* training studies, a less robust effect of training was observed in this work. Although the protocol used for the *in vitro* training in this study was based on the previously published work by *Bekkering et al.* (205), there were some differences that may have contributed to a weaker effect of training as observed in this

work. One of these differences could be due to previous BCG exposure by the participants of this and the previous studies. In the Netherlands, TB incidence is low (12), the estimated rates being 5.3 case per 100,000 individuals before the COVID-19 pandemic (7,387). The National Immunisation Programme in the Netherlands therefore does not include universal BCG vaccination and only recommends BCG vaccination for infants at an elevated risk of TB (387,388). Similarly, in the UK, TB incidence has also been low in recent years, with 8 TB cases per 100,000 individuals reported in 2018, and the BCG vaccine is now recommended selectively (7,12,389,390). In the past, however, both countries applied different BCG immunisation strategies. The Netherlands used selective anti-TB immunisation approach from 1979, while the UK applied mass BCG vaccination until 2005, only then switching to selective BCG vaccination (18). In the study described in this thesis, blood samples from BCG-vaccinated adult volunteers were used, whereas the *in vitro* BCG training studies in the Netherlands likely came from individuals that had not been exposed to BCG through vaccination.

As discussed in *Chapter 2*, historical BCG vaccination status has been associated with improved inhibition of mycobacterial growth and NK cell responses (235,246), and increased expression of CD14, CD11b and TLR4 on circulating monocytes was observed for up to 1 year post BCG immunisation (213). If present in trained monocyte cultures, these cells could contribute to their responses to secondary stimulation. Alternatively, it is possible that cells that had already been exposed to BCG may have a limited ability to train upon subsequent encounters with this vaccine. Studies exploring if stimuli that induce trained immunity can be superimposed on one another frequently focus on attempts to overcome pathological immune under-responsiveness caused by such conditions as sepsis (211,212,338) or to modulate chronic inflammatory conditions (391,392). Although there is some evidence that priming monocytes with some metabolites, such as vitamin A, may negate the outcome of BCG-dependent training (225), it is not known whether repetitive BCG priming of monocytes enhances their responses with every encounter. Most evidence, however, suggests that

monocyte encounter with this organism on a single occasion is usually sufficient to reprogram cells epigenetically (191,217). It is yet unknown whether further training-inducing stimulation enhances epigenetic or metabolic reprogramming, however, it is possible that additional encounters of BCG, through vaccination or training may not influence the innate immune cells if they have already been trained through immunisation.

### *6.2.3 Viability of BCG and its effects on trained immunity or heterologous effects of BCG*

One of the ideas circulating around the concept of trained immunity is that responses to secondary stimuli can be enhanced in the absence of the original stimulus (215). While this may be possible for small, soluble compounds, such as metabolites, and *in vitro* training protocols include multiple cell wash steps to remove the agents that induce trained immunity (205,265), BCG is a viable microorganism that can establish long-term infection in monocytes or macrophages. Although it is known that intracellular BCG signals via autophagy related or cytosolic sensors, and the inhibition of histone methyltransferases during training can abrogate the effect of training in human monocytes (170,191,217), few studies, if any, track what happens to BCG (or other trained immunity inducing agents) inside the cells during or post innate training, suggesting that these findings do not exclude the possibility of viable, intracellular BCG synergistically contributing to monocyte responses to heterologous stimuli.

As described in *Chapter 3*, this study did detect viable BCG in monocyte cultures at the end of training. In this study, reducing the viability of BCG contributed to changes in monocyte IL-6 or TNFα responses to secondary stimulation and some weak correlations were found between viable CFUs and the concentrations of these cytokines in trained monocyte supernatants. Also, in this work, irradiation-killed BCG did not train human CD14+monocytes. These findings contrasted with the previous observations where irradiation-killed BCG was able to train the cells if applied at the same dose as the live vaccine (217). It should be noted, however, that in the study by *Arts et al.*, a weaker effect on training was observed where dead

BCG was used and a recently published protocol on monocyte training also warns against using stocks of BCG with reduced viability (265).

This has several important implications. First, live, replicating BCG inside monocytes may provide signals synergistic to heterologous stimulation, so enhancing cytokine responses, or it may also be critical for the maintenance or addition of new permissive epigenetic marks at the promoters or enhancers of training inducing genes. Second, because BCG vaccines can vary significantly in the amount of viable BCG CFUs (262,393), this could influence the ability of this vaccine to induce trained immunity or other heterologous effects in vaccinated individuals. Viable BCG was recoverable from the BM of intravenously and, in some cases, subcutaneously BCG-vaccinated mice and was associated with reprogramming of their HSCs and resulting protection from *M. tuberculosis* challenge (227). Of interest, intradermal immunisation of human adults with BCG was shown to modulate gene expression in their BM as well (218). Although the latter study did not test for the presence of viable BCG in the BM, there is evidence that viable BCG can be recovered from injection sites for up to 4 weeks, a period closely overlapping with trained immunity effects observed in BCG-vaccinated humans (394). In addition, some studies report traces of mycobacterial DNA in human blood in individuals without apparent TB disease (395), suggesting the possibility of mycobacterial breakthrough into the bloodstream. While other mechanisms or mediators are more likely to contribute, such as the increase in IL-1β in blood of individuals who receive the BCG vaccine (209), it is possible that viable, replicating BCG may amplify these mechanisms, so contributing to secondary responses *in vitro* or *in vivo*.

## 6.3 Innate immune responses to BCG and TLR agonistsin the UK and South African cohorts

### *6.3.1 Overview*

Despite variation or inconsistencies in studies examining immune responses to BCG immunisation, few studies compare innate immune responses to this vaccine or its heterologous effects in different populations directly, especially in infants. Although such a comparison could not be made in this study, described in *Chapter* 4, innate immune responses to BCG and TLR agonists were investigated using PBMC samples available from BCG-vaccinated UK and South African infants. This work investigated not only cytokine responses to BCG and TLR agonists in different infant cohorts that received this vaccine but also explored monocyte surface receptor expression, expression of genes associated with protection against TB and the ability of PBMCs from these two cohorts to inhibit mycobacterial growth. In addition, the work described in *Chapter 4* investigated the contribution of biological sex to these responses and the contribution of population, sex, stimuli or donor on the extent of these responses in BCG-vaccinated infants.

### *6.3.2 Innate immune responses in the UK and South African infant cohorts*

In this study, described in *Chapter 4*, CD14+ monocyte profiles were investigated in BCG-vaccinated UK and South African infants. This study could not examine the differences in terms of cell frequencies expressing various combinations of TNFα, IL-6, CD11b and TLR4 or in mRNA levels of genes encoding components associated with protection against TB or trained immunity with respect to the origin of each cohort. However, there was a clear stimulus-specific effect on the expression of these molecules and similar patterns of this effect were observed in each of the cohorts despite their differences. This suggested that stimulation with BCG or innate stimuli reflected the necessity for the cells to respond to flexibly to infection.

Although the data from cohorts could not be compared directly, there was a trend for higher innate cytokine levels produced by South African infant PBMCs, for cytokines previously associated with heterologous effects of BCG in the BM and TB-protective myeloid cell

differentiation, such as IL-1β, G-CSF or GM-CSF (221,227,242,253). Higher production of IL-1β in Uganda and of GM-CSF in Malawi was previously found in PPD-stimulated whole blood samples from BCG-vaccinated infants (130,306). High levels of EGF, IL-6 and, for selected TLR agonists, IL-1α and MCP-1 were also observed in the South African infant cohort. Higher concentrations of these cytokines were found in BCG-vaccinated infants compared to the controls in earlier BCG immunisation studies, and EGF and IL-6 were previously associated with heterologous effects of BCG in the BCG-vaccinated infant group (128,223). It is not clear whether high levels of EGF, G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, and MCP-1 in PBMC cultures from South African infants who received this vaccine might lead to higher rates of myelopoiesis, monocyte or neutrophil generation and stronger manifestation of heterologous effects, contributing to improved protection against non-mycobacterial infections in this cohort compared to the infants that have lower levels of these cytokines. There is a possibility for this, as most evidence on BCG-associated all-cause mortality or morbidity reduction comes from high infectious disease burden settings whereas studies conducted in other settings found little evidence on the effect of BCG on infant morbidity (159,163,164,301), and studies comparing cytokine responses in infants from the UK, Uganda and Malawi found increased production of cytokines associated with trained immunity or heterologous effects of BCG (130,306) (*Table S4.6, S4.7*).

## 6.4 Cytokine profiling of whole blood responses to BCG and TB vaccine candidates

### *6.4.1 Overview*

Most studies on antitubercular immune responses to BCG and TB vaccine candidates have so far focused on relatively narrow cytokine profiles associated with mycobacteria-specific single- or polyfunctional Th1 or Th17 cell responses, their effector or memory functions or the ability to proliferate. In this work, a 41-plex MBA was optimised to investigate broad cytokine signatures induced by BCG, 4 TB vaccine candidates and their components using whole blood

assay set-up, as described in *Chapter 5*, going beyond the traditional measurements of IFNγ, TNFα, IL-17 or IL-2. This work characterised the signatures induced by these vaccines and determined the contribution of their components to the cytokine signatures induced. In addition, this study also proposed which of the vaccine candidates would be most likely to have the heterologous effects if tested *in vitro* or *in vivo*.

### *6.4.2 Differences in cytokine profiles induced by BCG and TB vaccine candidates*

One of the findings of the work described in *Chapter 5* was that although overall broadly similar cytokine profiles were induced by BCG and 4 TB vaccine candidates investigated in this study, there were some distinct patterns, especially in production of cytokines previously associated with heterologous effects of BCG, or Type I IFN or IFNγ associated responses, probably reflecting some properties specific to the vaccine categories or the adjuvant properties of the carriers themselves. For instance, although MVA85A and Nano-FP1 induced highly similar cytokine profiles, MVA85A was a more potent inducer of IFNα2, a Type I IFN typically induced by viral elements. Spore-FP1 was a more potent inducer of cytokines associated with heterologous effects of BCG (*Table 5.4*) than Nano-FP1 or MVA85A, and thus probably more likely to induce trained immunity *in vivo* than the latter two vaccine candidates. IL-1β was associated with BM reprogramming and enhanced pro-inflammatory BMDM generation, while G-CSF was associated with neutrophil genesis and some protection against polymicrobial sepsis in BCG-vaccinated mice (221,227,242,253). Both were strongly induced by Spore-FP1 vaccine, indicating this vaccine may also exert similar non-specific effects. BCG and its derivative ΔBCG1419c were more potent at inducing IFNγ or IP-10 than FP1 vaccines, suggesting that perhaps these vaccines induce different T helper cell repertoires – while BCG or ΔBCG1419c might be more Th1 based, perhaps FP1 vaccines induce more diverse cell types. Although the literature suggests such a possibility, splenocytes from Spore-FP1 or Nano-FP1 vaccinated mice producing more IFNγ, IL-10, IL-17 or IL-4 upon stimulation with *M. tuberculosis* antigens and expanding CD4+ or CD8+ memory T-cell populations more than

those from BCG-vaccinated animals (363,364), the effect of these vaccines or different carriers on helper T-cell diversity needs further investigation.

Overall, in this work, a strong effect of a vaccine carrier was observed, possibly, reflecting the adjuvant properties of each vaccine candidate. *Bacillus subtilis* spores were identified as the most potent inducers of innate cytokines and were highly immunogenic on their own, irrespective of whether the FP1 protein was present in the vaccine formulation. Previous studies showed that the Spore-FP1 vaccine was immunogenic in mice and able to induce or expand *M. tuberculosis* antigen-specific CD4+ or CD8+ T-cell populations; however, although *B. subtilis* spores alone were able to induce co-stimulatory or antigen presenting molecule expression on murine dendritic cells, this effect was dependent on the quantity of spores used (363), and spores alone were less potent at inducing IFNγ or TNFα in human tonsil cells than when combined with Ag85A and/or bactenectin 5, despite being able to expand CD8+IFNγ+ cell populations (377). It is possible that strong cytokine responses to spores on their own were observed in this work because a broad range of innate cytokines was investigated in this study; whole blood cultures containing diverse innate immune cell types were used, and a relatively high dose of spores was applied. Apart from the innate cytokines, T-cell-associated cytokine responses were also examined. Production of Th2 associated cytokines was comparable to that of the full Spore-FP1 vaccine formulation, its other components or other TB vaccines. Spores induced comparable levels of IFNγ to the BCG-Pasteur strain used in this study and were a more potent stimulus for IL-1β or TNFα production than BCG or other TB vaccines or their components, suggesting a propensity for steering the immune responses towards Th1-type, in agreement with observations in murine immunisation studies (363). Spores on their own also induced cytokines previously associated with heterologous effects of BCG (*Figure 5.2, Table 5.3*), suggesting they may have similar non-specific effects, however, further work would be needed to test this hypothesis.

The adjuvant effect observed in this study was particularly apparent when *B. subtilis* spores were substituted for nanoparticles as the carriers for the FP1 protein in either full or partial

Nano-FP1 formulation. Not only the nanoparticles were less potent at inducing cytokines associated with heterologous effects in infant BCG vaccination studies or other innate cytokines, they reduced the ability of the adjuvant Quil A to induce the innate and some adaptive cytokine responses when used in the full Nano-FP1 vaccine formulation. This was partially supported by the literature, where nanoparticles did not enhance the expression of co-stimulatory or antigen presenting molecules in murine macrophages, or their proinflammatory cytokine production, possibly due to low NF-κB activity or differential priming through Type I IFN pathways (364). However, Nano-FP1 vaccine itself was able to enhance Ag85B or FP1 specific IFNγ, IL-10, IL-17 or IL-4 responses in vaccinated mice and expand polyfunctional CD4+ T-cell populations, suggesting it as an immunogenic vaccine (364). It should be noted that the possible suppressive effect of nanoparticles was only observed for some of the cytokines, mostly those previously associated with heterologous effects of BCG (*Figure 5.3*), especially, compared to adjuvant Quil A alone or adjuvanted Spore-FP1 formulation.

There were some discrepancies in findings from this and previous studies, however. While nanoparticles alone or in the presence of FP1 stimulated RANTES and suppressed IP-10 in this study, previous work on murine macrophages suggested a reverse effect (364). While the reasons for this are not clear, differences in human whole blood cytokine responses suggest differential priming of immune system by nanoparticles and *B. subtilis* spores, likely to influence downstream responses to vaccine or vaccine candidate formulations using these carriers, with nanoparticle vaccines probably less likely to induce innate immune training. While innate immune cell priming through different transcription factors – AP1, NF-κB and IRF-3 (363,364) - is likely to contribute to differences in innate cytokine responses to these carriers and vaccines containing them, the mechanisms leading to distinct cytokine responses would need to be investigated in further studies.

## 6.5 Interindividual variability in innate immune responses to BCG and TLR agonists

### *6.5.1 Overview*

In this study, high interindividual variability was observed both in the *in vitro* training assays (described in *Chapters 2* and *3*) and in the comparison of the innate immune responses to BCG and TLR agonists (described in *Chapter 4*). This observation is consistent with findings from previous *in vitro* studies on monocyte training using BCG and from infant or adult human BCG immunisation studies where significant variation in cytokine responses to mycobacterial or heterologous stimuli was detected (170,233,238,265). Multiple factors are considered to play a role in variation in the specific or heterologous effects of the BCG vaccine in human adults and infants, including polymorphisms of genes encoding PRRs, cytokines or components regulating metabolic pathways (170,177,215,217,257), monocyte to lymphocyte ratio variation across individuals (346), season of birth (305,307), maternal BCG immunisation status (159,233,238,353,354,396), maternal TB status (130,396), exposure to environmental mycobacteria (27,30), and strain of BCG used for vaccination (23), even though not all the studies confirmed the associations with these factors (163,355).

### *6.5.2 Sex-differential effects*

Some factors contributing to interindividual variation have been consistently reported across different studies, sex of the participants being one of the factors. Although not analysed due to small size of the datasets from the *in vitro* training experiments and the high frequency of female donors in the work described in *Chapters 2* and *3*, *Chapter 4* examined the possible effect of sex more closely for the UK infant PBMC responses to BCG and TLR agonists. In most comparisons no differences between male and female infant responses to these stimuli were detected, but some subtle differences were found in others. In this study, higher baseline levels of IL-6 by FACS analysis were detected in females at 6 h of PBMC culture, reminiscent of the Luminex findings in the MISBAIR study where higher IL-6 production was found in 7 month old female Australian infants upon heterologous stimulation with TLR agonists or *H.*

*influenzae* and *E. coli* (233), although no differences were found in male or female UK infant IL-6 responses to other stimuli in FACS or multiplex bead array analyses in the work described in *Chapter 4.*

Elevated IP-10 production was also observed in supernatants from male infants after PBMC stimulation with BCG for 24 h in this work. In addition, PBMCs from BCG-vaccinated female infants were found to inhibit the growth of BCG more strongly than those from male infants. This is consistent with the previous findings from BCG-vaccinated UK adults, where higher rates of mycobacterial growth inhibition were found in females and a trend for higher IP-10 production in males was observed (235). A recent study associated IP-10 responses with enhanced mycobacterial inhibition and protection against TB in exposed but uninfected individuals, however, the possible effect of sex was not taken into account and samples from male BCG-vaccinated donors only were used in an accompanying comparison of heterologous cytokine responses to BCG (131). In another BCG vaccination study, IP-10 levels in plasma from BCG-vaccinated males negatively correlated with IL-6 and IL-1β responses to PBMC stimulation with *S. aureus* or *M. tuberculosis* (259). These findings are of interest as 1) for reasons incompletely understood, males are more frequently affected by TB than females, and 2) it has been speculated that male infants, especially, those with low-birth-weight (LBW) may benefit from heterologous effects of BCG vaccination more than female infants (152,164).

The evidence from BCG immunisation studies paints a somewhat complex picture. Systematic reviews on heterologous effects of BCG and other childhood vaccines found no difference in the effect of BCG on all-cause mortality in girls and boys (136,144), however, there is some evidence that BCG immunisation can reduce cytokine responses to secondary challenges in males as opposed to females (259). Some studies on infant BCG immunisation also suggest that the difference in BCG-vaccinated LBW female infant responsiveness to PPD or heterologous stimulation in terms of IL-1β, TNFα or IFNγ compared to non-vaccinated LBW infants may be higher than that of BCG-vaccinated males, although, similarly to the work

described in *Chapter 4*, most differences between BCG-vaccinated male and female secondary cytokine responses were not significant (236). In another study that measured innate and adaptive cytokine production in control and BCG-vaccinated Gambian infants, no sex-differential effects were found on TNFα, although some sex- and stimulus-specific differences in were detected in heterologous Th1 cytokine or IL-17 responses (258). Elevated IFNγ and TNFα production was detected in 7 day old male Australian neonates in response to heterologous stimuli; however, this effect was not dependent on BCG *per se* (240). This is perhaps not surprising as estradiol and dihydrotestosterone have been shown to reduce direct monocyte responses to BCG but not the effect of training (260).

Together, data from this study and the previous work of other groups suggests that although BCG-vaccinated females may inhibit mycobacterial growth more strongly than males and although male and female infants may react differentially to the BCG vaccine and its heterologous effects, these differences and their influence on secondary responses to *M. tuberculosis* or heterologous stimuli are subtle and should be considered in the context of other factors.

### *6.5.3 The effect of different BCG strains*

One of the factors that may have influenced the differences observed in the results from the *in vitro* training work described in *Chapters 2* and *3* and previously described studies on monocyte training with BCG could be the vaccine strains used. While in this work the BCG-Pasteur strain from Aeras was used to train monocytes, the previous studies in the Netherlands reported the use of the Danish BCG (SSI) strain (170,177,191,205,215,217). Samples from infants vaccinated with different BCG vaccine strains were also used in the UK and South African infant innate immune response to BCG comparison described in *Chapter 4*: while the UK infants were immunised with the InterVax BCG (strain SL222 Sofia), the South African infants received BCG-Tokyo strain (113,310). All these vaccine strains induce some

degree of protection against TB, are generally considered comparable in terms of protection against TB and are used interchangeably to vaccinate the human population (310,318,397).

Immunological studies, however, suggest that there are some subtle differences in the innate or adaptive cytokine profiles induced by these vaccines *in vitro* or *in vivo* and that they may affect downstream mycobacteria-specific or heterologous immune responses to some extent (24,262,300,398,399). In some of these studies, the Danish BCG strain was associated with stronger Th1 or IL-10 responses to mycobacterial or unrelated stimuli (24,398,399). In newborn and adult human blood, the Danish BCG was also shown to induce trained immunity associated cytokines more strongly than other, non-Pasteur strain BCG vaccines (262). As described in *Chapters* 2 and *3,* in this work a weaker and less consistent effect of monocyte training with BCG-Pasteur was found compared to the previous studies in the Netherlands. While such a possibility would be consistent with the previous findings, it is not clear whether this means that the Danish BCG is more potent at training monocytes than BCG-Pasteur - the ability of BCG to grow and its viability vary across strains and in different BCG formulations and it can also affect the cytokine profiles induced (252,262,393).

This is also relevant to infant immunisation. Previous work associated BCG-Bulgaria with stronger GM-CSF and G-CSF responses in newborn blood compared to the Japanese strain of BCG when both strains were applied in equal CFU amounts; however, when vaccination-equivalent doses were applied, the pattern was reversed for GM-CSF and PDGF-AB/BB (262). As reported in *Chapter 4,* in this study high production of G-CSF, GM-CSF and other trained immunity associated cytokines was detected in the South African infant cohort, who received BCG-Tokyo. A tendency for lower production was observed in the UK infant cohort, who received the InterVax BCG. It should be noted, however, that a number of confounders may have influenced this observation, given the differences between the two cohorts. Cytokine responses aside, as described in *Chapter 4,* overall very similar patterns of responses to mycobacterial or innate stimuli were detected in this study in both cohorts, including the inhibition of mycobacterial growth, suggesting that further work with a study designed

specifically to compare innate immune responses in cohorts from different settings or cohorts receiving different vaccines would be needed to investigate this.

The larger implications of these subtle strain or formulation dependent differences in immune responses to mycobacteria-specific or heterologous stimuli in BCG-vaccinated individuals are also uncertain (27,400). Studies comparing differences in morbidity or clinical consultation rates in infants vaccinated with different strains of BCG found no significant differences in high infectious disease settings, although some effects on BCG scar formation were detected (398,400,401). It should be noted that such effects are likely to be small and susceptible to other modulating effects.

## 6.6 Limitations

### *6.6.1 Differences in the UK and South African infant comparison groups*

One of the challenges in this study was associated with the recruitment of the BCG-vaccinated and control infants, especially in the UK. As discussed previously, the UK is a low-TB-incidence country and the BCG vaccine is currently recommended to infants at an elevated risk of TB, including those whose immediate family members were born abroad, in countries with high TB incidence, or those born in areas with ≥40 TB cases per 100,000 individuals (12,390). The UK participants for this study were recruited in London, at the North Middlesex Hospital. As one of the largest European capitals, London hosts large and diverse migrant communities, including individuals from countries where TB is endemic. Even so, the rates of TB in London have been decreasing for several years, the estimated incidence being 19 cases per 100,000 individuals, with the London borough of Newham being the only area exceeding the 40 cases per 100,000 individuals rate in 2018 (402). In this study, most participants were recommended the BCG vaccine because their parents or guardians came from countries with TB incidences higher than that in the UK.

From the beginning of the study, the recruitment rates were low. Several factors may have contributed to this. First, the global supply of the BCG vaccine was interrupted in 2015-2016 due to the manufacturing issues by one of the largest suppliers – the Statens Serum Institute in Denmark (403). A replacement BCG vaccine (supplied by InterVax Ltd.) was used in the UK until the supply of the Danish strain was re-established; however, the replacement vaccine was unlicenced in the UK (404) and some of the early recommendations to health care professionals during the shortage included the use of the remaining stocks of the SSI BCG until this replacement vaccine became available, even if the lots of the old stocks were expired (405). Although by the time this work was started in 2017, BCG from InterVax Ltd. only was used in the North Middlesex Hospital, it is possible that some parents or guardians may have refused the replacement vaccine contributing to low recruitment rates. Second, there was a period when a specialist trained to administer BCG intradermally was not available at the North Middlesex Hospital, and no BCG vaccinated infants could be recruited until another trained specialist was found. Third, in this study, the infants in the UK arm of study were vaccinated within the first week of life, and their blood samples were scheduled to be taken at 10 weeks from when the vaccine was administered. Some parents or guardians may have felt that infants at this age were too young to have their blood samples taken. Fourth, the participants and their parents or guardians did not receive any gifts, payments or compensations for participation in this study, and no placebo vaccine or additional treatment was offered to those who were not scheduled to receive the BCG vaccine. It is possible that this may have discouraged parents or guardians, especially if they had to spend a long time travelling to the hospital and pay travel or parking expenses. Lastly, because of the COVID-19 pandemic and associated safety regulations and disruption to health services, it was not possible to organise additional participant recruitment. Overall, although the group's previous recruitment of BCG vaccinated and control infants in the UK had worked well, logistical challenges resulted in problems with recruitment for this study.

Regarding the South African infant PBMC samples, they were all from BCG vaccinated infants recruited at a SATVI study site in Worcester, near Cape Town for previous BCG vaccination and protection from TB studies (113,310). Considering the previously estimated TB incidence rate of 2,000 cases per 100,000 infants under 2 years of age in these studies and associated risk of TB infection, it would have been unethical not to vaccinate the infants, especially, considering their inherently higher susceptibility to TB due to differentially polarised immune system (86) and that there are no licenced alternatives to the BCG vaccine. However, because of the lack of the unvaccinated control infants in the UK and South African infant comparison it was not possible to address the influence of BCG vaccination on infant secondary immune responses to BCG and innate stimuli. In addition because of differences in the characteristics of two cohorts, this study could not directly compare the ability of cells from these infant groups to inhibit mycobacterial growth, innate cytokine profiles induced by BCG and non-mycobacterial stimuli, and the expression of genes associated with trained immunity and protection against TB or other mycobacterial infections.

Ideally, the limitations of this comparison would have been addressed by setting up a study designed for such comparisons across multiple study sites, standardised clinically and laboratory-wise. It would include recruiting a trained specialist to administer the vaccine, using the same strain and batch of BCG in the UK and South Africa, and the number of infants recruited would have been based on the power size calculation. In addition, a control group of unvaccinated UK infants would have been recruited. Although there were plans for this, the numbers of unvaccinated, control infants were too low to conduct an appropriate comparison of the innate responses to BCG and TLR agonists for the control and the vaccinated infants. These shortcomings resulted from a lack of grant funding and ending of TBVAC2020 consortium funding. Also, regarding the limitations of this comparison, the study was initially meant to investigate the *in vitro* training model, screen for novel markers associated with trained immunity or heterologous effects of BCG and then investigate their expression or

production in BCG-vaccinated and control infants. However, the project was redesigned when the *in vitro* model was considered insufficiently robust for further detailed investigations.

### *6.6.2 Contribution of non-monocytic cell lineages to innate immune training and/or heterologous effects of BCG*

When this project was initiated in 2017, most of the evidence on trained immunity or associated mechanisms behind the heterologous effects of BCG came from studies on monocyte training *in vitro* or from PBMCs from BCG-vaccinated human individuals (170,177,191,213,217,225). There was also evidence that NK cells, one of the first innate immune cell types reported to form memory-like responses, can be trained by human BCG immunisation too (190,197,406). This study focused on the investigation of monocytes as, upon infection with *M. tuberculosis*, these cells can be recruited from blood into the lungs and differentiate into inflammatory macrophages to help contain this pathogen in granulomas and prevent progression to active TB disease (84). Since 2017, however, more cell types have been implied as possible targets of innate training, with possible involvement in protection against infectious diseases or exacerbation of chronic, inflammatory disorders: respiratory epithelial cells (407,408), fibroblasts or endothelial cells (409), microglia (193–196), innate lymphoid (192) or smooth muscle cells (410).

Among these, HSCs and neutrophils have received more attention in the context of heterologous effects of BCG and protection against TB recently. In mice, BCG immunisation was associated with epigenetic reprogramming of HSCs in the BM, enhanced myelogenesis, and generation of BMDMs with improved antimycobacterial properties (227), and in another murine BCG immunisation model, neutrophil depletion was associated with reduced control of *M. tuberculosis* growth in the lungs (241). In a murine neonatal polymicrobial sepsis model, BCG was also able to partially protect infected pups reducing microbial load in their spleens and blood in a neutrophil and GM-CSF dependent manner. Of note, human BCG vaccination was also associated with a neutrophil gene signature in the BM at 90 days post immunisation

in adults and with an increase in neutrophil blood counts in BCG-vaccinated infants (218). These findings reveal an interesting pattern on the possible role of neutrophils in mycobacterial infections or heterologous effects of BCG. So far, the involvement of neutrophils in the responses against *M. tuberculosis* has been considered complex at best, with a possible contribution to active disease through incomplete control of this pathogen in granulomas, its dissemination, and induction of Type I IFN responses favoured by this pathogen (84,411,412) but it is possible that BCG, being a live attenuated mycobacterium, may enhance their protective properties *in vitro* or *in vivo*. In this study, this possibility was not investigated but as described in *Chapter 4* in the investigation of the UK and South African infant innate cytokine profiles, consistently high levels of cytokines associated with the antimycobacterial and heterologous effects of BCG, that is, IL-1β, G-CSF and GM-CSF were found in supernatants from the South African infants, suggesting a possibility for influence on myelopoiesis or neutrophil responses to *M. tuberculosis* or other pathogens in these infant populations and that these differences may be worth investigating in future studies.

## 6.7 Future work

Although in this study a weak effect of human monocyte training with BCG *in vitro* was observed, only CD14+ monocytes were investigated. There is a possibility that different subsets of monocytes have different capacities to be trained and the future work should investigate not only classical but also intermediate and non-classical monocytes. Also, in this work, relatively few secondary stimuli were used, with most secondary challenging conducted using LPS. A narrow profile of cytokines was tested as well, focusing on markers associated with trained immunity in previous studies. Future work should include both a broader range of secondary stimuli and the cytokines or chemokines analysed, especially, where secondary responses of different monocyte subsets are investigated. In addition, in this study, cells from BCG-vaccinated donors were used. Because it is now known that historical BCG vaccination can influence NK cell or macrophage responses upon later infections or challenges, future

work may need to examine differences in whether trained immunity or heterologous effects of BCG depend on prior BCG status, using cells from both the vaccinated and unvaccinated donors. It is also interesting why live BCG may be more effective at inducing trained immunity *in vitro* than comparable dose of irradiation-killed BCG and whether that is due to synergistic signals from BCG live, replicating BCG or if other mechanisms are involved. This may also be relevant to heterologous effects of BCG *in vivo* and may need further investigation as dead BCG has been previously shown not to induce heterologous effects in the vaccinated individuals and the mechanisms for live BCG reprogramming BM of intravenously vaccinated mice or intradermally immunised humans are not fully clear.

Generally, studies on infant BCG vaccination in infancy or on heterologous effects of BCG often lack standardisation and frequently vary in study designs, including age at BCG vaccination or sampling, childhood immunisation schedules, strains of BCG used to vaccinate the infants, amount of viable BCG received, or the outcomes measured. Future studies need to be more standardised in order to effectively compare studies. There is also a lack of studies on side-by-side comparisons of mycobacteria-specific protection or heterologous protection against TB and other infectious diseases in BCG-vaccinated infants. Although the comparison of UK and South African infant innate antimycobacterial or TLR agonist responses reported in this thesis provided valuable information on similarities and differences in infant innate immune responses to BCG and other stimuli in different populations, there were some limitations. In this study, only PBMC responses with a focus on monocyte responses were investigated, and only samples from infant cohorts that had been administered BCG vaccine were available, limiting the breadth of responses studied and the ability to determine whether the observed differences in innate immune responses of the UK and South African infants were associated with heterologous effects of BCG or trained immunity or reflected more general differences.

To address these issues and investigate similarities and differences in innate antimycobacterial immune responses in BCG-vaccinated infants from different populations more extensively, future work could include a larger scale follow-up: a randomised controlled

trial in UK and South Africa or other countries, especially, where similar childhood immunisation schedules are similar. When the project described in this thesis was started in 2017, heterologous effects of the BCG vaccine were mostly associated with enhanced monocyte or NK cell responses post BCG vaccination. However, it has been discovered since then that BCG exerts its heterologous effects on the BM of the vaccinated humans or mice, and influences BMDM or neutrophil generation and their antimicrobial properties. Since in the investigation of the UK and South African infant innate immune responses to BCG broad PBMC or CD14+ monocyte responses were investigated, future work may need to include more extensive studies of more diverse innate cell populations, especially those involved in granuloma responses, including neutrophils, different subsets of monocytes (e.g. classical, intermediate or non-classical monocytes) or innate lymphoid cells. High levels of GM-CSF were found upon BCG or TLR agonist stimulation of PBMC cultures in South African infants in this study, so it would be interesting to compare whether this influences the frequencies of innate immune cell populations in BCG-vaccinated infants and if so, whether this could be associated with the population. There is also increasing evidence that antimycobacterial antibodies may contribute to BCG-vaccinated infant or adult protection from TB, so it may be important to investigate B-cell and antimycobacterial antibody responses or, if B-cells, having phagocytic properties and able to present antigens via MHC class II molecules, could also be epigenetically modified by exposure to BCG and contribute to its heterologous effects. Finally, to explore whether heterologous effects by BCG are induced differentially in human populations, the aforementioned randomised control trial would need to include a control group of samples from unvaccinated infants or blood specimens from the same individuals obtained prior to immunisation. These investigations should also be accompanied by functional studies, especially those on mycobacterial growth inhibition or killing – most infant BCG immunisation studies, especially those on heterologous effects of BCG rarely explore the association of observed differences in heterologous cytokine production with mycobactericidal properties, so little is known about their contribution to protection against TB or other infections.

In this study, some sex-differential effects were observed, with PBMCs from BCG-vaccinated female infants inhibiting mycobacterial growth more strongly than males. This was reminiscent of previous findings in BCG-vaccinated UK adults. It would be interesting to investigate this on a larger scale and also whether this effect would extend to BCG-vaccinated female PBMCs in other populations. Regarding other modifiers of innate immune responses to BCG or other stimuli, maternal TB or BCG status has been associated with stronger BCG-vaccinated infant immune cytokine responses to mycobacterial or heterologous stimulation. It was not investigated in this study but would need deeper examination in future, for instance, to find out whether this effect extends to the bactericidal properties of cells from BCG-vaccinated infants and if so, which mechanisms could be involved.

Future work would also need to investigate which other TB vaccines apart from BCG or MTBVAC can induce trained immunity or display heterologous effects. So far MVA has been shown to induce trained immunity *in vitro*, however, at a lesser capacity than *Vaccinia* and this effect was dose dependent (201). MVA85A, its derivative tested as a booster in previous TB vaccine trials, would also be expected to have similar training or heterologous effects. They have not yet been investigated. Spore-FP1 vaccine examined in this study was able to induce cytokines previously associated with heterologous effects of BCG in infant immunisation studies, an effect probably driven by *B. subtilis* spores used as a carrier for this vaccine. It would therefore be interesting to explore whether these vaccines also induce trained immunity *in vitro* and whether they can also epigenetically or metabolically reprogram the BM in mice or humans and if so, whether they also enhance myeloid cell progenitors or if they stimulate expansion of other cell populations. Also, data from previous studies on nanoparticles used as carriers for FP1 instead of *B. subtilis* spores found differential priming of murine macrophages by these vaccine carriers. Of interest, in this work, nanoparticles were also found less potent at inducing cytokines previously associated with heterologous effects of BCG. It would be interesting to explore how Type I IFN signalling, associated with nanoparticle-induced phenotype in murine macrophages interplays with training, and whether

it results in differentially trained cells and what consequences it could have for antimycobacterial properties of innate immune cells.

## 6.7 Concluding remarks

This study examined the heterologous effects of the BCG vaccine. The effects observed in this work, however, were highly variable, so it remains to determine why innate immune cells from some individuals may be more likely to be trained than cells from others. It is not clear which mechanisms are exploited by BCG to reprogram monocytes or other cell populations *in vitro* or even more so, *in vivo* and why viability of BCG influences the effects of training when non-viable stimuli, such as β-glucan, induce training. Considering that in BCG-vaccinated infants it was the stimulus or the donor factors that determined the extent of the innate immune responses to BCG or TLR agonists, although other TB vaccines or their components alone may also have heterologous effects, it still remains to understand how these effects work to be able to exploit them for intentional, targeted, effective application in control, prevention or treatment of infectious or non-communicable diseases.

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# *Appendix*

## Supplementary figures and tables

**Table S1.1.** *Impact of BCG or other agents on surface receptor or activation marker expression by innate immune cells*

| Species | Cell type | Setting | Timing of observation | Trained or BCG-vaccinated vs control | Reference |
|---|---|---|---|---|---|
| ***Live microorganisms or vaccines*** | | | | | |
| **BCG** | | | | | |
| Human (adults) | PBMCs | Vaccination | 2 wks post immunisation | ↑ %CD14+ monocytes<br>↓TLR4, ↑CD11b | (191) |
| | | | 3 mos post immunisation | ↑TLR4, CD11b | |
| | | | 2 wks post immunisation | ↓TLR4, ↑CD11b | (213) |
| | | | 3 mos post immunisation | ↑CD11b, CD14, TLR4 | |
| | | | 1 yr post immunisation | ↑CD11b, CD14, TLR4, CD206 (MR/MRC1) | |
| Human (adults), BCG non-vaccinated | LPS-irresponsive macrophages | *In vitro* stimulation | 8 h post BCG pre-treatment | ↑TLR4 surface expression upon *M. leprae* or LPS stimulation | (214) |
| | LPS-responsive macrophages | | | ↓TLR4 surface expression upon LPS stimulation | |
| Human (adults) | Macrophages | Historical BCG vaccination | - | ↓TLR4 in *M. leprae* stimulated CD68+ macrophages from BCG-vaccinated individuals<br>↓TLR4 in in *M. leprae* stimulated CD16+CD68+ macrophages from BCG-vaccinated individuals | |
| Human (adults) | Monocyte-derived DCs | Historical BCG vaccination | - | ↑*LILRB5* mRNA compared to baseline in BCG-stimulated cultures | (234) |
| | Monocyte-derived macrophages | | | ↓*LILRA3* mRNA in BCG-vaccinated compared to unvaccinated in unstimulated, BCG- and *M. tuberculosis*-stimulated cultures cultures | |

| | | | | | |
|---|---|---|---|---|---|
| | | | | ↓*LILRB2* mRNA in BCG-vaccinated compared to unvaccinated and *M. tuberculosis*-stimulated cultures | |
| Human (adults) | Alveolar macrophages | Vaccination | 2 wks post immunisation | ↓CD11b, HLA-DR | (216) |
| | | | 3 mos post immunisation | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | ↓TLR10 | (215) |
| Human (adults) | Whole blood | Vaccination, *P. falciparum* challenge | 5 weeks post immunisation and 3 d post antimalarial treatment | ↓ CD86 on monocytes | (167) |
| | | | ***Microbial components*** | | |
| | | | **β-glucan** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | ↑*CD36, CD204 (SR-A/MSR-1)* mRNA | (204) |
| | | | 6 d post training | ↑*CD206 (MR/MRC1), CD163, TLR4, TLR2, HLA-DRB1, CD40, CD80, CD86, CCR1, CD83, CD208 (DC-LAMP)* mRNA | (200) |
| | | | 6 d post training | ↑TLR10 | (215) |
| | | | 5 d post training | ↑CD14+, CD68+ and CD11b+ monocyte frequencies | (203) |
| | | | **MDP** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | ↑CD14+ monocyte frequency | (203) |
| | | | **Flagellin** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | ↑CD14+ monocyte frequency | (203) |
| | | | **Pam3Cys** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | ↑CD14+ monocyte frequency | (203) |
| | | | **R848*** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | ↑CD14+ monocyte frequency | (203) |
| | | | ***Metabolites*** | | |
| | | | **oxLDL*** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | ↑*CD36, CD204 (SR-A/MSR-1)* mRNA | (204) |
| | | | **Lipoprotein A** | | |

| | | | | | |
|---|---|---|---|---|---|
| Human (adults), patients with elevated plasma lipoprotein A levels | Monocytes | *Ex vivo* observation | - | ↑CCR7, CD62L (SELL), CD11b (ITGAM), CD11c, CD29, CD36, CD204 (SR-A/MSR-1) | (206) |

* Dose-dependent effect on the induction of training or tolerance.

MRC1 – mannose receptor C-type 1, MR – mannose receptor, MSR-1 – macrophage scavenger receptor 1, oxLDL – oxidised low-density lipoprotein, SELL – Selectin L, SR-A – scavenger receptor A, R848 – resiquimod

**Table S1.2.** *Impact of BCG or other agents on cytokine production by innate immune cells*

| Species | Cell type | Setting | Timing of observation | Secondary stimulus | Trained or vaccinated vs control | Reference |
|---|---|---|---|---|---|---|
| ***Live microorganisms or vaccines*** | | | | | | |
| **BCG** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 7 d post training | LPS | ↑TNFα | (191) |
| | | | | *C. albicans* | | |
| | | | | *S. aureus* | | |
| | | | | *E. coli* | | |
| | PBMCs | Vaccination | 3 mos post immunisation | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | |
| | | | | *M. tuberculosis* | ↑*IL-1B, TNFA* mRNA | |
| | | | | *C. albicans* | | |
| | | | | *S. aureus* | | |
| Mice (SCID) | Splenic monocytes | | 3 wks post immunisation | LPS | ↑TNFα | |
| Human (adults) | NK cells | Vaccination | 2 wks post immunisation | *M. tuberculosis* | ↑IL-1β, IL-6 | (197) |
| | | | | *C. albicans* | ↑IL-1β | |
| | | | | *S. aureus* | ↑IL-1β, IL-6 | |
| | | | 3 mos post immunisation | *M. tuberculosis* | ↑IL-1β, TNFα | |
| | | | | *S. aureus* | | |
| Human (adults) | PBMCs | Vaccination | 2 wks post immunisation | *M. tuberculosis* | ↑IFNγ, IL-22, TNFα | (213) |
| | | | | LPS | ↑IL-1β | |
| | | | | *C. albicans* | ↑IL-17 | |
| | | | | *S. aureus* | ↑IFNγ, IL-17 | |
| | | | 3 mos post immunisation | *M. tuberculosis* | ↑IFNγ, IL-22 | |
| | | | | LPS | ↑IL-1β | |
| | | | | *S. aureus* | ↑IFNγ | |
| | | | 1 yr post immunisation | *M. tuberculosis* | ↑IL-17, IL-22 | |
| | | | | *C. albicans* | ↑IL-17, IL-22, TNFα | |
| | | | | *S. aureus* | ↑IFNγ, IL-17 | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS | ↑TNFα, IL-6 | (170) |
| | | | | *C. albicans* | | |
| | | | | *B. burgdorferi* | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Human (adults) | PBMCs | Vaccination | 3 mos post immunisation | *B. burgdorferi* | ↑IL-1β, TNFα | |
| | | Intravesicular instillation | post initial treatment – 6 instillations | LPS | ↑IL-1β, IL-6, TNFα | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at promoters of *TNFA*, *IL-6* | (217) |
| | | | | LPS | ↑IL-6 | |
| | | | | RPMI | ↑IL-1RA | (205) |
| | | | | LPS | ↑IL-6, a trend for ↑IL-10 | |
| | | | | Pam3Cys | ↑IL-1RA | |
| Mice (C57BL/6) | Serum | Vaccination | 4 wks post immunisation | LPS | ↑IL-1β, TNFα | (198) |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | (177) |
| | | | | LPS | ↑TNFα, IL-6 | |
| Human (adults) | PBMCs | Vaccination | 1 mo post immunisation | LPS | ↑IL-1β, IL-6, TNFα | (209) |
| | | | | *M. tuberculosis* | ↑IL-1β, IL-6 | |
| | | | | *C. albicans* | ↑IL-1β, IL-6, TNFα | |
| | Plasma | Vaccination | 5 d post immunisation | - | ↓IFNα, IFNγ, IL-1RA, IL-8, TNFα | |
| | Adherent monocytes | *In vitro* training | 6 d post training | Yellow fever vaccine | ↑IL-8 | |
| Human (adults) | PBMCs | Vaccination | 3 mos post immunisation | *C. albicans* | ↑IL-1β, IL-6 | (218) |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS | ↑IL-6 | (219) |
| Human (neonates) | Adherent cord blood monocytes | | | | ↑TNFα, IL-6 | |
| Human (adults) | Monocytes | *In vitro* training (low dose) | 6 d post training | LPS | ↑IL-10 | (222) |
| | | *In vitro* training (high dose) | 6 d post training | LPS | ↑CCL22, CXCL1, CCL4, CCL5, IL-1α<br>↓IL-12p70 | |
| Human (neonates) | Cord blood monocytes | *In vitro* training (low dose) | 6 d post training | LPS | n.s. | |
| | | *In vitro* training (high dose) | 6 d post training | LPS | ↓IL-12p40 | |
| Human (adults) | PBMCs | Vaccination | 1 d post immunisation | LPS | ↑IL-6, IL-1β | (220) |
| | | | | *M. tuberculosis* | ↑IL-1β | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | PHA | ↑IFNγ | |
| | | | 4 d post immunisation | LPS | A trend for ↑IL-1β | |
| | | | 2 wks post immunisation | *M. tuberculosis* | ↑IFNγ | |
| Human (adults) | PBMCs | Vaccination | 12 mos post immunisation | Influenza H1N1 California | ↑IL-1β, IL-6, TNFα | (249) |
| | | | | SARS-CoV-2 Wuhan Hu-1 | ↑IL-6<br><br>A trend for ↓TNFα | |
| Human (adults) | PBMC and BCG co-culture supernatants | Vaccination | 4 wks post immunisation | *M. tuberculosis* | ↑IFNγ, TNFα, IL-1β, IL-2, IL-6, IL-10, GM-CSF, MIF, CCL23, CCL17, CCL22, CCL20, CCL19, CCL1, CCL8, CCL15, CX3CL1, CXCL2, CXCL5, CXCL6, CXCL9, CXCL10, CXCL11, CXCL13, CXCL16 | (131) |
| | | | | LPS | ↑IFNγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, GM-CSF, MIF, CCL23, CCL24, CCL17, CCL22, CCL20, CCL21, CCL19, CCL1, CCL25, CCL27, CCL13, CCL11, CCL3, CCL8, CCL15, CX3CL1, CXCL1, CXCL2, CXCL5, CXCL6, CXCL9, CXCL10, CXCL11, CXCL13, CXCL16 | |
| | | | | *C. albicans* | ↑TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, MIF, CCL23, CCL7, CCL24, CCL26, CCL22, CCL20, CCL21, CCL19, CCL25, CCL27, CCL13, CCL11, CCL3, CCL2, CCL8, CCL15, CX3CL1, CXCL1, CXCL2, CXCL5, CXCL6, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL16 | |
| | | | | *S. aureus* | ↑IFNγ, TNFα, IL-1β, IL-4, IL-6, IL-10, IL-16, MIF, CCL23, CCL7, CCL24, CCL26, CCL22, CCL20, CCL21, CCL19, CCL25, CCL27, CCL13, CCL11, CCL3, CCL2, CCL8, CCL15, CX3CL1, CXCL1, CXCL2, CXCL6, | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL16 | |
| | | *Ex vivo* stimulation | - | - | ↑IFNγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8, CCL23, CCL7, CCL24, CCL26, CCL20, CCL1, CCL25, CCL13, CCL11, CCL3, CCL8, CXCL1, CXCL6, CXCL9, CXCL11, CXCL13, CXCL16 compared to controls | |
| | | | | ***M. tuberculosis*** | | |
| Human (adults) | PBMC and BCG co-culture supernatants | *Ex vivo* stimulation | - | - | ↑IFNγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, GM-CSF, MIF, CCL23, CCL7, CCL24, CCL26, CCL17, CCL22, CCL20, CCL21, CCL19, CCL1, CCL25, CCL27, CCL13, CCL11, CCL3, CCL8, CCL15, CX3CL1, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, CXCL13 compared to controls | (131) |
| IGRA -ve contacts of active TB patients | Whole blood | *Ex vivo* stimulation | - | *E. coli* | ↑TNFα, IL-6, IL-1β in IGRA -ve vs IGRA +ve individuals | (386) |
| | | | | *S. pneumoniae* | ↑IL-6 in BCG-vaccinated IGRA -ve vs IGRA +ve individuals | |
| | | | | **MTBVAC** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | (198) |
| | | | | LPS | ↑TNFα, IL-6 | |
| Mice (C57BL/6) | Serum | Vaccination | 4 wks post immunisation | | ↑IL-1β, TNFα | |
| Mice (SCID) | | | | | ↑TNFα | |
| | | | | ***Vaccinia*** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS / Pam3Cys | ↑TNFα, IL-6 | (201) |
| | | | | **MVA** | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS / Pam3Cys | ↓TNFα, IL-6 | (201) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | ***C. albicans*** | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS<br>Pam3Cys<br>*C. albicans*<br>*M. tuberculosis* | ↑TNFα, IL-6 | (200) |
| | | | ***E. coli*** | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS<br>Pam3Cys<br>*C. albicans* | ↓TNFα | (200) |
| | | | ***P. falciparum*** | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 3 d post training | unstimulated<br>Pam3Cys | ↑H3K4me3 at *TNFA*, *IL-6*<br>↑TNFα, IL-6<br>↓IL-10<br>↑*TNFA* mRNA | (202) |
| Human (children) | Monocytes | Malaria | - | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* in children with malaria compared to adult controls | |
| | | | ***Microbial components*** | | | |
| | | | **β-glucan** | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS<br>Pam3Cys<br>*C. albicans*<br>*M. tuberculosis* | ↑TNFα, IL-6<br>↑*TNFA, IL6* mRNA<br>↑TNFα, IL-6 | (200) |
| | CD14+ monocytes | | | LPS | | |
| | Adherent monocytes | | | LPS<br>*B. burgdorferi* | ↑TNFα | (170) |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 and 6 d post training | LPS<br>Pam3Cys | ↑TNFα, IL-6 | (203) |
| Human (adults) | Monocytes | *In vitro* training | 7 d post training | LPS<br>Pam3Cys<br>*S. aureus* | ↑TNFα, IL-6 | (224) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | *E. coli* | ↑TNFα | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | Pam3Cys | ↑TNFα, IL-6 | (210) |
| | | | | RPMI | ↑IL-1RA | (205) |
| | | | | LPS | ↑IL-6, IL-10, IL-1RA | |
| | | | | Pam3Cys | ↑IL-1RA | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS | ↑TNFα, IL-6, IL-8 (CXCL8), CCL2 (MCP-1)<br><br>↑*TNFA, IL-6, MCP-1, IL-18, IL-8* mRNA | (204) |
| | | | | Pam3Cys | ↑TNFα, IL-6, IL-8 (CXCL8), CCL2 (MCP-1) | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | Pam3Cys | ↑TNFα, IL-6 | (206) |
| | | | | LPS | | (198) |
| | | | 5 d post training | LPS | | (201) |
| | | | | Pam3Cys | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | (207) |
| | | | | LPS | ↑TNFα, IL-6 | |
| | | | | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | (208) |
| | | | | LPS | ↑TNFα, IL-6 | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | unstimulated | ↓H3K9me3 at *IL-1B* promoter | (221) |
| | | | | *M. tuberculosis* | ↑TNFα, IL-6<br><br>↑*CCL18, MCP-1* mRNA | |
| Human (adults) | PBMCs | *In vitro* training | 6 d post training | LPS | ↑TNFα, IL-6, IL-10 | (219) |
| Human (neonates) | Cord blood mononuclear cells | | | | | |
| **Pam3Cys** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS | ↓TNFα, IL-6 | (203) |
| | | | | Pam3Cys | ↓IL-6, a trend for TNFα | |
| **LPS** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS | ↓TNFα | (203) |
| | | | 6 d post training | Pam3Cys | ↓TNFα, IL-6 | (210) |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Flagellin** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS | ↓IL-6, a trend for TNFα | (203) |
| **Poly I:C*** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS | ↓TNFα, IL-6 | (203) |
| | | | | Pam3Cys | | |
| **R848*** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS | ↓TNFα, a trend for IL-6 | (203) |
| | | | | Pam3Cys | ↓TNFα | |
| **TriDAP** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS | ↑TNFα, IL-6 | (203) |
| | | | | Pam3Cys | A trend for ↑IL-6 | |
| **MDP♣** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | LPS | ↑TNFα, IL-6 | (203) |
| | | | | Pam3Cys | ↑IL-6 | |
| **Hemozoin** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 3 d post training | Pam3Cys | ↑TNFα, IL-6<br><br>A trend for ↓IL-10 | (202) |
| ***Metabolites*** | | | | | | |
| **ATRA** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K9me3 at promoters of *TNFA*, *IL-6, IL-8, IL-10*, a trend for *IL-1RA*<br><br>↓H3K4me3 at promoters of *IL-10, IL-1RA,* a trend for *IL-8* | (225) |
| | | | | *M. tuberculosis* | ↓TNFα, IL-6, IL-10, IL-1RA | |
| | | | | *E. coli* | | |
| | | | | LPS | ↓TNFα, IL-6, IL-8, IL-10, IL-1RA | |
| | | | | Pam3Cys | | |
| | | | | *S. aureus* | | |
| | | | | Poly I:C | ↓TNFα, IL-8, IL-1RA | |
| | | | | *C. albicans* | ↓TNFα, IL-8, IL-10 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | *S. typhimurium* | ↓TNFα, IL-10, IL-1RA | |
| **oxLDL** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 5 d post training | LPS | ↑TNFα, IL-6, IL-8 (CXCL8), CCL2 (MCP-1)<br><br>↑*TNFA, IL-6, MCP-1, IL-18, IL-8* mRNA | (204) |
| | | | | Pam3Cys | ↑TNFα, IL-6, IL-8 (CXCL8), CCL2 (MCP-1) | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | RPMI | ↑IL-1RA | (205) |
| | | | | LPS | ↑IL-6, IL-1RA | |
| | | | | Pam3Cys | ↑IL-1RA | |
| **Lipoprotein A** | | | | | | |
| Human (adults) | Monocytes | *Ex vivo* observation of patients with elevated plasma lipoprotein A levels | 24 hour stimulation | Pam3Cys | ↑TNFα, IL-1β, IL-6<br><br>↓IL-10 | (206) |
| | Adherent monocytes | *In vitro* training | 6 d post training | | ↑TNFα, IL-6 | |
| **Fumarate** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | (207) |
| | | | | LPS | ↑TNFα, IL-6 | |
| **Mevalonate** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at *TNFA*, *IL-6* | (208) |
| | | | | LPS | ↑TNFα | |
| ***Host elements*** | | | | | | |
| **IL-1β** | | | | | | |
| Human (adults) | Adherent monocytes | *In vitro* training | 6 d post training | unstimulated | ↑H3K4me3 at *TNFA*, *IL1B*<br><br>↓H3K9me3 at *TNFA*, *IL1B* | (209) |
| | | | | LPS | ↑TNFα, IL-6 | |

| | | | | |
|---|---|---|---|---|
| | | Yellow fever vaccine | ↑IL-8, TNFα, IL-6 | |
| | 5 d post training | *M. tuberculosis* | ↑TNFα, IL-6 | (221) |

ATRA – all-transretinoic acid, d – day, MDP – muramyl dipeptide, mo – month, oxLDL – oxidised low-density lipoprotein, Poly I:C – polyinosinic:polycytidylic acid, R848 – resiquimod, TriDAP - L-Ala-gamma-D-Glu-mDAP, yr - year.

* Dose-dependent effect on the induction of training or tolerance.

**Table S1.3.** *The effect of infant BCG immunisation on surface molecule or cytokine production in innate immune cells*

| Influence on surface receptor expression | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Cohort** | **Country** | **BCG strain** | **Timing of vaccination** | **Sample type** | **Timing of observation** | **Secondary stimulus** | **BCG-vaccinated vs. control** | **Reference** |
| Infants | UK | BCG-Denmark | 6 wks of age or no BCG | Whole blood | 4 mos post immunisation | *M. tuberculosis* lysate | ↑CD11b, CD206 (MR/MRC1), CD69 | (223) |
| | | | | | | Pam3Cys | ↑CD69 | |

| Influence on cytokine profiles | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Cohort** | **Country** | **BCG strain** | **Timing of vaccination** | **Sample type** | **Timing of observation** | **Secondary stimulus** | **BCG-vaccinated vs. control** | **Reference** |
| Infants | UK | BCG-Denmark | 5-10 wks or no BCG | Whole blood | 3 mos post immunisation | *M. tuberculosis* purified protein derivative | ↑IFNγ, TNFα, IL-2, IL-1α, IL-6, IL-17, IL-4, IL-5, IL-13, IL-10, IL-8 (CXCL8), CXCL10 (IP-10), CCL3 (MIP1α), G-CSF, GM-CSF | (128) |
| Infants | South Africa | Not specified | At birth | Monocytes | 10 wks post immunisation♠ | BCG | ↑TNFα, IL-12 | (68) |
| | | | | | 36 wks post immunisation♠ | | ↑TNFα, IL-6, IL-12 | |
| | | | | Plasma | 10 wks post immunisation♠ | | ↑TNFα, IL-6 | |
| | | | | | 36 wks post immunisation♠ | | | |
| | | | | | 10 wks post immunisation♠ | Pam3Cys | ↑TNFα | |
| | | | | | 36 wks post immunisation♠ | | ↑TNFα, IL-6, IL-12 | |
| | | | | | 10 weeks of age♠ | Pam3Cys | ↑IL-10 | |

♠ Post-vaccination samples were compared to cord blood cytokine production.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Peripheral or cord blood | 36 weeks of age♠ | LPS | | |
| Low-birth-weight infants | Guinea-Bissau | BCG-Denmark | 6 wks of age or at birth | Whole blood | 4 wks post immunisation | unstimulated | ↑IFNγ, TNFα | (236) |
| | | | | | | PMA | ↑IFNγ, IL-6 | |
| | | | | | | PPD | ↑IFNγ, TNFα, IL-1β, IL-6, IL-10 | |
| | | | | | | Pam3Cys | ↑IFNγ, IL-1β, IL-6 | |
| Infants | UK | BCG-Denmark | 6 wks of age or no BCG | Whole blood | 4 mos post immunisation | *M. tuberculosis* lysate | ↑IFNγ, TNFα, IL-6, IL-8 (CXCL8), IL-10, IL-1RA, CCL3 (MIP1α), MIP-1β, CCL11 (Eotaxin), IL-1α, IP-10, IL-12p40, MCP-3, GM-CSF (CSF2), IFNα2, CD40L | (223) |
| | | | | | | Pam3Cys | ↑IL-6, IL-10, CCL3 (MIP1α), CCL11 (Eotaxin), IL-12p40, sCD40L, EGF, PDGF-AB/BB | |
| | | | | | | *C. albicans* | ↑IL-6, MCP-3, EGF, PDGF-AB/BB<br>↓IP-10 | |
| | | | | | | LPS | ↑IL-8 (CXCL8), GRO (CXCL1)<br>↓ GM-CSF (CSF2) | |
| | | | | | | *E. coli* | ↑EGF, GRO (CXCL1)<br>↓GM-CSF (CSF2) | |
| | | | | | | *S. aureus* | ↑EGF, PDGF-AB/BB | |
| Infants | Denmark | BCG-Denmark | At birth or no BCG | Whole blood | 4 d post immunisation | *E. coli* | ↑TNFα, IL-6 if vaccinated at 2-7 days of age | (237) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | LPS | ↑TNFα, IL-6, IL-10 if vaccinated at 2-7 days of age | |
| | | | | | | PHA | ↑IL-6, IL-10 if vaccinated at 2-7 days of age | |
| | | | | | | *S. pneumoniae* | ↑IL-10 if vaccinated at 2-7 days of age | |
| | | | | | 3 mos of age | *C. albicans* | ↓IL-10, a trend for ↓IL-6, IFNγ, IL-22 | |
| | | | | | 13 mos of age | BCG | ↓IL-1β, a trend for IL-6<br>↑IFNγ, IL-22 | |
| Infants | Australia | BCG-Denmark | At birth or no BCG | Whole blood | 7 d post immunisation | *L. monocytogenes* | ↓IL-1RA, MIP-1β, CCL2 (MCP-1) | (238) |
| | | | | | | R848 | ↓IL-1RA, IL-10, CCL3 (MIP-1α), MIP-1β, CCL2 (MCP-1), IL-6 | |
| | | | | | | Peptidoglycan | ↓CCL3 (MIP-1α), MIP-1β, CCL2 (MCP-1), IL-6 | |
| | | | | | | *E. coli* | ↓MIP-1β, CCL2 (MCP-1) | |
| | | | | | | *S. pneumoniae* | ↓CCL2 (MCP-1) | |
| | | | | | | *C. albicans* | | |
| Infants | Australia | BCG-Denmark | At birth or no BCG | Whole blood | 7 mos post immunisation | BCG | ↑CXCL9 (MIG), IL-6, TNFα | (233) |
| | | | | | | *M. tuberculosis* | ↑CXCL9 (MIG) | |
| | | | | | | *C. albicans* | | |
| | | | | | | *S. aureus* | ↑CXCL9 (MIG)<br>↓MIP-1β | |
| | | | | | | *S. pyogenes* | ↑CXCL9 (MIG), MIF | |
| | | | | | | *E. coli* | ↑IL-8 | |
| | | | | | | *L. monocytogenes* | ↓IL-10, CCL3 (MIP-1α), MIP-1β | |
| | | | | | | Pam3Cys | ↑IL-1RA | |
| | | | | | | LPS | ↑IL-8 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Infants | South Africa | BCG-Denmark | At birth or 10 wks of age | PBMCs | 10 wks post immunisation | unstimulated | ↑IL-8 (CXCL8), CCL2 (MCP-1), MIP-1β, EGF, IFN-α2, a trend for CXCL10 (IP-10) | (239) |
| | | | | | | *M. tuberculosis* HN878 | ↑TNFα, CXCL10 (IP-10), CCL2 (MCP-1), EGF<br>↓IL-1RA | |
| | | | | | | *M. tuberculosis* CDC1551 | ↑CCL2 (MCP-1), EGF, a trend for TNFα | |
| Infants | Australia | BCG-Denmark | BCG alone, BCG + HBV or HBV at birth or no vaccine | Whole blood | 7 d post immunisation | R848 | ↓IFNγ | (240) |
| | | | | | | *E. coli* | ↓CCL2 (MCP-1) overall and in females | |
| | | | | | | *L. monocytogenes* | ↑IFNγ in females | |
| | | | | | | Peptidoglycan | ↓CCL2 (MCP-1) in females | |

d – day, MRC1 – mannose receptor C-type 1, mo - month, MR – mannose receptor, MSR-1 – macrophage scavenger receptor 1, PHA – phytohemagglutinin, R848 – resiquimod, SR-A – scavenger receptor A, yr – year.

**Table S1.4.** *The impact of BCG on innate immune cell populations*

| ***Murine immunisation*** | | | | | | |
|---|---|---|---|---|---|---|
| **Model** | **Strain** | **BCG strain** | **Sample type** | **Timing of observation** | **BCG-vaccinated vs control** | **Reference** |
| Mice | C57BL/6 | BCG-Tice (intravenous) | Bone marrow | 14 and 30 days post immunisation | ↑ total LKS+ cells, ST-HSCs, MPPs | (227) |
| | | | | 4 weeks post immunisation | ↑ total and %LKS+ cells, total ST-HSCs, MPPs, MPP3 cells | |
| | | | | 1 month post immunisation | ↑ %MPP3 cells | |
| | | BCG-Pasteur | Lung tissue | 7 days post immunisation | ↓ %CD11b+F4/80+Ly6C+ macrophages<br>↑ %CD11b+F4/80+ and CD11b+CD14+ macrophages, CD11b+Ly6G+ neutrophils | (241) |
| | | BCG-Denmark | Spleen | 1 days post immunisation | ↑ mature neutrophils | (242) |
| | | | | 2 days post immunisation | ↑ total neutrophils, immature neutrophils | |
| | | | | 3 days post immunisation | ↑ total neutrophils, immature neutrophils, mature neutrophils | |
| ***Human immunisation*** | | | | | | |
| **Cohort** | **Country** | **BCG strain** | **Sample type** | **Timing of observation** | **BCG-vaccinated vs control** | **Reference** |
| Adults | The Netherlands | BCG-Denmark | PBMCs | 2 weeks post immunisation | ↑ %CD14+ monocytes | (191) |
| | | BCG-Bulgaria | Whole blood | 5 weeks post immunisation and 7 d post *P. falciparum* challenge | ↑% CD69+ NK cells, CD69+ γδ T-cells | (167) |
| | | | | 5 weeks post immunisation and 37 d post *P. falciparum* challenge | ↑ % granzyme B+ NK cells | |
| Infants | Denmark | BCG-Denmark | Whole blood | 4 days post immunisation | ↑ monocytes if vaccinated at 2-7 d post birth | (303) |
| Adults (females) | The Netherlands | | | 1 days post immunisation | ↑ complete blood counts, neutrophils | (220) |
| | | | | 4 days post immunisation | ↑ complete blood counts, neutrophils, monocytes, immature granulocytes | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Infants | Australia | | | 3 days post immunisation | ↑ neutrophil counts | (218) |
| Adults | The Netherlands | BCG-Bulgaria | Bone marrow | 3 months post immunisation | ↑ gene expression signature associated with neutrophil or granulocyte differentiation | |

***Human immunisation and exposure to M. tuberculosis***

| **Cohort** | **Country** | **BCG strain** | **Sample type** | **Timing of observation** | **Change from week 2 to week 14 of observation** | **Reference** |
|---|---|---|---|---|---|---|
| IGRA-ve household TB contacts | Indonesia | - | Whole blood | 2 and 14 weeks post IGRA testing | ↓ total and intermediate monocytes, immature granulocytes, total γδ T-cells | (386) |
| IGRA-ve household TB contacts (BCG-vaccinated only) | | | | | ↓ total, intermediate and non-classical monocytes, CXCR4+ granulocytes, total and CD56dim NK cells, total γδ T-cells | |
| IGRA+ve household TB contacts | | | | | ↑ CD56+ MAIT cells<br><br>↓ CXCR4+ granulocytes | |

MPP – multipotent progenitor, ST-HSC – short-term haematopoietic stem cell

**Table S1.5.** *Heterologous immune responses to BCG and their effect on infectious diseases*

| Murine studies | | | | | | |
|---|---|---|---|---|---|---|
| **Model** | **Strain** | **BCG-strain** | **Timing of observation** | **BCG-vaccinated vs control** | **Mechanism** | **Reference** |
| Mice (immunisation) | - | BCG-Brazil (intraperitoneal) | 14 days post immunisation, 50 day follow-up | ↓ Mortality of cyclophosphamide-treated mice upon *S. aureus* challenge | - | (247) |
| Mice (immunisation) | SCID | BCG-Denmark (intravenous) | 3 days post immunisation | ↓ CFU in kidney upon lethal *C. albicans* challenge | BCG-exposed TNFα+ splenic monocytes? | (191) |
| | | | 2 weeks post immunisation | | | |
| | | | 4 weeks post immunisation | ↓ Mortality of SCID mice upon lethal *C. albicans* challenge | | |
| Mice (parabiosis of BCG-vaccinated and control mice) | CD45.1 and CD45.2 | BCG-Tice (intravenous) | 14 weeks post immunisation (6 weeks parabiosis, 4 weeks *M. tuberculosis* challenge) | ↓ *M. tuberculosis* CFU in lungs in *CCR2-/-* CD45.2 parabionts conjoined with BCG-vaccinated CD45.1 mice | BCG-exposed leukocytes from CD45.1 mice | (227) |
| Mice (adoptive transfer of CD3+ T-cell depleted BM from BCG vaccinated mice) | | | 18 weeks post immunisation: CD3+ T-cell depletion at 4 weeks, followed by adoptive transfer of CD45.1 BM to irradiated CD45.2 mice, followed by *M. tuberculosis* challenge 14 weeks later | ↓ *M. tuberculosis* CFU in lungs, spleen and BM in unvaccinated CD45.2 recipients of BCG-vaccinated CD45.1 BM recipient) | BCG-exposed non-CD3+ T-cells | |
| Mice (adoptive transfer of *M. tuberculosis*-infected BMDMs from BCG-vaccinated mice) | C57BL/6 and *Rag1-/-* mice | | 4 weeks post immunisation: *M. tuberculosis* infected BMDM transfer from BCG-vaccinated or control C57BL/6 mice to unvaccinated *Rag1-/-* mice | ↓ *M. tuberculosis* CFU in lungs and spleen of *Rag1-/-* mice | BCG-exposed BMDM from C57BL/6 mice | |

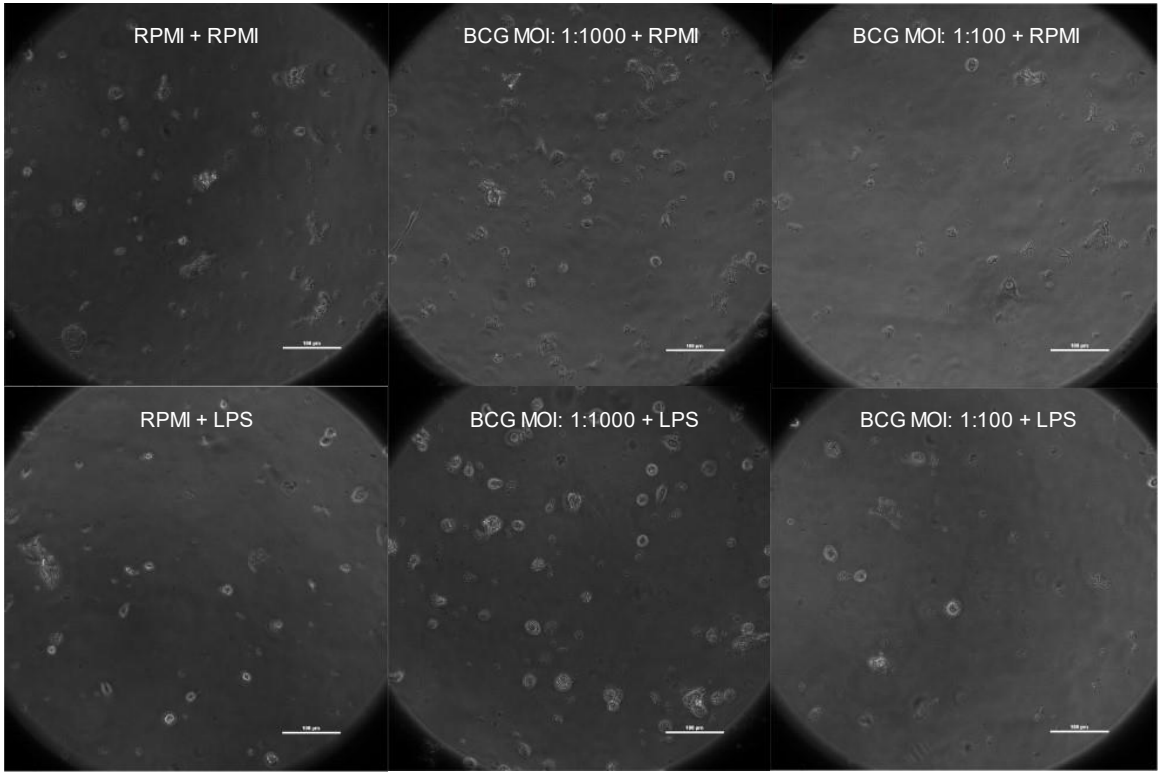
| | | | | | | |
|---|---|---|---|---|---|---|
| Mice (immunisation) | C57BL/6 | BCG-Denmark (subcutaneous) | 3 days post immunisation, 5 day follow-up | ↓ Polymicrobial CFU in spleen, blood, liver, lungs, peritoneal wash<br><br>↑ Survival of mice | BCG-dependent G-CSF increase in neutrophils | (242) |
| Mice (immunisation) | BALB/cAnNCrl | BCG-Denmark (intravenous) | 1 week post immunisation, 21 day follow-up | No difference in survival, weight or histological lung pathology scores of mice infected with influenza A/Anhui/1/2013 (H7N9) | - | (248) |
| Mice (immunisation) | C57BL/6 | BCG-Pasteur (subcutaneous) | 1 week post immunisation, 30 and 60 day follow-up | ↓ *M. tuberculosis* CFU | BCG-exposed neutrophils | (241) |
| | | | 1 week post immunisation, 30 day follow-up | ↓ *M. tuberculosis* CFU in lung and spleen | | |
| | C57BL/6 *cd4-/-* | | 1 week post immunisation, 30 and 60 day follow-up | ↓ *M. tuberculosis* CFU | | |
| | C57BL/6 *cd8-/-* | | 1 week post immunisation, 30 day follow-up | | | |

**Human studies**

| Cohort | Country | BCG-strain | Timing of observation | BCG-vaccinated vs control | Mechanism | Reference |
|---|---|---|---|---|---|---|
| Human (infants) | Uganda | BCG-Denmark | 6 weeks post immunisation | ↓ risk of physician diagnosed, non-tuberculous infectious disease<br><br>↓ risk of infectious presentations<br><br>A trend for ↓ risk of serious illness (significant for males) | ↓ fold accumulation of H3K4me3, H3K9me3 at the promoter of *TNFA* compared to baseline<br><br>A trend for ↓ fold accumulation of H3K4me3, H3K9me3 at the promoter of *IL-1B* compared to baseline | (164) |
| Human (adults) | The Netherlands | BCG-Denmark | 1 month post immunisation + 5 days post yellow fever vaccine | ↓ Yellow fever vaccine viral load | Viral load inversely associated with IL-1β levels | (209) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Human (adults) | The Netherlands | BCG-Bulgaria | 5 weeks post immunisation + ≥6 days post *P. falciparum* challenge | ↑ % of study participants with moderate or severe symptoms | Parasitemia inversely associated with CD69+ NK cell frequencies and expansion of HLA-DR+ monocytes | (167) |
| Human (adults) | The Netherlands | BCG-Denmark | 12 months post immunisation | No differences in SARS-CoV-2 infection, death, ICU admission, hospital stay or self-reported RTI rates | - | (249) |

CFU – colony forming unit, ICU – intensive care unit, RTI – respiratory tract infection

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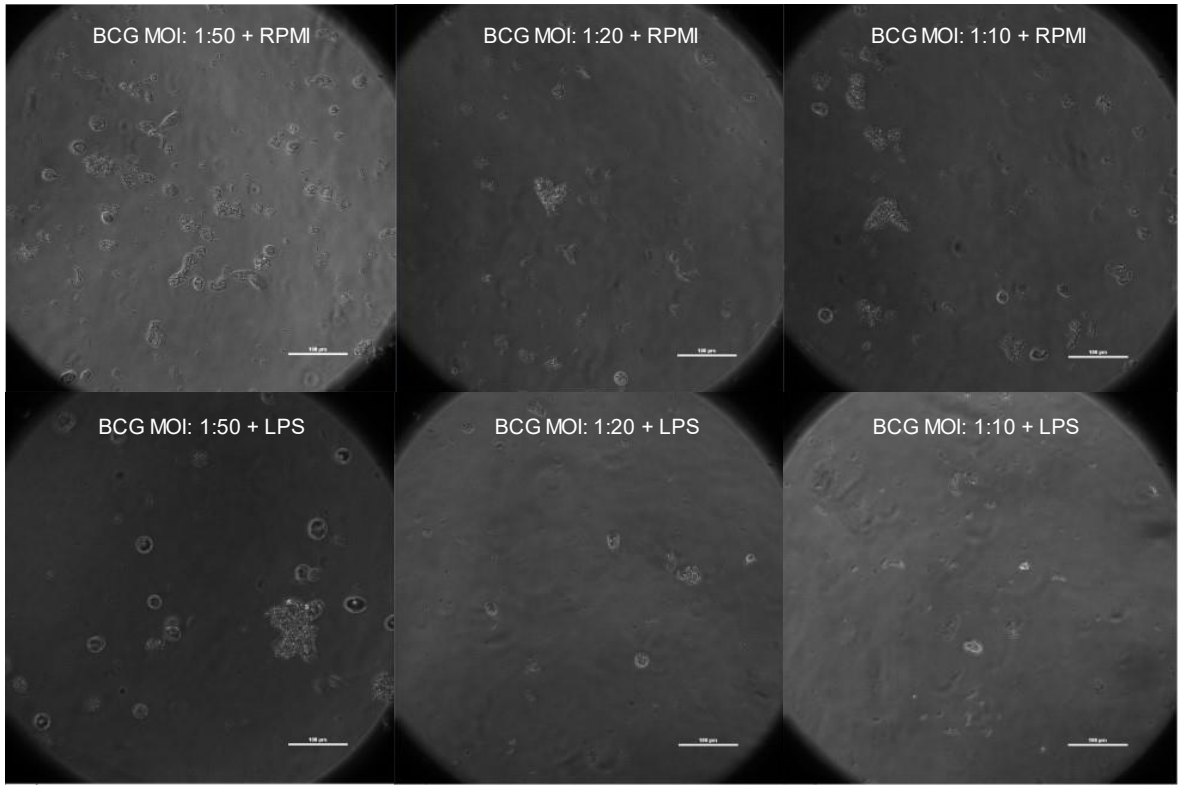


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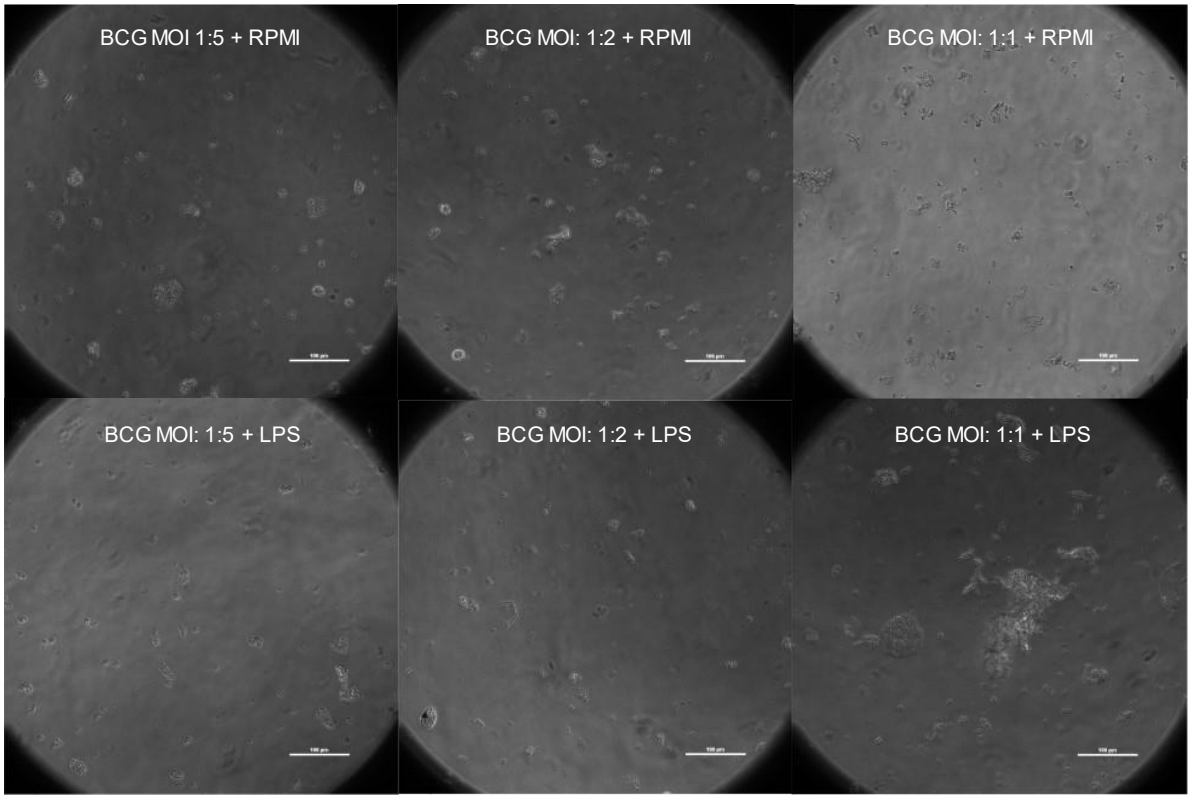


**Figure S2.1.** *Detrimental effect of BCG on CD14+ monocytes is dose-dependent*. 100,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at the indicated MOIs for 24 h on 96-well plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then re-stimulated with LPS or left unstimulated for 24 h. Inverted light microscopy images were taken at 20x magnification on day 7 post-training. RPMI + RPMI – training and re-stimulation double controls, RPMI + LPS – training controls challenged with LPS, BCG + RPMI – trained monocyte cultures in the absence of secondary stimulation, BCG + LPS – trained monocyte cultures challenged with LPS. Ratios indicate BCG MOIs used to train the monocytes. The images depict morphology of trained monocytes from a single donor in a representative experiment.

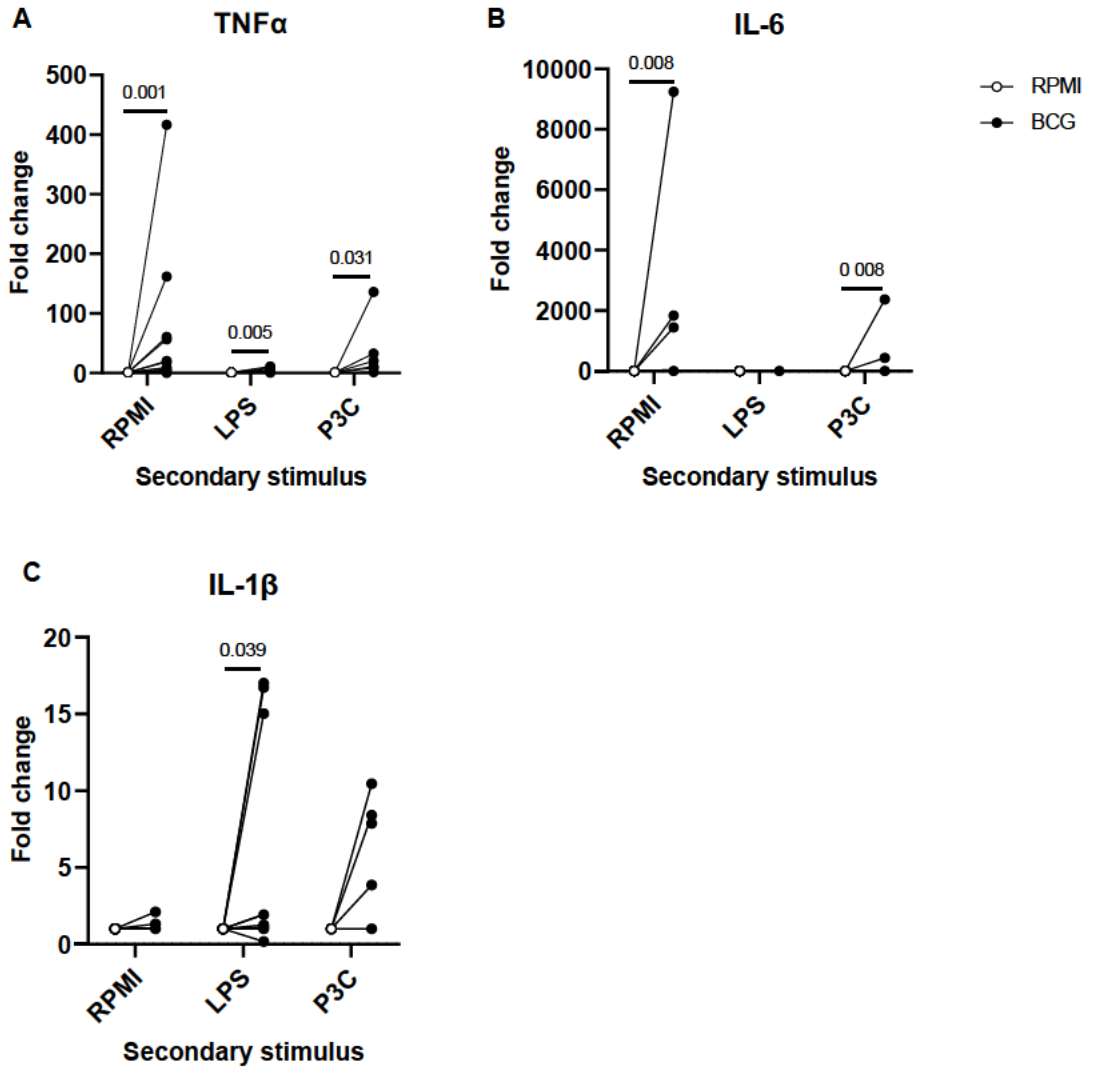


**Figure S2.2.** *Fold change in TNFα, IL-6 and IL-1β concentrations in trained monocyte cultures*. 300,000 human CD14+ monocytes per well were cultured in the presence or absence of BCG at MOI 1:50 for 24 h on 48-well tissue-culture plates and rested for 6 d with cell culture medium replaced on day 3 of training. The cells were then re-stimulated with LPS or Pam3Cys or left unstimulated for 24 h. Fold changes in concentrations of TNFα (**A**; n = 13), IL-6 (**B**; n = 12, and IL-1β (**C**; n = 11) compared were then measured in the supernatants of trained vs untrained monocyte cultures. Data points show fold changes in cytokine concentrations in individual donors. Numbers indicate p-values for significant differences. Wilcoxon matched-pairs signed-rank test was used to detect the differences between trained and control monocyte responses. BCG MOI indicates ratio of BCG CFUs to the numbers of cells per well.

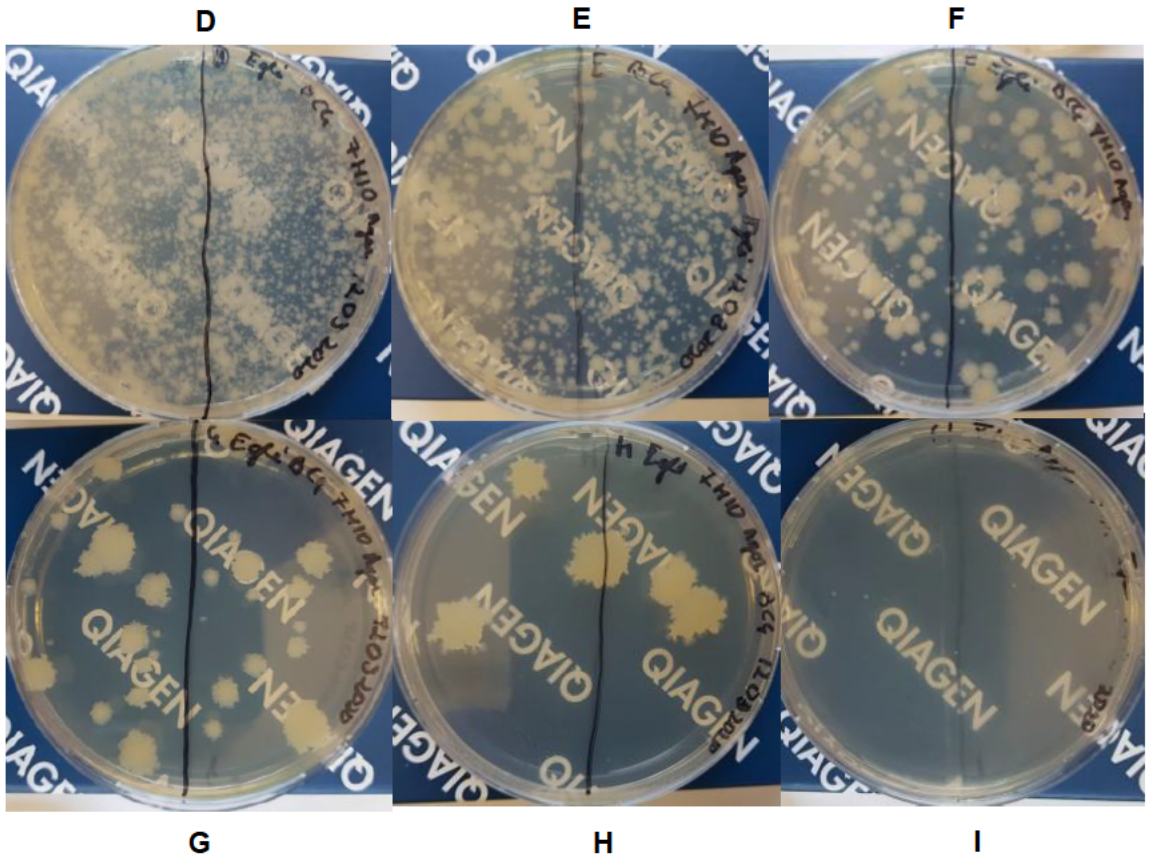


**Figure S3.1.** *The growth of BCG on 7H10 agar plates*. A series of ten-fold dilution of BCG standards used to quantify the BCG CFUs in MGIA experiments was also quantified on 7H10 agar culture plates. Two aliquots of 50 µl of each standard were spread over each plate and cultured at 37 °C for 21 days. CFUs were then counted and averaged for further comparison with the expected CFU concentrations. The expected CFU concentrations of MGIA BCG standards were: **D** – $2x10^5$ CFU/ml, **E** – $2x10^4$ CFU/ml, **F** – $2x10^3$ CFU/ml, **G** – $2x10^2$ CFU/ml, **H** – 2x10 CFU/ml, **I** – $2x10^0$ CFU/ml.

**Table S3.1.** *BCG standard concentrations as estimated by growth on 7H10 agar plates*

| **MGIA standard (CFU/500 µl)** | **Expected concentration (CFU/ml)** | **Replicate 1 (CFU)** | **Replicate 2 (CFU)** | **Mean CFU** | **Estimated concentration (CFU/ml)** |
|---|---|---|---|---|---|
| 100000 | *200000* | near confluent | near confluent | **-** | - |
| 10000 | *20000* | too dense | too dense | **-** | - |
| 1000 | *2000* | 104 | 80 | **92** | *1840* |
| 100 | *200* | 17 | 16 | **16.5** | *330* |
| 10 | *20* | 3 | 2 | **2.5** | *50* |
| 1 | *2* | 0 | 0 | **0** | *0* |

**Table S4.1.** *Antibodies and dyes used in FACS staining of infant PBMCs*

| Marker | Function | Fluorescent label | Antibody clone | Company | Titre* |
|---|---|---|---|---|---|
| | | *Viability staining* | | | |
| N/A | Viability indicator | LIVE/DEAD Fixable Aqua Dead Cell Stain | N/A | Life Technologies | 1:400 |
| | | *Cell surface marker staining* | | | |
| **CD14** | Classical / intermediate monocyte marker, LPS signalling | V450 | MΦP9 | BD | 1:20 |
| **CD16** | Non-classical / intermediate monocyte marker, Fc receptor | AF-700 | 3G8 | BD | 1:20 |
| **HLA-DR** | Antigen presenting molecule | PerCP-Cy5.5 | L243 | BioLegend | 1:20 |
| **TLR4** | Toll-like receptor, LPS signalling | PE | HTA125 | BioLegend | 1:20 |
| **CD11b** | Adhesion, complement receptor | BV711 | ICRF44 | BioLegend | 1:40 |
| | | *Intracellular marker staining* | | | |
| **IL-6** | Proinflammatory cytokine, associated with trained immunity | FITC | B-E8 | eBioscience | 1:20 |
| **TNFα** | Proinflammatory cytokine, associated with trained immunity | APC | Mab11 | BioLegend | 1:20 |

* Stain volume to final staining volume ratio

**Table S4.2.** *Cytokines investigated in UK and South African infant PBMC response to BCG and TLR agonist analyses*

| Cytokine | Function |
|---|---|
| **IL-1α** | IL-1 family cytokine, pro-inflammatory (226) |
| **IL-1β** | IL-1 family cytokine, pro-inflammatory, associated with heterologous effects of BCG, trained immunity and protection against TB (209,221,226) |
| **IL-1RA** | IL-1 family cytokine, receptor antagonist (226) |
| **EGF** | Associated with heterologous effects of BCG in infants (223) |
| **PDGF-AB/BB** | Associated with heterologous effects of BCG in infants (223) |
| **G-CSF / CSF3** | Elevated in BCG-vaccinated infants and mice (128,242,306) |
| **GM-CSF / CSF2** | Associated with heterologous effects of BCG in infants and in mice (223,242,253) |
| **IL-6** | Associated with trained immunity and heterologous effects of BCG in infants (191,223) |
| **TNFα** | Associated with trained immunity and protection against TB (191) |
| **CCL2 / MCP-1** | Associated with heterologous effects of BCG in infants (238) |
| **CXCL10 / IP-10** | Associated with trained immunity and protection against TB (131) |

**Table S4.3.** *Contribution of host factors to the to the total variation in infant innate immune responses to BCG and TLR agonists as indicated by two-way ANOVA analysis*

| Innate immune responses in the UK and South African infant cohorts | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cohort | | Stimulus | | Donor | | Cohort vs stimulus | |
| Parameter | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value |
| **CD14++CD16- cells** | | | | | | | | |
| %TNFα+IL-6- | 0.12 | 0.5571 | **70.61** | **<0.0001*** | **11.69** | **0.0033** | 0.41 | 0.4839 |
| %TNFα-IL-6+ | 1.73 | 0.2355 | **29.44** | **<0.0001** | **41.46** | **<0.0001** | 0.75 | 0.3974 |
| %TNFα+IL-6+ | 0.91 | 0.0662 | **79.73** | **<0.0001** | **8.87** | **<0.0001** | 0.36 | 0.2923 |
| %CD11b+TLR4hi | 0.73 | 0.4408 | **29.16** | **<0.0001** | **42.18** | **<0.0001** | 0.21 | 0.8543 |
| **Surface receptor on or intracellular cytokine expression in CD14++CD16- cells (MFI)** | | | | | | | | |
| TNFα | 0.43 | 0.3291 | **55.86** | **<0.0001** | **15.38** | **0.0247** | 0.57 | 0.5408 |
| IL-6 | 0.44 | 0.296 | **69.04** | **<0.0001** | **13.65** | **<0.0001** | 0.24 | 0.6791 |
| CD11b | **10.27** | **0.0352** | **3.99** | **<0.0001** | **74.85** | **<0.0001** | 0.70 | 0.0699 |
| TLR4 | 2.37 | 0.1323 | **42.38** | **<0.0001** | **33.93** | **<0.0001** | 0.85 | 0.2333 |
| **Cytokine concentration** | | | | | | | | |
| EGF | **19.19** | **0.0003** | **24.80** | **<0.0001** | **37.27** | **<0.0001** | **1.52** | **0.0238** |
| G-CSF | **11.43** | **0.0004** | **19.01** | **0.0001** | **23.89** | **0.0073** | **11.80** | **<0.0001** |
| GM-CSF | **9.28** | **0.0044** | **27.67** | **<0.0001** | **31.59** | **<0.0001** | **5.44** | **0.0007** |
| IL-12p40 | 0.17 | 0.7409 | 4.07 | 0.0573 | **49.09** | **<0.0001** | 0.71 | 0.6888 |
| IL-1RA | **13.92** | **0.0055** | **14.36** | **<0.0001** | **50.18** | **<0.0001** | **2.89** | **0.0044** |
| IL-1α | **5.52** | **0.003** | **36.01** | **<0.0001** | 17.16 | 0.1011 | **8.38** | **0.0002** |
| IL-1β | **8.41** | **0.0011** | **40.40** | **<0.0001** | **20.83** | **0.0003** | **9.74** | **<0.0001** |
| IL-6 | **14.83** | **0.0003** | **34.40** | **<0.0001** | **28.73** | **<0.0001** | **6.81** | **<0.0001** |

| Parameter | Cohort | | Stimulus | | Donor | | Cohort vs stimulus | |
|---|---|---|---|---|---|---|---|---|
| | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value |
| **IP-10** | 0.40 | 0.4574 | **24.04** | **0.0003** | 22.70 | 0.0937 | 2.92 | 0.1248 |
| **MCP-1** | **12.01** | **0.0020** | **26.53** | **<0.0001** | **33.95** | **<0.0001** | **11.67** | **<0.0001** |
| **TNFα** | 3.87 | 0.065 | **44.54** | **<0.0001** | **33.92** | **<0.0001** | **2.68** | **0.0024** |
| **Expression of genes associated with protection against TB** | | | | | | | | |
| ***GPR183*** | 2.40 | 0.2282 | **18.31** | **<0.0001** | **45.97** | **<0.0001** | 2.08 | 0.1291 |
| ***GPR68*** | 0.30 | 0.6645 | **22.59** | **<0.0001** | **44.74** | **<0.0001** | 0.68 | 0.6048 |
| ***SCARB2*** | 1.01 | 0.0911 | **77.92** | **<0.0001** | **9.62** | **0.0004** | 0.42 | 0.3682 |
| ***FBP1*** | 1.53 | 0.0907 | **64.69** | **<0.0001** | **14.47** | **0.0015** | 0.77 | 0.3152 |
| **UK male vs female infant innate immune response comparison** | | | | | | | | |
| **Parameter** | **Sex** | | **Stimulus** | | **Donor** | | **Sex vs stimulus** | |
| | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value |
| **CD14++CD16- cells** | | | | | | | | |
| **%TNFα+IL-6-** | 0.03 | 0.8516 | **64.24** | **<0.0001** | **13.98** | **0.0183** | 0.77 | 0.5720 |
| **%TNFα-IL-6+** | 0.84 | 0.5996 | **23.63** | **<0.0001** | **49.95** | **<0.0001** | 1.33 | 0.4148 |
| **%TNFα+IL-6+** | 0.50 | 0.3806 | **74.29** | **<0.0001** | **10.38** | **0.0015** | 0.12 | 0.8980 |
| **%CD11b+TLR4hi** | 0.43 | 0.6763 | **39.43** | **<0.0001** | **40.90** | **<0.0001** | 1.12 | 0.3323 |
| **Surface receptor on or intracellular cytokine expression in CD14++CD16- cells (MFI)** | | | | | | | | |
| **TNFα** | 0.69 | 0.4438 | **48.04** | **<0.0001** | **19.21** | **0.0401** | 0.33 | 0.9078 |
| **IL-6** | 0.87 | 0.3372 | **63.29** | **<0.0001** | **15.20** | **0.0051** | 0.25 | 0.8723 |

| Parameter | Sex | | Stimulus | | Donor | | Sex vs stimulus | |
|---|---|---|---|---|---|---|---|---|
| | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value | % of total variation | P-value |
| **CD11b** | 1.18 | 0.6300 | **5.58** | **0.0003** | **83.09** | **<0.0001** | 0.12 | 0.8830 |
| **TLR4** | 1.64 | 0.2635 | **51.10** | **<0.0001** | **20.84** | **0.0037** | 2.72 | 0.1308 |
| **Cytokine concentration** | | | | | | | | |
| **EGF** | 4.56 | 0.2193 | **22.31** | **<0.0001** | **47.71** | **<0.0001** | 0.63 | 0.6787 |
| **G-CSF** | 1.68 | 0.4050 | **25.46** | **0.0001** | **39.04** | **<0.0001** | 1.07 | 0.5906 |
| **GM-CSF** | 0.68 | 0.6036 | **25.57** | **<0.0001** | **41.05** | **<0.0001** | 0.88 | 0.6609 |
| **IL-12p40** | 4.42 | 0.2415 | 1.35 | 0.5582 | **51.02** | **0.0002** | 0.72 | 0.8310 |
| **IL-1RA** | 0.16 | 0.8316 | **12.61** | **0.0046** | **57.99** | **<0.0001** | 0.61 | 0.7745 |
| **IL-1α** | 0.07 | 0.8121 | **16.47** | **0.0452** | 21.17 | 0.4175 | 0.15 | 0.9885 |
| **IL-1β** | 2.30 | 0.2898 | **31.46** | **<0.0001** | **32.78** | **0.0002** | 1.27 | 0.5011 |
| **IL-6** | 4.31 | 0.1576 | **33.49** | **<0.0001** | **30.62** | **<0.0001** | 2.44 | 0.1418 |
| **IP-10** | 3.69 | 0.0889 | **20.85** | **0.0101** | 19.25 | 0.2101 | 5.17 | 0.1213 |
| **MCP-1** | 0.71 | 0.5662 | **20.09** | **0.0017** | **35.41** | **0.0008** | 4.29 | 0.1062 |
| **TNFα** | 4.44 | 0.2014 | **26.75** | **<0.0001** | **42.73** | **<0.0001** | 1.58 | 0.2487 |
| **Expression of genes associated with protection against TB** | | | | | | | | |
| ***GPR183*** | 0.28 | 0.7055 | **43.99** | **<0.0001** | **26.76** | **0.0015** | 0.36 | 0.8927 |
| ***GPR68*** | 2.75 | 0.3286 | **28.49** | **0.0002** | **37.51** | **0.0006** | 2.22 | 0.3970 |
| ***SCARB2*** | 2.24 | 0.1532 | **61.22** | **<0.0001** | **13.72** | **0.0053** | 1.41 | 0.2765 |
| ***FBP1*** | **7.18** | **0.0237** | **43.87** | **<0.0001** | **15.61** | **0.0347** | **6.33** | **0.0148** |

* Factors that were significantly associated with variation in infant innate immune responses are shown in bold.

***Table S4.4.*** *The effects of BCG and TLR agonists on BCG-vaccinated infant immune responses in the UK and South African cohorts as shown by P-values of Šídák's multiple comparisons test*

| Parameter | UK | | | | | | SA | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RPMI vs LPS | RPMI vs P3C | RPMI vs BCG | LPS vs P3C | LPS vs BCG | P3C vs BCG | RPMI vs LPS | RPMI vs P3C | RPMI vs BCG | LPS vs P3C | LPS vs BCG | P3C vs BCG |
| **CD14++CD16- cells** | | | | | | | | | | | | |
| **%TNFα+IL-6-** | 0.8882 | **0.0008** | **<0.0001** | **0.0033** | **<0.0001** | **<0.0001** | 0.0913 | **0.0007** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** |
| **%TNFα-IL-6+** | 0.075 | **<0.0001** | >0.9999 | **<0.0001** | 0.0707 | **<0.0001** | 0.9958 | **<0.0001** | >0.9999 | **<0.0001** | 0.9998 | **<0.0001** |
| **%TNFα+IL-6+** | **<0.0001** | **<0.0001** | 0.467 | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **0.0024** | **<0.0001** | **<0.0001** | **<0.0001** |
| **%CD11b+TLR4hi** | **0.0282** | **0.0007** | 0.7447 | **<0.0001** | **0.0004** | **0.0425** | 0.0834 | **<0.0001** | 0.3346 | **<0.0001** | **0.0002** | **0.0097** |
| **Surface receptor on or intracellular cytokine expression in CD14++CD16- cells (MFI)** | | | | | | | | | | | | |
| **TNFα** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **0.0054** | >0.9999 | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **0.0041** | 0.8878 |
| **IL-6** | **<0.0001** | **<0.0001** | **0.0279** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** | **0.0003** | **<0.0001** | **<0.0001** | **<0.0001** |
| **CD11b** | **0.0004** | 0.9865 | 0.4512 | **0.0013** | **0.0006** | 0.6203 | 0.3869 | 0.9389 | 0.9207 | 0.3992 | **0.0267** | 0.1241 |
| **TLR4** | **<0.0001** | **0.0245** | 0.9522 | **<0.0001** | **<0.0001** | 0.1589 | **<0.0001** | 0.7342 | 0.1988 | **<0.0001** | **<0.0001** | 0.9992 |
| **Cytokine concentration** | | | | | | | | | | | | |
| **EGF** | **0.0001** | **0.0007** | **0.0001** | 0.1386 | 0.9389 | 0.2405 | **<0.0001** | **<0.0001** | **<0.0001** | 0.6094 | 0.9674 | 0.8994 |
| **G-CSF** | **0.0005** | **0.0009** | **0.0009** | 0.0682 | **0.0071** | 0.0752 | **0.0181** | **0.0057** | **0.0021** | 0.1186 | **0.046** | **0.0364** |
| **GM-CSF** | **0.0005** | **0.0002** | **0.0002** | **0.0182** | 0.7797 | 0.9375 | **0.0006** | **0.0007** | **0.0004** | 0.8953 | 0.7911 | 0.2543 |
| **IL-12p40** | 0.9018 | 0.8589 | 0.8095 | 0.9997 | 0.9997 | >0.9999 | 0.0928 | 0.0734 | 0.6676 | 0.8752 | 0.9807 | 0.4592 |
| **IL-1RA** | 0.1618 | >0.9999 | **0.0275** | **0.0006** | **0.0076** | 0.2741 | **0.0267** | 0.391 | **0.0297** | **0.0046** | **0.001** | 0.2041 |
| **IL-1α** | 0.1664 | - | 0.91 | 0.1664 | 0.1655 | 0.91 | **0.0002** | 0.6919 | 0.4146 | **0.0002** | **0.0005** | 0.5257 |
| **IL-1β** | **0.0001** | **0.0021** | **0.0006** | **<0.0001** | **0.0095** | 0.9479 | **0.0002** | **0.0006** | **0.0003** | **0.0009** | **0.0021** | **0.016** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **IL-6** | **<0.0001** | **0.0003** | **0.0004** | 0.1109 | **0.0001** | **0.0006** | **0.0003** | **<0.0001** | **0.0003** | >0.9999 | **0.0013** | **0.0002** |
| **IP-10** | **0.0004** | **0.0158** | **0.0018** | **0.0286** | 0.2461 | **0.0425** | **<0.0001** | **0.0089** | **0.0004** | **0.0464** | 0.628 | 0.1307 |
| **MCP-1** | 0.1205 | **0.0463** | >0.9999 | **0.0005** | **0.0499** | 0.0506 | 0.9957 | **0.0007** | 0.3492 | **0.0004** | **0.0423** | **0.0007** |
| **TNFα** | **0.0001** | **<0.0001** | **<0.0001** | **0.024** | 0.9995 | 0.2187 | **<0.0001** | **<0.0001** | **<0.0001** | **0.0001** | 0.7839 | **0.0005** |
| **Expression of genes associated with protection against TB** | | | | | | | | | | | | |
| ***GPR183*** | **<0.0001** | **0.0002** | **<0.0001** | **0.0376** | >0.9999 | 0.636 | **0.0236** | 0.9989 | 0.3648 | **0.0023** | 0.9998 | 0.0825 |
| ***GPR68*** | 0.1177 | **0.0074** | 0.7265 | **0.0154** | 0.5082 | **0.0031** | 0.6761 | **0.0366** | 0.6441 | **0.0132** | 0.9177 | **0.0106** |
| ***SCARB2*** | **<0.0001** | **<0.0001** | **<0.0001** | **0.0063** | **0.0275** | >0.9999 | **<0.0001** | **<0.0001** | **<0.0001** | **0.0004** | **0.0001** | 0.8708 |
| ***FBP1*** | **<0.0001** | **<0.0001** | **0.0428** | 0.9557 | 0.1983 | 0.0591 | **<0.0001** | **<0.0001** | **<0.0001** | >0.9999 | **<0.0001** | **<0.0001** |

P-values <0.05 are shown in bold.

**Table S4.5.** *Differences in innate immune responses of the UK male and female infants or stimulated vs unstimulated PBMC cultures as indicated by p-values from Šidák's multiple comparisons test*

| UK male vs female infant innate immune response comparison | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Parameter** | **P-values for sex associated differences** | | | | **P-values for stimulated vs unstimulated** | | |
| | **RPMI** | **LPS** | **P3C** | **BCG** | **LPS** | **P3C** | **BCG** |
| **CD14++CD16- cells** | | | | | | | |
| **%TNFα+IL-6-** | >0.9999 | 0.7452 | 0.9996 | 0.8807 | 0.6656 | **0.0004** | **<0.0001** |
| **%TNFα-IL-6+** | 0.7933 | 0.9959 | 0.8140 | 0.9998 | 0.2226 | **<0.0001** | 0.9914 |
| **%TNFα+IL-6+** | >0.9999 | 0.8288 | 0.8730 | 0.9461 | **<0.0001** | **<0.0001** | 0.2877 |
| **%CD11b+TLR4hi** | 0.9692 | 0.9950 | >0.9999 | 0.6609 | **<0.0001** | **<0.0001** | 0.0983 |
| **Surface receptor on or intracellular cytokine expression in CD14++CD16- cells (MFI)** | | | | | | | |
| **TNFα** | 0.8781 | 0.9755 | 0.9553 | 0.9068 | **<0.0001** | **<0.0001** | **<0.0001** |
| **IL-6** | **0.0475** | 0.9131 | 0.9286 | 0.6575 | **<0.0001** | **<0.0001** | **0.0141** |
| **CD11b** | 0.9980 | 0.9799 | 0.9751 | 0.9949 | **0.0002** | 0.8840 | 0.2592 |
| **TLR4** | >0.9999 | 0.4535 | 0.4443 | 0.9923 | **<0.0001** | **0.0123** | 0.7815 |
| **Cytokine concentration** | | | | | | | |
| **EGF** | 0.3110 | 0.7702 | 0.8334 | 0.5533 | **<0.0001** | **0.0003** | **<0.0001** |
| **G-CSF** | 0.7914 | 0.7808 | 0.9938 | 0.7472 | **0.0003** | **0.0004** | **0.0004** |
| **GM-CSF** | 0.9865 | >0.9999 | 0.9641 | 0.8668 | **0.0003** | **<0.0001** | **<0.0001** |
| **IL-12p40** | 0.8963 | 0.8426 | 0.6637 | 0.2557 | 0.6866 | 0.6243 | 0.5636 |
| **IL-1RA** | 0.9972 | 0.3889 | 0.9634 | 0.9997 | 0.0844 | 0.9998 | **0.0138** |
| **IL-1α** | -* | 0.9991 | -* | 0.8089 | 0.0870 | -* | 0.7000 |
| **IL-1β** | 0.7209 | 0.9102 | 0.9821 | 0.2523 | **<0.0001** | **0.0010** | **0.0003** |
| **IL-6** | 0.9709 | 0.6091 | 0.4572 | 0.5850 | **<0.0001** | **0.0001** | **0.0002** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **IP-10** | 0.5928 | 0.4658 | 0.2816 | **0.0478** | **0.0002** | **0.0080** | **0.0009** |
| **MCP-1** | 0.9871 | 0.5432 | 0.5038 | 0.9578 | 0.0622 | **0.0234** | 0.9986 |
| **TNFα** | 0.8361 | 0.7716 | 0.6349 | 0.4395 | **<0.0001** | **<0.0001** | **<0.0001** |
| **Expression of genes associated with protection against TB** | | | | | | | |
| ***GPR183*** | -** | >0.9999 | 0.9991 | 0.9063 | **<0.0001** | **<0.0001** | **<0.0001** |
| ***GPR68*** | -** | 0.9947 | 0.7779 | 0.7673 | 0.0607 | **0.0037** | 0.4770 |
| ***SCARB2*** | -** | 0.9847 | 0.7851 | 0.3840 | **<0.0001** | **<0.0001** | **<0.0001** |
| ***FBP1*** | -** | 0.8377 | 0.6407 | 0.3349 | **<0.0001** | **<0.0001** | **0.0216** |

P-values <0.05 are shown in bold.

* Not detected.

****Reference values with relative mRNA quantification – 1.00.

**Table S4.6.** *Infant cytokine responses to BCG vaccination*

| Cytokine | BCG vaccination | BCG strain | Blood sample obtained | Effect | Assay | Country | Study |
|---|---|---|---|---|---|---|---|
| **EGF** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to Pam3Cys, *C. albicans*, *S. aureus, E. coli* | 48 h whole blood stimulation | | (223) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in the absence of stimulation<br><br>↑in BCG+ vs BCG- infants in response to *Mtb* HN878, *Mtb* CDC1551 | 24 h PBMC stimulation | South Africa | (239) |
| **FGF-2** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| **Eotaxin / CCL11** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys | 48 h whole blood stimulation | | (223) |
| **G-CSF** | 5-10 wks of age | BCG-Danish | | | | UK | (128) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3-13 wks of age | | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| **Flt-3L** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| **GM-CSF** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate<br><br>↓in BCG+ vs BCG- infants in response to *E. coli*, LPS | 48 h whole blood stimulation | | (223) |
| **Fractalkine / CX3CL1** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| **IFNα2** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in the absence of stimulation | 24 h PBMC stimulation | South Africa | (239) |
| **IFNγ** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to culture medium, PMA, PPD, Pam3Cys | 24 h whole blood stimulation | Guinea-Bissau | (236) |
| | 0-7 d | | 13 months post immunisation | ↑in BCG+ vs BCG- infants in response to BCG | | Denmark | (237) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | UK | (223) |
| | 0-10 d of age | | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to BCG, *Mtb*<br><br>↓in BCG+ vs BCG- infants in response to *E. coli, H. influenzae*, PEPG, LPS | 20 h whole blood stimulation | Australia | (233) |
| **GRO / CXCL1** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 6 wks of age | | 4 months post BCG immunisation | ↓in BCG+ vs BCG- infants in response to *E. coli*, LPS | 48 h whole blood stimulation | | (223) |
| **IL-10** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to PPD | 24 h whole blood stimulation | Guinea-Bissau | (236) |
| | | | | A trend for ↓in BCG+ vs BCG- LBW females in response to CL075 | | | |
| | 0-7 d | | 3 months post immunisation | A trend for ↓in BCG+ vs BCG- infants in response to *C. albicans* | | Denmark | (237) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys | 48 h whole blood stimulation | UK | (223) |
| | 0-10 d of age | | 7 days post immunisation | ↓in BCG+ vs BCG- infants in response to R848 | 20 h whole blood stimulation | Australia | (238) |
| | | | 7 months post immunisation | ↓in BCG+ vs BCG- infants in response to *L. monocytogenes* | | | (233) |
| **MCP-3 / CCL7** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys, *C. albicans* | 48 h whole blood stimulation | | (223) |
| **IL-12p40** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys | 48 h whole blood stimulation | | (223) |
| **MDC / CCL22** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| **IL-12p70** | 3-13 wks of age | BCG-Danish | 12 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| **PDGF-AA** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| **IL-13** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | At birth or 4.5 mos of age | BCG-Russia | 4.5 months of age | | 5 d whole blood stimulation | The Gambia | (413) |
| | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate<br><br>↓BCG+ vs BCG- in response to *C. albicans* | 48 h whole blood stimulation | | (223) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **PDGF-AB/BB** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to Pam3Cys, *C. albicans, S. aureus* | 48 h whole blood stimulation | | (223) |
| **sCD40L** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys | 48 h whole blood stimulation | | (223) |
| **IL-17** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | At birth or 4.5 mos of age | BCG-Russia | 4.5 months of age | | 5 d whole blood stimulation | The Gambia | (413) |
| | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to PPD | 24 h whole blood stimulation | Guinea-Bissau | (236) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate<br><br>↓in BCG+ vs BCG- infants in response to *C. albicans* | 48 h whole blood stimulation | UK | (223) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **IL-1RA** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| | 0-10 d of age | | 7 days post immunisation | ↓in BCG+ vs BCG- infants in response to *L. monocytogenes,* R848 | 20 h whole blood stimulation | Australia | (238) |
| | 0-10 d of age | | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to Pam3Cys | 20 h whole blood stimulation | | (233) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in response to *Mtb* HN878 | 24 h PBMC stimulation | South Africa | (239) |
| **IL-1α** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| **IL-1β** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to PPD and Pam3Cys<br><br>↑in BCG+ vs BCG- LBW females in response to culture medium | 24 h whole blood stimulation | Guinea-Bissau | (236) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0-7 d | | 13 months post immunisation | ↓in BCG+ vs BCG- infants in response to BCG | 24 h whole blood stimulation | Denmark | (237) |
| **IL-2** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate<br><br>↓in BCG+ vs BCG- infants in response to *C. albicans* | 48 h whole blood stimulation | | (223) |
| **IL-4** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| **IL-5** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to PPD | 24 h whole blood stimulation | Guinea-Bissau | (236) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | UK | (223) |
| **IL-6** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | At birth or 4.5 mos of age | BCG-Russia | 4.5 months of age | | 5 d whole blood stimulation | The Gambia | (413) |
| | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | | 6 d whole blood stimulation | UK | (306) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | 12 months post BCG immunisation | | | | |
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to PMA, PPD, Pam3Cys | 24 h whole blood stimulation | Guinea-Bissau | (236) |
| | 0-7 d | | 3 months post immunisation | A trend for ↓in BCG+ vs BCG- infants in response to *C. albicans* | 24 h whole blood stimulation | Denmark | (237) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys, *C. albicans, S. aureus* | 48 h whole blood stimulation | UK | (223) |
| | 0-10 d of age | | 7 days post immunisation | ↓in BCG+ vs BCG- infants in response to PEPG, R848 | 20 h whole blood stimulation | Australia | (238) |
| | | | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to BCG | | | (233) |
| **IL-7** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys | 48 h whole blood stimulation | | (223) |
| **IL-8** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, LPS | 48 h whole blood stimulation | | (223) |
| | 0-10 d of age | | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to *E. coli*, LPS | 20 h whole blood stimulation | Australia | (233) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in the absence of stimulation | 24 h PBMC stimulation | South Africa | (239) |
| | 5-10 wks of age | BCG-Danish | | | | UK | (128) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **IP-10 / CXCL10** | 3-13 wks of age | | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate<br><br>↓in BCG+ vs BCG- infants in response to *C. albicans* | 48 h whole blood stimulation | | (223) |
| | At birth | | 10 wks of age | A trend for ↑in BCG+ vs BCG- infants in the absence of stimulation<br><br>↑in BCG+ vs BCG- infants in response to *Mtb* HN878 | 24 h PBMC stimulation | South Africa | (239) |
| **MCP-1 / CCL2** | 0-10 d of age | BCG-Danish | 7 days post immunisation | ↓in BCG+ vs BCG- infants in response to *S. pneumoniae, E. coli, L. monocytogenes, C. albicans,* PEPG, R848 | 20 h whole blood stimulation | Australia | (238) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in the absence of stimulation<br><br>↑in BCG+ vs BCG- infants in response to *Mtb* HN878, *Mtb* CDC1551 | 24 h PBMC stimulation | South Africa | (239) |
| **MIP-1α / CCL3** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate, Pam3Cys | 48 h whole blood stimulation | | (223) |
| | 0-10 d of age | | 7 days post immunisation | ↓in BCG+ vs BCG- infants in response to PEPG, R848 | 20 h whole blood stimulation | Australia | (238) |
| | | | 7 months post immunisation | ↓in BCG+ vs BCG- infants in response to *L. monocytogenes* | | | (233) |
| **MIP-1β / CCL4** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| | 0-10 d of age | | 7 days post immunisation | ↓in BCG+ vs BCG- infants in response to *E. coli, L. monocytogenes,* PEPG, R848 | 20 h whole blood stimulation | Australia | (238) |
| | | | 7 months post immunisation | ↓in BCG+ vs BCG- infants in response to *S. aureus, L. monocytogenes* | | | (233) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in the absence of stimulation | 24 h PBMC stimulation | South Africa | (239) |
| **RANTES / CCL5** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| **TNFα** | 5-10 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (128) |
| | 3-13 wks of age | | | | | | (306) |
| | | | 12 months post BCG immunisation | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | At birth | | 4 weeks post immunisation | ↑in BCG+ vs BCG- LBW infants in response to culture medium, PPD, Pam3Cys | 24 h whole blood stimulation | Guinea-Bissau | (236) |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | UK | (223) |
| | 0-10 d of age | | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to BCG | 20 h whole blood stimulation | Australia | (233) |
| | At birth | | 10 wks of age | ↑in BCG+ vs BCG- infants in response to *Mtb* HN878<br><br>A trend for ↑in BCG+ vs BCG- infants in response to *Mtb* CDC1551 | 24 h PBMC stimulation | South Africa | (239) |
| **TNFβ** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| | 6 wks of age | | 4 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to *Mtb* lysate | 48 h whole blood stimulation | | (223) |
| | At birth | | 10 wks of age | A trend for ↑in BCG+ vs BCG- infants in the absence of stimulation | 24 h PBMC stimulation | South Africa | (239) |
| **VEGF** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |
| **sIL-2Rα** | 3-13 wks of age | BCG-Danish | 3 months post BCG immunisation | ↑in BCG+ vs BCG- infants in response to PPD | 6 d whole blood stimulation | UK | (306) |
| | | | 12 months post BCG immunisation | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **IL-22** | 0-7 d | BCG-Danish | 3 months post immunisation | A trend for ↓in BCG+ vs BCG- infants in response to *C. albicans* | 24 h whole blood stimulation | Denmark | (237) |
| | | | 13 months post immunisation | ↑in BCG+ vs BCG- infants in response to BCG | | | |
| **MIG** | 0-10 d of age | BCG-Danish | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to BCG, *Mtb*, *S. aureus, S. pyogenes, C. albicans* | 20 h whole blood stimulation | Australia | (233) |
| **MIF** | 0-10 d of age | BCG-Danish | 7 months post immunisation | ↑in BCG+ vs BCG- infants in response to *S. pyogenes* | 20 h whole blood stimulation | Australia | (233) |

*BCG-Danish – also referred to as BCG Denmark 1331 or the SSI strain in the descriptions of these studies.

**Table S4.7.** *Population associated differences in cytokine production in BCG-vaccinated infants*

| Cytokine | BCG vaccination | BCG strain | Blood sample obtained | Effect | Assay | Study |
|---|---|---|---|---|---|---|
| | | | *Population associated differences detected* | | | |
| **EGF** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to RPMI, LPS, Pam3Cys, BCG | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | No difference | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | | | (307) |
| **G-CSF** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to LPS, Pam3Cys, BCG | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↓UK vs Malawi to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | No difference | | (307) |
| **GM-CSF** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to LPS, BCG | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↓UK vs Malawi to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | No difference | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | | | (130) |
| **IL-1α** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to LPS | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↓UK vs Malawi infants to PPD | 6 d whole blood stimulation | (306) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | ↑The Gambia vs Malawi to PPD | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | No difference | | (130) |
| **IL-1β** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to LPS, BCG | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | No difference | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | ↓UK vs Uganda to PPD | | (130) |
| **IL-6** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to LPS, Pam3Cys, BCG | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↑in UK vs Malawi to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | ↑The Gambia vs Malawi to PPD | | (307) |
| **MCP1** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | ↓UK vs SA infants to Pam3Cys | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | Above detection range | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | ↓The Gambia vs Malawi to PPD | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | No difference | | (130) |
| *No population associated differences detected* | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **IL-12p40** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | No difference | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↑in UK vs Malawi to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | No difference | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | | | (130) |
| **IL-1RA** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | No difference | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↓UK vs Malawi infants to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | ↑The Gambia vs Malawi to PPD | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | No difference | | (130) |
| **IP-10** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | No difference | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↑in UK vs Malawi to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | ↓The Gambia vs Malawi to PPD | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | ↑UK vs Uganda to PPD | | (130) |
| **TNFα** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | No difference | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | | 6 d whole blood stimulation | (306) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | | | (307) |
| | UK: 0-7 d post birth<br>Uganda: at birth | UK and Uganda: BCG-Danish | 10 wks post BCG | | | (130) |
| **PDGF-AB/BB** | UK: 0-7 d post birth<br>SA: at birth | UK: InterVax BCG<br>SA: BCG-Tokiyo 172 | 10 wks of age | Not detected | 24 h PBMC stimulation | UK vs SA comparison |
| | UK and Malawi: 3-13 wks of age | UK and Malawi: BCG-Danish 1331 | 3 mos post BCG | ↓UK vs Malawi to PPD | 6 d whole blood stimulation | (306) |
| | Malawi and The Gambia: 0-7 d post birth | Malawi: BCG-Danish 1331<br>The Gambia: BCG-Russia | | ↑The Gambia vs Malawi to PPD | | (307) |

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| **Thesis Title** | Studies on innate immune responses to Mycobacterium bovis BCG | | |
| **Primary Supervisor** | Prof Hazel M Dockrell | | |

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# Heterologous effects of infant BCG vaccination: potential mechanisms of immunity

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The current antituberculosis vaccine, BCG, was derived in the 1920s, yet the mechanisms of BCG-induced protective immunity and the variability of protective efficacy among populations are still not fully understood. BCG challenges the concept of vaccine specificity, as there is evidence that BCG may protect immunized infants from pathogens other than *Mycobacterium tuberculosis* – resulting in heterologous or nonspecific protection. This review summarizes the up-to-date evidence for this phenomenon, potential immunological mechanisms and implications for improved childhood vaccine design. BCG induces functional changes in infant innate and adaptive immune compartments, encouraging their collaboration in the first year of life. Understanding biological mechanisms beyond heterologous BCG effects is crucial to improve infant protection from infectious diseases.

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**Keywords:** BCG • childhood immunization • heterologous vaccine effects • humoral responses • infant immunity • innate memory • monocytes • NK cells • T cells • trained immunity

The BCG is a live attenuated strain of *Mycobacterium bovis* and is the only currently licensed vaccine against TB. It is routinely administered to infants at or shortly after birth in regions where TB is endemic BCG vaccination confers consistent efficacy against disseminated forms of TB in childhood, such as TB meningitis and miliary TB, however, its protective efficacy against adult-type pulmonary TB varies [1,2]. Factors that have been implicated include BCG strain, route of administration, geographical location, exposure to environmental mycobacteria and helminth infection [3]. There is increasing evidence, especially in regions affected by a high infectious disease burden, that apart from protecting against TB, BCG may reduce infant mortality from unrelated infections. Here we review the evidence for this phenomenon, discuss potential mechanisms and outline the possible implications for future vaccine candidates.

## All-cause infant mortality reduction

Observational studies reported that BCG, alone or in combination with other vaccines, might decrease the all-cause mortality risk up to 30–50% for up to 2 years of age in West Africa [4–6], extending to up to 5 years of age in Uganda [7]. More specifically, it was shown that immunizing low-birth-weight infants with BCG at birth could significantly improve their survival for the first month of life because of decreased infection risk [8]. Similar findings were reported in India, where mortality rates were lower in BCG-vaccinated infants for up to 6 months of age, compared with the unvaccinated infant group [9]. Studies in Malawi and Guinea-Bissau also found a trend for reduced mortality among infants vaccinated with BCG [10,11]. Although the extent of BCG-dependent nonspecific reduction in infant mortality is difficult to evaluate, with the efficacy estimates reaching 6–72% in clinical trials

or 2–95% in observational studies [12,13], this evidence suggests that BCG may exert a beneficial, heterologous influence on infant survival, reducing mortality unrelated to TB.

### Impact on acquisition of infectious diseases

BCG may also reduce the acquisition of nonmycobacterial infections. In Uganda, BCG-vaccinated HIV-positive adults had lower risk of intestinal nematode infection than unvaccinated individuals [14]. BCG vaccination also decreased risk of heterologous infections in infants. In Guinea-Bissau, BCG-immunized infants had lower rates of neonatal sepsis and respiratory infection [8]. Similarly, hospitalization rate due to nontubercular respiratory infections and sepsis in Spain was lower among the BCG-vaccinated children [15]. A recent analysis of infant immunization with BCG in 33 countries suggested BCG vaccination may reduce acute lower respiratory infection incidence by 17–37% [16]. In contrast, a randomized trial in Denmark found no association between neonatal BCG vaccination and infection incidence [17]. The reasons for the discrepancies are not clear, although it has been suggested that benefits of infant BCG immunization in low-income settings may be partially accounted by lowered undiagnosed mycobacterial infection rate [18]. Such infections would be less likely in a setting with low infectious disease burden. Together, these studies imply that nonspecific BCG effects may be particularly beneficial in countries with high infectious disease load, reducing both the all-cause mortality and the disease incidence.

### Factors potentially contributing to the heterologous effects of BCG vaccination

#### BCG timing & interaction with other vaccines

Some studies suggested that diphtheria-tetanus-pertussis (DTP) vaccine might affect the impact of BCG on childhood mortality [4,9]; implicating that other vaccines may modulate the nonspecific effects of BCG immunization (Table 1). In contrast, a study in Burkina Faso found that risk of mortality before 2 years of age was reduced to a similar extent in infants vaccinated with BCG-only or both BCG and DTP [5]. Vaccination timing and sequence were suggested as potentially important to the nonspecific effects of vaccines, as studies in Senegal and Philippines found that immunizing infants with DTP at or following BCG administration was associated with enhanced survival [6,19–21]. Proposals were made that BCG following DTP might reduce all-cause infant mortality even further [22–24]. The WHO Strategic Advisory Group of Experts addressed the controversy of nonspecific BCG and DTP interactions in 2014 and concluded that the evidence for such effects was insufficient [13].

#### Infant age & time post-BCG vaccination

The extent to which heterologous effects of BCG vaccination are apparent may depend on age of the infant. BCG-dependent reduction in overall infant mortality may be the most evident in the first few months of life, before the nonspecific infant protection becomes influenced by the administration of subsequent vaccines [4,8]; however, in other studies heterologous effects are still apparent for up to 24 months of age [5,6]. Some studies reported BCG-associated reduction in overall mortality of children aged up to 5 years [7,25] or decrease in hospitalization rates due to nonmycobacterial infections in children up to 14 years of age [15]. This may be a consequence of improved early childhood survival as BCG-related reduction in hospitalization due to sepsis was the most significant in children aged 1–4 years, diminishing in older children [15]. This implies that the heterologous BCG effects manifest soon after immunization, but wane over time and may be the most apparent for neonates vaccinated at birth. Another possibility is that as infants grow older, they become exposed to pathogens more frequently, with the resulting development of the classical immunity against infectious diseases eventually overcoming the heterologous beneficial effects of BCG.

#### Sex-differential effects of BCG vaccination

Some studies indicate that BCG-dependent nonspecific infant mortality reduction may be influenced by male or female sex, females possibly benefitting from heterologous BCG effects more than males [9,26,27]. A review of female–male twin pair datasets from Guinea-Bissau and Senegal found that the survival benefit of BCG vaccination in females was variable, possibly due to low death rates observed among the vaccinated twin pairs [28]. No BCG-associated survival benefit in female infants was observed over the first 8 months of life in an Indian infant cohort [29], although this could be attributable to excess background female neonatal mortality in this region. No sex-related differences in heterologous BCG protection were observed in Burkina Faso [5]. SAGE addressed this issue in 2014; however, no evidence for differences in BCG-immunized female or male heterologous mortality reduction was found [13]. Of interest, some studies indicated that BCG-vaccinated females may produce higher

**Table 1. Nonspecific infant mortality reduction by BCG and interaction with diphtheria-tetanus-pertussis.**

| Study | Study type | Vaccine schedule[†] | BCG-vaccinated vs unvaccinated infants | DTP-vaccinated vs unvaccinated infants | BCG vs BCG & DTP | Observed age group | Ref. |
|---|---|---|---|---|---|---|---|
| Guinea-Bissau | Cohort | BCG & OPV at birth; DTP at 6, 10& 14 weeks; MV at 9 months | Mortality rate: At 6–12 months of age 3 9% among BCG-vaccinated 4 9% among BCG not vaccinated | Mortality rate: At 7 5-12 months of age 4 8% among DTP-vaccinated 4 0% among DTP not vaccinated | Mortality rate: At 7 5–9 months of age 3 9% among BCG & DTP; 2 5% among BCG only At 10–12 months of age 5 6% among BCG & DTP; 4 1% among BCG only | Up to 5 years of age | [4] [5] |
| Burkina Faso | Cohort | BCG at birth; DTP at 6, 10& 14 weeks[‡] | Mortality before 2 years of age risk ratio[§]: 0 37 | Mortality before 2 years of age risk ratio[§]: 0 23 | Mortality before 2 years of age risk ratio[§]: BCG & DTP vs unvaccinated 0 34 | Up to 2 years of age | |
| Senegal | Cohort | DTP-IPV at 2, 4 & 6 months[¶]; BCG administered with the first DTP-IPV; MV at 9–10 months | Not analyzed | | BCG & DTP vs unvaccinated mortality before 2 years of age ratio: cohort 1[¶]: 0 70 cohort 2[¶]: 0 59 (0 46–0 74) | Up to 2 years of age | [6] |
| Senegal | Cohort | Recommended schedule: BCG at birth; DTP& OPV at 6, 10& 14 weeks; MV at 9 months BCG first: BCG vaccinated, DTP1 not yet received; DTP1, DTP2 or DTP3 following BCG BCG & DTP first: DTP2 or DTP3 following BCG & DTP simultaneously DTP first: BCG following DTP1, DTP2 or DTP3 | Mortality rate ratio: 0 98– BCG-vaccinated, DTP1 not yet received | Mortality rate ratio: 1 33–DTP1, no BCG 1 41–DTP2, no BCG 0 63–DTP3, no BCG | Mortality rate ratio: 0 98 – BCG-vaccinated, DTP1 not yet received 0 96 – BCG first 0 69 – BCG & DTP first 1 10 – DTP first | Up to 24 months of age | [21] |
| Guinea-Bissau | Randomized trial | BCG & OPV at birth; DTP& OPV at 6, 10& 14 weeks; MV at 9 months; DTP & OPV booster at 18 months; BCG revaccination at 19 months | BCG revaccination vs no revaccination HR: 1 20 - the whole study period | | BCG revaccination vs no revaccination HR: 0 36 – DTP booster given prior to the trial 1 78 – no DTP booster prior to the trial | Up to 5 years of age | [23] |
| India | Cohort | BCG at 0–12 months; OPV at birth, 6, 10 & 14; DTP at 6, 10 & 14 weeks | HR[#]: 0 62 – for BCG-vaccinated vs no BCG 0 44 – BCG only, no DTP 0 72 – BCG & DTP | HR[#]: 0 70 - DTP prior to BCG 0 44 – DTP only | | Up to 6 months of age | [9] |
| Philippines | Cohort | BCG at 0–11 weeks; DTP & polio vaccine at 6, 10 & 14 weeks[††]; MV at 9 months | BCG-vaccinated infants only | | HR[‡‡]: 0 18 – females, no DTP 0 27 – DTP-vaccinated females 0 32 – DTP-vaccinated males | Up to 30 months of age | [19] |

[†]Vaccination timings correspond to infant age at the time of vaccination.
[‡]Infants were considered unvaccinated until the age of immunization with a specified vaccine.
[§]Adjusted for the area, dispensary in a village, use of health services, diarrhea in the first year of life and birth season.
[¶]Cohort 1 vaccinated as indicated in the Vaccination Schedule section. Cohort 2 received OPV instead of PV.
[#]Assumed HR for unvaccinated infants equals 1.
[††]Type of polio vaccine was not specified.
[‡‡]Assumed HR for infant males not vaccinated with DTP equals 1. The cited HR rates exclude two deaths of infants with an unknown DTP vaccination status.
[§§]Mortality rate ratio adjusted for most recent weight and controlled for age.
DTP1, 2 or 3: First, second or third dose of diphtheria-tetanus-pertussis vaccine; HR: Hazard ratio; IPV: Inactivated polio vaccine; MV: Measles vaccine; OPV: Oral polio vaccine.

**Table 1. Nonspecific infant mortality reduction by BCG and interaction with diphtheria-tetanus-pertussis (cont.).**

| Study | Study type | Vaccine schedule[†] | BCG-vaccinated vs unvaccinated infants | DTP-vaccinated vs unvaccinated infants | BCG vs BCG & DTP | Observed age group | Ref. |
|---|---|---|---|---|---|---|---|
| India | Cohort | Recommended schedule:<br>BCG at birth;<br>DTP & OPV at 2, 3 & 4 months;<br>MV at 9 months;<br>DTP & OPV booster at 18 months<br>Altered sequence of BCG and DTP vaccination:<br>BCG& DTP simultaneously<br>BCG following DTP | Mortality rate to 1 year of age:<br>2.2% in BCG only group<br>3.6% in the unvaccinated group | Mortality rate to 1 year of age:<br>2.4% in DTP only group<br>3.6% in the unvaccinated group | Mortality rate ratio in the first 9-12 months prior to receiving MV[§§]:<br>0.11 - BCG & DTP simultaneously and the last in the sequence vs DTP following BCG<br>0.14 - BCG & DTP simultaneously and the last in the sequence vs DTP only<br>0.13 - BCG & DTP simultaneously and the last in the sequence vs DTP as the last vaccine in the sequence<br>0.27 - BCG alone or BCG & DTP simultaneously and the last in the sequence vs DTP as the last vaccine in the sequence | Up to 5 years of age | [24] |

[†]Vaccination timings correspond to infant age at the time of vaccination.
[‡]Infants were considered unvaccinated until the age of immunization with a specified vaccine.
[§]Adjusted for the area, dispensary in a village, use of health services, diarrhea in the first year of life and birth season.
[¶]Cohort 1 vaccinated as indicated in the Vaccination Schedule section. Cohort 2 received OPV instead of IPV.
[#]Assumed HR for unvaccinated infants equals 1.
[††]Type of polio vaccine was not specified.
[‡‡]Assumed HR for infant males not vaccinated with DTP equals 1. The cited HR rates exclude two deaths of infants with an unknown DTP vaccination status.
[§§]Mortality rate ratio adjusted for most recent weight and controlled for age.
DTP1, 2 or 3: First, second or third dose of diphtheria-tetanus-pertussis vaccine; HR: Hazard ratio; PV: Inactivated polio vaccine; MV: Measles vaccine; OPV: Oral polio vaccine.

levels of inflammatory cytokines in response to nonmycobacterial stimuli than males at 4 weeks [30] or 1 and 12 weeks [31] postvaccination. Therefore, immunological mechanisms of heterologous effects of BCG vaccination may be sex-dependent; however, their contribution to heterologous infant mortality is not clear.

## Mechanisms implicated in heterologous BCG-vaccinated infant protection

### BCG-inducible trained innate immunity

A tempting candidate to explain the heterologous effects of BCG is the phenomenon of innate immune response training [32–34]. Originally identified in NK cells, innate memory or training enables the innate cells to respond more rapidly and strongly to antigens unrelated to the original stimulus and was shown to be BCG-inducible in monocytes. Pre-exposure of murine macrophages to BCG was demonstrated to increase their ability to cope with *Candida albicans* infection both *in vitro* and *in vivo* [35]. In humans, monocytes of BCG-vaccinated adults had increased expression of surface markers of activation and produced more IL-1β, IL-6, IFN-γ and TNF-α in response to *Staphylococcus aureus* or *C. albicans* for up to 3 months postvaccination compared with monocytes isolated before vaccination from the same adults (Figure 1) [36,37]. Interestingly, while surface receptor expression on monocytes from BCG-vaccinated adults was upregulated for up to a year, IL-1β and TNF-α production upon nonmycobacterial antigen stimulation diminished by this time [38]. This suggests that the most potent effects of heterologous BCG-trained immunity manifest over the first few months post-BCG vaccination. Apart from inducing functional, lasting monocyte changes, BCG enhanced the vaccinated adult NK cell IL-1β and IL-6 production in response to *C. albicans* and *S. aureus* for up to 3 months, also, improving T- and B-cell deficient mice, infected with *C. albicans,* survival [36,39]. This is consistent with the observations that BCG-associated reduction in all-cause infant mortality is the most significant during the first few months of life [4–5,8], implying that BCG-trained monocyte and NK cell immunity may contribute to broad infant protection from infectious diseases when they are the most susceptible.

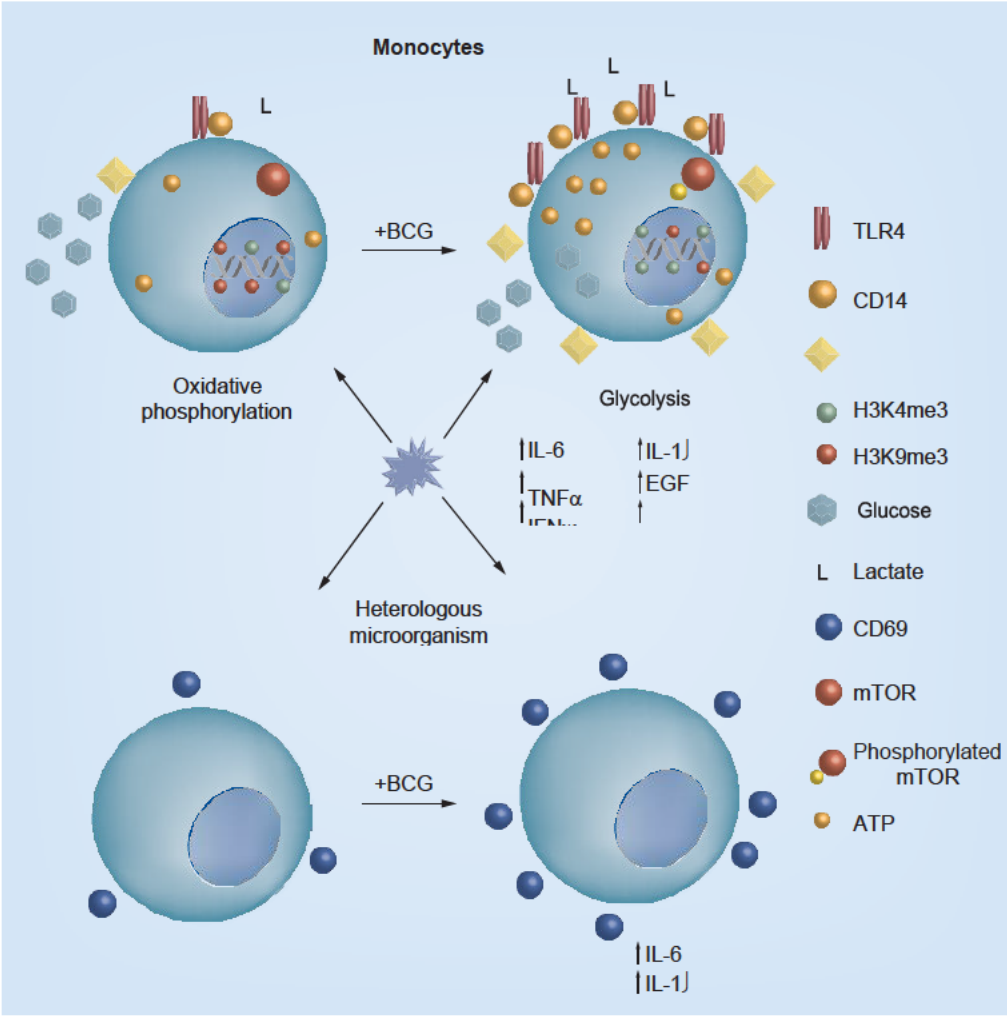


**Figure 1. BCG training-induced phenotype changes in monocytes and NK cells.** BCG training of human monocytes *in vitro* or by vaccination increases their surface marker expression and cytokine production in response to heterologous antigen stimulation [36,40]. In monocytes, these changes are regulated by metabolic shift from oxidative phosphorylation to glycolysis and histone modifications [41], with increased frequency of permissive H3K4me3 and reduced presence of inhibitory H3K9me3 at the promoters of cytokine, receptor and metabolic pathway component encoding genes [36,37,41-43]. The left side of the diagram depicts model innate immune cells prior to the BCG training and the right side - post-training. Enhanced cytokine production post-training is indicated by arrows. Heterologous microorganism - secondary, nonmycobacterial infectious agent.

+ BCG - *in vitro* or *in vivo* cell training with BCG.

TLR4: Toll-like receptor 4; H3K4me3: Trimethylation of lysine at position 4 on histone 3; H3K9me3: Trimethylation of lysine at position 9 on histone 3.

## Metabolic changes induced by innate immune training

Functional monocyte changes induced by BCG training have been associated with a metabolic shift from oxidative phosphorylation to aerobic glycolysis (Figure 1). First demonstrated in monocyte *in vitro* training with β-glucan phosphorylation [44]. Similar changes were observed in BCG-dependent monocyte training [41]. Peripheral blood mononuclear cells upon heterologous stimulation than cells obtained prior to the vaccination [41]. This correlates with the previous findings that monocytes obtained from BCG-immunized donors at these time points produce higher cytokine levels than monocytes isolated before the immunization [36,37,42]. Of note, inhibition of mTOR and glycolysis pathways diminished *ex vivo* BCG-trained human monocyte production of lactate, TNF and IL-6 upon lipopolysaccharide (LPS) challenge, supporting the role for glycolysis in the innate immune training [41]. Importantly, polymorphisms of HK2 and PFKP were associated with the ability of monocytes to be trained and produce cytokines in response to LPS [41]. This implies BCG-inducible training may be ineffective in some

**Table 2. Impact of BCG training on histone modification patterns at the promoters of the immune and metabolic genes.**

| Gene | Product | Function | Histone modification | Impact on gene expression | Cell type | Model | Ref. |
|---|---|---|---|---|---|---|---|
| *TNF-a*<br>*IL-6* | TNF-a<br>IL-6 | Immune responses | ↑H3K4me3 | Permissive | Monocytes | *In vivo*/BCG vaccination | [36] |
| | | | | | | *In vitro*/μ BCG training | [42] |
| | | | | | | *In vitro*/BCG training | [37] |
| | | | ↑H3K4me3<br>↓H3K9me3 | Permissive<br>Inhibitory | Monocytes | *In vitro*/BCG training | [41] |
| | | | ↓H3K9me3 | Inhibitory | Monocytes/ macrophages | *In vitro*/BCG training | [43] |
| *mTOR*<br>*HK2*<br>*PFKP* | mTOR<br>Hexokinase 2<br>Platelet phospho-fructokinase | Glycolysis | ↑H3K4me3<br>↓H3K9me3 | Permissive<br>Inhibitory | Monocytes | *In vitro*/BCG training | [41] |
| *GLS*<br>*GLUD*† | Glutaminase<br>Glutamate dehydrogenase | Glutaminolysis | ↑H3K4me3<br>↓H3K9me3 | Permissive<br>Inhibitory | Monocytes | *In vitro*/BCG training | [41] |

†H3K4me3 pattern change not significant upon BCG training.
H3K4me3: Trimethylation of histone 3 at lysine 4; H3K9me3: Trimethylation of histone 3 at lysine 9; μ BCG: μ-irradiated BCG.

individuals with metabolic component polymorphisms. Other pathways may be involved in innate immune training. Monocytes trained *in vitro* with BCG or oxidized low-density lipoprotein (oxLDL) were shown to increase reactive oxygen species production upon stimulation with zymosan, a yeast-derived ligand of TLR2 [45]. BCG enhanced IL-6 and TNF-α production in histone 3 lysine 4 trimethylation dependent manner, this effect is also demonstrated for oxLDL [36,42]. Interestingly, oxLDL stimulated monocyte scavenger receptor and CD36 expression and differentiation to foam cells [46]. Mycobacteria can interfere with the host's lipid metabolism and drive foam cell formation [47], suggesting that BCG may also exploit lipid metabolism to induce monocyte training.

## Epigenetic regulation of innate immune training

Epigenetic mechanisms, largely, histone modifications, regulate monocyte training (Figure 1 and Table 2). For example, enhanced surface activation marker and inflammatory cytokine expression upon nonmycobacterial stimulation of monocytes from BCG-vaccinated adults was associated with intracellular nucleotide sensor NOD2 dependent H3K4 trimethylation of promoters of genes encoding these monocyte markers and cytokines [36,42]. In addition, active promoters of β-glucan-trained monocytes contained higher levels of permissive histone modifications, such as H3K4me3 and histone 3 lysine 27 acetylation than promoters in untrained monocytes [44]. The accumulation of these epigenetic markers of promoter activation at the glycolysis and mTOR pathway component genes implied cellular metabolism in innate immune training [44]. BCG-inducible monocyte training enriched the activating H3K4me3 modification at mTOR, glycolytic enzyme, *tnf* and *il-6* gene promoters [41]. However, the regulatory patterns of training-related histone modifications seem to be complex as mTOR or glutamine pathway inhibition cancelled H3K4me3 accumulation at the cytokine promoters [41]. Importantly, not only the permissive, but also inhibitory histone modifications, such as histone 3 lysine 9 trimethylation regulate glycolysis and mTOR pathway component or inflammatory cytokine expression in BCG-trained cells [41,43]. BCG training was shown to suppress H3K9me3 mark while inhibition of glutamine or mTOR pathways enhanced the accumulation of this mark at the inflammatory cytokine promoters [41]. This suggests that enzymes managing histone modification patterns may respond to intracellular metabolite changes, coordinating cytokine or other gene expression accordingly.

## Infant BCG immunization & innate immune training

The evidence on whether BCG induces trained innate immunity in infants and if it contributes to their protection from nonmycobacterial pathogens is somewhat controversial. Although adult BCG vaccination or *in vitro* training models suggest that BCG primes monocytes to increase surface activation markers and type 1 cytokine production in response to heterologous antigen stimulation [36,37,42], infant immune responses to BCG seem more difficult to define. Differently from adults, no differences in monocyte surface activation marker expression were observed upon whole blood stimulation with heterologous stimuli in the BCG-immunized infant group versus unvaccinated

controls [40]. However, Pam3CSK4 stimulation upregulated NK cell activation marker CD69 in the vaccinated infant samples, implying that NK cells may mediate heterologous BCG effects in infants, similar to the NK cells of the BCG-vaccinated adults [39]. Likewise, in agreement with the adult studies, whole blood samples from BCG-immunized low-birth-weight infants produced more TNF-α, IFN-γ, IL-1β and IL-6 upon Pam3CSK4 stimulation compared with the unvaccinated infants (Table 3) [30]. Yet, different cytokine profile was identified in BCG-vaccinated UK infant whole blood cultures, with higher levels of EGF, IL-6, PDGF-AB/BB in response to Pam3CSK4, *C. albicans* and *S. aureus* challenge compared with the control group [40]. Previous studies explored narrower cytokine profiles [30,36,37], so it is not clear if discrepancies reflect differences in the adult and the infant immune systems or diverse study design.

Other studies did not confirm the association between infant BCG vaccination status and heterologous immune responses. In contrast to previous findings, no TNF-α production changes at 1 and 12 weeks postimmunization were found in BCG-vaccinated Gambian infants upon their PBMC stimulation with heterologous microorganisms (Table 3) [31]. In addition, no significant changes in cytokine responses to nonspecific stimuli were observed at 3 and 13 months postrandomization in whole blood samples obtained from the BCG-vaccinated infants compared with the controls in Denmark [51]. The reasons for the discrepancies among the findings from different studies are not clear, although potentially low immunogenicity of BCG used in some studies was suggested as a possible cause [31,51,52]. In Uganda, maternal BCG scar was associated with stronger inflammatory responses in infants upon whole blood culture stimulation with TLR agonists [53], suggesting that maternal BCG status could affect infant responses. Differences in the vaccination schedules, study design or infant populations may also contribute to diverse outcomes in such studies.

### BCG & other innate immune responses

Other mechanisms may contribute to the heterologous BCG effects. BCG-dependent immune training was shown to elevate levels of IL-6, TNF-α and IL-1β in BCG-vaccinated adults and infants 2 weeks to several months postvaccination in response to heterologous stimuli [30,36,37,40,42]. These cytokines can mediate the acute phase responses, suggesting that BCG-primed immune system might exploit plasma iron regulation upon encounter with infectious microorganisms. However, a study of Gambian neonates found no association between the vaccination status and plasma iron, hemoglobin, hepcidin, ferritin or IL-6 levels in the unvaccinated controls and neonates vaccinated with oral polio vaccine, HBV and BCG at birth or given BCG at 5 days of age [54]. The authors argued that early responses were measured, potentially missing out BCG-dependent nonspecific effects and that the observed neonate plasma levels of IL-6, hepcidin and ferritin were elevated irrespective of immunization status as a consequence of the birth process, potentially masking the nonspecific effects of BCG [54]. Further studies exploring a possible relationship between the acute phase responses in infants and nonspecific effects of BCG would be of interest.

### BCG-enhanced heterologous T-cell responses

BCG may steer the immune system toward Th1-type proinflammatory cytokine production, activating monocytes and alveolar macrophages, so mediating classical antimycobacterial effects. However, this effect may extend beyond mycobacterial specificity. In mice, BCG immunization enhanced protection from vaccinia virus via increased $CD4^+$ T-cell IFN-γ production [55]. Studies on human infant responses to BCG show similar effects (Table 3). BCG-Denmark improved IFN-γ and IL-10 responses to tetanus toxoid at 12 months of age in a Ugandan infant cohort [49]. In Philippines, infants, given BCG at birth, had higher frequencies of tetanus toxoid specific PBMCs producing IFN-γ and $CD4^+$ memory T cells secreting IFN-γ and TNF-α upon phytohemagglutinin stimulation [52]. In Guinea-Bissau, BCG-vaccinated infants produced more IFN-γ than unvaccinated controls upon whole blood stimulation with phorbol myristate acetate [30]. PBMCs from Gambian infants vaccinated with BCG at birth produced higher levels of IFN-γ, IL-5 and IL-13 in response to hepatitis B surface antigen, and their lymphocytes were more proliferative compared with the cells from control infants [48]. Increased IFN-γ-producing $CD8^+$ T-cell frequency upon *C. albicans* stimulation at 1 week post-BCG immunization was observed in another Gambian infant cohort, although this effect subsided by 12 weeks postvaccination [31]. This study also reported reduced IL-10 production in response to LPS and increased IFN-γ/IL-10 ratio upon *S. pneumoniae* stimulation in BCG-vaccinated females at 12 weeks postimmunization [31]. Together, these studies suggest that BCG vaccine may enhance maturation of Th1 cells with diverse specificities, improving responses to a broad range of microbial or childhood vaccine antigens. As infant immune responses shift from Th17-like toward Th1-type in the first year

**Table 3. Heterologous effects of BCG on cytokine production, cell surface marker expression and proliferation.**

| Study | Vaccine schedule[†] | Assay | Age at observation | Secondary stimulus | BCG vs control | | | Ref. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Cytokine production | Surface marker expression | Proliferation | |
| The Gambia | BCG-Pasteur at birth or BCG at 2 months HBV at birth, 2 & 4 months; OPV at birth, 1, 2 & 3 months; DTP at 2, 3 & 4 months control - BCG at 4.5 months | PBMCs | At birth, 2 & 4 months | HBsAg | ↑IFN-μ, IL-5 and L-13 at 2 and 4.5 months of age in infants given BCG at birth ↑IL-5 and IL-13 at 4.5 months of age in infants given BCG at 2 months | | ↑Lymphocyte proliferation at 2 and 4.5 months in infants given BCG at birth ↑lymphocyte proliferation at 4.5 months in infants given BCG at 2 months | [48] |
| | | | | TT | ↑IL-5 at 4.5 months of age in infants given BCG at 2 months ↑IL-13 at 4.5 months of age in infants given BCG at birth or 2 months | | No lymphocyte proliferation changes | |
| Uganda | BCG-Bulgaria, BCG-Denmark or BCG-Russia at birth; OPV at birth, 6, 10 & 14 weeks; DTP, Hib and HBV MV at 9 months | Whole blood | 12 months | TT | ↑IFN-μ in infants given BCG-Denmark ↑IL-10 in infants given BCG-Bulgaria or BCG-Denmark | | | [49] |
| | | | | PHA | ↑IFN-μ, IL-10, IL-13 in infants given BCG-Denmark | | | |
| South Africa | BCG-Denmark at birth control - BCG at 8 weeks | Whole blood | 8 & 14 weeks | SEB | n.s. | | ↑$CD4^+$ T-cell proliferation at 14 weeks | [50] |
| | | | | BP | ↑IL-2 + $CD8^+$ T cells at 8 weeks ↑IL-13 + $CD4^+$ and $CD8^+$T cells at 14 weeks | | | |
| Guinea-Bissau | OPV at birth; BCG-Denmark at birth; Penta at 6 weeks control - BCG at 6 weeks | Whole blood | 4 weeks | Pam3CSK4 | ↑IL-1þ, IL-6, TNF-a, IFN-μ | | | [30] |
| | | | | PMA & ionomycin | ↑IL-6, FN-μ | | | |
| The Gambia | OPV & HBV at birth; BCG-Russia at 6 weeks; Penta, PCV-13 & OPV at 8, 12 & 16 weeks; control - BCG at 18 weeks | PBMC | 6, 7 & 18 weeks | LPS | ↓IL-10 in females at 18 weeks[‡] | | | [31] |
| | | | | PMA & ionomycin | ↓IFN-μ in females at 18 weeks[‡] | | | |
| | | | | *Candida albicans* | ↑IFN-μ + $CD8^+$ T cells in males and females at 7 weeks[‡] | | | |
| | | | | *S. pneumoniae* | ↑IFN-μ /IL-10 ratio in females at 18 weeks[‡] | | | |

[†]Vaccination timings correspond to infant age at time of vaccination.

[‡]These timings correspond to the timing before the BCG vaccination, 1 week and 12 weeks post-BCG vaccination, respectively.

Pam3CSK4: S)-(2,3-bis(palmitoyloxy)-(2RS) -propyl)- N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH trihydrochloride, PCV-13, Penta: Diphtheria-pertussis-tetanus-*Haemophilus influenzae b* – HBV. Due to DiTeKiPol/Act-Hib availability issues, 26 BCG-vaccinated and 48 control infants received Infanrix Hexa. DiTeKiPol/Act-Hib contains diphtheria toxoid, TT, polio virus types 1-3, H. influenzae type b polysaccharide. Infanrix Hexa also contains hepatitis B surface antigen and lower content of pertussis toxoid and aluminium [63].

BP: Whole cell *Bordetella pertussis*; CL075: TLR7/8 agonist; DTP: Diphtheria-tetanus-pertussis; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B vaccine; MV: Measles vaccine; n s.: Not significant; OPV: Oral polio vaccine; PBMC: peripheral blood mononuclear cells; PHA: Phytohaemagglutinin; PMA: Phorbol myristate acetate; Polio 1–3: Poliovirus types 1–3 antigen; PPD: Purified protein derivative; Prevenar 13: 13-valent pneumococcal conjugate vaccine; SEB: Staphylococcal enterotoxin B; TT: Tetanus toxoid.

Table 3. Heterologous effects of BCG on cytokine production, cell surface marker expression and proliferation (cont.).

| Study | Vaccine schedule[†] | Assay | Age at observation | Secondary stimulus | BCG vs control | | | Ref. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Cytokine production | Surface marker expression | Proliferation | |
| United Kingdom | BCG-Denmark at 6 weeks control - no BCG | Whole blood | 4 months postvaccination | LPS | ↑IL-8<br>↓GM-CSF, GRO | | | [40] |
| | | | | Pam3CSK4 | ↑EGF, L-6, PDGF-AB/BB, MCP-3, IL-7, IL-10, IL-12p40, sCD40L, eotaxin, MIP-1a | ↑CD69 on NK cells | | |
| | | | | *C. albicans* | ↑EGF, L-6, PDGF-AB/BB, MCP-3<br>↓IL-2, IL-13, IL-17, IP-10 | | | |
| | | | | *Staphylococcus aureus* | ↑EGF, IL-6, PDGF-AB/BB | | | |
| | | | | *Escherichia coli* | ↑EGF<br>↓GM-CSF, GRO | | | |
| Denmark | BCG-Denmark at 0-7 days; DiTeKiPol/Act-Hib[§] & Prevenar 13 at 3, 5 & 12 months control - no BCG | Whole blood | 4 days, 3 & 13 months postrandomization to BCG or control groups | *C. albicans* | ↑TNF-a/IL-10 at 13 months | | | [51] |
| Philippines | BCG at 0-2 weeks r<br>BCG after the first DTP & OPV dose | PBMCs | 2-3 months | TT | ↑IFN-μ + PBMCs in infants vaccinated at 0-2 weeks | | | [52] |
| | | | | Polio 1-3 | ↑IFN-μ + PBMC trend in infants vaccinated at 0-2 weeks | | | |
| | | | | PMA & ionomycin | ↑IFN-μ + TNF-a + CD45RO + $CD4^+$ T cells in infants vaccinated at 0-2 weeks | | | |

[†]Vaccination timings correspond to infant age at time of vaccination.
[‡]These timings correspond to the timing before the BCG vaccination, 1 week and 12 weeks post-BCG vaccination, respectively.
Pam3CSK4: S)-(2,3-bis(palmitoyloxy)-(2RS) -propyl)- N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH trihydrochloride, PCV-13, Penta: Diphtheria-pertussis-tetanus-*Haemophilus influenzae b* – HBV. Due to DiTeKiPol/Act-Hib availability issues, 26 BCG-vaccinated and 48 control infants received Infanrix Hexa. DiTeKiPol/Act-Hib contains diphtheria toxoid, TT, polio virus types 1-3, H. influenzae type b polysaccharide. Infanrix Hexa also contains hepatitis B surface antigen and lower content of pertussis toxoid and aluminium [63].
BP: Whole cell *Bordetella pertussis*; CL075: TLR7/8 agonist; DTP: Diphtheria-tetanus-pertussis; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B vaccine; MV: Measles vaccine; n.s.: Not significant; OPV: Oral polio vaccine; PBMC: peripheral blood mononuclear cells; PHA: Phytohaemagglutinin; PMA: Phorbol myristate acetate; Polio 1–3: Poliovirus types 1–3 antigen; PPD: Purified protein derivative; Prevenar 13: 13-valent pneumococcal conjugate vaccine; SEB: Staphylococcal enterotoxin B; TT: Tetanus toxoid.

of life [56], intensifying this process through BCG vaccination may contribute to heterologous infant protection from infectious diseases (Figure 2). However, this effect may be limited as no difference in HBsAg-specific IFN-γ producing PBMC frequencies was found in BCG-vaccinated and control infants in Philippines [52], suggesting that BCG did not affect responses to hepatitis B vaccine in this population.

BCG may modulate heterologous responses in other T-cell populations – *C. albicans* and *S. aureus* boosted IL-17 and IL-22 production at 2 weeks and 1 year postimmunization in BCG-vaccinated adults [38]. Whole blood samples from BCG-immunized infants produced less IL-13 and IL-17 upon *C. albicans* stimulation at 4 months postimmunization than samples from unvaccinated controls [40]. Increased fraction of IL-2-producing, proliferating $CD8^+$ *Bordetella pertussis*-specific T cells was found in BCG-vaccinated HIV-exposed uninfected South African infants compared with the control group [50]. This suggests that BCG may also regulate Th17, Th22 or cytotoxic T-lymphocyte subsets. It is not clear how BCG might exert this effect, but, persisting in an infant, it may prolong activation of the innate system and provide continuous cytokine signals for T-cell activation. Dendritic cells, innate lymphocytes and conventional T cells can make IL-22, while IL-1β or IL-6 can promote its secretion, mediating immune responses to respiratory pathogens and fungal infections [57], implying that trained immunity

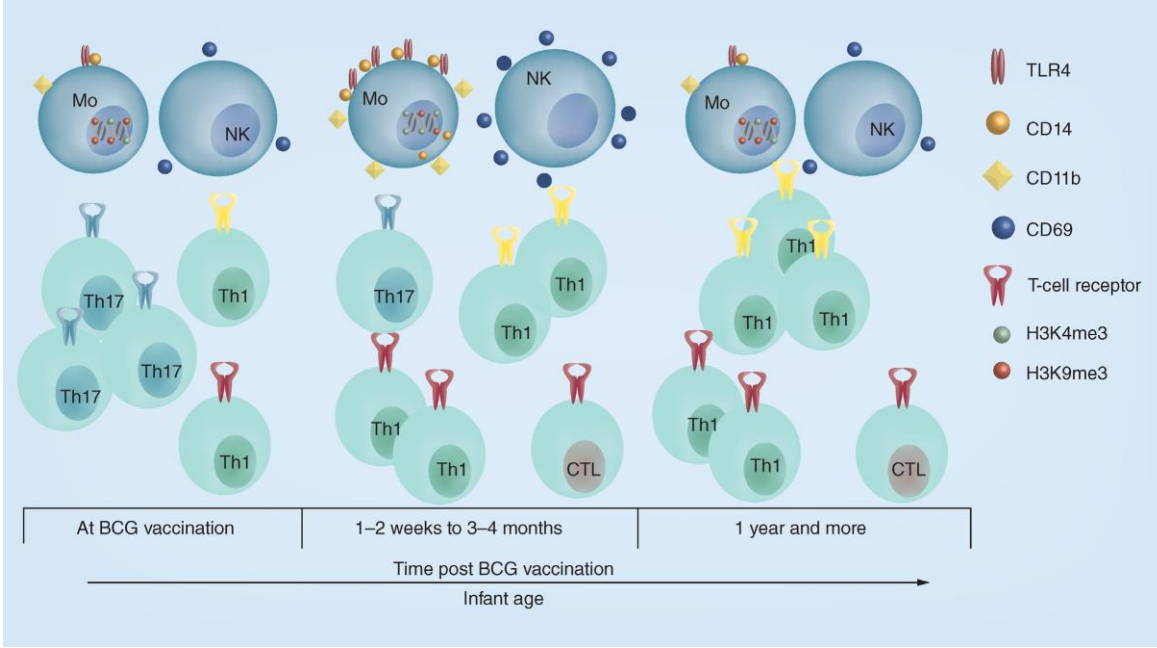


**Figure 2. A model of cell populations mediating BCG-vaccinated infant heterologous responses.** The diagram shows the innate and adaptive immune cells implicated in nonspecific infant protection and the likely timings for their involvement with respect to BCG vaccination and infant age. At of or immediately after BCG-vaccination, monocytes and NK cells of young infants are 'untrained', by low surface receptor expression or cytokine production. Once these cells become 'trained' by BCG, they increase surface receptor expression and inflammatory cytokine production and may cope with childhood infections more readily [30,36,40]. This effect diminishes over time, subsiding by 1 year postvaccination [38]. BCG, however, induces mycobacteria-specific Th1 or CTL responses [30,40]. BCG-supported heterologous T-cell responses may enhance trained innate immune responses from several weeks postimmunization and provide heterologous protection from childhood infections once trained innate immunity fades. The impact of BCG on heterologous B-cell responses is not yet clear, the current evidence being contradictive.

CTL: Cytotoxic T-cell; Mo: Monocyte; Th1: T-helper cell 1; Th17: T-helper cell 17. The role of other cells in trained immunity or heterologous adaptive responses is not well characterized yet and is therefore not presented.

and heterologous T-cell responses may complement one another mediating BCG-dependent heterologous infant protection from infections. Importantly, trained innate immunity wanes over time: PBMCs from BCG-vaccinated adults produce less TNF-α and IL-1β in response to *C. albicans* and LPS at 1 year postimmunization [38]. However, BCG-vaccinated infant protection from all-cause mortality extends for several years [4–7] or until adolescence [15]. Although improved neonatal or infancy survival can contribute to the long-term survival rates, the adaptive immune responses may take over heterologous infant protection from childhood infections once the trained immunity effect diminishes (Figure 2).

## Potential mechanisms beyond heterologous protection from infectious diseases & cancer

As well as reducing all-cause infant mortality, BCG may decrease the development of some cancers. A case-cohort study in Denmark suggested that BCG may reduce a risk of lymphoma [58]. Applied as a therapy against bladder cancer, BCG reduced patient mortality, tumor progression and recurrence for up to 10 years, however, this effect tended to decrease over time [59]. Infant protection from nonmycobacterial infections may share some mechanisms with BCG-dependent antitumor effects, with trained immunity implicated in BCG immunotherapy against bladder cancer [37]. Polymorphisms of autophagy gene *ATG2B* limited the ability of BCG-trained monocytes to improve IL-1β, IL-6 and TNF-α production upon heterologous stimulation *in vitro* and *in vivo* and correlated with increased tumor progression and recurrence in bladder cancer patients treated with intravesical BCG [37]. Cytokines promoted by innate training, for example, IL-1β, IL-6 and TNF-α were suggested to mediate the anticancer effects of BCG [59], implicating overlap between BCG-mediated nonspecific protection from infectious diseases and cancer. Increased frequencies of T helper cells and IL-2, IL-12, TNF-α, IFN-γ and IL-10 production may also mediate the anticancer

**Table 4. Influence of BCG on antibody responses to Expanded Program for Immunization vaccines.**

| Study | Vaccine schedule[†] | Age at observation | BCG vs Control | Ref. |
|---|---|---|---|---|
| The Gambia | BCG-Pasteur at birth<br>or<br>BCG at 2 months<br>HBV at birth, 2 & 4 months;<br>OPV at birth, 1, 2 & 3 months;<br>DTP at 2, 3 & 4 months control - BCG at 4.5 months | At birth, 2 & 4 months | ↑aHBs at 2 and 4.5 months of age in infants vaccinated with BCG at birth<br>↑aPV1 at 4.5 months of age in infants vaccinated at 2 months of age | [48] |
| Australia | BCG-Denmark, BCG-Japan or BCG-Russia at birth;<br>HBV at birth;<br>PCV-7, Infanrix Hexa[‡] & Rota Teq[§] at 2, 4 & 6 months;<br>control - no BCG | 4 weeks after the last immunization | ↑aPn against serotypes 9v & 18c trend for<br>↑aPn against serotype 6b<br>↓aHBs | [61] |
| South Africa | BCG-Denmark & OPV at birth;<br>TETRActHib, HBV & OPV at 6, 10 & 14 weeks;<br>MV at 9 months control - BCG at 14 weeks | 14, 24 and 52 weeks | No differences in levels of aHib, aPT, aTT and aHBs antibodies | [62] |
| The Gambia | OPV & HBV at birth;<br>BCG-Russia at 6 weeks of age;<br>Penta[¶], PCV-13 & OPV at 8, 12 & 16 weeks control - BCG at 18 weeks | 6, 7 & 18 weeks | No differences in levels of aPV1, aPV2, aHBs, aDP, aPT and aTT antibodies | [31] |
| Denmark | BCG-Denmark at 0-7 days;<br>DiTeKiPol/Act-Hib[#] & Prevenar 13 at 3, 5 & 12 months control - no BCG | 13 months | No differences in levels of IgG against aPT, aDP, aTT, aHib or aPn against serotypes 4, 6b, 9v, 14, 18c, 19f, 23f | [63] |

[†]Vaccination timings correspond to infant age at time of vaccination.
[‡]DTP-hepatitis B-inactivated polio virus-*Haemophilus influenzae* type b vaccine.
[§]Oral pentavalent rotavirus vaccine.
[¶]Penta – diphtheria-pertussis-tetanus-*H. influenzae b* – HBV.
[#]Due to DiTeKiPol/Act-Hib availability issues, 44 BCG-vaccinated and 51 control infants received Infanrix Hexa.
aDP: Antidiphtheria; aHBs: Anti-HBsAg; aHib: Anti-*H. influenzae*; aPV1: Antipoliovirus type 1; aPn: Antipneumococcal; aPT: Antipertussis; aTT: Antitetanus; DTP: Diphtheria-tetanus-pertussis vaccine; HBV: Hepatitis B vaccine; MV: Measles vaccine; OPV: Oral polio vaccine; PCV-7: 7-valent pneumococcal vaccine.

effects of BCG [58,59], suggesting the involvement of heterologous T-cell responses, similar to the observations on heterologous infant protection from infectious diseases (Table 3).

## Influence of BCG on humoral responses to nonmycobacterial stimuli

Few studies explored the impact of BCG vaccination on humoral responses to heterologous antigens, however, in adults, BCG was shown to boost antibody titres against influenza vaccine [60]. Infants given BCG at birth also had higher antibody levels to HBsAg and to polio antigens than infants whose BCG vaccination was delayed (Table 4) [48]. Elevated serum antibody concentrations to pneumococcal antigens were found in BCG-immunized Australian infants compared with the control group, although, contrary to the previous findings, lower anti-HBsAg IgG levels were detected in the BCG-vaccinated group [48,61]. This study also observed a trend for increased concentrations of IgG against *Haemophilus influenza* and tetanus toxoid antigens [61]. Although these studies suggest BCG may have nonspecific effects on antibody production to other childhood vaccines, other findings maintain the controversy over the influence of BCG on heterologous antibody titres or function. No differences in levels of antibodies to Expanded Program for Immunization (EPI) vaccine antigens were found at 12 weeks postimmunization in BCG-vaccinated Gambian infants compared with the controls [31]. Similarly, no differences in titres of antibodies against *H. influenza*, pertussis, tetanus and hepatitis B antigens were found in South African infants immunized at birth compared with the group in which BCG immunization was delayed [62]. A recent trial in Denmark found no association between the BCG vaccination status and antibody titres against other childhood vaccine antigens at 13 months of age [63]. Possibly, timing of BCG vaccination during early immune system development phases may influence its nonspecific effects on antibody responses, as BCG may have contributed to elevated antibody titres against *H. influenza,* pertussis and several pneumococcal antigens in infants vaccinated at 2–7 days postbirth [63]; however, the extent of this effect is not clear. Variation in BCG strains, EPI vaccines or immunization schedules applied in individual studies may also influence infant humoral responses. Reducing their impact in future studies may be necessary to establish whether BCG influences antibody responses to other EPI vaccines or childhood infections.

## Conclusion & future perspective

A large body of evidence suggests that BCG vaccination provides protection from diseases other than TB and that it may modulate the immune responses to other childhood vaccines. Several important implications for BCG and other vaccines that may exert similar beneficial heterologous effects arise from these findings.

First, the immunological mechanisms beyond heterologous infant protection from infectious diseases are not understood. Although BCG or other live vaccines, such as MMR vaccine may broadly enhance monocyte activation status and function or proinflammatory, Th1-polarizing responses [64], or modulate antibody responses [48,61], the data on immune mechanisms beyond heterologous infant protection from infections are inconsistent.

To overcome this, future immunological studies or randomized trials exploring the heterologous effects of BCG and their immunological mechanisms in infants may need to reduce variation in vaccination schedules or observation timings. Although this is difficult to conduct in real-life settings, it would allow for more comparability between the studies and their outcomes. In addition, despite the controversies, future studies need to address the issue of potential vaccine interactions to ensure optimal infant protection from infectious diseases.

Further work needs to address how BCG or other childhood immunizations regulate T- and B-cell subsets of diverse antigen specificities and which memory or effector cell fractions they maintain or promote to proliferate. In parallel, the role of the innate immune responses in mediating the nonspecific effects of BCG, MMR or other vaccines needs to be studied more extensively. It would also be interesting to test if other cell types could be involved in heterologous infant protection from all-cause mortality or infections. Together, this knowledge may be exploited for improving anti-TB and other childhood vaccine design.


### Financial & competing interests disclosure

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## Executive summary

**All-cause infant mortality reduction**

- BCG reduces all-cause infant mortality, unaccounted by reduction in mycobacterial infections alone.

**Impact on acquisition of infectious diseases**

- BCG may decrease the risk of nonmycobacterial sepsis and respiratory infections among the vaccinated infants.

**Factors potentially contributing to the heterologous BCG effect manifestation**

- Heterologous effects of BCG may be modified by interactions with other vaccines, such as diphtheria-tetanus-pertussis; however, the evidence for this is controversial.
- BCG can significantly decrease all-cause infant mortality and infectious disease acquisition in young infants; however, this effect can extend throughout childhood and adolescence.
- The sex of an infant may influence heterologous BCG protection from infectious diseases, with some studies reporting that female infants may benefit more from BCG-immunization; however, the evidence for such an effect is mixed.

**Mechanisms implicated in heterologous BCG-vaccinated infant protection**

- BCG may enhance maturation of infant Th1 responses and monocyte and NK cell ability to cope with a broad spectrum of pathogens for prolonged time periods.
- It may do so via innate immune training, a process characterized by metabolic, cytokine production and surface marker changes in monocytes and NK cells.
- During innate immune training with BCG, trained cells undergo a metabolic shift from oxidative phosphorylation to glycolysis.
- In parallel, changes in epigenetic regulation occur in BCG-trained cells, with accumulation of gene expression activating histone modifications accumulating at the promoters of genes encoding IL-1β, IL-6 and TNF-α, glycolytic pathway components and surface receptors.
- The evidence for the presence of immunological mechanisms associated with trained immunity in BCG-vaccinated infants is mixed, with some studies reporting enhanced NK cell activation, elevated TNF-α, IL-1β, IL-6, IFN-γ, EGF or PDGF-AB/BB production upon heterologous stimulation; however, other studies found no association between BCG immunization and heightened innate immune responses.
- Improved heterologous Th1-like responses, with increased TNF-α and IFN-γ production in response to Expanded Program for Immunization vaccine antigens were reported in multiple sites, including Uganda, Guinea-Bissau, The Gambia or Philippines.
- As BCG-vaccinated individual IL-1β and TNF-α production in response to innate immunity stimuli subsides by 1 year postimmunization, heterologous BCG-dependent T-cell activation can contribute to or maintain nonspecific BCG effects once trained immunity benefits wane.
- BCG may modulate infant humoral responses to other immunizations, elevating or decreasing antibody levels to such vaccines as hepatitis B or pneumococcal conjugate vaccines; however, further studies are needed to determine the extent of this effect.

**Conclusion & future perspective**

- Multiple epidemiological and immunological studies confirm that BCG exerts broad, beneficial effects in the vaccinated individuals protecting them from diseases other than TB.
- Trained immunity enhanced heterologous T-cell responses and, possibly, modulated antibody responses to other vaccines may mediate the nonspecific effects of BCG.
- Further work is needed to address the role of these factors on BCG and other childhood vaccine dependent heterologous effects and define the underlying mechanisms.

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| **Surname/Family Name** | Butkeviciute | | |
| **Thesis Title** | Studies on innate immune responses to Mycobacterium bovis BCG | | |
| **Primary Supervisor** | Prof Hazel M Dockrell | | |

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## SECTION B – Paper already published

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# Can what have we learnt about BCG vaccination in the last 20 years help us to design a better tuberculosis vaccine?

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abstract

The BCG vaccine will, in 2021, have been in use for 100 years. Much remains to be understood, including the reasons for its variable efficacy against pulmonary tuberculosis in adults. This review will discuss what has been learnt about the BCG vaccine in the last two decades, and whether this new information can be exploited to improve its efficacy, by enhancing its ability to induce either antigen-specific and/or non-specific effects. Many factors affect both the immunogenicity of BCG and its protective efficacy, highlighting the challenges of working with a live vaccine in man, but new insights may enable us to exploit better what BCG can do.



## 1. The BCG vaccine is capable of inducing protection against tuberculosis in some groups and settings

It is often stated that the BCG vaccine does not provide protection against tuberculosis. This is not true. The systematic review published by Mangtani et al. [1] included randomised controlled trials investigating whether BCG vaccination induced protection against tuberculosis. BCG vaccination is protective in some age groups and in some settings – in neonates against pulmonary and disseminated forms of tuberculosis, and at latitudes of 40° and above it gives better protection against pulmonary disease than in vaccinees living closer to the equator. In school age children protection was stronger if vaccination was restricted to those who were skin test negative to PPD using the Mantoux skin test [1]. These observations support the generally held view that exposure to other mycobacteria can reduce the protection induced with BCG vaccination through either masking the protection that BCG induces, or by blocking multiplication of the live BCG thereby preventing it from inducing protection [2], a consensus that has strengthened in the last 20 years. Overall, it is not correct to say that the BCG vaccine is unable to protect – as it can protect infants and young children against disseminated forms of TB, and adults against pulmonary TB in some circumstances [1]. Most of the world's children receive BCG vaccination with two-thirds of those countries giving BCG vaccination estimated to have >90% vaccine coverage [3]. BCG vaccination is recommended by the WHO to be given shortly after birth, however, when vaccine coverage is usually assessed at 1 year of age, in some settings many infants have been vaccinated later than the WHO recommends [4,5]. It is also clear that this wide vaccine coverage has been insufficient to control the spread of tuberculosis. Given that in 2019, there were 10 million individuals diagnosed with tuberculosis (TB) and 1.4 million deaths [6], we need an improved TB vaccine or vaccination regimen [7].

## 2. BCG-induced protective immunity can be long-lived – but this may depend on the type of immunity being measured

Another comment often made about the BCG vaccine is that it fails to induce long-lasting immunity, generally assumed to be mediated by classical T-cell memory. Again, this is not correct, as despite concerns about the induction of long-term immunological memory by BCG in mice, in certain settings, BCG can induce very long-lived protection in man – for as long as 50–60 years in Alaskan natives and American Indians [8]. Although many studies have not included longer term follow-up, the metanalysis by Abubakar et al. [9] identified one trial and four observational studies where protection lasted for 15 years or more. A recent retrospective population-based cohort analysis of BCG vaccination studies in Norway also found 58% protection against pulmonary tuberculosis 10–19 years after vaccination; however, this effect was diminished at later time-points [10].

Such longevity might be possible for antigen-specific immune responses as a result of antigen-specific T-cell memory but so far there is no evidence that non-specific protection as discussed below can last so long. The effects of non-specific trained immunity have so far only been shown to last for several months and

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to wane by 12 months after BCG vaccination [11], although longer effects might result from the epigenetic reprogramming of cells in the bone marrow [12]. Observational studies have suggested that longer-term non-specific effects can persist in individuals for more than a few years after vaccination; one Danish case-cohort study found that BCG vaccination was associated with protection against natural deaths (but not against accidental deaths, murders or suicides) for decades [13].

## 3. BCG vaccines are variable in composition

There is evidence that different strains of the BCG vaccine can induce varying degrees of T-cell immunity to mycobacterial or heterologous antigens. Infant BCG vaccination studies in Uganda, Nigeria, South Africa and Australia suggested that BCG Denmark may induce higher proportions than BCG Bulgaria or BCG Russia of single or multiple cytokine producing CD4+ T-cells responding to PPD, BCG or heterologous antigens and higher cytokine produc- tion by these cells [14–16]. In Australia, vaccination with BCG Japan outperformed both BCG Russia and BCG Denmark in terms of Th1 cytokine, IL-10, MCP-1 or MIP-1b production in response to mycobacterial antigens [16]. In Brazil, not only was the extent of cytokine production by healthy adult peripheral blood and umbilical cord mononuclear cells different in response to BCG Mor- eau, BCG Denmark and BCG Pasteur but also the rates of apoptosis: BCG Moreau induced the strongest cytokine production and the greatest degree of apoptosis [17]. Collectively, these studies sug- gest that different strains of BCG can induce differing classical immune responses.

There are some subtleties here though: any potential strain-specific antimycobacterial or heterologous effects of BCG might be susceptible to confounding, such as delivery route (discussed below) or the number of viable bacilli in the vaccine. *Mycobacterium bovis* BCG can be tricky to grow, even for experienced vaccine producers, and the proportion of live and dead bacilli can vary in different vaccine batches. This makes it hard to compare different BCG strains directly. Even if grown and prepared in exactly the same way, which not all BCG vaccines are, the rate of growth can also vary. A study by Biering-Sørensen et al. [18] showed that slower growing batches resulted in greater vaccina- tion site scarring and increased cytokine production in response to mycobacterial or heterologous stimuli. In another study, differ- ent strains of BCG were found to differ in proportions of viable bacilli and to induce divergent cytokine profiles in whole blood from newborns and adults [19]. Interestingly, the number of viable BCG bacilli in this study correlated with levels of GM-CSF, PDGF- AB/BB, IL-1b, TNFa and IFNc, cytokines known to have roles in antimycobacterial and trained immunity [19], suggesting that the viability of BCG might affect the degree of innate training. Indeed, gamma-irradiation of BCG decreased its ability to induce trained immunity and related cytokine production *in vitro,* although it did not abolish training completely [20].

Potential influences of BCG strains on heterologous downstream effects may be even more difficult to capture. While in normal birth weight infants from Guinea-Bissau BCG Denmark was associated with higher rates of scar formation compared to BCG Russia, there was no significant difference in rates of health consultations between infant groups vaccinated with these two BCG strains [21]. In another study in Guinea-Bissau, no significant differences in morbidity or mortality by 6 weeks of age were observed in newborns given BCG Russia, BCG Denmark and BCG Japan [22]. This suggests that if different strains of BCG affect the non-specific effects induced by BCG, the impact is likely to be limited, although more studies are needed.

In summary, whatever BCG does, live BCG usually does it better than dead bacilli – and this includes not only protection but induction of non-specific trained immunity. Despite these differences in immunogenicity and in composition, the different strains of BCG were not found to be associated with protection against TB in the Mangtani systematic review [1].

## 4. What has been learnt about the immunogenicity of BCG vaccination?

If the BCG vaccine is given to either adolescents or infants in the UK, strong T-cell responses to cross-reactive mycobacterial antigens such as PPD are induced. The last two decades have largely been the era of cytokines for measurement of immunogenicity, with a focus on the measurement of IFNc secretion.

Comparisons of different geographic settings in a series of trials in adolescents and young adults showed that whereas BCG vaccination induced protection against pulmonary tuberculosis in the UK, in Malawi it failed to induce any protection against tuberculosis (although it did induce some protection against leprosy) [23]. PPD stimulation of diluted whole blood samples from UK adolescents showed minimal IFNc production prior to BCG vaccination, and a marked increase that was greatest at 3 but that remained strong at 12 months following vaccination [24]. In contrast, in Malawi most adolescents and young adults were pre-sensitised to PPD before BCG vaccination and did not show significant increases in response following BCG vaccination. The ability to make a strong IFNc response in such assays is associated with changes in DNA methylation [25]. When South African infants progressing to a diagnosis of TB were stratified into groups of high, medium or low IFNc responders using an ELISPOT assay in which PBMC were stimulated with BCG, the high responders showed the slowest rate of progression to TB [26]. BCG vaccination of UK infants can induce polyfunctional T-cells making IFNc, TNFa and IL-2 [27], a cell type also attracting much interest as a possible correlate of protection, but in a cohort of South African infants there was no association of these responses with progression to TB disease [28]. T-cell responses are needed though, as shown by how susceptible those with HIV infection are to *M. tuberculosis* infection, or the rapidly progressive infections seen in SCID mice, as well as the increased mycobacterial growth in mice lacking the ability to produce or respond to IFNc; in mice and in man there are similar examples of genetic mutations in the IFNc-IL-12 axis resulting in susceptibility to mycobacterial disease [29]. IFNc provides valuable information about immunogenicity and may play a role in protection but measuring it alone has not delivered a confirmed correlate of protection. A number of other immunological components, such as various cell types, antibodies and cytokines have been proposed to be associated with protection against tuberculosis (Table 1), but confirmed correlates of protection are still needed.

## 5. Can measuring mycobacterial growth inhibition directly provide a better estimate of protection?

Mycobacterial growth inhibition (MGI) assays have recently been exploited to investigate the association between immunity and the ability to restrict mycobacterial growth following BCG vac- cination. They can also provide a system in which the contributions of various cells and cytokines can be dissected.

UK infants showed a marked induction of MGI following BCG vaccination [27]. In healthy adults, historical BCG vaccination was associated with improved mycobacterial growth inhibition *ex vivo* on its own or in the presence of isoniazid or rifampicin [30]. Interestingly, this study detected a possible association between NK cell frequency and inhibition of mycobacterial growth,

**Table 1**
Immunological components associated with protection against TB

| Immunological component | Association with protection against TB | Study | References |
|---|---|---|---|
| | ***Cellular components*** | | |
| BCG-responsive high-IFNc producing PBMCs | Lower risk of progression to TB disease | BCG-vaccinated infants | [26] |
| **CD4+IFNc+TNFa+IL-2+ T-cells** | Enhanced inhibition of BCG growth in MGIT | BCG-vaccinated infants | [27] |
| **Th17 cells** | | | |
| **CD4+ central memory T-cells** | Enhanced inhibition of BCG growth in MGIT | *M. tuberculosis* exposed uninfected individuals | [34] |
| | | BCG-vaccinated NHPs | [53] |
| **CD4+IFNc+TNFa+ T-cells** | Control of *M. tuberculosis* induced lung pathology at study week 6 | | |
| **CD4+ T-cells:** | Reduction of thoracic *M. tuberculosis* burden | BCG-vaccinated NHPs | [54] |
| **CD154+IFNc+IL-2+TNFa+** | | | |
| **CD154+IL-2+TNFa+** | | | |
| **CD8+ T-cells:** | | | |
| **IFNc+TNFa+IL-2+** | | | |
| **IFNc+TNFa+** | | | |
| **Peak CD4+ and CD8+ T-cell counts** | | | |
| **CD4+PD-1+KLRG1- T-cells** | Reduction of *M. tuberculosis* burden in lungs and spleen | BCG-vaccinated mice | [57] |
| **Epigenetically reprogrammed monocytes** | Reduction of *M. tuberculosis* burden in lungs, spleen and bone marrow | BCG-vaccinated and non-vaccinated murine parabiont and adoptive bone marrow transplant models | [12] |
| **NK cells** | Enhanced inhibition of BCG growth in MGIT | Historically BCG-vaccinated adults | [30,31] |
| **B-cells, CXCL10+ CD14$_{dim}$ monocytes** | Enhanced inhibition of BCG growth in MGIT | *M. tuberculosis* exposed uninfected individuals | [34] |
| | | BCG-vaccinated mice | [83] |
| **Neutrophils** | Reduction of *M. tuberculosis* burden in the lungs | | |
| | ***Soluble components*** | | |
| **IFNc** | Control of mycobacterial infection | Human and mice gene deficiencies | [29] |
| **CXCL9, CXCL10** | Enhanced inhibition of BCG growth in MGIT | *M. tuberculosis* exposed uninfected individuals | [34] |
| **IL-1b, TNFa, IL-6** | Elevated | | |
| **IL-10** | Reduction of pulmonary and extrathoracic *M. tuberculosis* burden | BCG-vaccinated NHPs | [59] |
| **aPPD-IgA** | | | |
| **aAg85A-IgG** | Lower risk of progression to TB disease | BCG-vaccinated infants | [26] |
| **aAM-IgG, aAM-, aLAM-IgM** | Enhanced survival of mice infected with *M. tuberculosis* | *M. tuberculosis* infection in mice | [103] |
| | | Exogenous LAM challenge in mice | |
| | Improved clearance of LAM from the circulation and spleen | Factory workers unexposed to TB | [104] |
| **a19-kDa-IgG** | Negative correlation between DTH responses to PPD and a19-kDa-IgG levels | | |
| **aAM-, aLAM-, aHBHA-, a16-kDa-a-crystalin-, and aMPB83-IgG, anti-mycobacterial IgG, IgA** | Reduced susceptibility to TB or progression to disease | Murine or NHP *M. tuberculosis* infection, passive serum or polyclonal IgG transfer, B-cell deficiency models, functional assays | [105] |

**Abbreviations:**
AM – arabinomannan
DTH – delayed-type hypersensitivity
HBHA – heparin binding hemagglutinin
MPB83 – mycobacterial cell surface lipoprotein
LAM – lipoarabinomannan
NHP – non-human primate

NB: *The list of studies or reviews presented in this table is not comprehensive.*

with a tendency for higher proportions of NK cells in BCG-vaccinated individuals. Further analyses showed that while overall and cytotoxic NK cell frequencies were associated with *ex vivo* inhibition of mycobacterial growth in BCG-naïve individuals, cytokine-producing NK cell responses correlated with control of mycobacterial growth in BCG-vaccinated individuals [31]. BCG vaccination has been shown to enhance NK cell activation and cytokine production in response to mycobacterial or heterologous stimuli in infants and adults, an effect that lasted 3 to 4 months [32,33]. BCG also protected SCID mice from lethal *Candida* infection with a partial role demonstrated for NK cells [33].

Activated monocytes or macrophages are considered protective against TB and their efficiency in containing mycobacterial infections has also been explored in mycobacterial growth inhibition assays. A study by Joosten et al. found that enhanced secretion of CXCL9 and CXCL10 by non-classical monocytes was associated with greater mycobacterial growth inhibition in individuals who were exposed to TB but not infected, compared to TB patients or healthy controls, although central memory T-cell responses and B-cell frequencies were also associated with control of mycobacterial growth [34]. PBMCs from those exposed to TB also showed some features consistent with innate immune training, e.g. elevation of innate immune cytokines IL-1b, TNFa or IL-6 in response to BCG stimulation and higher CXCL10 production in response to heterologous stimuli, although monocyte TNFa was not associated with improved mycobacterial growth inhibition.

## 6. Other explanations for variable responses to BCG

The complexity of measuring such vaccine-induced immune responses in the "real world" is very considerable. Immune status

is affected by our environment, health, nutrition, microbiome, age, and more [35]. Marked differences in the IFNc and broader cytokine responses following *in vitro* stimulation of diluted whole blood with PPD were observed between Malawian and UK infants who were BCG vaccinated 3 or 12 months previously [36,37]. A study of cytokine responses in diluted whole blood cultures stimulated with PPD for 6 days in Ugandan infants given BCG at birth showed the development of immune responses that peaked 4–10 weeks post vaccination, but with considerable individual variation, and some infants failed to make a detectable cytokine response [38]. The literature on how delaying BCG vaccination affects the immunogenicity of BCG vaccination has not shown any consistent improvement with delayed vaccination [39]. The genetics of the vaccinees, other vaccines they are given, nutrition, seasonality and more, will influence these responses. However, certain additional factors have received more attention in the last decade.

## 7. Maternal influences on the response to vaccination in their infants

Newborn infants receiving BCG vaccination should be immunologically naïve, and any confounding effects of environmental or non-tuberculous mycobacteria should not be present. However, young infants may have an immature immune system that has been influenced by their mothers' immune or infection status [40,41]. For example, latent TB infection (LTBI) in a woman might influence how her baby responds to BCG vaccination. In Uganda, cytokine responses in BCG-vaccinated infants showed no associa- tion with the mothers' LTBI status [38]. This is perhaps surprising, as mycobacterial antigens might have crossed the placenta and induced either sensitisation or tolerance in the infant. Other com- mon infections, such as malaria or other parasitic infections in the mothers during pregnancy can have broad immunomodulatory effects on the immune system of the newborns/infants. Although helminth infections in the mother had limited effects on the response to BCG in Uganda [42], viral infections such as CMV alter CD8 T-cells and rate of progression to TB in infants [26,43].

The BCG vaccination status of the mother may also have some effects on Th2 cytokine responses in BCG vaccinated infants to *M. tuberculosis* culture filtrate proteins or cord blood IL-10, IFNc or immune cell growth factor responses to innate stimuli [42,44]. A possible beneficial association of previous maternal immunisation with BCG and lower rates of parent-reported infections was found in infants at 0–3 months of age in the Danish BCG study [45], and maternal BCG scar was also associated with lower infant mortality risk in Guinea-Bissau [46]. Whether this reflects an as yet unknown biological mechanism or was associated with confounding health-care practices within a family is not clear.

## 8. Would BCG be more protective if given by another route?

Although when first used in 1921 the BCG vaccine was given orally, it is now given intradermally. One area of recent and active research is whether BCG (and other novel TB vaccines) might be more protective if given by routes other than the standard intradermal route. Intradermal vaccination is tricky and well-trained staff are needed to give intradermal vaccines such as BCG. Some countries have therefore used a multipuncture device to deliver BCG; this has the added benefit of reducing scarring which in some cul- tures is regarded by parents/guardians as of major importance. For example, in Japan and South Korea BCG Japan has been deliv- ered percutaneously with a multi-puncture device, but skin test responses and IFNc responses to PPD in Korean children aged 4– 7 years given BCG Pasteur intradermally or BCG Tokyo by multi-puncture device were comparable [47]. A larger trial of BCG given percutaneously or intradermally in South Africa found that there was no difference in the protective efficacy of BCG given by these routes [48,49]. A strength of this South African study was that the same BCG strain (Japan 172) was given by both routes. Presence of a BCG scar is often used as a proxy for BCG vaccination history, although scars can disappear over time and not all of those vaccinated develop a scar. Both the presence and the size of BCG scar in children that have received BCG vaccination has been associated with improved survival indicating non-specific protection [50,51].

Studies in animal models have shown that BCG can be more protective if given intravenously rather than by other routes. Early studies in which BCG was given to non-human primates (NHPs) were published as long ago as 1970 [52] but there has been a recent revival of interest in giving BCG by this route. Vaccinating Rhesus macaques by the intravenous route induced better protection than giving BCG intradermally, or intradermally with boosting by intratracheal administration [53]. A larger study in Rhesus macaques used positron emission tomography–computed tomography (PET–CT) imaging with $^{18}$F-fluorodeoxyglucose, confirming the improved protection given by intravenous BCG, and showed that 9/10 animals given BCG intravenously failed to show any lung lesions [54]. In another study, giving BCG to mice intravenously was shown to alter the differentiation of haematopoietic stem cells, promoting myelopoiesis and enhancing the activation status of bone marrow-derived macrophages [12]. In addition, compared to subcutaneous immunization, BCG delivered intravenously could be detected in the bone marrow for 7 months after BCG vaccination, suggesting prolonged interaction with the immune system. However, giving BCG intravenously in man is not likely to be practical and could induce adverse events including disseminated disease in immunosuppressed individuals.

Alternative delivery routes delivering BCG directly into the mucosa or lungs may also be worth exploring [55]. Compared to intravenous immunization, aerosol vaccination with BCG gave less bacterial dissemination and reduced bacterial counts in the lungs [56]. In mice, intranasal BCG induced better protection in the lungs than the standard intradermal vaccination, with induction of antigen-specific tissue-resident T-cells expressing a PD-1$^{+}$ KLRG1$^{-}$ cell-surface phenotype [57]. BCG can also induce protection in mice when given by the sublingual route [58]. In NHPs, mucosal delivery was associated with improved Th17 cell and IL-10 responses, slower IGRA conversion, lower pathology in the lungs and better control of *M. tuberculosis* growth in the lungs or lymphoid tissues compared to the intradermal route [59]. We still need a better understanding of how to maximise beneficial immune responses in the lungs while avoiding excessive immune activation.

## 9. Do different routes of administration also affect induction of innate training?

BCG vaccination can also induce non-specific protection against unrelated pathogens [60,61] and reduce all-cause mortality in infants [62–66]. Importantly, this vaccine can induce a phe- nomenon known as "trained immunity", resulting in epigenetic or metabolic reprogramming of the innate immune cells and enhanced surface marker expression or cytokine responses upon secondary stimulation [33,67,68], suggesting that this mechanism can contribute to the non-specific effects of BCG and protection against infectious diseases [69]. Adults vaccinated with BCG and then given yellow fever vaccine were shown to have reduced virae- mia compared to BCG-naïve controls and this effect was associated with enhanced IL-1b production [70]. Infant BCG vaccination stud- ies in Guinea-Bissau and the UK, as well as Australia demonstrated that production of cytokines associated with innate immunity was

enhanced in BCG vaccinated infants compared to unvaccinated controls upon secondary stimulation with heterologous stimuli [32,71–73]. This phenomenon is not restricted to BCG alone as another live mycobacterial vaccine – MTBVAC has been shown to enhance innate cytokine responses to LPS in human monocytes and in mice to improve resistance to an otherwise lethal infection with *S. pneumoniae* [74].

BCG revaccination was able to increase reversion of interferon-gamma release assay (IGRA) positivity in South African adolescents who had been BCG vaccinated at birth [75], which has led to renewed interest in giving a repeat BCG vaccination. In children, two randomised trials have provided some evidence that a repeat BCG vaccination may reduce all-cause mortality [76]; for example, in infants in Guinea Bissau who had received their diptheria, pertussis and tetanus (DPT) booster before their BCG revaccination at 19 months, there was some evidence of a reduction in mortality between 19 and 60 months [77]. In the South African H4/BCG trial, it was observed that the BCG revaccinated group had a lower rate of upper respiratory tract infections than in either the H4:IC31 group or the placebo group [75]. In an Indonesian study in which BCG was given monthly for 3 months to elderly individuals, the prevalence of acute respiratory infections was reduced [78]. These studies indicate that revaccination or boosting as well as primary vaccination with BCG may be able to induce or enhance innate memory with beneficial effects on survival; similar effects have also been observed with other live attenuated vaccines such as smallpox or oral polio vaccine [76].

The route of BCG administration can affect mycobacteria- specific immune responses and efficacy of the BCG vaccine. How- ever, can different routes of BCG delivery affect the extent of innate immune training? So far, most studies of BCG-dependent innate immune training in humans have used intradermal BCG vaccina- tion. However, recent exposure to tuberculosis has also been asso- ciated with innate immune training, suggesting that aerosol or mucosal interaction with mycobacteria can imprint innate immune responses [34]. Immunising calves with aerosolised BCG was associated with induction of trained immunity in PBMCs, although cytokine production by alveolar macrophages was not affected [79]. In humans, alveolar macrophages expressed lower levels of activation markers CD11b and HLA-DR after intradermal immunisation with BCG, although this study did not examine BCG-dependent changes in alveolar macrophage cytokine responses [80]. It is possible that induction of trained immunity in the lungs might be regulated or contributed to by adaptive immune cells, as adenovirus-dependent priming of alveolar macro- phages in mice was found to be dependent on IFNc produced by CD8+ T-cells in a model of *S. pneumoniae* infection [81].

Is this different if the BCG vaccine is delivered by other routes? In mice, intravenous delivery of BCG induced stronger haematopoietic cell expansion and differentiation compared to subcutaneously injected vaccine and was capable of priming bone marrow derived macrophages (BMDMs), enhancing their ability to control *M. tuberculosis* growth *in vitro* [12]. Intradermal BCG vaccination of humans also polarised haematopoietic stem cell differentiation into myeloid cells [82], suggesting that some BCG associated changes in the innate immune system can occur irrespective of the delivery route.

In another study, mice were vaccinated with BCG subcutaneously and their ability to control growth of *M. tuberculosis* in the lungs was compared with other routes of immunisation: intravenous, intranasal, aerosol or intramuscular [83]. A protective effect of similar extent on mycobacterial growth in the lungs was found for most immunisation routes despite varying colony form- ing units of BCG, except for low-dose aerosolised BCG which did not induce protection. In subcutaneously vaccinated mice, the pro- tection against *M. tuberculosis* growth in the lung was independent of T-cell responses, suggesting that BCG mediated protection via innate immune cells [83]. Interestingly, depletion of neutrophils in this model was associated with diminished protection by BCG [83], supporting findings in humans, where intradermal BCG was associated with a neutrophil transcriptional signature and elevated neutrophil counts in BCG-vaccinated infants [82].

There also seem to be differences in how BCG, delivered via the skin, affects the innate immune cells. In humans, intradermal BCG vaccination induced a trained phenotype in monocytes in NOD2 dependent manner, enhancing accessibility of proinflammatory genes for transcription and cytokine production upon secondary stimulation with mycobacterial or heterologous antigens [67,82], with similar changes happening in the NK cells [33] and NK cell cytokine responses associated with inhibition of mycobacterial growth years in these historically vaccinated individuals [31]. However, control of *M. tuberculosis* growth in lungs of subcutaneously BCG-vaccinated mice was not mediated by NOD2 dependent pathways, monocytes or NK cells [83]. Further investigation would be required to clarify whether these differences reflect the influence of route of vaccine delivery or differences in human and murine trained innate responses, as differences in regulation of trained immunity by long non-coding RNAs in human and mur- ine models have been reported previously [84].

## 10. How can what we have learnt about BCG accelerate the development of new TB vaccines?

There is a pipeline of candidate TB vaccines in development, of varying types. Some are recombinant BCG vaccines, designed to be safer in infants who are HIV infected, or to induce improved protection by inclusion of additional antigens from *M. tuberculosis*. Some are other live mycobacterial vaccines, including *M. tuberculosis* itself with mutations that reduce its virulence, or environmental non-tuberculous mycobacteria. It is likely that any issues that affect growth of BCG bacilli in a BCG vaccine, will similarly affect the growth of another live mycobacterial vaccine. Other vaccine candidates include subunit or recombinant proteins in adjuvant, which would be given as a booster vaccine following BCG vaccination, that would depend on BCG vaccination having induced an effective primary immune response. Similarly, the vaccines that consist of viral vectors that deliver one or more antigens, are usually intended to boost a pre-existing immune response rather than induce a primary immune response. The TB vaccine portfolio is therefore very dependent on what BCG vaccination does or does not do. It may also be beneficial if primary vaccination (for exam- ple with a live mycobacterial vaccine) can induce non-specific innate training.

One surprising result from a recent vaccine trial of the subunit H4 vaccine to prevent infection rather than disease, was that repeat BCG given as the control arm, was more effective at inducing reversion to IGRA-negative status than the subunit vaccine (although neither vaccine provided significant protection against IGRA conversion, taken as indication of *M. tuberculosis* infection) [75]. This was surprising because in a number of earlier studies performed in different settings such as Malawi [23] or Brazil [85], there was no improvement seen with a repeat BCG vaccina- tion although with data from a longer-term follow-up of the BCG-REVAC trial there was some evidence that repeat BCG could be protective in an area of Brazil with low prevalence of non-tuberculous mycobacteria [86].

## 11. Could inducing greater innate training improve the protection given by BCG or these new vaccines?

Trained immunity has been associated with protection against heterologous infections and has been implicated in the non-

specific effects of BCG. However, improving innate immunity may also be able to enhance the protective efficacy of new TB vaccines [87,88]. BCG could be detected in the bone marrow 7 months after BCG vaccination and where it could reprogram haematopoietic stem cells (HPSCs) inducing their differentiation into epigenetically primed myeloid cells capable of reducing growth of *M. tuberculosis* [12]. In another mouse immunisation model, BCG vaccination induced protection against *M. tuberculosis* in a neutrophil-dependent manner [83]. Of interest, BCG vaccination of human adults induced transcriptional signatures consistent with myeloid cell differentiation or neutrophil responses, epigenetically imprinting both the HPSCs and CD14+ cells [82]. Not only BCG, but the recombinant *M. tuberculosis* vaccine, MTBVAC, has also been shown to induce trained immunity *in vitro*, resulting in elevated production of IL-1b, TNFa or IL-6 upon secondary stimulation [74]. BCG-dependent enhancement of these cytokines could be exploited, as the cytokines could act as adjuvants to induce improved Th1 or Th17 responses that are considered protective against TB. Mycobacterial component-based vaccines, such as RUTI have also been shown to improve inhibition of mycobacterial growth *ex vivo* in association with phenotypic changes in monocytes from vaccinated mice [89]. Metabolites, such as fumarate, or the fungal component b-glucan, can also induce innate immune training *in vivo* [90–92]. BCG and b-glucan can induce features of trained immunity in cells from both neonates and adults [93], suggesting that microbial components, and metabolites might be exploited in combination with BCG or other anti-TB vaccines to enhance innate immunity and possibly protective T-cell responses not only in adults, but also in neonates, the main target group for immunisation against TB.

It may also be necessary to optimise vaccine regimens to maximise innate training. Just as for adaptive T-cell (and antibody) responses, BCG-dependent innate training (or that induced by other live vaccines such as MTBVAC [74] may be susceptible to external factors, resulting in variability. In healthy adults, circadian rhythms have been shown to modulate both heterologous and mycobacteria-specific cytokine production, with individuals administered BCG vaccine in the morning showing higher differences from baseline at 2 weeks or 3 months post-immunisation than individuals vaccinated in the evening [94]. What is learnt from BCG may help in the design of better vaccination strategies for both tuberculosis and other diseases [95].

It is also possible that trained innate or heterologous effects of BCG might be sex-specific, enhancing some immune responses more in males than in females or vice versa [96,97]. It should be noted that such effects are subtle and often result in trends rather than large-scale effects on all-cause morbidity or mortality [98]. While neither the systematic review by the WHO SAGE committee in 2014, nor its update in 2016 found sex-differential effects on all- cause mortality in BCG-vaccinated infants [63,64], some recent studies showed that beneficial effects of BCG on all-cause mortality can be observed at different time points since vaccination in males and females [65].

Finally, it is not fully clear for how long the effects of trained immunity last. Some studies in healthy adults showed that enhancement of cytokine production is transient and unlikely to last beyond a few months after vaccination [11], although as noted above some longer term protection may be induced [13]. However, while variability and lack of longevity of trained immunity might limit prime-boost strategies, vaccines, compounds and metabolites inducing this phenomenon could still be exploited as adjuvants. This rationale underpins new trials of BCG vaccination as an interim protective measure against COVID-19, for example in front-line healthcare workers [99,100].

## 12. Conclusions

The BCG vaccine has been used for almost one hundred years but we still have a lot to understand about it [101]. BCG vaccina- tion can induce long-lasting protection against tuberculosis, and induces T-cell responses, but there is considerable variability in individual responses to vaccination between and within different settings, which may result in both BCG itself and other factors affecting responses to vaccination [102]. Measuring growth inhibi- tion of BCG or *M. tuberculosis* itself may be more informative but we still lack proven correlates of protection. New routes of administration are being investigated such as giving BCG intravenously or by aerosol. BCG revaccination is also attracting interest. We need to understand better what BCG does and does not do, in order to develop more effective vaccination regimes to protect against tuberculosis, using either BCG, a modified BCG vaccine, or a new TB vaccine. We also need to investigate whether increasing innate training might enhance the efficacy of BCG vaccination. Finally, when developing new vaccines, we need to avoid the loss of any beneficial non-specific protective effects that BCG vaccination provides to infants.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.01.068.

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