

INVESTIGATING THE ROLE OF INNATE IMMUNITY IN MEDIATING THE NON-SPECIFIC EFFECTS OF BACILLUS CALMETTE-GUÉRIN VACCINE

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ABSTRACT

Background

Epidemiological evidence suggests that Bacillus Calmette-Guérin (BCG) vaccine exerts non-specific (heterologous) effects in infants; decreasing neonatal mortality in high-mortality settings and preventing allergy and morbidity from infection in developed countries. New tuberculosis (TB) vaccines could potentially lack these beneficial effects. Immune mechanisms underlying the non-specific effects of BCG vaccine have been linked to 'trained immunity' or innate immune memory.

Aims

Part 1: To investigate whether neonatal BCG vaccination alters the immune response to heterologous pathogens and Toll-like receptor (TLR) ligands in (i) the neonatal period and (ii) infancy. Part 2: To use a systems vaccinology approach to identify innate immune signatures underlying the non-specific effects of BCG vaccine.

Methods

Part 1: In the Melbourne Infant Study: BCG for allergy and infection reduction (MIS BAIR), 1272 infants were randomised to receive BCG vaccine or no BCG vaccine within the first 10 days of life. A subset of participants was recruited to an immunological sub-study. A whole blood stimulation assay of 'specific' mycobacterial antigens, heterologous bacterial or fungal antigens and TLR ligands was used to interrogate cytokine responses at 7 days (n=212) and 7 months (n=167) post randomisation.

Part 2: In the BabyBAIR study, 44 infants who were BCG vaccinated prior to travel to a TB-endemic area were recruited. Blood was collected from participants prior to BCG vaccination, and at 7 days and 3 months post vaccination. Cytokine responses and cell populations were analysed following *in vitro* stimulation of whole blood as above. RNASeq was also done on whole blood and the transcriptome was analysed for differentially expressed genes. Pathway analysis was done using functional gene set enrichment analysis (fGSEA) at each time point compared to baseline.

Results

In the MIS BAIR study, infants who were BCG-vaccinated had significant differences in their heterologous cytokine responses at both 7 days and 7 months post randomisation compared to BCG-naïve infants. At 7 days post randomisation, compared to BCG-naïve neonates, BCG-vaccinated neonates had evidence of a pro-inflammatory bias in RPMI-stimulated (nil) samples. Following heterologous stimulation, BCG-vaccinated neonates had decreased chemokine (MCP-1, MIP-1 α , MIP-1 β), IL-1RA, IL-6 and IL-10 responses following stimulation of TLR2 (PEPG) and TLR7/8 (R848). At 7 months post randomisation, compared to BCG-naïve infants, BCG-vaccinated infants, had decreased IFN- γ responses to stimulation with heterologous pathogens. Decreased IFN- γ responses in the BCG-vaccinated group were attributable to a reduction in the proportion of individuals mounting an IFN- γ response. Heterologous cytokine responses were modified by sex and maternal BCG vaccination status in both neonates and older infants.

In the BabyBAIR study, longitudinal cytokine analysis showed BCG vaccination to be associated with a pro-inflammatory bias at baseline (prior to BCG vaccination), a robust pro-inflammatory heterologous response at 7 days post BCG vaccination and downregulation of pro-inflammatory cytokines 3 months post BCG vaccination compared to baseline. Flow cytometry suggested that both myeloid and monocyte derived dendritic cells were associated with the observed heterologous cytokine responses. RNASeq analysis of the whole blood transcriptome following BCG vaccination indicated widespread changes in innate immune signalling pathways and identified several potential mechanisms by which BCG vaccine could mediate its beneficial heterologous effects.

Conclusions

Neonatal BCG vaccination leads to significant changes in the immune phenotype of vaccinated individuals. Following *in vitro* stimulation with heterologous pathogens, BCG-vaccinated infants have altered immune responses which might improve regulation of the inflammatory response during acute infection and a subsequent

reduction in all-cause mortality. These results support the paradigm of BCG-induced trained immunity and provide additional information regarding the nature of the response in neonates and between different classes of pathogens.

DECLARATION

I, Bridget Freyne, certify that:

- This thesis comprises my original work except where indicated in the preface.
- Due acknowledgement has been made in the text to all other material used.
- The thesis is fewer than 100,000 words in length exclusive of tables, appendices and bibliography.

Signed: 

Date: 27th July 2019

PREFACE

The opportunity for my PhD candidature arose from the Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR). MIS BAIR is a randomised controlled trial of BCG vaccine or no BCG vaccine at birth on the development of atopic sensitisation, eczema, asthma and infection at one and five years of age. MIS BAIR recruited 1272 newborn infants at the Mercy Hospitals for Women, Heidelberg and Werribee and Geelong Hospital. Recruitment was completed in September 2016 and the results of the clinical outcomes will be published in 2019. The MIS BAIR trial and immunological sub-study was funded by two NHMRC project grants. The BabyBAIR study was supported by a Murdoch Children's Research Institute (MCRI) theme grant. My PhD candidature was supported by a Human Rights Scholarship from the University of Melbourne and a postgraduate fellowship awarded by the European Society of Paediatric Infectious Diseases (ESPID). This thesis includes four papers published in peer-reviewed journals and one publication in preparation.

A section of chapter 1: 'The non-specific effects of BCG Vaccine - clinical context' is adapted from the paper "**Freyne B**, Curtis N Archimedes: Neonatal BCG vaccination for the prevention of childhood allergy. *Arch Dis Child*. 2014 Feb;99(2):182-4". The PhD candidate contributed substantially to this paper having conceived the design of the literature review with Prof Nigel Curtis, completed the review, interpreted the data, wrote the first draft of the manuscript and prepared it for submission. The co-author contributed to the interpretation and editing of the manuscript.

Two sections of chapter 2: 'BCG vaccine & heterologous immunity' are adapted from a pair of peer-reviewed publications of which the PhD candidate was first author; "**Freyne B**, Marchant A, Curtis N, BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms. *Trans R Soc Trop Med Hyg*. 2015 Jan;109(1):46-51." and "**Freyne B**, Marchant A, Curtis N, BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases. *Trans R Soc Trop Med Hyg*. 2015 Jan;109(1):52-61." The PhD candidate contributed substantially to these papers having designed and completed the reviews, interpreted the data and written the first drafts of the

manuscripts. The co-authors assisted with the interpretation of the data and editing of the manuscripts.

The results from chapter 3 have been published in the peer-reviewed paper "**Freyne B,** Donath S, Germano S, Gardiner K, Casalaz D, Robins-Brown RM, Amenyogbe N, Messina N, Netea M, Flanagan K, Kollmann T, Curtis N. Neonatal BCG vaccination influences cytokine responses to Toll-like receptor ligands and heterologous antigens. *J Infect Dis.* 2018 May;217(11):1798-1808. Under the supervision of Prof Nigel Curtis, Dr Christel Zufferey and Ms Susie Germano, The PhD candidate developed the optimisation protocols and did the optimisation experiments during the development of the whole blood assay and was trained in laboratory protocol design, whole blood assay methods and luminex cytokine analysis by Dr Christel Zufferey and Ms Susie Germano. The PhD candidate reviewed the literature on BCG vaccine-associated cytokine responses and Toll-like receptor ligand interactions which, along with the results of the optimisation experiments and discussion with collaborators (Prof Toby Kollmann, Prof Katie Flanagan) was used to inform the final cytokine / stimulant choices in this and subsequent chapters. Under the supervision of Ms Susie Germano, the PhD candidate cultured the bacteria and fungus which was used to make the stock concentrations used in all final assays and was trained in the use of the VITEK automated bacterial identification instrument by Ms Susie Germano. The PhD candidate was trained in pneumococcal serotyping by Ms Monica Cooper (MCRI) and serotyped the pneumococcal isolates used in the whole blood assay. The PhD candidate visited participants at their home to collect the blood samples along with midwives Ms Nathalie Papagiorcopulo, Ms Lea Renihan and Ms Judith Spotswood and provided a paediatric review at the time of the home visit and provided a call-in Paediatric Advice Line for participants in the study. Ms Susie Germano and Ms Snezana Spillane did the majority of the whole blood stimulations and luminex analysis. Together with Dr Nicole Messina and Ms Susie Germano, The PhD candidate was responsible for the quality control of the cytokine data, data cleaning and development of the final dataset. Under the supervision of A/Prof Susan Donath, the PhD candidate did the statistical analysis, developed the figures and prepared the first draft of the manuscript together with Prof Nigel Curtis, and Dr Nicole Messina. All co-

authors commented on the manuscript and final submitted manuscript was authored by the PhD candidate.

The results from chapter 4 have been published in the peer-reviewed paper “**Freyne B**, Messina, Donath S Germano S, Bonnici R, Gardiner K, Casaz D, Robins-Browne RM, Netea MG, Flanagan KL, Kollmann T, Curtis N and the MIS BAIR group, Neonatal BCG vaccination reduces interferon-gamma responsiveness to heterologous pathogens in infants from a randomised controlled trial ”, J Infect Dis. 2020 Jan 28. pii: jiaa030. doi: 10.1093/infdis/jiaa030. [Epub ahead of print]. The PhD candidate contributed substantially to this paper having been involved in the design of the whole blood assay and statistical analysis plan as outlined in the previous chapter. For this part of the project, the participants visited the Mercy Hospital in Heidelberg for blood collection, which was done by Ms Judith Spotswood. Ms Susie Germano, Ms Snezana Spillane and Ms Rhian Bonnici did the whole blood stimulations and luminex analysis. The PhD candidate was responsible for data cleaning, development of the final dataset, the statistical analysis, development of the figures and preparation of the first draft of the manuscript under the supervision of Prof Nigel Curtis, A/Prof Susan Donath and Dr Nicole Messina. All co-authors commented on the manuscript and the final submitted manuscript was authored by the PhD candidate.

The PhD candidate contributed substantially to the results presented in chapter 5. The study was conceived and the protocol developed by the PhD candidate under the supervision of Prof Nigel Curtis. Funding for the RNASeq analysis came from a successful grant application (MCRI theme grant) on which the PhD candidate was associate investigator. The PhD candidate managed all ethics and regulatory considerations for this study. The laboratory standard operating procedure (SOP) was developed by the PhD candidate with assistance from Ms Susie Germano and Dr David Martino. The PhD candidate oversaw all elements of recruitment and follow-up and was assisted in these procedures by Drs Ben Forbes and Rae Gordon, as well as the staff of the Royal Children’s Hospital Infectious Diseases out-patient service and Immunisation clinic. Blood samples were collected by the PhD candidate, Drs Ben Forbes and Rae Gordon or Judith Spotswood at the Royal Children’s Hospital or in

participants' homes. The whole blood stimulation assays were done by the PhD candidate, Dr Rae Gordon, Ms Susie Germano, Ms Rhiann Bonnici, Mr Pedro Ramos and Ms Snezana Spillane. Dr Ben Forbes did the flow cytometry and associated analysis and presented these results as a dissertation for his 'Scholarly selective'. Ms Susie Germano and Ms Rhiann Bonnici did the luminex and ELISA analyses. With Dr Nicole Messina, the PhD candidate optimised the protocol for RNA extraction and subsequently extracted and quality checked all RNA samples under the supervision of Dr Nicole Messina and with the assistance of Dr David Martino. The RNASeq analysis was done at the Victorian Clinical Genetic Service (VCGS) and the bioinformatics analysis was done in collaboration with A/Prof Helder Nakaya and Dr Frederico Ferreira at the University of Sao Paulo. The methodology for the analysis of RNASeq data was kindly provided by A/Prof Helder Nakaya. The cytokine data was cleaned and analysed under the supervision of A/Prof Susan Donath. Hierarchical clustering of the cytokine data was done in collaboration with Dr Michael Poidinger (MCRI). The PhD candidate did the unsupervised analysis of the RNASeq data and provided biologic interpretation of the results which were used to inform subsequent rounds of analysis and the interpretation of the findings presented in chapter 5.

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Since enrolling in my PhD, I have gotten married to my wonderfully long-suffering husband Richard, become a mother to two amazing daughters, Grace and Winnie, and have lost my original academic inspiration, my father, Prof Sean Freyne. Combining clinical training, these life events and a PhD candidature led to many challenging moments. The submission of this thesis is only possible because of the love and support I received from my husband, my mother Gail Grossman Freyne, my sister Sarah Freyne and a group of friends, which although occasionally distant were always available. In addition, this thesis would not have been possible without the unprecedented support, encouragement and care I received from supervisors and colleagues. In this regard, I have to acknowledge particularly Nigel Curtis, Kaya Gardiner, Nicole Messina, Christel Zufferey Susie Germano, Susan Donath, Rhiann Bonnici, Penelope Bryant and Andrew Steer. Finally, there was a small army of people involved in the MIS BAIR study clinical and laboratory teams, without whom this PhD would not have been possible and whose contributions are described in the Preface and relevant publications.

The content of this thesis was much improved through the input of my advisory committee, which included Paul Monagle, Susan Donath, Andy Giraud and Pierre Smeesters. There were many colleagues on the 5th Floor of the Murdoch Children's Research Institute who were generous with their time, knowledge and support at various stages of this PhD including Frances Oppesidiano, Eileen Dunne, Monica Cooper, Ciara Baker, Kristy Azzopardi, Paul Licciardi and David Martino.

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not least, I would like to thank Pamela Morrison for being a calm, collected and caring presence and for always knowing where Nigel was.

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LIST OF ABBREVIATIONS

ARI	acute respiratory infection
BCG	Bacille Calmette-Guérin
CBMC	Cord blood mononuclear cells
CFU	colony forming units
DC	dendritic Cell
DEGS	differentially expressed genes
DTH	delayed-type hypersensitivity
DTP	Diphtheria – Tetanus – Pertussis vaccine
ELISA	enzyme linked immunosorbent assay
fgSEA	functional gene set enrichment analysis
GAS	group A streptococcus
GBS	group B streptococcus
GCSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte monocyte colony stimulating factor
GO	gene ontology
HIV	human immunodeficiency virus
HR	hazard ratio
HREC	human research ethics committee
IFN	interferon
IL	interleukin
IP-10	interferon-inducible protein-10
IPV	inactivated poliovirus vaccine
IRR	incidence rate ratio
LBW	low birth weight
LPS	lipopolysaccharide
LRTI	lower respiratory tract infection
MCP-1	Monocyte chemoattractant protein-1
MCRI	Murdoch Children's Research Institute
MDP	muramyl dipeptide
MFI	Mean fluorescent intensity
MGIA	mycobacterial growth inhibition assay
MHW	Mercy Hospital for Women
MIF	macrophage inhibitory factor
MIG	monocyte induced by gamma
MIP-1 α	Macrophage inhibitory protein-1 α
MIP-1 β	Macrophage inhibitory protein-1 β
MIS BAIR	Melbourne Infant Study: BCG For Allergy and Infection Reduction
MMR	measles mumps rubella vaccine
MMRV	measles mumps rubella varicella vaccine
MRI	magnetic resonance imaging
MRR	mortality rate ratio

MS	multiple sclerosis
MTBk	heat killed <i>M. tuberculosis</i>
NES	normalised enrichment score
NK cell	natural killer cell
NLR	NOD-like receptor
NOD	nucleotide oligemirisation domain
NOD mice	non-obese diabetic mice
Pam3CYSK4	(S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride
PBMC	Peripheral blood mononuclear cells
PEPG	peptidoglycan
PF	preventive fraction
pg/mL	picogram/mL
PHA	phytohaemagglutinin
PNG	Papua New Guinea
PPD	purified protein derivative
PRR	pattern recognition receptor
R848	resiquimod
RCH	Royal Children's Hospital Melbourne
RCT	randomised controlled trials
RIG	retinoic acid –inducible gene
RLR	RIG-like receptor
RNA	ribonucelic acid
RNASeq	RNA sequencing
RPMI	Roswell Park Memorial Institute medium
RR	relative risk
RSV	Respiratory Syncitial Virus
RT	room temperature
SAGE	special advisory group of experts
SD	standard deviation
SNPs	single nucleotide polymorphisms
SOP	standard operating procedure
SSI	Serums Statens Instituit (NL)
TB	tuberculosis
Th1/2	T helper cell type 1/2
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UV	ultraviolet
VCGS	Victoria clinical genetics service
WHO	World Health Organisation

INTRODUCTION

This thesis presents data obtained from participants in two studies done in Melbourne between January 2013 and July 2017 and focuses on BCG-induced heterologous immunity in infants. A whole blood assay of heterologous pathogens and Toll-like receptor (TLR) ligands was developed to interrogate the infant immune response post BCG vaccination.

Analysis of pilot cytokine data from the 7 day old participants of the MIS BAIR study gave rise to two observations. Firstly, several chemokines showed consistently elevated expression in RPMI-stimulated samples. Secondly, there was marked inter-individual variability in cytokine expression in both RPMI-stimulated and antigen-stimulated cytokine levels. This led to the development of a novel regression analysis methodology using the RPMI-stimulated values as a covariate. This methodology was used in the two peer reviewed publications of cytokine data from the MIS BAIR study presented in chapters 3 and 4. This methodology was also used by other members of our group in a peer reviewed paper of which I am co-author (Appendix 1).

The results of the cytokine analysis from the MIS BAIR study confirmed an effect of BCG vaccine on innate immune responses to heterologous pathogens and TLR ligands in neonates and infants. The BabyBAIR study was conceptualised with the aim of understanding the underlying mechanisms through application of a systems vaccinology analysis. The BabyBAIR study is the first to use systems vaccinology to understand the mechanisms of BCG-vaccine induced heterologous immunity in infants.

Ancillary publications (in press and in progress) arising from this PhD are presented in Table 1.

Publication details	Thesis chapter
Completed first author papers in peer reviewed journals	
Archimedes: Neonatal BCG vaccination for the prevention of childhood allergy. <i>Arch Dis Child</i> . 2014 Feb;99(2):182-4.	Chapter 1 (p9-13)
BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms. <i>Trans R Soc Trop Med Hyg</i> . 2015 Jan;109(1):46-51.	Chapter 2 (p27-32)
BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases. <i>Trans R Soc Trop Med Hyg</i> . 2015 Jan;109(1):52-61.	Chapter 2 (p40-49)
Neonatal BCG vaccination influences cytokine responses to Toll-like receptor ligands and heterologous antigens. <i>J Infect Dis</i> . 2018 May;217(11):1798-1808	Chapter 3(R)
Neonatal BCG vaccination induces a dichotomous interferon-gamma response as part of an altered infant immune response to heterologous pathogens.	Chapter 4(R)
Co-authored papers arising from additional related work during candidature	
Innate immune responses following Kawasaki disease and toxic shock syndrome. <i>PLoS One</i> . 2018 Feb 15;13(2):e0191830.	Appendix 1
Melbourne Infant Study for prevention of allergy and infection (MIS BAIR). A randomised, controlled trial to determine if BCG vaccination at birth prevents the development of allergy and infection in infants. (<i>BMJ Open</i> . 2019 Dec 15;9(12):e032844)	Appendix 2
Biological sex influences antibody responses to routine vaccinations in the first year of life. (<i>Acta Paediatr</i> . 2020 Jan;109(1):147-157. doi: 10.1111/apa.14932.)	Appendix 3

Table 1. Publications arising from PhD candidature

(A) adapted text (R) results chapters

RESEARCH PLAN

HYPOTHESIS

BCG vaccination in infancy has a beneficial effect on the developing immune system, which in turn leads to decreased mortality from infection in vulnerable populations. This may occur either by enhancing the function and memory of immune cells for heterologous pathogens or by enhancing immune-regulatory mechanisms preventing severe immune-mediated pathology in response to infection.

AIMS

Part 1: To investigate whether neonatal BCG vaccination alters the immune response to heterologous pathogens and Toll-like receptor (TLR) ligands in (i) the neonatal period and (ii) infancy.

Part 2: To use a systems vaccinology approach to identify innate immune signatures underlying the non-specific effects of BCG vaccine.

STUDY SETTING & PARTICIPANTS

This thesis presents results from two complimentary studies done in Melbourne, Australia:

1. The Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR): was done at the Mercy hospital for women, Heidelberg, The Mercy Hospital Werribee and Geelong Hospital.
2. Baby BAIR: Investigating the immunological basis for the non-specific effects of BCG in infants was done at the Royal Children's Hospital Melbourne, Parkville.

THE MELBOURNE INFANT STUDY FOR ALLERGY AND INFECTION REDUCTION (MIS BAIR)

Recruitment of pregnant women to participate in MIS BAIR started in July 2014 at the Mercy Women's Hospital (MHW), Heidelberg. MIS BAIR is a randomised controlled trial (RCT) of BCG vaccine in newborn babies with primary clinical endpoints of allergic food sensitisation, eczema and lower respiratory tract infection. Secondary outcomes

include challenge proven allergy and hospital admission for lower respiratory tract infection. Infants (n=1272) were block randomised as soon as possible after birth. BCG Denmark (SSI) 0.05 mL was administered intradermally to the intervention group within 24 hours of randomisation. Participants also received hepatitis B vaccine at birth as per the Australian immunisation guidelines. As BCG results in a visible scar in most individuals, a placebo vaccine was not used however, outcome assessors were blinded to the vaccination status. Infants were followed up until one year of age with three-monthly questionnaires and a skin prick test at one year. A subset of patients (determined by parental consent) in each group provided a blood sample at 7 (± 4) days, 7 (4-8) months, and between 12 and 24 months post randomisation. The results of cytokine analysis in supernatants following whole blood stimulation with heterologous pathogens and TLR-ligands for the first two time points are presented in this thesis. A RCT, such as MIS BAIR, would be unethical in settings of high TB endemicity. Thus, the immunological sub-study nested within MIS BAIR provides a unique opportunity to describe heterologous immune responses in BCG-vaccinated term infants compared to BCG-naïve controls.

INVESTIGATING THE IMMUNOLOGICAL BASIS FOR THE NON-SPECIFIC EFFECTS OF BCG IN INFANTS (BABY BAIR)

This was a longitudinal cohort study that recruited infants and children referred to the Royal Children's Hospital Immunisation centre and Infectious Diseases out-patient clinics for BCG vaccination prior to travel to a TB-endemic country. Eligible children whose parents consented to participation in the study had blood samples taken prior to BCG vaccination (on the same day), at 7 (± 4) days and 3 months (± 2) weeks post BCG vaccination. A systems vaccinology framework combined (i) cytokine analysis in supernatants following stimulation with heterologous pathogens and TLR-ligands (ii) flow cytometry following stimulation with heterologous pathogens and TLR-ligands and (iii) RNASeq analysis of whole blood.

ETHICAL APPROVAL

The MIS BAIR study was approved by the human ethics research committees of the RCH (HREC 33025) and the MHW (HREC R12/28). MIS BAIR was registered as a clinical trial (clinical trials registration NCT01906853). Baby BAIR was approved by the research committee at RCH (HREC 33128G).

Figure 1.1 shows the timing of blood samples for immunological assays relative to BCG vaccine administration in the MIS BAIR and BabyBAIR studies.

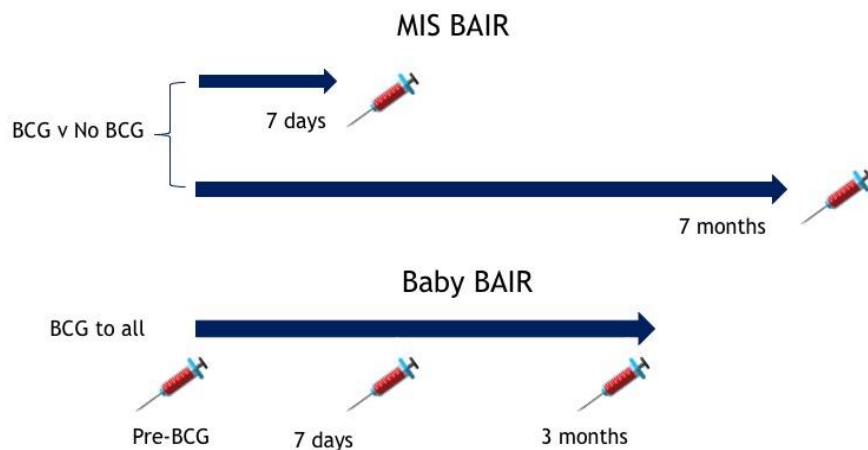


Figure 1.1 Timing of blood samples in relation to BCG vaccination

CHAPTER SUMMARY

Chapter 1 provides an overview of the relevant clinical literature describing the non-specific effects of BCG vaccine. This chapter provides the clinical context and details of the factors relevant to data analysis and interpretation. This chapter includes a brief review published in Archives of Disease in Childhood.

Chapter 2 is a literature review of the published scientific literature relating to the potential mechanisms for the non-specific effects of BCG vaccine. This chapter includes two review publications published in Transactions of the Royal Society of Tropical Medicine.

Chapter 3 is the analysis of heterologous cytokine responses in participants in the MIS BAIR trial 7 days following randomisation to BCG vaccination or no BCG vaccination. It comprises a peer-reviewed manuscript published in the Journal of Infectious Diseases in 2018. This chapter also includes additional methods relevant to the whole blood assay and the statistical methodology.

Chapter 4 is the analysis of the heterologous cytokine responses in participants in the MIS BAIR trial 7 months following randomisation to BCG vaccination or no BCG vaccination. This chapter includes a publication in progress which is author reviewed and was submitted to the Journal of Infectious Diseases in 2019.

Chapter 5 describes the methods and results of the BabyBAIR study.

Chapter 6 discusses the main results of this thesis, outlines the strengths and limitations of the research, and suggests directions for future research.

Chapter 1 THE NON-SPECIFIC EFFECTS OF BCG VACCINE - CLINICAL CONTEXT

This chapter presents the clinical and epidemiological evidence for the non-specific effects of BCG vaccine. Firstly, the evidence for BCG vaccine as an immune modulator with the potential to prevent the development of allergic disease and thus the rationale for the MIS BAIR study is presented. Secondly, the evidence for the beneficial non-specific effects of BCG vaccine on all-cause mortality and morbidity from infection is discussed. This chapter concludes with a summary of the clinical effect modifiers of the non-specific effects of BCG vaccine.

1.1 BCG & ATOPY

Allergic disease including food allergy, eczema and asthma is a public health priority in high-income settings and particularly in Australia. The hygiene hypothesis proposes that diminished microbial exposure in early life prevents the switch from the T helper 2 (Th2) immune response that predominates in neonates, to a T helper 1 (Th1) response [1]. A persistent Th2 dominant immune response is associated with the development of allergic disease. The hypothesis of the MIS BAIR trial is that BCG vaccine can act as an early life microbial exposure, encourage Th1 immune development and prevent the development of allergic disease in a high prevalence setting [2, 3]. The clinical evidence supporting this hypothesis was reviewed in the publication presented in section 1.2(pages 9-13) [4] of this thesis and the key findings of this review are discussed here. The results of this review informed the design of the MIS BAIR study with respect to (i) choice of eczema and food allergy as primary outcomes at 12 months of age and the (ii) definition of outcome measures.

This review included one randomised controlled trial and four systematic reviews (three with meta-analyses) of observational studies. Excluded articles included ten randomised controlled trials of BCG as an immune therapy for asthma and allergic rhinitis in childhood as they did not relate to the question of prevention.

The randomised controlled trial of neonatal BCG vaccination as a preventive intervention for allergic disease included neonates with a family history of allergic disease who were vaccinated with BCG or saline placebo at 6 weeks of age [5]. At 18 months of age, there was significantly less use of eczema medication in the BCG-vaccinated group and a trend towards less eczema. However, the study population was small and powered only to detect a 50% difference in prevalence of allergic disease between the two groups. The four systematic reviews of observational studies highlighted the clinical and methodological heterogeneity of the available observational evidence. The search strategy varied between reviews depending on the research question. Two early reviews included studies of the potential positive and negative effects of all childhood vaccines on the development of allergic disease [6, 7] However, two more recent systematic reviews of observational studies were relevant as they focused solely on the potential protective effect of BCG vaccination [8, 9]. Both reviews highlighted the limitations of the data but concluded that a protective effect of BCG vaccination was likely for the outcome of asthma.

Results from the Danish Calmette study, which ran in parallel to the MIS BAIR study and posed the same clinical question, were published in 2018. In this RCT of neonatal BCG vaccine in Danish infants there was no observed reduction in the rate of food allergy, recurrent wheeze and only a moderate effect on the development of atopic dermatitis (NNT=21) [10-12].

1.2 PUBLISHED REVIEW PAPER

QUESTION 2

Does neonatal BCG vaccination prevent allergic disease in later life?

CLINICAL SCENARIO

You are working on the postnatal ward of a busy maternity hospital. While doing a routine discharge check of a newborn baby, the mother asks you about the availability of BCG vaccination. Her two older children have severe eczema, asthma and food allergies, and she has read on the internet that BCG may reduce the risk of allergic disease.

STRUCTURED CLINICAL QUESTION

In a newborn infant (patient), does BCG vaccination (intervention) prevent the development of allergic disease in later life (outcome)?

SEARCH STRATEGY AND OUTCOME

Medline and Embase were searched (1946 to current date) using the keywords ((bacille Calmette-Guérin) OR (BCG)) AND (allergy OR asthma). Restrictions were human subjects, randomised controlled trials, systematic reviews and meta-analyses. This search yielded 64 articles. A search of the Cochrane Library revealed a further five articles. Of these 69 articles, one randomised controlled trial and four systematic reviews (three with meta-analyses) of observational studies were relevant and selected for full text review. Excluded articles included 10 randomised controlled trials of BCG as an immune therapy for asthma and allergic rhinitis in childhood as they did not relate to the question of prevention.

COMMENTARY

The hygiene hypothesis proposes that diminished microbial exposure in early life prevents the switch from the T helper 2 (Th2) immune response that predominates in neonates to a T helper 1 (Th1) response.¹ A persistent Th 2-dominant immune response is associated with the development of allergic disease. BCG has strong immunomodulatory properties that include the induction of Th1 cytokines,^{2 3} as well as significant effects on innate immunity.⁴ BCG is the most widely administered neonatal vaccine worldwide and has a well-documented safety profile. It is an attractive potential intervention for the prevention of allergic disease.

We found only one randomised controlled trial of neonatal BCG vaccination as a preventive intervention for allergic disease (table 1).⁵ In this study, neonates with a family history

of allergic disease were vaccinated with BCG or saline placebo at 6 weeks of age. At 18 months of age, there was significantly less use of eczema medication in the BCG-vaccinated group and a trend towards less eczema. However, the study population was small and powered only to detect a 50% difference in prevalence of allergic disease between the two groups. Although there was no difference between the groups in respiratory symptoms, the trial was too short to provide a definitive assessment of asthma. The results also need to be interpreted in light of the selection of infants at high risk of allergic disease.

There have been a large number of observational studies addressing the influence of BCG vaccination on allergic disease. These have been qualitatively evaluated in no less than four systematic reviews, each of which highlights the clinical and methodological heterogeneity of the available evidence. The search strategy varied between reviews depending on the research question. Two early reviews included studies of the potential positive and negative effects of all childhood vaccines on the development of allergic disease.^{6 7} However, two more recent systematic reviews of observational studies were relevant as they focused solely on the potential protective effect of BCG vaccination.^{8 9}

El-Zein *et al*⁸ examined the effect of BCG vaccination on the development of asthma (23 studies) while Arnoldussen *et al*⁹ looked at multiple allergic outcomes (17 studies) (table 1). There are important differences in the 28 individual studies included in the two reviews. A key difference is the age at

which BCG vaccine was administered. To exert a protective immunomodulatory effect, it is likely that BCG vaccination would have to be administered in the early neonatal period. However, BCG was given in the studies up to 12 years of age. BCG was described as 'neonatal' in only 11 of the 28 studies, the age at vaccination varying between 1 day and 3 months. Revaccination at up to 12 years of age occurred in two studies. There were also differences in the reliability of the method used to assess BCG vaccination status, which varied between health records (15 studies), scar (7 studies), and positive tuberculin skin test (4 studies). The method used was not mentioned in two studies. There was also considerable heterogeneity between the included studies in the duration of follow-up. El-Zein *et al* pooled their asthma data in a random effects model ($I^2=39\%$) to give an OR of 0.82 (95% CI 0.72 to 0.92) for the protective effect of BCG vaccination on asthma. For the six studies of children vaccinated at less than 1 year of age, the OR was 0.86 (95% CI 0.79 to 0.94). The more homogenous data, from the 16 studies in which vaccination was confirmed by health records, were combined in a fixed effects model to give an OR of 0.86 (95% CI 0.79 to 0.93). In contrast, Arnoldussen *et al* did meta-analyses for multiple outcomes without any stratification by age at vaccination (table 1). Both reviews highlighted the limitations of the data but concluded that a protective effect of BCG was likely for the outcome of asthma.

In summary, only eight studies addressed BCG administered in the neonatal period confirmed by the medical record or a

Table 1 Does neonatal BCG vaccination prevent allergic disease in later life?

Citation	Study group	Study type (level of evidence)	Outcome	Key results	Comments
Steenhuis <i>et al</i> , 2008 ⁵	121 infants in Denmark with a first-degree relative with previous or present allergic disease. BCG-vaccinated at 6 weeks of age, n=62; Non-BCG-vaccinated, n=59	Prospective single blind, randomised placebo-controlled trial (Level 2)	Validated questionnaires for symptoms of allergic disease at 6 weeks (baseline), 4 months and 18 months of age	<i>Eczema (ever)</i> 44% vs 61% RR 0.72 (0.5–1.0) p=0.07 NNT=5.6* <i>Medication use for eczema</i> 25% vs 43% RR 0.58 (0.3–1.0) p=0.04 NNT=5.9*	Study was only powered to detect a reduction of greater than 50% in the prevalence of allergic disease in the intervention group. 18 month follow-up inadequate to assess asthma
El-Zein <i>et al</i> , 2010 ⁸	23 studies: 10 cohort 5 case control 8 cross-sectional Total vaccinated, n=41 003	Systematic review and meta-analysis of observational studies (Level 3)	Asthma diagnosis or presence of wheeze	<i>Asthma/wheeze</i> BCG confirmed by scar or positive TST or vaccine record (23 studies) REM OR 0.82 (0.72 to 0.92). BCG confirmed by vaccine record (16 studies) FEM OR 0.86 (0.79 to 0.93)	Marked heterogeneity between studies. Significant protective effect; confirmed in subset of 16 homogenous studies ($I^2=0$) with documented BCG vaccination
Arnoldussen <i>et al</i> , 2011 ⁹	17 studies: 1 randomised controlled trial ⁵ 7 cohort 2 case control 7 cross-sectional Total vaccinated, n=39 403	Systematic review and meta-analysis of observational studies (Level 3)	Sensitisation (IgE/SPT), eczema, allergic rhino-conjunctivitis, asthma	<i>IgE</i> (5 studies; $I^2=0\%$) FEM OR 1.31 (1.07 to 1.60) <i>SPT</i> (5 studies; $I^2=57\%$) REM OR 0.87 (0.67 to 1.13) <i>Atopic dermatitis/eczema</i> (7 studies; $I^2=89\%$) REM OR 0.84 (0.64 to 1.09) <i>Allergic rhino-conjunctivitis</i> (9 studies; $I^2=67\%$) REM OR 1.07 (0.89 to 1.28) <i>Asthma</i> (12 studies; $I^2=91\%$) REM OR 0.73 (0.56 to 0.95)	Modest protective effect only for asthma. Results include a sensitivity analysis to reduce identified publication bias

*Calculated from the data provided.

FEM, fixed effects model; NNT, number needed to treat; REM, random effects model; RR, relative risk; SPT, skin prick test; TST, tuberculin skin test.

scar and had adequate follow-up to detect relevant clinical outcomes. Methodological differences preclude pooling of the data from these studies and the results are conflicting, including one retrospective cohort study of 20 383 participants that found an association between BCG vaccination and increased eczema.

In summary, there is some evidence that BCG vaccination at birth protects against allergic disease. However, this is based on only one randomised controlled trial and the conclusions of systematic reviews of observational studies that have significant methodological weaknesses, heterogeneity and conflicting results. While BCG vaccination is generally safe, adverse effects can occasionally occur. Therefore, in the absence of an adequately-powered, randomised controlled trial of BCG vaccination at birth for the prevention of allergic disease is completed, there is insufficient evidence to recommend this intervention.

Clinical bottom lines

- One randomised controlled trial suggests that neonatal BCG vaccination in those with a family history of allergic disease reduces the development and severity of eczema measured up to 18 months of age (Level 2).
- Two meta-analyses of observational studies found a protective effect of BCG vaccination on the development of asthma (Level 3).
- In the absence of adequately powered randomised controlled trials, there is insufficient evidence to recommend routine BCG vaccination for the prevention of allergy.

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REFERENCES

- 1 Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;299:1259–60.
- 2 Rook GA. Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis. *Immunology* 2009;126:3–11.
- 3 Marchant A, Goetghebuer T, Ota MO, *et al*. Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guérin vaccination. *J Immunol* 1999;163:2249–55.
- 4 Kleinnijenhuis J, Quintin J, Preijers F, *et al*. Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA* 2012;109:17537–42.
- 5 Steenhuis TJ, van Aalderen WM, Bloksma N, *et al*. Bacille-Calmette-Guérin vaccination and the development of allergic disease in children: a randomized, prospective, single-blind study. *Clin Exp Allergy* 2008;38:79–85.
- 6 Koppen S, de Groot R, Neijens HJ, *et al*. No epidemiological evidence for infant vaccinations to cause allergic disease. *Vaccine* 2004;22:3375–85.
- 7 Balicer RD, Grotto I, Mimouni M, *et al*. Is childhood vaccination associated with asthma? A meta-analysis of observational studies. *Pediatrics* 2007;120:e1269–77.
- 8 El-Zein M, Parent ME, Benedetti A, *et al*. Does BCG vaccination protect against the development of childhood asthma? A systematic review and meta-analysis of epidemiological studies. *Int J Epidemiol* 2010;39:469–86.
- 9 Arnoldussen DL, Linehan M, Sheikh A. BCG vaccination and allergy: a systematic review and meta-analysis. *J Allergy Clin Immunol* 2011;127:246–53, 53 e1–21.

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Does neonatal BCG vaccination prevent allergic disease in later life?

Bridget Freyne and Nigel Curtis

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1.3 BCG & *M. TUBERCULOSIS*; THE 'SPECIFIC' EFFECTS OF BCG

BCG vaccine has been administered for the prevention of *M. tuberculosis* infection since the 1920s [13]. It is the most widely administered vaccine worldwide [14]. BCG vaccine reduces the risk of all tuberculosis (TB) infections by 50%, TB meningitis by 64% and TB related deaths by 74% [15]. BCG vaccine does not prevent sufficient cases of pulmonary TB to halt the global epidemic of the disease but remains a cost-effective strategy for the prevention of severe TB disease, particularly in children [16, 17]. The prevention of TB transmission and reduction in associated mortality is a global health priority with the World Health Organisation (WHO) committing to a 90% reduction in TB deaths by 2030. Multiple new vaccine candidates are in development although to date none have shown efficacy over and above that offered by BCG [18, 19]. The efficacy of BCG vaccine against *M. tuberculosis* is highly variable and poorly understood. This variability is hypothesised to be secondary to environmental influences on the vaccine response (helminth exposure, environmental mycobacterial exposure, sunlight) and individual level variability in the immune response and associated tendency to develop a scar following BCG vaccination [20].

1.4 BCG VACCINE AND A REDUCTION IN ALL-CAUSE MORTALITY

The initial report of BCG vaccine efficacy published in 1931 in the Proceedings of the Royal Society of Medicine and authored by Albert Calmette gives a fascinating background to the development of the vaccine and the first suggestion that BCG might confer beneficial non-specific effects [21]. BCG vaccine was developed from a culture of *Mycobacterium bovis* isolated from a tuberculous lesion on the udder of an infected cow. Between 1905 and 1913 the initial isolate underwent 230 passages in order to attenuate virulence and prepare for testing in humans. In 1923, the vaccine's developer, Albert Calmette was approached by a paediatrician treating an orphaned infant, exposed to TB by their mother and to be left in the care of a grandmother who also suffered from pulmonary TB. This child received the first dose of BCG given to a human and went on to survive without contracting the disease. Based on this, a BCG vaccine campaign was commenced and this publication also includes analysis of 8075 infants between 1 month and 1 year of age who received an oral dose of the vaccine. Given the limitations of diagnosing TB in children at the time, an outcome of all-cause mortality was reported. This was 4.6% in vaccinated children and 16% in unvaccinated

children. These results prompted the author, Albert Calmette to hypothesise that either the prevalence of TB in children was much higher than previously thought or the vaccine “conferred on the organism a special aptitude to resist those other infections which are so frequent in young children.”

Following this report, BCG vaccine went into widespread use with the aim of controlling the spread of TB. All-cause mortality continued to be used as an outcome for vaccine efficacy and a positive effect was evident in controlled trials from the UK and the USA done in the 1940s [22]. More recently, multiple observational and interventional studies from low income countries reported similar beneficial effects on all-cause mortality [20, 23-32]. In 2017, a WHO special advisory group of experts (SAGE) on immunisations did a systematic review of the available evidence. They concluded that there was a beneficial effect of BCG vaccine on mortality with an average relative risk of 0.7 (0.49-1.01) from four clinical trials considered at low risk of bias and 0.47 (0.32- 0.69) in nine observational studies [33]. Of note, was the authors conclusion regarding the clinical implications. Although the absolute risk reduction of between 5-10 cases per 1000 childhood deaths is small in absolute numbers, the withdrawal of this effect in the context of a billion vaccinations a year with a safe and established product would be significant. The overall conclusion was that research should turn to understanding the mechanism of any effect so that it might be preserved.

A subsequent randomised controlled trial of BCG vaccine on low birth weight (LBW) infants in India using BCG-Russia did not show a protective effect on all-cause mortality in the setting of a neonatal intensive care unit [34]. The results of this trial have called into question the generalisability of the non-specific effects outside of the African setting or with BCG vaccine strains other than BCG-Denmark.

1.5 BCG VACCINE AND PROTECTION FROM INFECTION

In two clinical trials included in the WHO SAGE committee meta-analysis the excess mortality in the BCG-naïve infants was attributable to sepsis, fever and lower respiratory tract infection as determined by verbal autopsy [25, 27, 35]. A third trial with the outcome of neonatal mortality (<28 days), showed a non-significant reduction in all-cause mortality (mortality rate ratio 0.7 (0.47-1.01)) in infants weighing <2500g (LBW) who received BCG vaccine at birth compared to those in whom BCG vaccine was delayed. However, secondary

analysis of infectious causes of death did show a significant reduction in mortality in BCG-vaccinated infants compared to controls (mortality rate ratio 0.57 (0.35-0.93)) [35]. The most frequent cause of death in this analysis was neonatal sepsis. In a meta-analysis of three clinical trials done to examine the effect of BCG vaccine at birth on in-hospital mortality, there was a significant reduction in neonatal (<28 days) mortality attributable to sepsis (relative risk 0.46 (0.22-0.98)) and a non-significant reduction in sepsis associated mortality less than 1 year of age (relative risk 0.55 (0.28-1.06)) [36].

All-cause hospitalisation was used as an outcome measure of the beneficial non-specific effects of BCG vaccine in clinical trials from high and low income settings (Table 1.1) [36, 37]. BCG vaccine at birth had no effect on all-cause hospitalisation up to 15 months of age in a randomised controlled trial of 6189 Danish infants [37]. The authors reported a non-significant effect of BCG vaccine on the primary outcome in the 17% of infants in their trial whose mothers reported a history of BCG vaccination (hazard ratio 0.81(0.69-1.09)). In a separate analysis of infants from the same trial, BCG vaccine at birth led to no reduction in hospitalisations secondary to infection (hazard ratio 0.99 (0.85-1.15)). However, having a mother who was BCG-vaccinated or being born by Caesarean section significantly modified the effect of BCG vaccine on the primary outcome [38]. Similarly, a meta-analysis of three randomised controlled trials from Guinea Bissau also showed no effect of BCG vaccine at birth on the rate of both all cause hospitalizations or hospitalizations secondary to infection up to 12 months of age [36].

It is somewhat counter-intuitive that BCG vaccine at birth reduces all-cause infant mortality while having no effect on the rate of hospitalisation due to infection in the same age group. There are several possibilities which might explain this scenario. Firstly, it is possible that BCG vaccine is not protective against the pathogens responsible for non-fatal infectious hospitalisations in infants. Alternatively, it is possible that the beneficial effect of BCG vaccine is syndrome specific rather than pathogen specific. This hypothesis suggests that BCG vaccine reduces mortality from the inflammatory syndrome secondary to infection which could arise from sepsis, severe pneumonia or complicated malaria. Finally, it is possible that the hospitalisation data suffers from sampling bias. If the effect on mortality is predominantly from the immediate post-natal period, it would occur prior to post-natal discharge and would not be counted as a hospitalisation. This final supposition is supported

by the results of a secondary meta-analysis of data from three randomised controlled trials which is included in the publication of trial data of the protective effect of BCG vaccine on neonatal mortality by Biering-Sorensen *et al* [35]. In this fixed-effects model, protection from BCG vaccine is highest at 3 days post BCG vaccination (mortality rate ratio=0.17 (0.02-1.35), less at 28 days post BCG vaccination (mortality rate ratio 0.28 (0.06-1.37)) and non-significant by 12 months (mortality rate ratio 0.41 (0.14-1.18)).

Two approaches have been used to investigate aetiology specific effects of BCG vaccine (Table 1.2). Firstly, secondary analysis of health systems datasets found an association between BCG vaccination status and risk of hospitalisation secondary to sepsis or pneumonia in Spain and hospitalisation for acute respiratory infection (ARI) in a multinational cohort [39, 40]. A limitation of the second study was the inability to definitively exclude cases of ARI secondary to *M. tuberculosis* infection [40]. Secondly, epidemiological studies sought an association between being BCG-naïve with the development of subsequent illness. A case-control study from Guinea Bissau found that a positive BCG vaccine history was protective against the development of all-cause acute lower respiratory tract infection and respiratory infection due to RSV [41]. A prospective cohort study in the same setting found a non-significant protective effect of having a BCG vaccine scar on the development of all-cause gastroenteritis and diarrhoea due to Rotavirus [42]. Finally, a retrospective cohort study found that having a BCG scar was protective for the outcome of death from malaria 0.37 (95%CI 0.13-0.76) but not from sepsis, pneumonia, meningitis, diarrhoea or encephalitis [29].

There is evidence from animal challenge models that BCG vaccine alters the immune response to and subsequent mortality from a variety of pathogens including bacteria, fungi, parasites and viruses [43]. Two recent human challenge models of BCG vaccine showed clinically relevant changes in the immune response to yellow fever viraemia and malaria parasitaemia [44, 45]. These studies are reviewed in detail in the next chapter, however taken together with the epidemiological evidence it is unlikely that the beneficial effect of BCG vaccine on mortality secondary to infection is due to protection against a single pathogen or even class of pathogen.

Location	Design	Intervention	Age	Sample size	Result
Denmark [37]	RCT	BCG at birth	0-15m	4,262	All cause: HR 1.05 (0.93-1.18) 2° infection: HR 0.99 (0.85-1.15)
Guinea Bissau [36]	MA RCT	BCG at birth	0-12m	6,583	All cause: IRR 0.97 (0.72-1.31) Neonatal sepsis IRR 0.75 (0.50-1.13) Infant sepsis: IRR 0.78 (0.55-1.11)
Greenland [46]	RC	BCG y/n (VR)	3m-3y	19,363	2° infection: IRR 1.07(0.96-1.20) ARI: IRR 1.10 (0.98-1.24)
Spain [39]	Eco CC	BCG y/n (VR)	0-12m	464,611	ARI: PF 41.4% (40.3-42.5) Sepsis: PF 52.8% (43.8-60.7)
Multinational [40]	RC	BCG y/n (VR)	0-5y	151,322	ARI: arrow 0.83 (0.74-0.94)

Table 1.1 BCG vaccination and rate of all-cause or pathogen specific hospitalisations

RCT=Randomised controlled trial, MA RCT= metanalysis of randomised controlled trials, RC=Retrospective cohort, Eco CC= ecological case control, HR=Hazard ratio, IRR=incidence rate ratio, PF=preventative fraction. ARI= acute respiratory infection

Aetiology	Location	Design	Intervention	Age	Sample size	Result
ARI [41]	Guinea Bissau	CC	BCG y/n (VR)	3m-5y	376	aOR 2.87 [1.31-6.32]
RSV [41]	Guinea Bissau	CC	BCG y/n (VR)	3m-5y	48	aOR 2.22 [0.66-7.44]
Rotavirus [42]	Guinea Bissau	PC	BCG y/n (VR)	6m-2y	200	IRR 0.45 (0.14-1.46)
Malaria [29]	Guinea Bissau	PC	BCG scar	3m-5y	1617	MR 0.32 (0.13-0.76)
Sepsis [47]	Malawi	CC	BCG scar	0m-6m	48	p<0.001 (KW)*

Table 1.2 BCG vaccination and morbidity due to specific infectious aetiologies

ARI= acute respiratory infection, RSV= respiratory syncytial virus, VR=vaccine record, CC=Case control, PC=Prospective cohort, trial, aOR=adjusted odds ratio, IRR=incidence rate ratio, MR=mortality ratio. * direct comparison between groups only, no risk ratio as 0% incidence in scar group

1.6 EFFECT MODIFIERS OF THE NON-SPECIFIC EFFECTS OF BCG VACCINE

Variability in the protection offered by BCG against *M. tuberculosis* is well documented but poorly understood. The relevance of this observation for the non-specific effects of BCG vaccine are unknown. Documented effect modifiers of the non-specific effects of BCG vaccine include (i) sex (ii) timing of BCG administration (iii) effect of concomitant vaccinations and vitamin supplementation (iv) BCG vaccine characteristics including vaccine strain and batch and (v) maternal BCG vaccination status.

1.6.1 SEX

Multiple epidemiological studies have reported differential non-specific effects of BCG vaccine between boys and girls on both mortality and susceptibility to infection. The beneficial effect on all-cause mortality is reported as being greatest in girls [48]. Recent meta-analysis of nine studies where the effect of BCG vaccine on all-cause mortality was examined separately in boys and girls found no significant difference between the sexes (relative risk ratio 1.02 (0.73-1.41) [33]. In Danish infants, sex had no effect on the development of atopic wheeze or dermatitis or rate of hospitalisations at 12 months post neonatal BCG vaccination [10-12, 49].

Hypothetical explanations for differential immune responses between boys and girls include the effect of sex hormones on immune cells and sex differential expression of proteins, cytokine receptors and TLRs with their genetic loci on the X chromosome [50]. Darboe *et al* compared the immune response between BCG-vaccinated boys (n=33) and BCG-vaccinated girls (n=31) in the Gambia. These infants were vaccinated at 6 weeks of age and cytokine responses following *in vitro* whole blood stimulation with heterologous pathogens and TLR ligands were measured at 1 week and 11 weeks post vaccination. They reported minimal sex differential effects apart from a decline in IFN- γ responses to *S. pneumoniae* over time in female participants. Of note, there was minimal IFN- γ response to PPD (specific control) and the authors hypothesised this could be due to the use of the relatively less immunogenic strain BCG-Russia in this study [51].

The available evidence suggests that sex may not influence heterologous clinical outcomes following BCG vaccination at a population level however the effect of sex on immune responses and immune regulation during infection remains poorly understood.

1.6.2 TIMING OF BCG VACCINE ADMINISTRATION

The importance of the time of administration of BCG vaccine is complex and ultimately confounded by age and concomitant vaccination status. Secondary analysis of the data from randomised controlled trials of early (at birth) versus delayed (at 6 weeks) BCG vaccination suggests that BCG vaccination at birth is more beneficial [35]. It is unclear however whether this is due to waning heterologous efficacy of the vaccine, a decrease in the susceptibility of infants to mortality from infection over time or a change in the infant immune response to BCG vaccine which is dependent on the time of administration. Two cohort studies and one randomised controlled trial reported the effect of BCG vaccine between different age groups and concluded that the effect was greater the earlier the vaccine was administered [30, 52, 53].

1.6.3 EFFECTS OF INTER-CURRENT IMMUNISATIONS AND VITAMIN SUPPLEMENTATION

The literature on the non-specific effects of infant vaccination is dominated by the paradigm that there are two opposing effects of existing vaccines (i) the beneficial effect of live vaccines (BCG and Measles vaccine) and (ii) the detrimental effect of killed vaccines particularly Diphtheria-Tetanus-Pertussis (DTP). This implies that the order of vaccinations in the immunisation schedule may result in antagonistic or competing clinical effects i.e. that the beneficial immune response initiated by BCG vaccination at birth may be subsequently dampened by administration of DTP containing vaccines up to six months. The literature pertaining to these effects was synthesised as part of the WHO SAGE review. These authors compared the routine administration sequence (BCG followed by DTP), with situations in which BCG and DTP were administered together or BCG was administered following DTP. There was a tendency towards a more beneficial effect on mortality when BCG was given with DTP or before it however, the authors noted a high degree of bias and heterogeneity and data was not combined as a result [33].

In Sub-Saharan Africa, vitamin A supplementation is routine but can vary with availability. Epidemiological evidence suggests that co-administration of vitamin A may increase the beneficial non-specific effects of live vaccines on all-cause mortality [54]. This was not however considered of adequate strength to assign an association in the WHO SAGE review [33]. There is evidence however, for an effect of vitamin A on immune responses following BCG vaccination [55, 56]. In Australia, vitamin D is the only routinely administered micro-nutrient in infancy. Vitamin D is a known immune-modulator with effects on the immune response to Tuberculosis infection and the efficacy of BCG treatment of bladder cancer [57-61].

1.6.4 BCG VACCINE CHARACTERISTICS

The original attenuated *M. bovis* BCG strain was kept in culture for several years before final preservation. Secondary lots were distributed worldwide and further culture was done prior to lyophilization. There is evidence of significant genomic differences between vaccine strains [62]. There is further evidence of differences in the immune response following neonatal vaccination with different BCG strains [63]. There is however insufficient evidence to suggest that one strain offers significantly greater protection against Tuberculosis [64] [65]. The implications for the non-specific effects of BCG are unknown. There is evidence to suggest that development of a scar is more likely following the use of BCG-Denmark rather than BCG-Russia (72-92% v 52%) [29, 66, 67] and that scar development is associated with improved all-cause mortality and morbidity due to respiratory infection [31]. A recent randomised controlled trial of early versus delayed BCG-Russia vaccination on all-cause mortality in Indian neonates showed no effect [34]. The authors concluded that the use of BCG-Russia may have influenced these results and that further evidence is needed to assess the effect of BCG strain on all-cause mortality [68]. There is further evidence that difference in mycobacterial growth rate between batches of an individual strain can affect scar formation, reactivity to the mycobacterial antigen “purified protein derivative” (PPD) and the resulting immune response including the production of pro-inflammatory cytokines TNF- α [69].

1.6.5 MATERNAL BCG

There is immunological and clinical evidence that maternal BCG vaccination can affect subsequent infant immune responses to BCG vaccine. Mawa *et al* examined the innate immune response in cord blood from infants whose mothers were either positive or negative with respect to the presence of BCG vaccine scar [70]. Infants born to BCG scar positive mothers had increased IFN- γ , IL-12, IL-10, TNF- α and IL-1 β production when compared to infants of mothers with no BCG scar. This was coupled with upregulation of pro-inflammatory and IFN- γ pathways measured on RNA isolated from whole blood at both one and six weeks post BCG vaccination. In the Danish-Calmette randomised controlled trial of BCG-Denmark, hospitalization prior to 15 months of age was significantly reduced in infants who had a BCG-vaccinated mother (hazard ratio 0.65 (0.45-0.94 95%CI) compared to those whose mother had no history of BCG vaccination (hazard ratio 1.10 (0.93-1.41 95%CI) [38]. More recently the presence of maternal BCG vaccine scar has been associated with reduced all-cause mortality in BCG-vaccinated infants in Guinea-Bissau [71].

1.7 CONCLUSION

The hypothesis for this thesis is that BCG vaccination in infancy has a beneficial effect on the developing immune system, which in turn leads to decreased mortality from infection in vulnerable populations. Review of the clinical evidence related to the beneficial non-specific effects of BCG vaccine thus provides essential context for this thesis in two ways. Firstly, review of the literature related to BCG vaccination and a reduction in atopic disease in childhood provides the rationale for the MIS BAIR study. Secondly, although it is clear that the magnitude of the BCG vaccine effect on all-cause mortality is small, in high mortality settings it provides a safe, acceptable and affordable contribution to prevention of infant mortality. Finally, there are several important observations which informed the design and analysis of the research presented in this thesis. Since the effect of BCG vaccination is not confined to protection from a single clinical syndrome, there is a need to assess the BCG-induced immune response following stimulation with multiple pathogens. Furthermore, factors such as sex, timing of BCG vaccination, maternal BCG vaccination status, mode of

delivery may modify the effect of BCG vaccine. The effect of concurrent vaccinations on the heterologous immune response to BCG vaccine could not be definitively assessed in this thesis as all infants were vaccinated according to the Australian National Immunisation guidelines. However, participant selection for the immunological sub-study was controlled to ensure homogeneity of vaccination status. The BabyBAIR study which included children of different ages and vaccine histories provided an opportunity for limited evaluation of the effect of the last vaccine administered.

Chapter 2 BCG VACCINE & HETEROLOGOUS IMMUNITY

2.1 INTRODUCTION

This chapter presents the literature on BCG-induced heterologous immunity with emphasis on the role of the innate immune system. The literature is presented in four parts. Firstly, the experimental evidence for BCG-associated heterologous immunity from animal and *in vitro* studies, which spans seven decades. Secondly, the existing literature on the heterologous immune response to BCG vaccine in humans. Thirdly, an overview of the evidence for BCG as a clinically relevant immune modulator in the fields of oncology and auto-immunity. The search strategy which was used for the two published reviews in this chapter is presented in (Appendix 4). Finally, a brief overview of potential effect modifiers of BCG-induced heterologous immunity which are relevant to the interpretation of the results of this thesis.

2.2 BCG-INDUCED HETEROLOGOUS IMMUNITY; *IN VITRO* & ANIMAL STUDIES

Heterologous immunity, the concept that exposure to one antigen can lead to an altered immune response to a subsequent unrelated antigenic challenge is well described [48, 72, 73]. The early literature on BCG-induced heterologous immunity runs in parallel to the literature underpinning our understanding of cell mediated immunity. BCG was one of the first routine vaccinations in use. Scientists trying to understand the mechanism of action of BCG had difficulty measuring immune responses to *M. tuberculosis*, as it is a difficult organism to culture. Instead, experimental models were developed using unrelated intracellular organisms such as *L. monocytogenes* and *S. enteritidis* as surrogates, thus indirectly providing evidence of BCG-induced heterologous immunity [74].

This literature is presented in two parts: (i) experimental studies (*in vitro* and animal models) done with the aim of describing immune mechanisms following BCG vaccine and (ii) animal models of infection where BCG was used as a therapeutic intervention against a heterologous pathogen. Review of this literature resulted in two publications

which are included as sections 2.2.2 (p27-32) and 2.2.4 (p40-49) [43, 75] and which are summarised below.

2.2.1 BCG-INDUCED HETEROLOGOUS IMMUNITY: EXPERIMENTAL MODELS AND IMMUNOLOGICAL MECHANISMS

Throughout the 1950s-70s, scientists trying to understand the basis for BCG-induced protection against TB succeeded in describing the components of cell-mediated immunity including phagocyte migration, antigen presentation, cytokine production and activation of cytotoxic and helper T cells. This was achieved through a series of *in vitro* and animal experiments which initially utilised heterologous intracellular organisms such as *S. enteritidis* and *L. monocytogenes* as surrogates for *M. tuberculosis*. As interest grew in BCG as an immune-modulator, experimental models expanded to include a wide variety of bacterial, viral, fungal and parasitic pathogens.

The use of heterologous pathogens in experimental models aimed at describing cell-mediated immunity following BCG vaccination thus provided the first experimental evidence of BCG-induced heterologous immunity. The heterologous, 'non-specific' effects of BCG discussed in the following review publication refer to both specific heterologous immunity, i.e. cross-reaction of antigenic compounds from different organisms [76], and non-specific heterologous immunity, i.e. where BCG leads to a modification of the immune system that results in heterologous effects without involving the recognition of antigenic determinants. In the studies of non-specific macrophage activation, enhanced heterologous immunity was only evident in the case of intracellular organisms [77-79]. Conversely, there was evidence of 'trained' immunity following challenge with *E. coli*, *S. aureus* and *C. albicans* [80]. This raises the interesting notion that BCG may exert beneficial heterologous effects against disparate pathogens by different mechanisms and may be influenced by particular conditions i.e. heterologous immunity secondary to non-specific macrophage activation required on-going antigenic stimulation with viable BCG, as apparently occurs in infants who may have local reactions to BCG vaccine up to nine months of age but 'trained' immunity was demonstrated in the context of inactivated BCG.

2.2.2 PUBLISHED REVIEW PAPER

BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms

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Randomized controlled trials indicate that bacille Calmette-Guerin (BCG) has beneficial heterologous ('non-specific') effects on mortality in high mortality settings. These findings have stimulated interest in understanding the immunological mechanisms underlying these effects in the hope of harnessing them to reduce all-cause mortality. This line of investigation is especially important in light of BCG being discontinued in some countries as the prevalence of TB falls. Stopping routine BCG in this situation may have the unintended consequence of depriving children of the beneficial immune modulating effects of this vaccine. BCG has been recognized as a potent immunomodulator for decades. This review details experimental studies involving BCG and any heterologous antigen that aimed to interrogate potential immunological mechanisms. To provide a historical perspective, the evidence is presented chronologically. The lines of immunological enquiry can be seen to mirror the evolution of our understanding of cell-mediated immunity and its components. As new clinical trials to investigate the heterologous effects of BCG are undertaken, an understanding of the history of BCG-induced immunity against heterologous antigens may provide information on immunological pathways worthy of further interrogation using modern immunological methods.

Keywords: BCG, Heterologous, History, Immunology, Non-specific

Introduction

Substantial evidence supports the concept that bacille Calmette-Guerin (BCG) vaccine has a beneficial effect in reducing infant mortality in high mortality settings.^{1–4} A similar effect on all-cause mortality was observed in the UK and the USA following the introduction of universal BCG immunization for the prevention of TB in the 1950s.⁵ Epidemiological studies suggest that BCG also protects against the development of allergic disease.⁶ More recently, studies have reported a clinical benefit with BCG vaccination in the prevention of pneumonia,⁷ and the treatment of diabetes⁸ and multiple sclerosis.⁹ The immunological and molecular mechanisms underlying these beneficial heterologous ('non-specific') effects remain uncertain.

This review of the history of the proposed mechanisms underlying BCG-associated heterologous immunity from the 1950s to the present day is focused on the laboratory studies that have provided insight into the immunological mechanisms. These studies were not specifically designed to answer the hypothesis that BCG exerts heterologous effects but nonetheless provide relevant immunological evidence in relation to phagocyte migration, antigen presentation, cytokine profiles, activation of cytotoxic and

helper T cells, and epigenetic reprogramming of innate immune pathways. Our other review outlines the evidence for BCG-associated heterologous immunity from animal studies of BCG as an intervention against infectious disease.¹⁰

The evidence presented in this review is organized according to the immunological mechanisms that were under investigation. Presented chronologically, the history of the heterologous effects of BCG parallels developments in the understanding of cell mediated and, more recently, innate immunity. This substantial body of evidence provides biological plausibility to the results of clinical studies showing non-specific effects of BCG vaccine. In addition, understanding the historical progression of this work may highlight areas worthy of further enquiry in future clinical studies in humans.

Macrophage mediated non-specific antibacterial activity

Macrophage activation is the continuum from bone marrow-derived monocytes through to mature phagocytic cells exhibiting either tumoricidal or bactericidal properties.¹¹ Historically, the

study of macrophage differentiation and 'activation' is closely tied to the study of BCG. Early work on BCG and its interaction with heterologous antigens focused on demonstrating the non-specific 'activation' of macrophages leading to enhanced bactericidal properties.

In the 1950s, Howard et al.¹² showed a five-fold increase in the 'phagocytic index' of mice receiving intravenous BCG. This index was designed as a measure of 'reticuloendothelial system' activity and was based on blood clearance of colloidal carbon over time. Radiolabelled *Salmonella enteritidis* was cleared from the blood of BCG infected mice more rapidly than controls and these mice had improved survival (13 vs 5.5 days).¹² In contrast, Pletsityi et al.¹³ found a decreased 'phagocytic index' in the blood of rabbits immunized with intradermal BCG 14 days earlier. They defined this index as the proportion of leukocytes exhibiting ingested *Escherichia coli* in vitro. A similar effect was seen following smallpox, diphtheria-tetanus-pertussis (DPT) and 'anti-rabies' administration.¹³ The low phagocytic index seen in this study may also represent a migration of monocytes out of the bloodstream in intradermally immunized mice. Yarkoni et al.¹⁴ found increased acid phosphatase activity of mouse peritoneal macrophages after intraperitoneal, 'local', injection of BCG. They subsequently showed that these BCG-stimulated peritoneal macrophages exhibited tumoricidal activity and that this effect was replicated in hamsters.¹⁴ The contradictory findings in these early studies of macrophage activation may have been due to variability in BCG dose or route of administration and to the lack of standardisation of outcome measures.

Throughout the 1960s, Mackaness et al.¹⁵ did multiple studies aimed at describing the basis for cell-mediated immunity. They observed that intracellular pathogens exhibited cross-reactive immunity that could not be passively transferred and which was associated with morphological changes to macrophages, which they determined to be 'the effector cell'. They intravenously injected groups of mice with *Brucella abortus*, *Listeria monocytogenes* and BCG and subsequently challenged them with the same bacteria. The outcome measures were viable bacterial counts in the spleens, footpad delayed type hypersensitivity (DTH), bactericidal properties of infected peritoneal macrophages and animal survival. They found that prior injection with all three bacteria led to decreased growth of the challenge bacterium in the spleens of infected mice. This effect paralleled the development of DTH. Furthermore, they found that peritoneal macrophages harvested after intraperitoneal immunization with BCG showed inhibitory activity against heterologous bacteria in vitro. These effects were short lived and required re-immunization to improve survival in vivo. They concluded that acquired cellular immunity could be non-specific in its expression but required a state of prior or ongoing homologous sensitization. Furthermore, due to the temporal relationship between peak resistance to infection and the development of DTH, the authors concluded that a macrophage-bound antibody was likely responsible for both phenomena.¹⁵

Blanden et al.¹⁶ used heterologous bacteria such as *S. enteritidis* and *L. monocytogenes* to characterize the host response to intracellular infection in BCG-immunized animals. This circumvented the difficulties associated with growing and inactivating *Mycobacterium tuberculosis*. They confirmed that BCG immunization resulted in lower counts of viable *L. monocytogenes* in the spleen of infected animals following challenge. They also validated the work by

Mackaness¹⁵ showing that resistance peaked when viable BCG levels were highest. Furthermore, they found this resistance to be dose dependent, terminated by administration of isoniazid and not induced by heat-killed BCG.¹⁶ Blanden and Dannenberg attributed their findings to the theory of 'local immunity', stating that activated macrophages only exerted systemic effects during periods of intense antigenic stimulation, subsequently becoming confined to local granulomata.¹⁷

Blanden¹⁶ and Mackaness¹⁵ attributed the effect of BCG on non-specific intracellular organisms to enhanced antibacterial activity of 'immunologically' activated macrophages. However, some authors questioned the notion that BCG-induced macrophage activation resulted in 'systemic', heterologous antibacterial activity.

Van Dissel et al.¹⁸ compared the rates of bacterial killing of *L. monocytogenes*, *S. enteritidis*, *E. coli*, *Staphylococcus aureus* and *Toxoplasma gondii* by peritoneal macrophages harvested from three experimental groups: mice who received intravenous BCG alone, mice who received intravenous BCG and subsequent intraperitoneal purified protein derivatives (PPD) and control mice who received intraperitoneal peptone (a substance known to induce peroxidase positive cells). The three groups were given an intraperitoneal challenge of the heterologous bacteria and were sacrificed shortly after to allow macrophage harvest. Macrophages harvested from BCG/PPD-stimulated mice had increased bactericidal activity against *L. monocytogenes*. The same macrophages had non-significant increased intracellular killing of *Salmonella typhimurium* but no effect on *S. aureus* and *E. coli*. The intracellular multiplication but not viability of *T. gondii* was inhibited in both BCG and BCG/PPD-infected mice. The in vivo response to infection with these organisms was measured in identical experimental groups by determining viable bacterial counts in the blood, liver and spleen together with relative organ size following infection. Viable counts of *L. monocytogenes* were decreased by 20% in the tissues of BCG/PPD mice without an increase in organ size. In the case of *S. typhimurium* viable counts were reduced by 90% in BCG/PPD mice over the first 2 days and then increased exponentially with splenic size increasing six-fold. The investigators confirmed that macrophages were activated by showing increased hydrogen peroxide production and control of intracellular toxoplasma proliferation. The authors concluded that although macrophages were activated this led to differential activity against disparate heterologous bacteria in vivo. In the case of *S. typhimurium*, BCG/PPD-infected mice exhibited an initial dramatic reduction in viable bacterial counts in the tissues and clearance from the blood followed by an exponential bacterial proliferation and death with a concurrent six-fold increase in splenic size. The authors concluded that in the case of *S. typhimurium*, BCG/PPD led to marked macrophage proliferation and clearance to tissues in the absence of any enhanced intracellular killing. The conclusions of this work supported a non-specific antibacterial activation of BCG-stimulated macrophages against *L. monocytogenes*, however this effect was not sufficient to confer heterologous control of disparate bacteria.¹⁸

'Immunologically committed' lymphoid cells

Macrophage activation, the 'effector' mechanism of cell-mediated immunity, was identified well in advance of the afferent stimulatory cascade induced by BCG. In 1945, Merrill Chase

showed that resistance to 'tubercle bacilli' could be transferred with lymphocytes but not serum.¹⁹ Investigators became increasingly interested in 'immunologically committed lymphoid cells', which contributed to DTH,²⁰ conferred specificity on antibacterial resistance^{21,22} and could augment the effect of macrophages by the secretion of immunologically active soluble mediators.²³

Infection with BCG was known to induce local lymph nodes to undergo proliferation in the paracortical region housing thymocyte-derived cells. Radiolabelling of lymphocytes showed that they circulated from spleen to periphery repeatedly and could freely enter exudates.^{21,22} Mackaness et al.²¹ found that splenic cells from *Listeria*-immunized mice conferred 100% protection against *Listeria* challenge. This effect was diminished if radiosensitive macrophages were ablated or if a chemical substance such as mitomycin was used to stop lymphoid cell proliferation in the tissues. BCG-immunized mice also displayed resistance to *Listeria* infection, however the contribution of splenic cells from these animals was not found unless a second dose of BCG was administered concurrently. The authors concluded that lymphoid cells were important but not the final effector cells in heterologous immunity between BCG and *Listeria*. In further experiments to explore this immune pathway, they showed that intravenously administered BCG and BCG-induced splenic cells activated peritoneal macrophages (i.e., cells remote from the site of injection), leading to the idea that soluble mediator(s) could play an important role.²¹

Kelly²⁴ aimed to provide further evidence that lymphocytes and associated soluble mediators contributed to BCG-induced killing of *Listeria*. Kelly harvested peritoneal macrophages after local injection of BCG cell wall and derived a supernatant capable of killing 94% of *Listeria* in vitro within one hour. Isolation of lymphocytes from the same animals could cause activation of normal macrophages in vitro leading to antibacterial activity against *Listeria* of 74%.²⁴

T cells and antigen presentation

We now recognize the 'immunologically committed' lymphoid cells described by Mackaness²¹ as T helper cells. T helper 1 (Th1) cells are defined by the production of IFN- γ and IL2 and are central to host defence against bacteria, viruses and tumour cells. BCG is a powerful inducer of a Th1 phenotype in infants,²⁵ thus providing a hypothetical mechanism for the reduction in allergy and asthma (diseases associated with a Th2 cell phenotype) that has been described following BCG immunization.²⁶ Th1 cells remain a potentially important focus for the heterologous protection against infection associated with BCG.

T cell mediated heterologous immunity is the term assigned to changes in T cell repertoire and T cell receptor epitope specificity that are induced following prior exposure to a pathogen. Examination of T cell repertoires following viral infection in mice showed heterologous protection against subsequent, unrelated viral challenge. This protection was less than that seen after homologous infection, and was not always reciprocal. In the same set of experiments, heterologous viral immunity was associated with a combination of cross-reactive T cell triggering and an altered cytokine milieu.²⁷

T cell mediated heterologous immunity has been shown between BCG and Vaccinia virus (VV) in mice. BCG-immunized

mice and controls were treated with isoniazid to induce a resting immune state to avoid the possibility that live BCG may induce persistent innate immune activation. The mice were subsequently infected with lymphocytic choriomeningitis virus (LCMV) and VV and viral titres were assessed in organs 1 and 3 days after infection. Vaccinia viral titres were reduced by a factor of between 10-fold and 100-fold in BCG-vaccinated mice whereas LCMV titres were unchanged. These results were replicated in BCG immune B10 mice and major histocompatibility complex (MHC)-congenic B10 mice, indicating that this heterologous immunity exists across different MHC-classes. BCG and VV-infected mice had marked increases in splenic size 6 days following infection and statistically significant increases in both CD4 and CD8 cells in both the spleen and peritoneal cavities. This protective effect was blocked by cyclosporine and by anti-CD4 antibodies but was unaffected by anti-CD8 antibodies, leading the authors to conclude that this was a unique example of CD4-mediated heterologous immunity. Notably the protective effect of BCG on viral titres was seen within 2 days, suggesting a direct effector role of CD4 T cells. The role of cross-reactive T cell receptors (TCR) in the observed heterologous immunity was explored. However, none of the eight characterized immunodominant epitopes of VV were associated with heterologous protection. The authors concluded that these experiments showed a unique case of CD4+ memory type cells as the mediators of heterologous immunity in the absence of ongoing immune activation. They could not define whether this process involved cross-reactive TCR or was TCR-independent.²⁸

T helper and T regulatory cell pools and the subsequent effect on memory T cell repertoires are mediated and maintained by the function of antigen presenting cells. Larsen et al. showed that BCG-stimulated dendritic cells had increased IL-10 production in vitro in comparison to controls.²⁹ Furthermore, BCG stimulation led to the elaboration of T cells without a Th1/Th2 bias and which predominantly produced IL-10. They proposed that decreased mortality post BCG vaccination was the result of down-regulation of harmful pro-inflammatory cytokines.

Cytokines

In 1974, Rocklin et al. showed that migration inhibitory factor (MIF) was released by PPD-sensitized lymphocytes in vitro.³⁰ Re-stimulation of the same lymphocyte population led to the release of multiple factors including macrophage-activating factor, now known as IFN- γ . Nathan et al. originally identified IFN- γ as a macrophage-activating cytokine produced by T cells in 1983.³¹ Salvin et al. had isolated MIF from animal models following BCG immunization and proposed that a balance between MIF, IFN- γ and other unidentified inhibitory factors might control the host response to *M. tuberculosis*.³² However, the addition of IFN- γ to macrophage models in which BCG was the primary stimulant, failed to enhance antibacterial activity against either specific or heterologous antigens.¹⁸ Similarly, Moulton and Gerner³³ showed BCG-vaccinated mice subsequently challenged with PPD had high levels of serum IFN- γ and a bacteriostatic effect against *S. aureus*. In the absence of PPD challenge, the bacteriostatic activity was maintained despite no increase in IFN- γ levels. They attributed this bacteriostatic effect to an as yet unidentified mediator.³³

Clark et al. repeatedly showed a protective effect of BCG against *Babesia* and *Plasmodium* infection in mice.³⁴ The

protective immunity induced by BCG in these models occurred within 12 hours, was as complete as that conveyed by previous homologous infection and led to intra-erythrocytic death of the parasites. The authors stated that since erythrocytes express no MHC molecules and as the heterologous killing was intra-erythrocytic, the effect of BCG was attributable to a soluble mediator rather than a heterologous antibody. Contemporaneously, scientists at the Memorial Sloan Kettering hospital described a soluble molecule that was cytotoxic to tumour cells.³⁵ This was tumour necrosis factor (TNF) that is now recognized to be central to the host response to, and subsequent pathogenesis of, a large number of pathogens known to exhibit heterologous immunity (BCG, *Plasmodium*, *Babesia*, *Brucella*, *Coxiella*, *Salmonella*, *Listeria*, *Leishmania* and *Toxoplasma*).³⁶

Antibody mediated heterologous immunity

BCG has been shown to promote antibody responses but this phenomenon and its role in heterologous immunity have not been studied in detail. In 1930, Dienes and Schoenheit injected antigens including timothy grass pollen and egg albumin into a mouse tuberculous lesion and documented a DTH reaction and an increase in antibody titres.³⁷ Using sheep red blood cells as an antigen, Miller et al.³⁸ showed that BCG increased both B and T cell responses. This was dose dependent with DTH developing at low doses but becoming inhibited at higher doses. This was postulated to be secondary to an accumulation of antigen antibody complexes.³⁸

Minden et al.³⁹ hypothesized that the heterologous protection seen in animal models of infection was due to cross-reactive antibodies recognising different antigenic components of pathogens. They measured binding of radiolabelled antigens from rabbits immunized with BCG, *Listeria* spp., *Salmonella* spp., *E. coli*, *Staphylococcus epidermidis* and *Brucella abortus* and control animals before and after BCG vaccine. All six rabbits immunized with *Listeria*, 4 of 6 *Brucella*-immunized and 3 of 6 *Salmonella*-immunized showed increased binding of BCG.³⁹

Trained immunity

'Trained immunity' is the term given to the phenomenon of immune memory in innate cells and pathways and may be specific or non-specific.⁴⁰ The non-specific up-regulation of innate cells and effector mechanisms resulting in enhanced protection against any secondary infection has been studied in the context of the beneficial, non-specific effects of BCG seen in humans.

Kleinijenhuis et al.⁴¹ recently showed that BCG-induced epigenetic modifications in monocytes play an important role in 'trained immunity'. They showed BCG induced increased trimethylation of histone H3 at lysine 4 (H3K4) at the level of cytokine production and TLR4 binding. To further characterize BCG-induced innate immune regulation, adult peripheral blood mononuclear cells (PBMC) were cultured with BCG in vitro. Following heterologous stimulation with TLR ligands and bacteria, there was increased production of TNF- α . This effect was duplicated when NOD2 ligand MDP was added to the assay. PBMCs from an individual known to be dectin-1 deficient were capable of producing this effect whereas PBMC from a NOD2 deficient individual were not.

The authors concluded that the epigenetic modification of monocytes seen after BCG most likely mediated effects through the NOD2 pathway.⁴¹

Conclusions

BCG has powerful effects on many components of the immune system. The studies included in this review fall into two broad categories. Early studies aimed to elucidate the immune response induced by BCG in relation to its protective effect against TB. The use of heterologous antigens in these studies provided the first evidence that BCG might exert protective effects against organisms other than mycobacteria and that cell mediated immunity or its components may be involved. Later studies investigating the potential role of cross reactive T cell epitopes, soluble mediators and epigenetic modifications induced by BCG were designed to test the hypothesis that BCG exerts heterologous effects via the specific immunological mechanisms studied. These studies in general used larger numbers of animals and more rigorous reporting methods thus providing useful information about the individual mechanisms studied.

Although the studies reviewed here provide biologic plausibility for the significant reduction in mortality seen in BCG-immunized infants in high mortality settings this evidence needs to be considered in light of some methodological weaknesses and also in the historical context of the methods used. These studies were exploratory in design and vulnerable to bias and confounding. Although controls were used in most experiments, the studies were small in size and numbers in each group not defined a priori making assessment of attrition or exclusion of outlier variables impossible. In addition, there was no randomization of animals to groups. There was also significant heterogeneity in the dose, strain and mode of administration of BCG making comparison between studies difficult. In light of current knowledge about the influence of sex on immunological responses it is difficult to draw conclusions from studies done using a single-sex group of animals or where sex was not specified. Furthermore, animal models need to be considered in terms of the induced pathologic response. Some animal models of infections differ from human disease. For example, *Listeria* infection in mice is uniformly fatal, never leading to a chronic stage.

None of the experimental models provide a definitive explanation of the immunological mechanisms underlying heterologous immunity. This might be considered unsurprising in light of the fact that to date the immunological mechanisms underlying the specific effect of BCG remain uncertain and no immunological correlate of protection against TB has been identified following BCG immunization. The data presented here however suggests that multiple mechanisms are likely to be involved.

The heterologous ('non-specific') effects of BCG discussed in this review refer to both specific heterologous immunity, i.e., cross-reaction of antigenic compounds from different organisms, and non-specific heterologous immunity, i.e., where BCG leads to a modification of the immune system that results in heterologous effects without involving the recognition of antigenic determinants. Evidence of specific heterologous immunity is illustrated by the work of Salvin et al.³² and the work outlined in the 'Antibody mediated heterologous immunity' section above. A much larger body of evidence suggests that the heterologous

effects of BCG are mediated through ‘training’ or ‘activating’ various immune pathways, modulating their response to subsequent challenges. In the studies of non-specific macrophage activation, enhanced heterologous immunity was only evident in the case of intracellular organisms. Conversely, there is evidence of ‘trained’ immunity following challenge with *E. coli*. This raises the interesting notion that BCG may exert beneficial heterologous effects against disparate pathogens by different mechanisms. However, the division between these mechanisms is not clear-cut. Early work on macrophage activation highlighted the need for on-going antigenic stimulation with viable BCG, as apparently occurs in infants who may have local reactions to BCG up to 9 months of age. However, both CD4-mediated ‘specific’ cross-reactivity and epigenetic ‘trained’ immunity were both demonstrated in the context of inactivated BCG.

Animal models will continue to help increase our understanding of the mechanisms involved in heterologous immunity provided by BCG. However, studies using animal models should be designed to reflect clinically relevant parameters, including age, sex and pre-existing immunity. In addition, immunological studies should be performed in relevant human populations, including newborns and infants, to identify potential mechanisms that can be further tested in improved animal models.

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References

- 1 Aaby P, Roth A, Ravn H et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis* 2011;204:245–52.
- 2 Shann F. The nonspecific effects of vaccines and the expanded program on immunization. *J Infect Dis* 2011;204:182–4.
- 3 Biering-Sorensen S, Aaby P, Napirna BM et al. Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. *Pediatr Infect Dis J* 2012;31:306–8.
- 4 Kristensen I, Aaby P, Jensen H. Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa. *BMJ* 2000;321:1435–8.
- 5 B.C.G. AND vole bacillus vaccines in the prevention of tuberculosis in adolescents; first (progress) report to the Medical Research Council by their Tuberculosis Vaccines Clinical Trials Committee. *Br Med J* 1956;1:413–27.
- 6 Arnoldussen DL, Linehan M, Sheikh A. BCG vaccination and allergy: a systematic review and meta-analysis. *J Allergy Clin Immunol* 2011;127:246–53, 253 e1–21.
- 7 Holm-Delgado MG, Stuart EA, Black RE. Acute lower respiratory infection among Bacille Calmette-Guerin (BCG)-vaccinated children. *Pediatrics* 2014;133:e73–81.
- 8 Yagi H, Matsumoto M, Kishimoto Y et al. Possible mechanism of the preventive effect of BCG against diabetes mellitus in NOD mouse. II. Suppression of pathogenesis by macrophage transfer from BCG-vaccinated mice. *Cell Immunol* 1991;138:142–9.
- 9 Ristori G, Romano S, Cannoni S et al. Effects of Bacille Calmette-Guerin after the first demyelinating event in the CNS. *Neurology* 2014;82:41–8.
- 10 Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases. *Trans R Soc Trop Med Hyg* 2015; 109:52–61.
- 11 Campbell PA, Czuprynski CJ, Cook JL. Differential expression of macrophage effector functions: bactericidal versus tumoricidal activities. *J Leukoc Biol* 1984;36:293–306.
- 12 Howard JG, Biozzi G, Halpern AC et al. The effect of *Mycobacterium tuberculosis* (BCG) infection on the resistance of mice to bacterial endotoxin and *Salmonella enteritidis* infection. *Clinique Medicale Propedeutique d l'Hopital Broussais*; 1959.
- 13 Pletsity DF, Fomina VG. Effect of vaccination on nonspecific phagocytic activity of peripheral blood leukocytes. *Bull Exp Biol Med* 1973;75:299–301.
- 14 Yarkoni E, Wang L, Bekierkunst A. Stimulation of macrophages by cord factor and by heat-killed and living BCG. *Infect Immun* 1977;16:1–8.
- 15 Mackaness GB. The immunological basis of acquired cellular resistance. *J Exp Med* 1964;120:105–20.
- 16 Blanden RV, Lefford MJ, Mackaness GB. The host response to Calmette-Guérin bacillus infection in mice. *J Exp Med* 1969;129:1079–107.
- 17 Dannenberg AM Jr. Cellular hypersensitivity and cellular immunity in the pathogenesis of tuberculosis: specificity, systemic and local nature, and associated macrophage enzymes. *Bacteriol Rev* 1968;32:85–102.
- 18 van Dissel JT, Stikkelbroeck JM, van den Barselaar M et al. Divergent changes in antimicrobial activity after immunologic activation of mouse peritoneal macrophages. *J Immunol* 1987;139:1665–72.
- 19 Chase MW. The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc Soc Exp Biol* 1945;59:134–5.
- 20 Bloom BR, Bennett B. Migration inhibitory factor associated with delayed-type hypersensitivity. *Fed Proc* 1968;27:13–5.
- 21 Mackaness GB. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J Exp Med* 1969;129:973–92.
- 22 North RJ. Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. *Cell Immunol* 1973;7:166–76.
- 23 van Furth R. Mycobacteria and macrophage activation. *Res Microbiol* 1990;141:256–61.
- 24 Kelly MT. Activation of guinea pig macrophages by cell walls of *Mycobacterium bovis*, strain BCG. *Cell Immunol* 1976;26:254–63.
- 25 Marchant A, Goetghebuer T, Ota MO et al. Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guerin vaccination. *J Immunol* 1999;163:2249–55.
- 26 Strachan DP. Family site, infection and atopy: the first decade of the ‘hygiene hypothesis’. *Thorax* 2000;55(Suppl. 1):S2–10.
- 27 Welsh RM, Che JW, Brehm MA et al. Heterologous immunity between viruses. *Immunol Rev* 2010;235:244–66.

- 28 Mathurin KS, Martens GW, Kornfeld H et al. CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses. *J virol* 2009;83:3528–39.
- 29 Madura Larsen J, Benn CS, Fillie Y et al. BCG stimulated dendritic cells induce an interleukin-10 producing T-cell population with no T helper 1 or T helper 2 bias in vitro. *Immunology* 2007;121:276–82.
- 30 Rocklin RE, MacDermott RP, Chess L et al. Studies on mediator production by highly purified human T and B lymphocytes. *J Exp Med* 1974;140:1303–16.
- 31 Nathan CF, Murray HW, Wiebe ME et al. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med* 1983;158:670–89.
- 32 Salvin SB, Ribi E, Granger DI et al. Migration inhibitory factor and type II interferon in the circulation of mice sensitized with mycobacterial components. *J Immunol* 1975;114(1 Pt2):354–9.
- 33 Moulton RG, Gerner GD. Antibacterial activity of BCG-induced, interferon-containing sera. *Can J Microbiol* 1986;32:442–5.
- 34 Clark IA, Allison AC, Cox FE; Protection of mice against *Babesia* and *Plasmodium* with BCG. *Nature* 1976;259:309–11.
- 35 Old LJ, Clarke DA, Benacerraf B. Effect of Bacille Calmette Guerin infection on transplanted tumours in the mouse. *Nature* 1959;184:291–2.
- 36 Clark IA. Heterologous immunity revisited. *Parasitology* 2001;122(Suppl):S51–9.
- 37 Dienes L, Schoenheit EW. Certain characteristics of the infectious process in connection with the influence exerted on the immunity response. *J Immunol* 1930;19:41–6.
- 38 Miller TE, Mackaness GB, Lagrange PH. Immunopotentiality with BCG. I. The immune response to different strains and preparations. *J Natl Cancer Inst* 1973;51:1655–67.
- 39 Minden P, McClatchy JK, Farr RS. Shared antigens between heterologous bacterial species. *Infect immun* 1972;6:574–82.
- 40 Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe* 2011;9:355–61.
- 41 Kleinnijenhuis J, Quintin J, Preijers F et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA* 2012;109:17537–42.

2.2.3 BCG-INDUCED HETEROLOGOUS IMMUNITY, A HISTORICAL PERSPECTIVE: INTERVENTION STUDIES IN ANIMAL MODELS OF INFECTIOUS DISEASES

The use of BCG as an intervention against infectious diseases in animal models of disease was a popular line of scientific enquiry in the 1960s and 1970s. Systematic review of the literature identified nineteen studies which reported a protective effect of BCG as an intervention in animal models of infectious diseases. Five studies involving bacterial challenge, two a fungal challenge, eight with parasite challenge and four with viral challenge. No studies identifying a negative effect were identified.

These studies have several important methodological limitations which prevent extrapolation of the results to humans. Firstly, although controls were used in most experiments, the numbers of animals in each experimental group were often small and, critically, not defined *a priori* making attrition or exclusion of outlier variables impossible to assess [81-83]. Secondly, where authors did not specify the length of follow up, overall mortality could not be assessed [83, 84]. Thirdly, there was no randomisation of experimental animals indicated in any study [80, 84, 85]. Finally, animal models of disease are often designed to achieve high rates of infection and thus have limited capacity to detect beneficial effects of an intervention at low rates of infectivity which may be seen in humans [86, 87]. Despite these limitations, the results from the animal studies presented here provide strong evidence for a protective effect of BCG against a wide variety of pathogens.

In each study, the authors attributed the protective effect to a particular mechanism i.e. non-specific macrophage activation [88] or increased interferon production [83]. However, the experiments described had limited capacity to differentiate between the relative contribution of macrophages, cytokines or lymphocytes. More recent studies did quantify the relative importance of different effector mechanisms but were unable to definitively correlate them with protective efficacy. Parra *et al* associated the presence of CD8+ T cells with control of *Plasmodium* parasitaemia [89] and Kleinnenjenhuis *et al* concluded the innate immune function was sufficient for control of fatal candidaemia [80]. However, both of these studies also noted increases in

innate immune genes and IFN- γ production respectively suggesting that BCG vaccine induced both innate and cell-mediated immunity.

An interesting observation from this review was that in the case of intracellular organisms i.e. *S. enteritidis*, *C. albicans* and the parasites, the pathological changes associated with improved survival related to enhanced pathogen clearance from the blood and increased sequestration in the tissues. In contrast, in the case of bacteria such as *S. aureus* and *E. coli* and the viruses the outcome measures were development of disseminated disease i.e. encephalitis and mortality or bacterial growth. This observation supports the idea suggested in the previous review publication that BCG vaccine may exert beneficial heterologous effects through different effector mechanisms for disparate pathogens. An alternative explanation is that different elements of the BCG-induced immune response are highlighted when disparate challenge models are used. For example, protection from mortality secondary to intracellular organisms may be due to enhanced clearance from the blood i.e. prevention of dissemination whereas for bacteria and viruses, there was evidence of dissemination but decreased overall pathogenicity and mortality. These results indicate that studies of the BCG-induced heterologous immune response should not be limited to challenge with a single pathogen or immune mechanism.

2.2.4 PUBLISHED REVIEW PAPER

BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases

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The WHO Special Advisory Group of Experts (SAGE) review of the available epidemiological and trial evidence in humans concluded that bacillus Calmette-Guérin (BCG) vaccination leads to beneficial heterologous ('non-specific') effects, specifically on all-cause mortality. Randomized controlled trials showing this beneficial effect suggest improved survival is the result of enhanced protection against infection. This paper reviews the available evidence for the attenuating effects of BCG vaccine on experimental infections in animal models, including protection from bacteria, viruses, parasites and fungi. The reviewed studies suggest that BCG activates multiple immune pathways and that the basis for BCG-associated heterologous immunity may vary by pathogen. Modern immunological and molecular methods, exemplified by 'vaccinomics', are well placed to further investigate the basis of BCG's heterologous effects using a systems biology approach.

Keywords: Animal, BCG, Heterologous, Non-specific, Vaccine

Introduction

The reduction in mortality in bacillus Calmette-Guérin-vaccinated infants observed in high mortality settings has been attributed to the heterologous ('non-specific') effects of BCG on host immunity. The WHO Special Advisory Group of Experts (SAGE) review of the evidence supporting a protective heterologous effect of BCG focused on epidemiological studies and specifically did not include any evidence from animal studies. This paper outlines the evidence from animal studies of BCG as an intervention against infectious diseases. This was a popular line of scientific enquiry in the 1960s and 1970s and followed the studies summarized in our companion review,¹ which provide biological plausibility for the ability of BCG to protect against heterologous infections.

Extrapolation of findings from animal models of disease have a controversial history. Considerable effort has been put into standardizing translational animal research to maximize its relevance to understanding interventions for human disease. Such guidelines include familiar concepts from human research including hypothesis-driven questions, sample size calculations, use of

controls, randomization of subjects and full reporting of attrition and adverse events. Specific to animal studies are the reporting requirements relating to the animals (including age and sex) and environmental conditions under which they were kept. Repeated validation of findings in different animals, at different time points contributes to the 'robustness' of any identified effect.² Of particular importance in assessing the strength of evidence in animal studies, is acknowledgement of publication bias with neutral or negative effects much less likely to be published.^{3–5}

While meta-analysis of animal studies of therapeutic interventions is considered extremely difficult and often inappropriate, critical reviews of systematically-acquired literature are encouraged. Although the majority of studies discussed in this paper pre-date guidelines on animal experimentation, we have applied the criteria from these guidelines to assess the strength of existing evidence. In contrast to the studies detailed in our companion review¹ that explore the underlying immunological mechanisms of BCG-associated heterologous immunity, the studies in this review report morbidity and mortality outcomes in BCG-treated or BCG-vaccinated animals compared to controls. The aim of

this review is to assess the strength of evidence for a clinically relevant protective effect of BCG in animal models of disease. The evidence in this article is presented chronologically and by the proposed immunological mechanism of action.

Non-specific immune activation of the reticuloendothelial system including macrophage activation

There are 10 studies in which the authors cited macrophage activation or non-specific activation of the reticuloendothelial system as the most likely mechanism by which they expected BCG to protect against heterologous infection (Table 1). Four of these studies addressed heterologous protection against bacteria,^{6–9} five against parasites,^{10–14} two against fungi^{8,15} and one against a virus.¹⁶

In studies investigating BCG's ability to protect against bacterial infection, three studies showed prolonged survival.^{6–8} The fourth was a model of surgical infection and showed reduced growth of *Escherichia coli* in tissues.⁹ These studies are heterogeneous in terms of the methods used. Details of the dose and route of administration of BCG, as well as the interval between BCG and bacterial infection, are outlined in Table 1. Dubos et al.⁶ aimed to confirm their previous finding in mice that heterologous protection existed between *E. coli*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Mycobacterium fortuitum*. On this occasion the authors tested protection against *S. aureus* following BCG infection. They hypothesized that it was related to activation of the reticuloendothelial system. They showed increased survival at day 17, post intravenous infection with *S. aureus* for both viable and heat-killed BCG, with the effect more marked with the latter. They validated the protective effect of heat-killed BCG by repeating the experiments with different doses and different preparations. Furthermore, they showed that increasing the time between BCG vaccination and staphylococcal infection to 2 months did not diminish the survival benefit.⁶

Howard et al.⁷ demonstrated a prolongation of survival time following experimental infection with *S. enteritidis*. This paralleled a marked increase in generalized reticuloendothelial activation and a rapid clearance of bacteria from the bloodstream. They further observed a marked increase in susceptibility to endotoxin despite increased bacterial clearance and survival.

Sher et al.⁸ aimed to explore the protective effects of BCG against bacterial and fungal infections in normal mice and mice immunosuppressed with cyclophosphamide. As BCG was gaining momentum as a treatment for malignancy, proving that it could have anti-infective properties in immune compromised hosts was of interest. Compared with controls, increased survival time was observed in both groups of BCG-vaccinated mice (immunologically normal and cyclophosphamide-treated) after intravenous challenge with *S. aureus* and *Candida albicans*. Van t'Wout et al.¹⁵ were also interested in the clinical relevance of BCG for treating candidal infections in immune compromised hosts. They concluded that BCG inhibits dissemination of candida following concomitant intravenous administration of BCG. They demonstrated increased metabolic activity of macrophages and subsequent restriction of intracellular germ tube growth and hypothesized that these were the likely mechanisms of protection.

Five papers addressed parasitic infection following BCG administration; three with trypanosomiasis,^{10,11,14} one with

toxoplasmosis¹² and one with schistosomiasis.¹³ Ortiz-Ortiz et al.¹⁰ conducted a simple interventional study, which showed overwhelmingly positive results for control of *Trypanosoma cruzi* infection in neonatal mice using high doses of BCG. They concluded that based on contemporaneous evidence that cells from mice repeatedly immunized with BCG had an increased capacity for killing *T. cruzi* in vitro, cell-mediated immunity was the most likely mechanism. Kuhn et al.¹¹ did similar experiments with intravenous BCG and experimental *T. cruzi* infection in adult mice and found no difference in survival time or level of parasitaemia. Similarly, Hoff et al.¹⁴ found no effect of intraperitoneal BCG infection on the mortality of adult mice with experimental trypanosome infection. Despite the different modes of administration of BCG in these two studies, it is interesting that no protective effect was found in adult mice whereas an overwhelmingly positive effect was observed in neonatal mice.

Civil et al.¹³ tested the protective effect of different doses and modes of administration of BCG against various measures of disease progression in schistosomiasis including mean schistosomula count, day of peak resistance and mean adult worm number. They found the response to be dose dependent. It was also dependent on mode of administration, with subcutaneous, intraperitoneal, intradermal and scarification methods having no benefit, while intravenous administration reduced mortality by 40%.

Werner et al.¹⁶ reported increased survival of BCG-vaccinated mice compared with controls when challenged with vaccinia virus. Although the authors stated that macrophage activation was the recognized primary mode of action of BCG at the time of their study, it is interesting that they observed a four-fold increase in antibodies against vaccinia virus in the BCG-vaccinated mice. They concluded that BCG, which had been recently shown to have anti-tumour effects, might also provide anti-infectious protection in cancer patients.

'Immunologically-committed' lymphoid cells

Larson et al.¹⁷ studied BCG protection against infection with herpesviridae, which at the time were postulated to be associated with the development of cervical cancer. The authors reported that BCG protected against the development of encephalitis and reduced mortality in a rabbit model that involved corneal scarification with *Herpesvirus hominis* (simplex) type 2. One of the strengths of this study was that protection was shown in two distinct strains of rabbit. The authors concluded that their clinical findings could be explained by recently uncovered immunological mechanisms of non-specific resistance such as central nervous system antibodies in a rabies virus model¹⁸ or immunologically active lymphocytes as described by Mackaness et al.¹⁹

Nakamura et al.²⁰ confirmed the concept that BCG increases susceptibility to endotoxin, as originally proposed by Howard et al.⁷ They also showed that BCG-immunized rabbits had lower bacterial counts in eye wash fluid than controls following infection with *Shigella*. The net effect was that the severity of conjunctivitis was unchanged between infected animals and controls.

Smrkovski et al.²¹ concluded that BCG altered the progression of visceral leishmaniasis in mice and that the observed effect was dose and time dependent. These authors drew heavily on the work of Mackaness et al.¹⁹ to provide a potential immunological mechanism for these effects, specifically that viable BCG in the spleen of immunized animals leads to sustained lymphocyte

Table 1. Animal studies investigating BCG heterologous protection from infection with macrophage activation as a proposed mechanism

Author	Hypotheses	Population	BCG	Outcome measures	Results	Sample size	Random	Controls	Report LTFU attrition	Detail of animal model and care
Dubos 1958 ⁶	BCG (viable, heat killed and extracts) protect against IV <i>S. aureus</i>	Mice Neonatal Male or Female	IP Viable BCG Phila (0.1 ml) vs IP HK BCG Phila (2.5 mg/2 ml)	Cumulative death up to day 17	HK BCG 3 deaths day 17 Viable BCG 8 deaths day 17 Saline 8 deaths day 12 (0 survival day 17)	Immunized (n=8) Controls (n=8)	✓	✓	✗	✓ Albino mice/ Rockefeller Swiss strain
Howard 1959 ⁷	BCG alters resistance to endotoxin and <i>S. enteritidis</i> infection at 12–14 days	Mice 22 g Male	IV BCG Pasteur (0.25 mg)	Phagocytic index	5x fold increase	Immunized (n=11) Controls (n=7)	✗	✓	✗	✓ Albino mice Swiss strain
				Susceptibility to endotoxin	LD50 BCG 4.5×10^7 vs controls 5×10^9	Immunized (n=99) Controls (n=41)				
				Mean survival	BCG 13d vs controls 5.5d	Immunized (n=41) Controls (n=37)				
Ortiz-Ortiz 1973 ¹⁰	BCG protects against IP <i>T. cruzi</i> at 10 days	Mice 4–6 weeks Sex NS	IV BCG Mexico (4×10^6)	Mortality	Controls 100% mortality BCG 60% mortality	Immunized (n=10) Controls (n=10)	✗	✓	✗	✓ Carworth Farm strain (CF1)
				Mean survival	Controls 19.4d (SEM ± 0.83) BCG 31d (SEM ± 3.1)					
				Parasite count	Trypanomastigotes in blood reduced in BCG group ($p < 0.05$)					
Kuhn 1974 ¹¹	BCG protects against IP <i>T. cruzi</i> at 21 days	Mice Adult Female	IV Viable BCG Strain not specified (3 mg/0.2 ml wet weight)	Mean survival	BCG 23.2d (SD ± 1.64) (range 10–25) Controls 23.4d (SD ± 5.26) (range 15–28)	Immunized (n=10) Controls (n=10)	✗	✓	✓	✓ C3H(He)
				Peak parasitaemia	No difference					
				Radiolabelled parasite distribution	Significantly more organisms in spleen and kidneys in BCG group					
Sher 1975 ⁸	BCG protects immune competent and immune deficient mice from infection with IV <i>S. aureus</i> and <i>C. albicans</i> at 3, 7, 14, 28 days in a dose- dependent manner	Mice Adult >23 g Male	IP BCG Brazil (10^6 / 10^4 / 10^2 cfu/0.1 ml)	Mean survival in immune-competent (<i>S. aureus</i>)	BCG 23.2d Controls 15d	Immunized (n=25) Controls (n=25)	✗	✓	✓	✓ CDF1
				Mean survival in immune-deficient (<i>S. aureus</i>)	At 14 days BCG 13.4d (SEM ± 3) Control 7d (SEM ± 0) ($p < 0.001$) At 21 days BCG 10.2d (SEM ± 0.7) Controls 5.4d (SEM ± 0.5) ($p < 0.0001$)					

				Effect of isoniazid on mean survival (<i>S. aureus</i>)	BCG alone 38d (SEM ± 3.5) BCG+INH 23.9d (SEM ± 4) INH alone 23d (SEM ± 4) Control 7.8d (SEM ± 2)					
				Mean survival time in immune-deficient (<i>C. albicans</i>)	BCG 7d (SEM ± 0) Controls 13.4d (± 0.3 SEM)					
Hoff 1975 ¹⁴	BCG protects against <i>T. cruzi</i> infection at 3 and 18 days	Mice Female 14–16 g	IP BCG Glaxo 10 ⁵	Cumulative mortality 32d	BCG 100% mortality Controls 100% mortality	Immunized (n=6) Controls (n=6)	X	X	X	X
Tabbara (Abstract) 1975 ¹²	BCG protects against <i>Toxoplasma</i> chorioretinitis	Rabbits Age NS Sex NS	IV and retrobulbar BCG (strain not specified)	Disease onset and severity	IV BCG delayed onset and severity	NS	X	X	X	X
				Toxoplasma in tissue	Toxoplasma isolated from BCG and controls					
Civil 1978 ¹³	BCG protects mice from infection with <i>S. mansoni</i> at 14 days	Mice Neonatal Female	IV/IP/SC/ Viable BCG Tice vs HK BCG Tice	MPS $\pm$ SEM	MPS BCG immunized vs controls (p<0.01)	Immunized (n=6) Controls (n=6)	X	✓	X	X
				Day of peak resistance	Peak d5. Reduction of infection if BCG 14, 7, 1d pre or up to 4d post challenge.					
				Mean adult worm number $\pm$ SEM	Adult worm 15 $\pm$ 3 vs 32 $\pm$ 3 (p<0.01)					
				Dependent on strain	BCG Tice 53% MPS (p<0.01) BCG Pasteur 63% (p<0.05)					
				Dependent on dose and route of administration	>10 ⁶ HK BCG Tice conferred protection. Scarification, IP, SC no protection; IV 60% protection					
Werner 1979 ¹⁶	BCG provides protection against VV at 7–12 days	Mice Adult 15–25 g Male	IP/NG BCG Connaught 10 ⁷	Mortality	BCG 3/25 Controls 25/25	Immunized (n=25) Controls (n=25)	✓	X	✓	✓
				Efficacy of NG BCG	NG BCG had no effect					
				VV antibodies	VV antibody titres increased post BCG vaccine (1:128 vs 1:512)					
Fagelman 1981 ⁹	BCG provides non-specific antibacterial activity against <i>E. coli</i> surgical wound infection at 13 days	Mice Adult Male	SC BCG (strain not specified) 10 ⁸	Growth curves of viable <i>E. coli</i> from infected muscle	BCG reduced growth of <i>E. coli</i> (p<0.04)	Immunized (n=8) Controls (n=8)	X	X	✓	X

Continued

Table 1. Continued										
Author	Hypotheses	Population	BCG	Outcome measures	Results	Sample size	Random	Controls	Report LTFU attrition	Detail of animal model and care
Van t'Wout 1992 ¹⁵	BCG/PPD stimulated macrophages cause increased phagocytosis and intracellular growth of <i>C. albicans</i>	Mice Adult Male	IV BCG Denmark 5×10 ⁶ + IP PPD 50 µg	Growth of Candida in organs H ₂ O ₂ production by macrophages Intracellular growth of <i>C. albicans</i>	Candida in liver and spleen was less in BCG immunized mice at 1–7 days (p<0.01) BCG/PPD stimulated peritoneal macrophages 6x increase in H ₂ O ₂ production Germ tube length was decreased in BCG/PPD stimulated macrophages (p<0.01)	NS	✗	✓	✗	✓ SPF Swiss mice
BCG: bacillus Calmette–Guérin; CFU: colony forming units; HK: heat killed; H ₂ O ₂ : hydrogen peroxide; INH: isoniazid; IP: intraperitoneal; IV: intravenous; LD50: lethal dose at which 50% mortality would be expected; LTFU: lost to follow-up; NG: nasogastric; NS: not stated; MPS: mean pulmonary schistosomula; PPD: purified protein derivative; SC: subcutaneous; SEM: standard error of the mean; W: vaccinia virus.										

stimulation, release of soluble mediators and subsequent control of intracellular organisms. In terms of BCG as therapy for leishmaniasis, it was more effective at lower levels of amastigote infection, which the authors attributed to less antigenic competition.

Cytokine production

At the start of the 1970s, macrophage inhibition factor and interferon type II had been isolated in vitro.^{22,23} Salvin et al.²⁴ went on to prove that they could be isolated in vitro from mice infected with BCG. When these mice were subsequently challenged with an antigen, such as old tuberculin, the yield of these factors was increased three- to four-fold.

The experimental work by Clarke et al.²⁵ published in 1976, provides some of the strongest evidence that BCG exerts heterologous effects. Mice were infected intraperitoneally with high doses of one of three species of *Babesia* or two of *Plasmodia*. Sampling of blood at intervals after infection allowed comparison of parasite growth curves between BCG-infected and non-infected mice. BCG-infected mice had lower levels of parasitaemia. These studies were replicable in numerous animals and with a variety of parasitic species. Furthermore, experiments were done to exclude the involvement of macrophages and antibodies in the protective effect by giving the BCG-immunized mice intravenous silica either before or after babesial challenge. The authors concluded that soluble mediators were required for the effective killing of intra-erythrocytic parasites.

Suenaga et al.²⁶ reported that intra-peritoneal BCG infection protects mice from subsequent ectromelia (mouse pox) viral infection, leading to decreased mortality. They went on to show increased production of interferon in peritoneal exudates of BCG-infected mice and lower interferon levels in the liver, spleen and blood compared with controls. In a further experiment comparing peritoneal and splenic cells of BCG-immunized mice and controls, they showed that both cell types had an eight-fold increase in interferon production in vitro.

Sakuma et al.²⁷ progressed the research on the antiviral properties of interferon produced post BCG infection. They confirmed the effect of BCG in reducing mortality from ectromelia virus. They showed increased interferon production, which was at least partially dependent on splenic function. They went on to conclude that BCG-induced resistance to viral infection involved the production of interferons by macrophages and T lymphocytes. This was further supported by the finding that treating mice with anti-thymocyte and anti-macrophage serum reduced the protective effect.

Trained immunity

The work of Kleinnijenhuis et al.²⁸ described in detail in our companion review,¹ showed increased blood cytokine production in response to heterologous antigens following BCG immunization in humans. These authors showed that these effects were likely due to epigenetic reprogramming of monocytes and specifically the NOD2 pathway. To confirm the importance of innate immunity in mediating the heterologous effects of BCG vaccine, they did an additional experiment in which 15 SCID (severe combined immune deficiency) mice were treated with intravenous BCG or control solution and 14 days later were inoculated with a lethal dose of *C. albicans*. At 1-month post infection, survival was

Table 2. Animal studies investigating BCG heterologous protection from infection with immunologically committed lymphoid cells as a proposed mechanism

Author	Hypotheses	Population	BCG	Outcome measures	Results	Sample size	Random	Controls	Report LTFU attrition	Detail of animal model and care
Larson 1972 ¹⁷	BCG immunization protects rabbits from HHV2 infection	Rabbits Adult Male/ Female	IV BCG Pasteur 10 ⁷	Encephalitis post corneal scarification	Immunized 24/30 Controls 30/30	Immunized (n=30) Controls (n=30)	✗	✓	✗	✓ NZ Dutch belted (DB)
				Mortality post corneal scarification	Immunized 8/30 Controls 25/30	Immunized (n=30) Controls (n=30)				
				Vaginitis	Immunized 8/12 Controls 9/11	Immunized (n=13) Controls (n=11)				
Nakamura 1972 ²⁰	BCG protects against <i>Shigella</i> kerato-conjunctivitis at 22 days	Rabbits Age NS Sex NS	IV BCG Pasteur 10 ⁷	DTH PPD DTH endotoxin	DTH response to endotoxin 3x DTH response to PPD in BCG immunized	Immunized (n=8) Controls (n=8)	✗	✓	✗	✗
				Severity of conjunctivitis	Incidence and severity unchanged					
				Isolation of mean bacterial counts from eye	Unvaccinated 3x bacterial counts of vaccinated					
Smrkovski 1975 ²¹	BCG reduces severity of visceral <i>Leishmaniasis donovani</i> The effect is dependent on parasite burden and route of administration	Mice 6 weeks Female	IV BCG Pasteur 10 ⁷	Determination of TPB	BCG immunized reduced TPB on d16 and d30 compared to controls (p<0.001)	NS	✗	✓	✓	✓
			IV BCG Pasteur 10 ⁷		Spleen TPB peak at d30 10 ⁵ amastigotes (p<0.01) 10 ⁶ amastigotes (p<0.05)	Immunized (n=21) Controls (n=21)				
			IV or IP BCG Pasteur 10 ⁷		IP BCG significant decreases in spleen TPB at d30 and d45. IV BCG no effect	IV Immunized (n=21) IP Immunized (n=21) Controls (n=21)				
					IP BCG no effect on liver TPB IV BCG decrease in liver TPB (p<0.001)					

BCG: bacillus Calmette-Guérin; DTH: delayed type hypersensitivity; HHV2: *Herpes hominis (simplex)* virus type 2; IP: intraperitoneal; IV: intravenous; LTFU: lost to follow-up; PPD: purified protein derivative; TPB: total parasite burden.

Table 3. Animal studies investigating BCG heterologous protection from infection with cytokine production as a proposed mechanism

Author	Hypotheses	Population	BCG	Outcome measures	Results	Sample size	Random	Controls	Report LTFU attrition	Detail of animal model and care
Salvin 1974 ²²	BCG/OT immunization leads to production of lymphokines with anti-infective properties	Mice Female Adult	IV BCG No Strain 3×10 ⁶ +IV 50 OT µg	Culture of <i>S. aureus</i> , <i>S. faecalis</i> , <i>P. aeruginosa</i> <i>C. albicans</i>	MIF/IFN serum bacteriostatic <i>in vitro</i> - inhibition of growth of bacteria but not yeast	BCG serum (n=3) Control serum (n=3)	✗	✓	✗	✓ Swiss Webster
Clarke 1976 ²⁵	BCG protects against babesial infection	Mice Female/ Male Adult	IV BCG Glaxo 2×10 ⁷	Parasitaemia	In BCG mice low-level parasitaemia and cleared quickly. Controls 50% parasitaemia	Immunized (n=57) Controls (n=NS)	✗	✗	✗	✓ CBA
	Effect maintained at high parasite loads			Parasitaemia	Maintained at IP parasite loads of 10 ⁶ , 10 ⁷ , 10 ⁸					
	Effect not due to babesial antibodies			Babesial antibodies	Negligible antibody levels in serum					
	BCG protects against <i>Plasmodia spp.</i>			Parasitaemia and survival	100% survival in 4 mice	Immunized (n=4)				
Suenaga 1978 ²⁶	BCG protects against ectromelia virus	Mice Female Adult	IP BCG Tokyo (1 mg wet weight)	Mortality	BCG mortality=6/18 Control mortality=14/21	Immunized (n=18) Controls (n=21)	✗	✓	✗	✓ DDN
				Ectromelia Virus liver, spleen, peritoneal exudates (TCID50)	Higher TCID50 in controls in all areas but difference greatest for liver and spleen cell exudates					
	BCG increases IFN production			IFN production by peritoneal splenic cells	8x production of IFN in BCG immunized mice					

Sakuma 1976 ²⁷	BCG protection against ectromelia Virus	Mice Female Adult	IP BCG Tokyo (1 mg wet weight)	Mortality	BCG+Splenectomized=14/25 Splenectomized only=20/25 BCG alone 7/25 Controls=23/25	Immunized (n=25) Controls (n=25)	✓ ✓ ✓ ✗	✓ ✓ ✓ ✓	DDN
	BCG leads to increased IFN production			Relative IFN production	BCG+splenectomized mice had 1/8 production of IFN compared to BCG+sham operated				
	Protective effect is due to IFN activity via lymphocytes and macrophages			Effect of splenectomy on RES function (CC)	BCG enhanced CC in splenectomized and non-splenectomized				
				Survival	BCG alone 100% survival day 10, BCG+ATS 40%, BCG+AMS 0%				
AMS: anti macrophage system; ATS: antithymocyte serum; BCG: bacillus Calmette-Guérin; BM: bone marrow; CC: carbon clearance; IFN: interferon; IP: intraperitoneal; IV: intravenous; LTFU: lost to follow-up; MIF: macrophage inhibitory factor; NS: not stated; OT: old tuberculin; TCID 50: tissue culture infectious dose that will infect 50% of cells at a defined inoculum.									

100% in the BCG group and 30% in the control group. The median survival time was significantly prolonged in the BCG group and the fungal burden of disease (*C. albicans* log¹⁰ cfu/g of kidney tissue) was significantly decreased at both day 3 and day 14.^{14,28}

Parra et al.²⁹ showed a consistent reduction in parasitaemia levels in mice infected with *Plasmodium yoelli* following subcutaneous BCG immunization compared with controls. Interestingly, this effect was significant at 2 months post immunization, but was not observed at 2 weeks. The role of CD4 and CD8 T cells was investigated with depletion experiments. CD4 cells were shown to be essential for parasite control. Depletion of CD8 cells did not influence parasite proliferation, but abolished the protective effect of BCG. The authors also showed increased gene expression of several innate antibacterial peptides. Incubation of parasites with the peptides lactoferrin and cathelicidin LL-37 prior to infection, led to significant decreases in peak parasitaemia.

Conclusions

The evidence in this review has been presented under the headings: macrophage activation, immunologically-committed lymphoid cells and cytokine production to complement and provide continuity with our companion review. This division is based on the way that the investigation of potential theories of BCG-mediated heterologous immunity occurred over several decades, paralleling popular lines of immunological enquiry. However this separation is somewhat artificial, as we now understand that BCG induces an interplay of several elements of cell-mediated immunity. We found no studies attributing a clinical heterologous effect to humoral immunity, although several authors found pathogen-specific antibodies were increased in BCG-immunized animals.^{12,16} Interestingly, Clark et al.²⁵ found that, despite consistent protection against babesia infection in mice, babesial antibodies were absent.

It is difficult to extrapolate the evidence presented here to the clinical benefit seen in humans following BCG immunization. We now appreciate the importance of sex on the observed reduction of mortality effect following BCG and on postulated underlying immunological mechanisms. One limitation is therefore that the majority of experiments were done in a single-sex group of animals or sex was not specified. In addition, the results from animal models (the majority of which were in mice) need to be interpreted in terms of the induced pathologic response and its relevance to human infections. Moreover, there was also significant heterogeneity in the dose, strain and mode of administration of both BCG and experimental pathogens, further contributing to the difficulty in making meaningful comparisons between studies and interpreting their clinical relevance.

Many of the studies had methodological limitations or flaws. This is underlined by the fact that no study fulfilled all four recommended quality criteria for animal studies (Tables 1–4). Although controls were used in most experiments, the numbers of animals in each experimental group were often small and, critically, not defined ‘a priori’ making attrition or exclusion of outlier variables impossible to assess.^{7,20,26} Where authors did not specify the length of follow-up, overall mortality could not be assessed.^{17,26} There was no randomization of experimental animals indicated in any study. Assessor bias was controlled for in one study in which animals in a control group for splenectomized animals

Table 4. Animal studies investigating BCG heterologous protection from infection with innate immunity as the proposed mechanism

Author	Hypotheses	Population	BCG	Outcome measures	Results	Sample size	Random	Controls	Report LTFU attrition	Detail of animal model and care
Kleinnijenhuis 2012 ²⁸	BCG protects SCID mice from <i>C. albicans</i> infection	Mice Age NS Sex NS	IV BCG Pasteur 10 ⁶	Mortality Fungal disease burden in kidney tissue CFU/g TNF α production from splenic cells after LPS stimulation	BCG=70% Controls=100% Difference in fungal burden p<0.001 Difference in TNF α production from spleen monocytes p<0.001	Immunized (n=15) Controls (n=15)	X	✓	✓	✓ Prkdc ^{SCID}
Parra 2013 ²⁹	BCG provides partial protection against <i>P. yoelii</i> CD4/CD8 cell depletion reduced BCG-mediated protection Innate gene expression was altered by BCG Antibacterial peptides are stimulated by BCG and confer protection against <i>P. yoelii</i>	Mice Age NS Sex NS	SC BCG Pasteur 10 ⁶	Parasitaemia Parasitaemia following CD4/CD8 cell depletion Differential gene expression profiles Parasitaemia post lactoferrin or cathelicidin pretreatment	At 2 months past BCG: parasitaemia decreased (p<0.05) At 2 weeks past BCG: no significant protection CD4 depleted: BCG peak parasitaemia =75% Controls peak parasitaemia=75% CD8 depleted: BCG peak parasitaemia =35% Controls peak parasitaemia=25% 2–6x upregulation of 4 antibacterial peptide genes normally down regulated by plasmodia infection Reduction in parasitaemia Lactoferrin: p<0.05 Cathelicidin LL-37, peptide: p=0.03	Immunized (n=30) Controls (n=30)	X	✓	✓	✓ C57BL/6

BCG: bacillus Calmette-Guérin; IP: intraperitoneal; IV: intravenous; LPS: lipopolysaccharide; LTFU: lost to follow-up; NS: not stated; SC: subcutaneous; SCID: severe combined immune deficiency.

received sham operations. Several studies had increased validity as a result of repeated experiments to replicate results.^{17,25,28}

Overall, the results from the animal studies presented here provide strong evidence for a protective effect of BCG against a wide variety of pathogens. We have highlighted the limitations that hinder extrapolation of these findings to humans. Furthermore, the evidence suggests that BCG exerts wide-ranging effects involving both innate and cell-mediated immunity, and that the predominant mechanisms may vary by pathogen. Future research in humans to further explore the mechanisms underlying the heterologous effects of BCG should focus on a systems biology approach. Animal models of infectious diseases will remain important to complement human studies and help validate potential mechanisms.

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References

- 1 Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms. *Trans R Soc Trop Med Hyg* 2015;109:46–51.
- 2 Kilkenny C, Browne WJ, Cuthill IC et al. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010;8:e1000412.
- 3 Hirst JA, Howick J, Aronson JK et al. The need for randomization in animal trials: an overview of systematic reviews. *PLoS One* 2014;9:e98856.
- 4 Sena ES, van der Worp HB, Bath PM et al. Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. *PLoS Biol* 2010;8:e1000344.
- 5 Tsilidis KK, Panagiotou OA, Sena ES et al. Evaluation of excess significance bias in animal studies of neurological diseases. *PLoS Biol* 2013;11:e1001609.
- 6 Dubos RJ, Schaedler RW. Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections I. Protective effects. *J Exp Med* 1957;106:703–17.
- 7 Howard JG, Biozzi G, Halpern AC et al. The effect of mycobacterium tuberculosis (BCG) infection on the resistance of mice to bacterial endotoxin and salmonella enteritidis infection. *Clinique Medicale Propedeutique d l'Hopital Broussais*; 1959.
- 8 Sher NA, Chaparas SD, Greenberg LE, Bernard S. Effects of BCG, *Corynebacterium parvum*, and methanol-extraction residue in the reduction of mortality from *Staphylococcus aureus* and *Candida albicans* infections in immunosuppressed mice. *Infect Immun* 1975;12:1325–30.
- 9 Fagelman KM, Flint LM Jr, McCoy MT et al. Simulated surgical wound infection in mice: effect of stimulation on nonspecific host defense mechanisms. *Arch Surg* 1981;116:761–4.
- 10 Ortiz-Ortiz L, Gonzalez-Mendoza A, Lamoyi E. A vaccination procedure against *Trypanosoma cruzi* infection in mice by nonspecific immunization. *J Immunol* 1975;114:1424–5.
- 11 Kuhn RE, Vaughn RT, Herbst GA. The effect of BCG on the course of experimental Chagas' disease in mice. *Int J Parasitol* 1975;5:557–60.
- 12 Tabbara KJ, O'Connor GR, Nozik RA. Effect of immunization with attenuated *Mycobacterium bovis* on experimental toxoplasmic retinochoroiditis. *Am J Ophthalmol* 1975;79:641–7.
- 13 Civil RH, Warren KS, Mahmoud AA. Conditions for bacille Calmette-Guerin-induced resistance to infection with *Schistosoma mansoni* in mice. *J Infect Dis* 1978;137:550–5.
- 14 Hoff R. Killing in vitro of *Trypanosoma cruzi* by macrophages from mice immunized with T. cruzi or BCG, and absence of cross-immunity on challenge in vivo. *J Exp Med* 1975;142:299–311.
- 15 van 't Wout JW, Poell R, van Furth R. The role of BCG/PPD-activated macrophages in resistance against systemic candidiasis in mice. *Scand J Immunol* 1992;36:713–9.
- 16 Werner GT. The effect of BCG-vaccination on vaccinia virus infections in mice. *Experientia* 1979;35:1514–5.
- 17 Larson CL, Ushijima RN, Karim R et al. Herpesvirus hominis type 2 infections in rabbits: effect of prior immunization with attenuated *Mycobacterium bovis* (BCG) cells. *Infect Immun* 1972;6:465–8.
- 18 Bell JF, Lodmell DL, Moore GJ, Raymond GH. Brain neutralization of rabies virus to distinguish recovered animals from previously vaccinated animals. *J Immunol* 1966;97:747–53.
- 19 Mackaness GB. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J Exp Med* 1969;129:973–92.
- 20 Nakamura M, Cross WR. Susceptibility of rabbits immunized with *Mycobacterium bovis* (BCG) or *Mycobacterium phlei* to Shigella keratoconjunctivitis. *Infect Immun* 1972;6:1025–30.
- 21 Smrkovski LL, Larson CL. Effect of treatment with BCG on the course of visceral leishmaniasis in BALB/c mice. *Infect Immun* 1977;16:249–57.
- 22 Salvin SB, Nishio J. Cellular origin of migration inhibitory factor released into the circulation of intact tuberculous mice. *Federation Proceedings* 1975;34:4355.
- 23 Salvin SB, Youngner JS, Nishio J, Neta R. Discussion paper: a tumor-inhibiting lymphokine from tuberculous mice. *Ann NY Acad Sci* 1976;276:369–72.
- 24 Salvin SB, Nishio J, Shonnard JT. Two new inhibitory activities in blood of mice with delayed hypersensitivity, after challenge with specific antigen. *Infect Immun* 1974;9:631–5.
- 25 Clark IA, Allison AC, Cox FE. Protection of mice against Babesia and Plasmodium with BCG. *Nature* 1976;259:309–11.
- 26 Suenaga T, Okuyama T, Yoshida I, Azuma M. Effect of *Mycobacterium tuberculosis* BCG infection on the resistance of mice to ectromelia virus infection: participation of interferon in enhanced resistance. *Infect Immun* 1978;20:312–4.
- 27 Sakuma T, Suenaga T, Yoshida I, Azuma M. Mechanisms of enhanced resistance of *Mycobacterium bovis* BCG-treated mice to ectromelia virus infection. *Infect Immun* 1983;42:567–73.
- 28 Kleinnijenhuis J, Quintin J, Preijers F et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA* 2012;109:17537–42.
- 29 Parra M, Liu X, Derrick SC et al. Molecular analysis of non-specific protection against murine malaria induced by BCG vaccination. *PLoS One* 2013;8:e66115.

2.3 BCG-INDUCED HETEROLOGOUS IMMUNITY; HUMAN STUDIES

The immunological basis for the non-specific effects of BCG vaccine in humans were the subject of a WHO SAGE review [90]. This group concluded the data was too heterogeneous to draw any conclusion on underlying mechanisms. In the following section, only studies which report heterologous cytokine response following BCG vaccination in infants are presented. Methodological considerations which may contribute to the observed heterogeneity between studies and which are relevant to the interpretation of this thesis are discussed. Finally, the systems vaccinology studies of the immune response to BCG vaccine which were unpublished at the time of the aforementioned review are discussed.

2.3.1 INFANT CYTOKINE RESPONSES FOLLOWING BCG VACCINATION

The WHO SAGE observed a systematic difference in the results reported from cohort and case-control studies with cohort studies more likely to report increases in pro-inflammatory cytokine responses. An explanation for this observation was the effect of immune ontogeny in longitudinal studies involving infants [91, 92]. However, this effect was also seen in adult participants, whose immune response should not be susceptible to ontogenic change over the course of a longitudinal study [93].

2.3.1.1 Interpretation of multiplex cytokine data in human studies

Between-group comparisons of multiplex cytokine data are influenced by the inter-individual variability of cytokine responses which can adversely affect the ability to detect a statistically significant difference due to an external exposure such as a vaccine. The power to detect a difference between groups with a particular sample size is also affected by the strength of the stimulation induced by the antigen being studied. Therefore, cohort studies, which control for inter-individual variability have increased power to detect a significant difference attributable to the vaccine, compared to case-controlled studies. This is particularly true where the cytokine response being measured is robust irrespective of the vaccine effect i.e. pro-

inflammatory responses following stimulation with LPS. Thus, statistically 'significant' cytokine/stimulant pairs may vary between studies depending on the study design, the sample size and the choice of antigen. Cytokine data output will also be influenced by duration of stimulation, duration of incubation and dilution factor of RPMI medium: blood. Thus, phenotypical patterns of cytokine response which are consistent between studies are more important than reproducible results for any one cytokine/stimulant pair.

Cytokine responses to BCG vaccination in infancy are broadly characterised under the following phenotypical groups to facilitate the description of results;

1. Pro-inflammatory cytokines – TNF- α , TNF- β , IL-6, IL-1 β .
2. Anti-inflammatory – IL-1Ra, IL-10.
3. Chemokines – MIP-1 α , MIP-1 β , MCP-1, MIF, IL-8, MIG, IP-10.
4. T-cell activator cytokines – IL-10, IL-12p40, sCD-40L.
5. Th1 cytokines – IFN- γ IL-2.
6. Th2 cytokines – IL-4, IL-5, IL-9, IL-13.
7. Growth factors – GCSF, GM-CSF, GRO.

These cytokine classifications are over-simplistic and ignore the pleiotropic nature of certain cytokines (IL-6, IL-10), and also the complexity of the cellular pathways from which they originate and operate. They are useful however to summarise the patterns of cytokine production in infants following BCG vaccination.

2.3.1.2 The specific, mycobacterial response to BCG vaccination

Infant cytokine responses to 'specific' mycobacterial stimulants are characterised by the production of pro-inflammatory and Th1 cytokines at birth with the production of Th2 cytokines (IL-5, IL-13) and T cell activation and regulatory cytokines (IL-12, IL-10 and sCD40L) from three to twelve months of age accompanied by a decrease in pro-

inflammatory cytokines such as TNF- α during this time period [94, 95]. This pattern of cytokine production was seen in both case-controlled and cohort studies of infant cytokine responses, however Th2 derived cytokines were more frequently reported as significantly altered in cohort studies.

2.3.1.3 The non-specific, heterologous cytokine response to BCG vaccination

To date there are four controlled studies of BCG-induced heterologous cytokine responses in infants, two of which report results from neonates (Table 2.2).

Differences in the cytokines measured, the age at BCG vaccination and the time between BCG vaccination and blood sample make direct comparisons difficult. In BCG-vaccinated neonates, at 1 month post vaccination, the heterologous response was characterised by increased pro-inflammatory cytokine production both in RPMI-stimulated (nil) samples and following stimulation of TLR 1 / 2 (Pam3CYSK4) [96]. In BCG-vaccinated infants, from 3 months post vaccination, the heterologous response was characterised by increased IL-6 and growth factor production following stimulation with heterologous bacteria [97], decreased IL-17, IL-13 and IL-6 production in response to *C. albicans* and decreased IL-6 following *C. albicans* stimulation [53].

Three of the four studies in Table 2.2 also reported heterologous cytokine responses following stimulation with innate immune ligands [96-98]. Stimulation of TLR 1/2 (Pam3CYSK4) induced the production of pro-inflammatory and T cell activator cytokines in BCG-vaccinated infants compared to BCG-naïve infants. While stimulation of TLR 4 (LPS) led to upregulation of IL-8 with concomitant downregulation of growth factors at 4 months post BCG vaccination in the study by Smith *et al.* but had no effect on cytokine production at either 3 or 13 month post vaccination in the Danish study or at three months in South African infants.

The available cohort studies of cytokine production following stimulation with heterologous 'non-specific' bacterial stimulants were done in adults using peripheral blood mononuclear cells (PBMCs). In these studies, heterologous cytokine responses were characterised by increased production of IFN- γ and pro-inflammatory cytokines response to stimulation with heterologous bacteria up to three months post

vaccination compared to baseline [93, 99-101]. They showed that pro-inflammatory cytokines other IFN- γ were largely produced by NK cells and that the effect persisted until one year post vaccination with the added contribution of IL-17 and IL-22 [99, 100].

2.3.1.4 Conclusions

In light of the methodological considerations outlined above it is difficult to compare changes in individual cytokine/pairs between studies. Identification of reproducible phenotypical patterns however are relevant, although it is not possible to attribute clinical significance to these changes. For example, the specific, anti-mycobacterial cytokine responses following BCG vaccination are characterised by Th1 polarization in infants however this has never been correlated with protective efficacy against *M. tuberculosis* infection. The existing studies of which measured heterologous cytokine responses following BCG vaccination suggest the induction of a pro-inflammatory phenotype however this appears variable between age groups and geographic locations and is more frequently identified in cohort studies.

Author	Design	Population	Age BCG	Age at blood	Assay	Stimulant	Cytokine	Results
Nissen 2018 Denmark [53]	RCT	4d BCG+ve (n=80) BCG-ve (n=78) 3m BCG+ve (n=78) BCG-ve (n=72) 13m BCG+ve (n=76) BCG-ve (n=68)	Birth	4d 3m 13m	Whole blood 1:5	1d & 4d incubate RPMI LPS PHA BCG <i>C. albicans</i> <i>E. coli</i> <i>S. pneumoniae</i>	1d IL-1 β IL-6 TNF- α 4d IFN- γ IL-17 IL-22	4d N/S 3m <i>C. albicans</i> $\downarrow$ IL-6, IL-10, IL-22.
Hanekom 2014 South Africa [98]	Case control	Cord BCG-ve (n=25) 10wk BCG+ve (n=36) 3mo BCG+ve (n=35)	Birth	Cord 10wk 3m	Whole blood 1:10	18hr incubate LPS Pam3CYSK4 BCG	TNF- α IL-6 IL-10 IL-12p40 IL-12p70	10wk Pam3CYSK4 $\uparrow$ TNF- α , IL-6, IL-12 36wk Kruskal-Wallis (overall effect at 36wk) $\uparrow$ TNF- α , IL-6 Pam3CYSK4 $\uparrow$ TNF- α , IL-6, IL-12 Kruskal-Wallis (overall effect at 36wk) $\uparrow$ TNF- α , IL-6, IL-12
Smith 2017 UK [97]	Case control	BCG+ve (n=10) BCG-ve (n=11)	6wk	4m	Whole blood 1:5	2d incubate <i>C. albicans</i> <i>S. aureus</i> MTBk LPS Pam3CYSK	42-plex	Pam3CYSK4 $\uparrow$ IL-10, EGF, PDGF, sCD40L, IL-12p40, IL-6, MCP-3, IL-7, eotaxin, MIP-1 α <i>C. albicans</i> $\uparrow$ EGF, IL-6, PDGF, MCP-3 $\downarrow$ IL-2, IL-13, IL-17, IP-10 <i>S. aureus</i> $\uparrow$ EGF, IL-6, PDGF <i>E. coli</i> $\uparrow$ EGF $\downarrow$ GM-CSF, GRO LPS $\uparrow$ IL-8 $\downarrow$ GM-CSF, GRO
Jensen 2015 [96]	RCT	BCG+ve (n=261) BCG-ve (n=217)	Birth	1m	Whole blood 1:9	24hr LPS Pam3CYSK4	7-plex	RPMI $\uparrow$ TNF- α , IFN- γ Pam3CYSK4 $\uparrow$ TNF- α , IFN- γ , IL-6, IL-1 β

Table 2.1 Multiplex cytokine responses to heterologous pathogens and TLR ligands following BCG vaccination in infants

RPMI=Roswell Park Memorial Institute Medium, LPS=Lipopolysaccharide, PHA=Phytohaemagglutinin, MTBk=Heat killed *M. tuberculosis*.

EGF=Epidermal growth factor, PDGF=Platelet derived growth factor, GM-CSF=Granulocyte-monocyte colony stimulating factor, RCT=randomised controlled trial, GRO=CXCL1

2.3.2 SYSTEMS VACCINOLOGY AND BCG

Systems vaccinology integrates data from classical immunological assays (ELISA, flow cytometry) and high-throughput technologies (transcriptomics, proteomics, metabolomics) [102]. It has been successfully used to correlate innate immune signatures with protective efficacy [103] to generate data-driven hypotheses and novel insights into how vaccines protect against disease [104]. Systems vaccinology has been used to investigate the immune response to BCG vaccine in the context of both mycobacterial (Table 2.3) and heterologous infection (Table 2.4).

The development of new *M. tuberculosis* vaccines has led to the development of surrogate correlates of protection following BCG vaccination. Table 2.3 shows two examples, mycobacterial growth inhibition assay (MGIA) and quantification of mycobacterial growth in biopsy material from the BCG scar [104, 105]. Joosten *et al* showed that inhibition of mycobacterial growth was strongest in BCG-vaccinated individuals who had a recent exposure to *M. tuberculosis*, variable following BCG vaccination alone and poor in cases of both active and latent tuberculosis infection [105]. Furthermore, inhibition of mycobacterial growth correlated with an increased monocyte: lymphocyte ratio and increased IP-10 from a population of CD14⁺ monocytes characterised by splice variants in CXCR3 (G-coupled chemokine receptor on effector T cells). Since this signature correlated with mycobacterial growth inhibition which was strongest in individuals with recent exposure to *M. tuberculosis*, the authors concluded that this phenotype was induced by BCG and activated on exposure to another mycobacterium. Of note, the immune response following stimulation with routine mycobacterial antigens such as PPD did not correlate with mycobacterial growth inhibition. This was due to inter-individual variability in the response of participants to PPD and underscores the utility of a comprehensive, hypothesis generating description of the immune response, particularly where there is a clinically relevant end-point.

Matsumiya *et al.* quantified the growth of *M. bovis* in biopsy material from the BCG scar of individuals at two weeks post vaccination and found that it correlated with

increased expression of innate immune genes of interest including *NOD2* (nucleotide-binding oligomerization domain) and *LILRB* (leukocyte immunoglobulin like receptor) [104]. Scar severity was associated with an increased pro-inflammatory cytokine response and rapid clearance of BCG growth from the scar site in the first two weeks post vaccination. Participants who had a history of prior BCG vaccination (median of 10 years) had an increase in differentially expressed genes following the experimental intradermal BCG challenge. The development of a scar is associated with improved heterologous protection following BCG vaccine [31] and the model in this study suggests that the more robust an individual's initial pro-inflammatory response post BCG-vaccination, the more marked the development of a scar. This provides an immune response phenotype which could be correlated with clinical endpoints in epidemiological trials.

The study design used by Fletcher *et al* is the gold standard systems vaccinology design. These authors correlated early post BCG vaccination immune signatures with development of clinical *M. tuberculosis* infection in infants exposed to the disease compared to controls [106]. The inability of this study to identify an early post BCG vaccination signature which correlated with protective immunity against *M. tuberculosis* is significant. Using an unbiased systems biology approach, this study found that BCG vaccination induced two distinct immune response patterns. This dichotomous immune response post BCG vaccination in both cases and controls reduced the overall power to detect a significant correlate of protection. The authors could identify no association between gender, ethnicity, birth weight or gestational age on the development of either of the observed patterns of immune response and the presence of an unmeasured environmental effect modifier cannot be ruled out. Variability in the protective efficacy of BCG vaccination against *M. tuberculosis* infection in different geographic locations is well reported [107], the results of this study suggest the existence of variability in individual level responsiveness which needs to be considered in the design and interpretation of future studies.

Human challenge models of yellow fever and *P. falciparum* infection following BCG vaccination support trained immunity as a potential mechanism for BCG-induced

heterologous immunity [44, 45]. In the case of yellow fever, control of viraemia was correlated with an initial increase in IL-1 β responses in RPMI (nil) stimulated samples prior to heterologous challenge and lower levels of all measured cytokines during the viral challenge period in BCG-vaccinated adults compared to controls. Interleukin-1 β is considered a marker of trained immunity and single nucleotide polymorphisms (SNPs) in genes of the IL-1 signalling pathway were associated with variability in the induction of trained immunity post BCG vaccination. Similarly, control of parasitaemia in the human challenge model of *P. falciparum* was associated with the induction of both NK and monocyte populations and the production of IFN- γ in only a subset of BCG-vaccinated patients. This pattern of immune responses was not evident in BCG-naïve participants. Although the numbers are small, this adds to the evidence that within a given population there may be distinct immune responses to BCG vaccine at an individual level. It is also interesting that BCG-vaccinated participants reported more severe symptoms in the early phase of malaria infection suggesting perhaps a more robust, early pro-inflammatory activation which although uncomfortable to the host was beneficial in terms of subsequent control of disease.

The studies discussed in this section highlight the strengths of systems biology approaches, particularly in a cohort design and where a controlled human challenge is possible. It is noteworthy that irrespective of whether the aim of the study was to explore control of mycobacterial or heterologous infection post BCG vaccination several consistent characteristics of the BCG immune response were seen including (i) the effect of priming following prior exposure to BCG vaccine (ii) the possibility of individual level genetic variation contributing to the immune response to BCG and (iii) the response to BCG vaccine is characterised by mixed innate and T cell responses and (iv) distinct patterns of BCG-induced immune response are seen with disparate pathogenic challenges.

Author	Population	Intervention or Exposure	Correlate	Outputs	Conclusions
Joosten 2018 Netherlands [105]	Adults	BCG TB exposed LTBI Active TB	MGIA with BCG	Cytokines (PBMC) Monocyte subsets T cell subsets	Inhibition of mycobacterial growth by exposure group: BCG-vaccinated= variable, active TB=none, LTBI=none, TB exposure(recent)=good Inhibitions of mycobacterial growth did not correlate with PPD and Quantiferon Inhibition of mycobacterial growth correlated with: - CD19⁺ B cells - CD4⁺ central memory cells - CD14 - CD14:CD3 (monocyte: lymphocyte) Inhibition of mycobacterial growth inversely correlated with: - CD3⁺ cells - CD4⁺/CD8⁺ effector cells
Fletcher 2016 South Africa [106]	Neonates	BCG	<i>M. tuberculosis</i> infection	Cytokines (PBMC) Flow cytometry RNA micro-array	No immune signature differentiated cases of <i>M. tuberculosis</i> (n=29) BCG immune response characterised by two distinct patterns (clusters) Clusters differed by proportion of M1 (classically activated) versus M2 (alternatively activated) macrophages Clusters differed by cytokines which matched macrophage type i.e. M1 (IFN-γ, IP-10, IL-6, MIP-1α, MIP-1β) and M2 (EGF, VEGF, IL-1Ra)
Matsumiya 2015 UK [104]	Adults	BCG	Mycobacterial growth in vaccine site biopsy at 2 weeks BCG scar severity	Cytokines (WB) Flow cytometry (WB) DNA micro-arrays	Mycobacterial growth correlated with: - NOD2 - LILRB Mycobacterial growth Inversely correlated with: - Inflammatory cytokines - Scar severity DEGS: innate immune, T cell immune, apoptosis, glycolysis. Group who were prior BCG-vaccinated had increased DEGS post BCG challenge

Table 2.2 Systems vaccinology approaches to investigate the mechanisms for protective efficacy of BCG against *M. tuberculosis*

BCG=Bacille Calmette-Guerin, TB=Tuberculosis, LTBI=latent tuberculosis infection, MGIA=Mycobacterial growth inhibition assay, PBMC=peripheral blood monocytes, PPD=purified protein derivative, WB=whole blood, DEGS=Differentially expressed genes NOD2=nucleotide oligomerization binding protein, LILBR=Leukocyte Immunoglobulin like receptor

Author	Population	Intervention or Exposure	Correlate	Outputs	Results / Conclusions
Arts 2018 Netherlands [44]	Adults BCG (n=15) Controls (n=15)	YF vaccine	YF viraemia	Cytokines Flow cytometry CHIP-Seq	BCG vaccination led to genome wide epigenetic reprogramming of monocytes predominantly associated with G-protein coupled receptors, MAP kinases, PI3K/AKT and VEGF signalling. BCG vaccination led to increased IL-1β at baseline and decreased cytokinaemia post yellow fever viraemia. BCG vaccination led to decreased yellow-fever viraemia and this was correlated with baseline IL-1β levels. Individuals with SNPs in genes associated with IL-1β signalling had changed in trained immunity profile.
Walk 2019 Netherlands [45]	Adults BCG (n=9) Controls (n=10)	Malaria HCI	Parasitaemia Adverse events	Monocyte subsets Neutrophil subsets T cell subsets Cytokines	BCG vaccination associated with - Increased symptom severity (patient report) - Greater drop in platelet count A subset of BCG-vaccinated volunteers had a distinct immune profile characterised by increased NK-cell (CD69⁺), monocyte (CD14⁺) subsets and IFN-γ responses. The same subset of volunteers showed decreased parasitaemia day 7. BCG-vaccinated volunteers had increased CD8⁺ lymphocyte responses to <i>P. falciparum</i> challenge at day 45.

Table 2.3 Systems vaccinology approaches to investigate the mechanisms for protective efficacy of BCG against heterologous infection

BCG=Bacille Calmette-Guerin, YF=Yellow fever, CHIP-Seq=chromatin immunoprecipitation sequencing, MAP=mitogen activated protein PI3K/Akt = phosphatidylinositol 3-kinase Akt/Protein Kinase B, HCI=Human challenge infection, NK=Natural killer cells

2.4 BCG VACCINATION AS A CLINICAL IMMUNE MODULATOR

BCG vaccine is a potent immune modulator. It is first line therapy in the treatment of transitional cell carcinoma of the bladder in adult patients [108] and recent randomised trial evidence demonstrate a significant reduction in pathological changes on MRI in patients with chronic relapsing Multiple Sclerosis [109] and C-peptide levels in patients with longstanding type 1 diabetes mellitus [110] following BCG therapy. The hypothetical mechanisms underlying these effects are presented to explore potential overlap with BCG-induced heterologous immunity in the context of infection.

BCG has been a mainstay in the treatment of uroepithelial cancer since 1976 [111]. Intravesical BCG therapy is the standard of care for carcinoma in situ, high-grade papillary tumours and lamina-propria-invasive tumours [112]. The mechanism of action of BCG in bladder cancer is incompletely understood but is associated with induction of an acute inflammatory response which requires live *M. bovis* to have direct contact with uroepithelial cells [113]. A suggested mechanism of action is internalisation of BCG by bladder cancer cells leading these cells to initiate antigen presentation and recruitment of immune cells [114]. Both CD4⁺ and CD8⁺ T cells increase in the urine and on pathological samples following BCG therapy however only NK cells have been associated with direct cytotoxicity against tumour cells [115-117]. The cytotoxic effect of BCG activated NK cells is enhanced *in vitro* by IFN- γ and IL-12p40 and inhibited by IL-10 and seems to be mediated partly by increased expression of perforin [118, 119]. In addition, NKT cells and $\gamma\delta$ -T cells are cytotoxic against bladder cancer cells *in vitro* when stimulated by BCG-pulsed dendritic cells [120]. Neutrophils are the most commonly identified cells in the urine of patients treated with intravesical BCG and along with NK cells producing the cytokine TRAIL (TNF related apoptosis inducing ligand) which contributes to the cytotoxicity of these cells [121, 122]. Toll-like receptors-2,4 and 9 have all been implicated in the production of pro-inflammatory cytokines via activation of NF- κ B signalling pathway [123]. The anti-tumour effects of BCG are dependent on both innate and T cell mediated immunity and a pro-inflammatory phenotype in the immediate post-therapy phase.

Clinical trials of BCG vaccine in patients with a variety of multiple sclerosis (MS) syndromes have shown improvement in the appearance of MRI-identified disease plaques and also prevention in the development of axonal loss on MRI scan which is associated with the development of disability [109, 124]. The mechanism of action is unclear and is thought to be due to increased systemic production of TNF- α . Unlike other auto-immune disorders treatment of Multiple sclerosis with TNF- α blockade is associated with worsening of disease [125]. A genetic variant of Multiple sclerosis is also associated with production of TNF receptor 1 which downregulates TNF signalling [126]. More recent work from a mouse model of Parkinson's disease has shown BCG-induced repair of neuro-inflammation through enhanced T regulatory cell functions [127].

BCG vaccination was associated with improved control of blood sugars and decreased C-peptide levels in patients with long-term type 1 diabetes mellitus [128]. The mechanism for this clinically beneficial effect is the induction of glycolysis [129]. Traditionally BCG vaccination is associated with induction of a Th1/Th17 phenotype however BCG vaccination also prevented development of diabetes mellitus in Non-obese diabetic (NOD) mice by a process which was dependent on a shift away from Th1/Th17 [130]. The discovery that BCG-induced trained immunity was due to the induction of glycolysis in monocytes and macrophages offered a possible explanation for this paradox [131]. Regulatory T cell development, controlled via induction of the Foxp3⁺ gene is dependent on glycolysis in T cells [132, 133]. In this paradigm, glycolysis induced by BCG would be responsible both for the development of the pro-inflammatory Th1/Th17 phenotype and also the production of inducible T regulatory cells with the capacity to protect the host from uncontrolled inflammation or excessive innate immune activation.

The notion that BCG vaccine can be an effective treatment for auto-immune diseases associated with relative TNF- α deficiency through induction of a pro-inflammatory response and simultaneous tolerance to hyper-inflammation is an attractive hypothetical mechanism by which BCG vaccine could reduce mortality in sepsis,

particularly during the neonatal period, a relatively TNF- α deficient time of life, the neonatal period.

2.5 BCG-INDUCED RESISTANCE & TOLERANCE IN THE CONTROL OF INFECTION & AUTO-IMMUNITY

The animal and human models described above indicate BCG vaccination can lead to (i) enhanced clearance of mycobacteria [104], intracellular organisms [45] and tumour cells [113], through the initiation of inflammation and (ii) enhanced tolerance to inflammation following widespread dissemination of bacteria, viruses [44] and autoimmune DAMPs [134]. This dual functionality, which varies by challenge, suggest that BCG vaccination may mediate its protective heterologous effects through a combination of (i) resistance and (ii) tolerance [135]. This underlies the importance of considering the choice of clinical correlate which will reflect the most likely underlying immune mechanism for any given pathogen [135].

Resistance and tolerance can be inter-dependent such as with innate immune receptors (TLRs) and effector molecules (reactive oxygen species) which when activated mediate resistance to an invading pathogen but which must also be controlled to prevent hyper-activation of the immune system [136]. Similarly, the relative activity or blockade of TNF can provide a trade-off between resistance and tolerance to infection and disease [137]. However, tolerance to disease can also be mediated several mechanisms which are unrelated to pathogen resistance such (i) repair (ii) reduction of tissue damage (iii) host or microbe energy utilisation and (iv) clearance of toxic compounds generated by infection [135].

The study of tolerance and resistance in animal and insect models of disease suggest that holistic hypothesis-generating enquiries into host-microbe interactions, which go beyond hypothesis-driven interrogation of immunological pathways, are necessary to understand complex host-pathogen interactions and the potential effect of immune-modulators on these relationships. In addition, an individual's capacity for resistance or tolerance to disease may be mediated by factors such as age, genetic predisposition

and environmental exposure necessitating consideration of these factors in the design and analysis of studies [138, 139].

2.6 POTENTIAL EFFECT MODIFIERS OF THE IMMUNE RESPONSE TO BCG VACCINE

In addition to methodological considerations in the interpretation of cytokine data following BCG vaccination, it is essential to consider both internal and external effect modifiers in experiments designed to interrogate the heterologous response following BCG vaccination. As discussed in chapter 1, sex, environment, maternal BCG status and the timing of BCG vaccination after birth are potentially important **external** effect modifiers for the clinical heterologous effects of BCG vaccine. Nissen *et al.* found no evidence of effect modification by timing of neonatal BCG, maternal BCG status or sex on heterologous cytokine responses in their study [53]. Of note, immune responses in this study were associated with variations in the growth of BCG vaccine from different vials and batches [69]. There is evidence that innate immune responses vary by geographic location [140] and the 'specific' anti-mycobacterial immune response following BCG vaccine varies between populations however the cause of this variation is unclear [94].

Internal effect modifiers on cytokine response data include host genetics and the developmental stage of the immune system. Host genetics and host microbiome may confer variability in the immune responsiveness to the vaccine [141] and to the stimulants in the assay [142]. Furthermore, the developmental stage of the immune system at the time of investigation must be considered in the interpretation of results [91, 143]. In the first weeks after birth, the neonatal innate immune system shifts from a tolerant phenotype *in utero* to a robust immune defence against multiple organisms both pathogenic and colonizing [144]. Neonates show a higher susceptibility to infection and also infectious related mortality due to dysregulated inflammatory responses [145, 146]. Several diseases of prematurity are attributed to dysregulation of the innate immune response including necrotising enterocolitis [147, 148] and periventricular leukomalacia [149, 150]. Whole blood stimulation assays with TLR ligands done in infants show changes in cytokine output over time [151, 152]. The newborn innate cytokine response is characterised by IL-23 and IL-6 production which

is replaced over time by TNF- α and IL-1 β . Also, notable in the newborn innate immune response is the absence of type I interferon production and the low levels of IL-12p40 production [153]. Mechanisms which have been associated with these variations include (i) epigenetic modifications (IFN- γ) [154] (ii) reduced expression of interferon regulatory factors-3,5,7 (IL-12p40) [155] and (iii) inhibition of cytokines by soluble mediators such as adenosine (TNF- α) [156]. Evaluation of the heterologous immune response following BCG vaccination therefore needs to be considered in light of the capacity of the immune system to mount or control a given response at a given age.

2.7 CONCLUSIONS

This review of the literature on BCG-induced heterologous immunity suggests that induction of innate immunity and specifically ‘trained immunity’ are important potential underlying mechanisms. Furthermore, the literature suggests that the manifestation of heterologous immunity may be variable between individuals and be dependent on the nature heterologous challenge. In addition to innate immunity, there is an apparent role for cell-mediated immunity including NK-cells, T regulatory cell function and cytotoxic T cells. These observations support the use of multiple pathogens and the measurement of both innate and T cell associated cytokines when designing experiments to interrogate the heterologous immune response following BCG vaccination. Systems vaccinology is clearly a powerful tool to understand the mechanisms of BCG-induced heterologous immunity.

Study design and participant factors can affect the measurement of the BCG-induced heterologous response in infants. A strength of this thesis is the combination of multiplex cytokine data from both cohort and case-controlled analyses. Participant factors including sex, timing of BCG vaccine, maternal BCG vaccination and inter-individual variability of the immune response are considered during both analysis and interpretation of the results in each chapter.

**Chapter 3 NEONATAL BCG VACCINATION INFLUENCES CYTOKINE
RESPONSE TO TOLL-LIKE RECEPTOR LIGANDS AND
HETEROLOGOUS PATHOGENS**

3.1 INTRODUCTION

This chapter presents the results of cytokine analysis from MIS BAIR trial participants at 7 days post randomisation to BCG vaccine or no BCG vaccine. The study participants, laboratory methods, results and discussion are detailed in the included publication. The following provides additional information on the development of the laboratory protocols which are also applicable to chapters 4 and 5. Additional information is provided on the development of the statistical methodology which is also applicable to chapter 5.

3.2 DEVELOPMENT OF THE WHOLE BLOOD ASSAY

A whole blood stimulation assay containing mycobacteria, bacteria, a fungus and TLR-ligands was developed and was used for the experiments presented in chapters 3-5.

3.2.1 CHOICE OF HETEROLOGOUS PATHOGENS

As discussed in chapter 1, the beneficial effect of BCG vaccination on all cause infant mortality was associated with a reduction in death secondary to sepsis and pneumonia. Group B streptococcus (*S. agalactiae*), *E. coli* and *L. monocytogenes* are the most prevalent pathogens associated with neonatal sepsis worldwide. Sepsis beyond the neonatal period but within the first six months of life is most commonly caused by *S. pneumoniae*, *S. aureus*, *S. pyogenes* and *H. influenzae*. *S. pneumoniae* remains the leading cause of bacterial lower respiratory tract infection (LRTI) worldwide with respiratory syncytial virus (RSV) and influenza accounting for the majority of hospitalisations and deaths from viral LRTIs. Thus, these pathogens were all considered for inclusion in the whole blood assay. *M. tuberculosis* and BCG were chosen as the most clinically relevant mycobacterial antigens to measure the 'specific' immune response.

Bacteria and fungi were donated by the RCH clinical microbiology laboratory. These pathogens were isolated from the blood of infants with a diagnosis of sepsis. Two isolates of each pathogen were provided for preparatory assays (Table 3.1). The WHO influenza collaborating centre (Parkville), contributed two inactivated influenza strains.

Inactivated RSV was unavailable for use in pilot assays and we did not have the capacity to work with live viruses.

Organism	Date of specimen collection	Age of patient
<i>E. coli</i> A	15/02/2012	12d
<i>E. coli</i> B	21/02/2012	18d
<i>L. monocytogenes</i> A	28/04/2008	0d
<i>L. monocytogenes</i> B	03/02/2009	0d
<i>C. albicans</i> A	20/05/2011	11d
<i>C. albicans</i> B	04/04/2010	4wk
GBS A (<i>S. agalactiae</i>)	01/06/2012	10d
GBS B (<i>S. agalactiae</i>)	02/09/2011	4d
<i>S. aureus</i> A	23/08/2011	13d
<i>S. aureus</i> B	22/03/2013	19d
<i>S. pneumoniae</i> (serotype 15C)	01/04/2012	2yr
<i>S. pneumoniae</i> (serotype 19A)	06/11/2014	1yr
<i>H. influenzae</i> type B	11/08/2012	3m
<i>H. influenzae</i> non-type B	20/12/2010	9m

Table 3.1 Candidate pathogens for inclusion in the whole blood assay.

3.2.2 OPTIMISATION EXPERIMENTS

A series of optimisation experiments was done using blood donated from adult volunteers and neonatal participants in the MIS BAIR study who consented to participation in the immunological sub-study. The aims of these experiments were to determine:

1. The minimum dilution fraction (RPMI: blood) which could be used (1:2, 1:5, 1:10).
2. The optimum length of incubation for the measurement of Th1 (IFN- γ) and innate (TNF- α) (4hr versus 18hr).
3. The optimum choice (isolate A versus isolate B) and strength (CFU/mL) for each pathogen to be included in the assay.

A summary of the optimisation experiments is given in table 3.2. Detailed laboratory protocols for these experiments are included as appendix 5a-e.

Selected optimisation data are shown in Figures 3.1 and 3.2. Optimisation experiment 3 (Figure 3.1) trialled several blood:RPMI dilutions to determine the maximum dilution factor at which a selection of cytokine responses could be consistently measured across all pathogen types. Figure 3.1 shows that apart from Gram-negative stimulations any dilution beyond 1:2 resulted in undetectable cytokine responses.

To determine the optimal incubation time at which to measure a combination of early and late cytokine responses, both 4 and 18 hour incubations were tested. In Figure 3.1 it is clear that 18 hours of incubation was required to detect cytokine responses consistently across all pathogen types.

Figure 3.2 shows combined neonatal and adult pilot data from optimisation experiments 4 and 5., stimulation with Gram-negative pathogens and *C. albicans* resulted in detectable cytokine responses at lower overall concentrations (CFU/mL) and this is reflected in the final assay concentrations.

In the MIS BAIR study, at 7 months post randomisation (Chapter 4), and in the BabyBAIR study (Chapter 5) group B streptococcus (*S. agalactiae*) was substituted with group A streptococcus (*S. pyogenes*) as the former is rarely a pathogen outside of the neonatal period. Inactivated influenza virus was poorly immunogenic and it was excluded from these experiments.

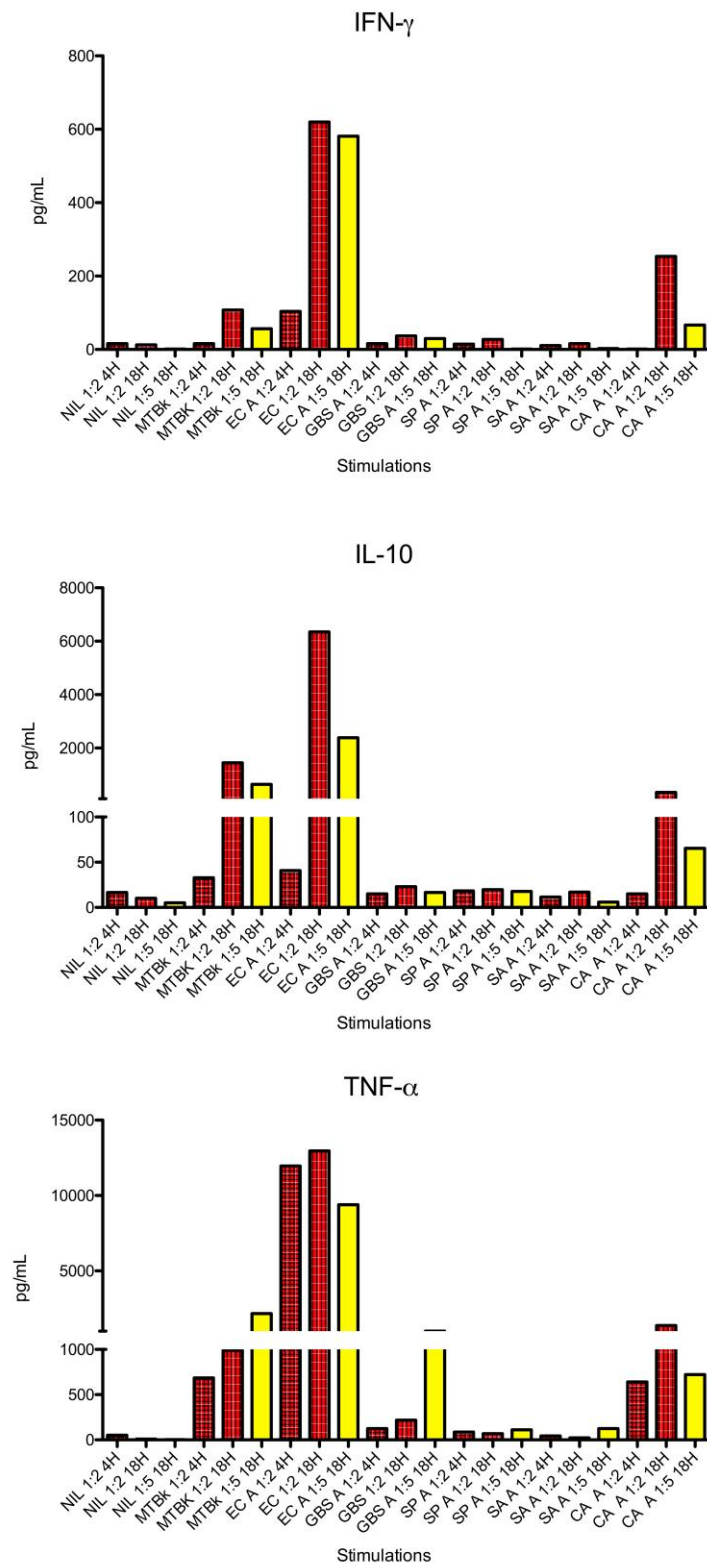


Figure 3.1 Optimisation experiment 3 (i) RPMI:blood dilution 1:2 / 1:5 and (ii) incubation time 4h / 18h. Cytokine concentrations in supernatants (pg/mL)

NIL=RPMI, MTBK=Heat killed *M. tuberculosis*, EC=*E. coli*, GBS=Group B streptococcus (*S. agalactiae*), SA=*S. aureus*, CA= *C. albicans*.

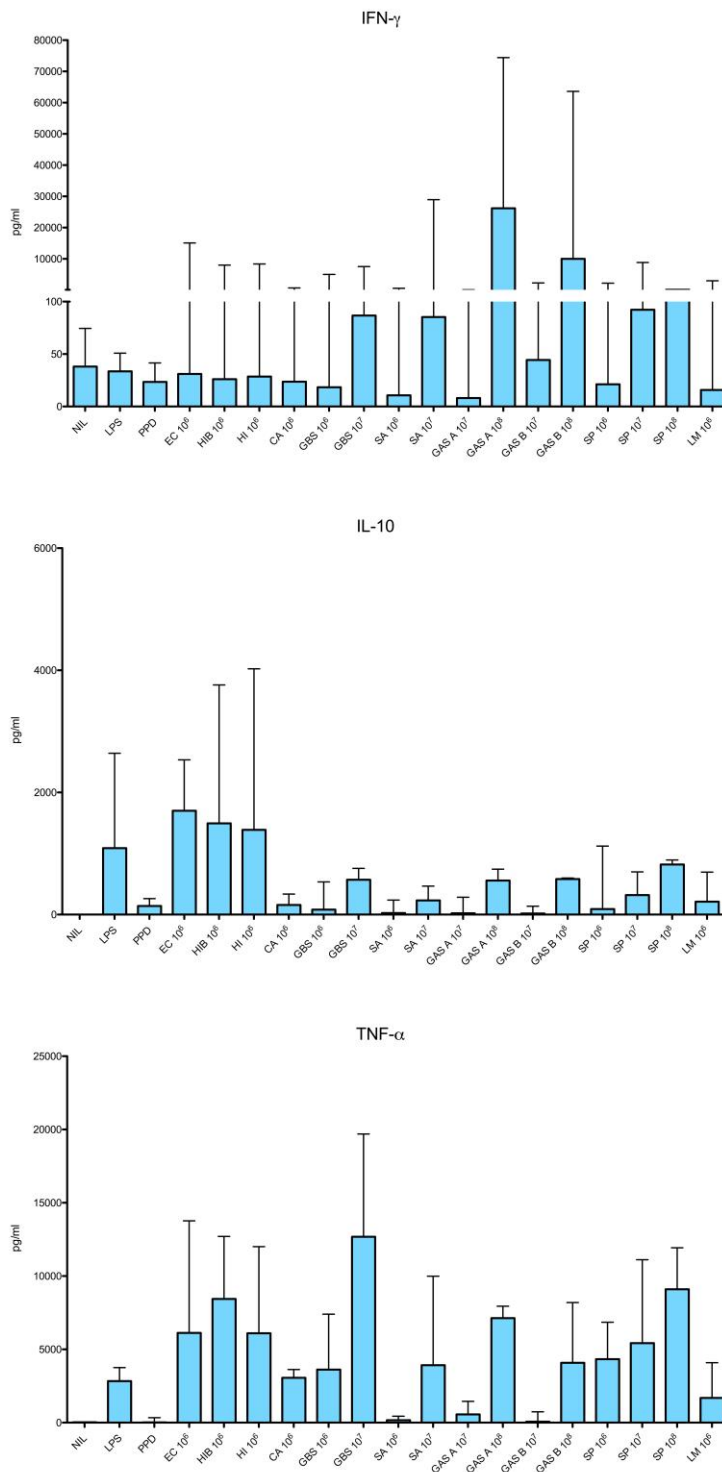


Figure 3.2 Combined cytokine response data from optimisation experiments 4 and 5 combined data: evaluation of cytokine production (median/IQR) at varying concentrations of pathogen (CFU/mL)

LPS= Lipopolysaccharide, PPD=Purified protein derivative, EC=*E. coli*, HI= *H. influenzae* type-B, CA= *C. albicans*, GBS=Group B streptococcus (*S. agalactiae*), SA=*S. aureus*, GAS= Group A streptococcus (*S. pyogenes*), SP= *S. pneumoniae*, LM=*L. monocytogenes*

Experiment	Participants	Dilution RPMI: WB	Stimulants (CFU/mL)		Incubate	Cytokines		Decisions
1	1 adult	1:10,1:5, 1:2	MTBk 10^5 10^6		4h, 18h	TNF- α , IL-6		Drop 1:10
2	2 adults	1:4,1:3,1:2	MTBk 10^6 10^7		4h, 18h	TNF- α , IL-6		Drop MTBK 10^7
3	1 adult	1:5, 1:2	<i>L. monocytogenes</i> 10^7 <i>E. coli</i> A 10^7 <i>S. agalactiae</i> A 10^7 <i>S. pneumoniae</i> A 10^7 <i>S. aureus</i> A 10^7 <i>C. albicans</i> A 10^7 <i>S. pyogenes</i> A 10^7	<i>E. coli</i> B 10^7 <i>S. agalactiae</i> B 10^7 <i>S. pneumoniae</i> B 10^7 <i>S. aureus</i> B 10^7 <i>C. albicans</i> B 10^7 <i>S. pyogenes</i> B 10^7	4h, 18h	2-plex IP-10 IL-13	6-plex TNF- α IL-1 β IFN- γ IL-10 IL-1ra IL-2	All bacterial isolates functional 1:2 dilution 18h incubation
4	2 adults	1:2	<i>L. monocytogenes</i> 10^8 10^9 <i>E. coli</i> A 10^6 10^7 <i>S. agalactiae</i> A 10^7 10^8 <i>S. pneumoniae</i> A 10^7 10^8 <i>S. aureus</i> A 10^7 10^8 10^9 <i>C. albicans</i> A 10^7 <i>S. pyogenes</i> A 10^7 10^8 10^9 Influenza A (H3N2) 10^5 10^6	<i>E. coli</i> B 10^6 10^7 <i>S. agalactiae</i> B 10^7 10^8 <i>S. pneumoniae</i> B 10^7 10^8 <i>S. aureus</i> B 10^7 10^8 10^9 <i>C. albicans</i> B 10^7 <i>S. pyogenes</i> B 10^7 10^8 10^9 Influenza A (H1N1) 10^5 10^6	4h, 18h	2-plex IP-10 IL-13	6-plex TNF- α IL-1 β IFN- γ IL-10 IL-1ra IL-2	Drop <i>E. coli</i> B <i>S. agalactiae</i> B <i>S. pneumoniae</i> B <i>S. aureus</i> B Influenza
5	3 neonates	1:2	<i>E. coli</i> A 10^6 GBS A 10^6 10^7 <i>S. pneumoniae</i> A 10^6 10^7 <i>L. monocytogenes</i> 10^7 <i>S. aureus</i> A 10^6 10^7 10^8 <i>C. albicans</i> A 10^6 <i>H. influenzae</i> type B 10^6 <i>H. influenzae</i> non-type B 10^6 LPS 10ng/mL <i>S. pyogenes</i> A 10^7 10^8	<i>S. pyogenes</i> B 10^7 10^8	18h	TNF- α , IP-10 MIP-1 β		Drop <i>S. pyogenes</i> B Final stimulant concentrations.

Table 3.2 Optimisation experiments done during development of whole blood assay

RPMI: WB= Roswell park memorial institute medium: whole blood, CFU=colony forming units, MTBk=Heat killed *M. tuberculosis*.

LPS=Lipopolysaccharide,

3.2.3 CHOICE OF TLR LIGANDS FOR USE IN ASSAY

A review of the literature on the TLR-dependent innate immune response to BCG vaccine informed the choice of TLR-ligands. Three animal studies and seven *in vitro* studies had assessed the effect of BCG vaccine on TLR signalling at the time of assay development (Table 3.3). TLR4 and TLR2 were identified as important for both activation and the control of the immune response to BCG vaccine and were therefore both included in the assay. TLR7/8 ligand (R848) and TLR1/2 (Pam3CYSK4) were included to interrogate intracellular TLR signalling. R848 was chosen as it is a potent innate immune stimulant in neonates and Pam3CYSK4 as it can also stimulate signalling via other intracellular innate immune pathways including Nod-like receptors. The whole blood assay was adapted from methods developed by our collaborators at the University of British Columbia, Vancouver [157]. All whole blood assays used in the MIS BAIR and BabyBAIR studies were made, sealed and shipped to Australia in a single batch to further enhance standardisation and comparability between studies.

Author	Model	TLR signalling pathway interrogated	Findings
Arko-Menash 2007 [158]	Murine macrophages	TLR2 TLR4	IFN- γ induced mycobacterial killing by macrophages was inhibited by selective TLR2 blockade.
D'Avila 2007 [159]	TLR2 ^{-/-} mice	TLR2	Blockade of eotaxin in TLR2 deficient mice stopped eosinophilic pleurisy.
Doz 2007 [160]	Murine macrophages	TLR2 TLR4	TLR4 is activated by LAM to inhibit inflammatory cytokine release following BCG vaccination.
Fremond 2003 [161]	TLR4 ^{-/-} mice	TLR4	TLR4 ^{-/-} mice controlled BCG infection but had increased inflammation characterised by organomegaly and neutrophil infiltration.
Heldwein 2003 [162]	TLR2 ^{-/-} mice TLR4 ^{-/-} mice	TLR2 TLR4	TLR2 ^{-/-} mice had high lung bacillary loads following BCG infection compared to TLR4 deficient mice while TLR4 deficient mice had pronounced pro-inflammatory cytokine response compared to TLR2 ^{-/-}
Lasunskiaia 2006 [163]	TLR2 ^{-/-} murine macrophages	TLR2	Changes to macrophage activity and cytoskeleton were dependent on stimulation of PIK3 pathway following BCG infection.
Von Meyenn 2006 [164]	Murine macrophages & DCs	TLR2 TLR4 TLR9	TLR2/4/9 ^{-/-} macrophages had decreased IL-6 and TNF production and TLR2/4/9 ^{-/-} DCs had decreased CD86 expression and IL-12 production.
Andersson 2012 [165]	Human epithelial cells & neutrophils	TLR2 TLR4	Mycobacterial infection induced TLR2 dependent secretion of IL-6 and IL-8 which correlated with neutrophil migration.
Kierner 2009 [166]	TLR9 ^{-/-} human alveolar macrophages	TLR9	TLR9 deficient mouse macrophages showed improved intracellular survival of BCG and increased cytokine signalling.
Mendez-Sampaio 2008 [167]	Human epithelial cells	TLR2 TLR4	The use of neutralizing antibodies against TLR2/4 signalling led to decreased phosphorylation of the ERK /APK pathway and IL-8.
Miyazaki .2005 [168]	Human uroepithelial cells	TLR2 TLR4	TLR 2/3/4/9 were detected in uroepithelium at 3 hours post intravesical BCG therapy with peak at 6 hours

Table 3.3 Experimental models of the innate immune response to BCG vaccine associated with TLR signalling

-/- = knockout mice

3.2.4 CHOICE OF CYTOKINES TO BE MEASURED IN SUPERNATANTS FOLLOWING WHOLE BLOOD STIMULATION.

A list of candidate cytokines was compiled following a 3-stage review of the literature:

1. Human studies which reported cytokine production following BCG vaccination for the prevention of TB or BCG immune therapy for bladder cancer.
2. Human, animal or *in vitro* studies reporting cytokine production in response to heterologous stimulation.
3. Paediatric studies reporting cytokine production in whole blood following innate ligand stimulation.

The aim of this review was to identify cytokines which were indicative of the innate immune response to BCG vaccine irrespective of the stimulant used and which could be successfully measured in infant blood.

Twenty-five candidate cytokines were identified and are summarised in Table 3.4. Th17 (IL-17) and Th2 (IL4, IL5) cytokines were frequently significantly changed in BCG-vaccinated infants compared to controls [94]. However, with the exception of IFN- γ a pragmatic decision was made to focus on cytokines produced primarily by cells of the innate immune system. Type-1-interferons were increased in BCG-vaccinated infants compared to controls in the blood of Malawian infants but they were low in blood from UK infants, so were not included [123]. The final cytokine panel for each study is included in the relevant publication.

Cytokine	Action	Reference
IL-1β	Pro-inflammatory	16-fold induction post BCG vaccination in whole blood from term infants [94].
TNF-α	Pro-inflammatory	5-fold induction post BCG vaccination in whole blood from term infants IFN- γ had a positive correlation with TNF- α [169].
IL-6	Pro-inflammatory	7-fold induction post BCG. IL-6 had a positive correlation with IFN- γ levels [94]. BCG-induced IL-6 production via TLR2 but not TLR4 [170]. Marker for sepsis severity in neonates [171].
IL-1ra	Anti-inflammatory	2-fold induction post BCG in whole blood from term infants [94].
IL-10	Anti-inflammatory	Increased IL-10 associated with better response to BCG in bladder cancer. IL-10 production in BCG bladder treatment follows TNF- α (with IL-6) and preceded IFN- γ [172]. 10-fold induction post BCG vaccination at 3 months [94].
IL-12p40	Innate	No changes seen in pre-and post-vaccinated infants in UK [94].
IL-15	Innate	IL-15 expression was unchanged before and after BCG vaccine at 3 months of age [94].
IL-18	Innate	IL-18 production induced via Nramp-IRF8-iNOS [169].
IP-10 (CXCL10)	Innate	IP-10 increased day 1 and peaked day 2 in the urine after BCG treatment [173].
IFN-α2	Innate	IFN- α 2 > at 3 months and 1 year post BCG vaccination in Malawian infants compared to UK infants. In UK infants 1.6 – 22-fold increase at 3 months [94].
IL-23	Innate	Produced by dendritic cells and monocytes and induces IFN- γ production [174].
MCP-1 (CCL2)	Chemokine	High background rate and little change at 3 months post vaccine in term infants [94].
MIP-1B (CCL4)	Chemokine	5-fold increase at 5 months post vaccination in term infants [94].
MCP-3 (CCL7)	Chemokine	10-fold increase post vaccine in term infants. Upregulated by IFN- γ / TNF- α [94].

Cytokine	Action	Reference
IL-8 (CXCL8)	Chemokine	3-fold induction post BCG vaccine in infants [94]. IL-8 produced in mononuclear cells derived from BCG lesions at day 1-3 post vaccine [175].
MIF	Chemokine	MIF leads to macrophage activation and non-specific bactericidal effects [176]. MIF levels correlated with therapeutic effect of BCG on recurrent herpes [177]. MIF identified as the primary lymphokine in delayed hypersensitivity to Tuberculin [178].
MIG (CXCL9)	Chemokine	Produced in response to IFN- γ stimulation of PBMCs in TB patients in Brazil [179].
IL-2	Th1	6-fold induction post vaccine in infants. Positive correlation with IFN- γ /TNF- α [94].
IL-4	Th2	IL-4 poorly detected [67]. IL-4 poorly detected [172]. IL-13 production much higher than IL-4 [94]. IL-4 production at 2 months & 4 months downregulated by BCG vaccination at birth [180].
IL-5	Th2	IL-5 3-fold increase post BCG vaccine in infants [94]. IL-5 and IL-13 decreased compared to IFN- γ at one year [180]. IL-5 increased at 5 months post BCG vaccine but decreased by 2 years in response to PPD [99].
IL-17A	Th17	15-fold increase of IL-17 at 3 months post BCG vaccine in infants [94]. IL-17 production waned by 9 months post BCG vaccine at birth [99].

Table 3.4 List of candidate cytokines identified from studies in humans following BCG vaccination or BCG therapy for bladder cancer

3.3 STATISTICAL METHODOLOGY

The statistical methodology used for cytokine analysis in the MIS BAIR study was developed under the supervision of A/Prof Susan Donath. These methods were used for the analyses presented in this chapter, chapter 4 and were adapted for use in a peer-reviewed publication for which I am co-author (appendix 1) [181]. The multiplex cytokine data from the MIS BAIR study was characterised by (i) measurable levels of chemokine expression in RPMI-stimulated (nil) samples and (ii) marked inter-individual variability in both RPMI and antigen stimulated samples. The development of the statistical methodology to address these features is discussed in the following.

3.3.1 RPMI-STIMULATED (NIL) CYTOKINE LEVELS

Measurable chemokine concentrations in RPMI-stimulated (nil) samples have been reported previously, and in the case of MIF (macrophage-inhibitory factor), are of functional importance for the neonatal response to infection [182]. Of note, sustained production of MIF was subsequently detrimental in a mouse model of neonatal sepsis and final outcomes were improved by the use of a recombinant MIF antibody [200], thus emphasising the importance of a robust but regulated inflammatory response during neonatal infection.

The distribution of RPMI-stimulated (nil) chemokine expression (MIF, MIG, IL8, MCP-1, MIP-1 β), although marked by inter-individual variability and the presence of outliers, approached a normal distribution suggesting these chemokines are constitutively produced (Figure 3.1). In contrast, IFN- γ , TNF- α , IL-10 and IL-1 β concentrations were generally low or not measurable in RPMI-stimulated (nil) samples

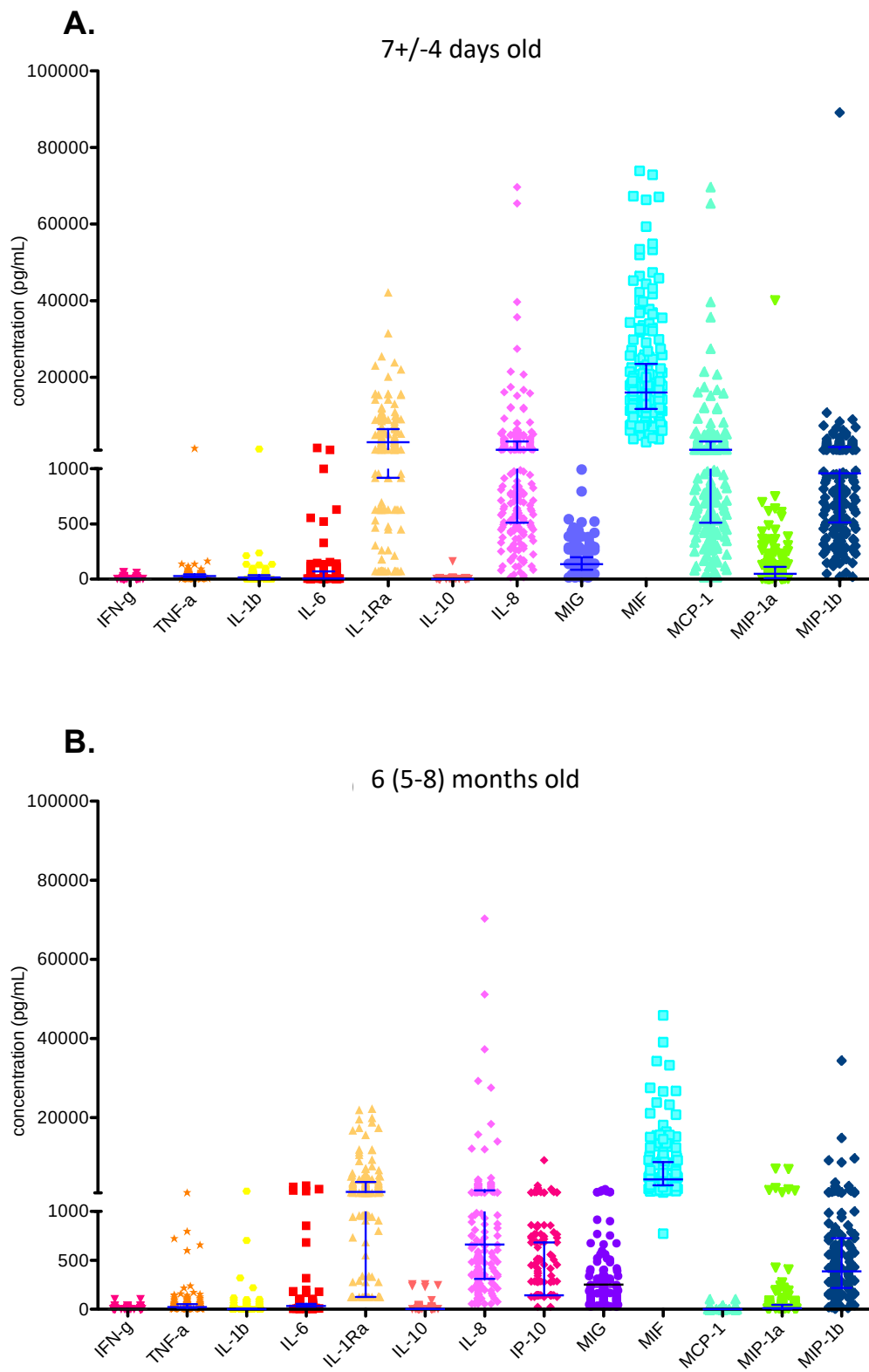


Figure 3.3 RPMI-stimulated (nil) cytokine responses in the MIS BAIR study at (A) 7+/-4 days post randomisation and (B) 6 (-1/+2) months post randomisation.

3.3.2 INTER-INDIVIDUAL VARIABILITY

The marked inter-individual variability in cytokine responses are a product of genetic factors, participant age, past or present environmental exposures, the stimulant used or the intervention i.e. a vaccine [142, 183-188]. In this analysis, the independent variable, BCG vaccine, represents an environmental exposure which was hypothesised to change subsequent cytokine responses in the context of infection. When comparing immune responses between BCG-vaccinated and BCG-naïve infants, the challenge was to minimise the impact of individual level variability on the ability to detect an effect of BCG vaccine.

A commonly employed method to reduce variability in cytokine response data is to 'baseline correct', i.e. the unstimulated cytokine value is subtracted from the stimulated value. Patients who have negative values as a result (i.e. higher cytokine levels in unstimulated samples than in stimulated samples) are often excluded from the analysis [189]. This approach negates the possibility that the independent variable (in this case BCG vaccine) could lead to significant downregulation of a given cytokine response.

Rather than baseline correct, the RPMI-stimulated (nil) value was included as a co-variate in the regression analysis, thus acknowledging that BCG vaccination could (i) independently effect the expression of this value prior to stimulation and that (ii) the nature of this effect would vary between individuals.

Table 3.5 shows that the inclusion of the RPMI-stimulated (nil) value as a covariate in the comparison of MCP-1 levels between BCG-vaccinated and BCG-naïve neonates improved the precision of the estimate of the BCG-vaccine effect.

	No covariate				Covariate			
	LCI	GMR	UCI	p-value	LCI	GMR	UCI	p-value
BCG	0.79	1.01	1.28	0.95	0.79	1.01	1.28	0.97
MTB	0.79	1.07	1.45	0.66	0.77	1.04	1.39	0.81
SA	0.66	0.92	1.26	0.59	0.63	0.85	1.15	0.28
SP	0.52	0.72	0.98	0.04	0.49	0.67	0.91	0.01
GAS	0.38	0.94	2.30	0.89	0.38	0.91	2.13	0.81
GBS	0.68	0.89	1.15	0.36	0.67	0.86	1.12	0.26
EC	0.64	0.79	0.98	0.04	0.63	0.78	0.97	0.02
HI	0.66	0.81	1.00	0.05	0.66	0.81	1.00	0.05
LM	0.57	0.79	1.11	0.17	0.51	0.68	0.92	0.01
PAM3	0.69	0.90	1.17	0.44	0.68	0.88	1.13	0.30
PEPG	0.63	0.79	0.99	0.04	0.62	0.79	0.99	0.04
LPS	0.60	0.87	1.28	0.49	0.58	0.85	1.23	0.38
R848	0.68	0.81	0.96	0.02	0.67	0.80	0.95	0.01

Table 3.5 Comparison of analysis of covariance with simple linear regression for the effect of BCG vaccine on MCP-1 responses

LCI / UCI = Lower / Upper 95% confidence intervals, GMR = geometric mean ratio,
 BCG = Bacille Calmette Guerin, MTB = *M. tuberculosis*, SA = *S. aureus*, SP = *S. pneumoniae*,
 GAS = Group A streptococcus (*S. pyogenes*) GBS = Group B streptococcus (*S. agalactiae*), EC =
E. coli, HI = *H. influenzae*, , CA = *C. albicans*, LM = *L. monocytogenes*, PEPG = Peptidoglycan,
 PAM = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-
 Lys4OH, trihydrochloride, LPS = lipopolysaccharide, R848 = resiquimod,

3.4 PUBLISHED RESULTS PAPER

Neonatal BCG Vaccination Influences Cytokine Responses to Toll-like Receptor Ligands and Heterologous Antigens

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Background. BCG vaccination is associated with a reduction in all-cause infant mortality in high-mortality settings. The underlying mechanisms remain uncertain, but long-term modulation of the innate immune response (trained immunity) may be involved.

Methods. Whole-blood specimens, collected 7 days after randomization from 212 neonates enrolled in a randomized trial of neonatal BCG vaccination, were stimulated with killed pathogens and Toll-like receptor (TLR) ligands to interrogate cytokine responses.

Results. BCG-vaccinated infants had increased production of interleukin 6 (IL-6) in unstimulated samples and decreased production of interleukin 1 receptor antagonist, IL-6, and IL-10 and the chemokines macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , and monocyte chemoattractant protein 1 (MCP-1) following stimulation with peptidoglycan (TLR2) and R848 (TLR7/8). BCG-vaccinated infants also had decreased MCP-1 responses following stimulation with heterologous pathogens. Sex and maternal BCG vaccination status interacted with neonatal BCG vaccination.

Conclusions. Neonatal BCG vaccination influences cytokine responses to TLR ligands and heterologous pathogens. This effect is characterized by decreased antiinflammatory cytokine and chemokine responses in the context of higher levels of IL-6 in unstimulated samples. This supports the hypothesis that BCG vaccination modulates the innate immune system. Further research is warranted to determine whether there is an association between these findings and the beneficial nonspecific (heterologous) effects of BCG vaccine on all-cause mortality.

Keywords. Bacille Calmette-Guérin; immunisation; heterologous; nonspecific; trained immunity.

Substantial evidence supports the concept that BCG vaccine reduces all-cause infant mortality in high-mortality settings [1–4]. The recent World Health Organization Special Advisory Group of Experts on Immunization systematic review of the nonspecific (heterologous) effects of BCG showed that neonatal BCG vaccination is associated with a 30% reduction in all-cause mortality (relative risk, 0.70; 95% confidence interval, .49–1.01) [5]. Epidemiological studies also suggest that BCG vaccine given at birth protects against the development of allergic disease [6, 7] and pneumonia [8].

Randomized controlled trials of BCG vaccination at birth report a reduction in neonatal mortality attributable to infection [1, 5]. Evidence suggests that the benefit of BCG vaccination is conferred as early as 7 days of life [4]. Clinical and demographic factors that influence vaccine responses include

sex [1, 9], coadministration and timing of other neonatal vaccines [10], and maternal BCG vaccination [11].

The need for further research into the immunological mechanisms underlying the heterologous effects of BCG vaccine has been highlighted [12]. In animal models, BCG vaccine improves survival against a wide range of pathogens [13]. Multiple pathways of innate, cellular, and adaptive immunity are proposed to underpin BCG vaccine's protective effect [14]. In a study done in West Africa, BCG vaccination altered neonatal cytokine responses following stimulation with Toll-like receptor (TLR) ligands [15]. Similarly, BCG vaccination influences cytokine responses to heterologous bacteria in peripheral blood mononuclear cells (PBMCs) from adults [16]. It is proposed that this results from induction of so-called trained immunity (ie, immune memory in the innate immune system) through epigenetic reprogramming of monocytes [16].

Our study aimed to investigate whether BCG vaccination influences neonatal cytokine responses to TLR ligands and clinically significant bacterial and fungal pathogens and, thus, induces heterologous innate immune effects.

METHODS

Participants were a subset of infants from the Melbourne Infant Study: BCG for the Prevention of Allergy and Infection (MIS

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BAIR), a randomized controlled trial of BCG vaccine in 1272 neonates, with the primary outcomes of allergic sensitization, eczema, and lower respiratory tract infection (clinical trials registration NCT01906853). Participants were randomized 1:1 to undergo vaccination with BCG-Denmark (0.05 mL) intradermally within 10 days of birth or to receive no BCG vaccine. All participants also received a birth dose of hepatitis B vaccine as per Australian guidelines. A subset of 447 participants recruited over an 18-month period (January 2014–June 2015) had blood samples collected for immunological analyses (Figure 1). Exclusion criteria for infants in the immunological study were suspected perinatal sepsis, no receipt of hepatitis B vaccine, or receipt of blood products.

Written informed consent was obtained from the parents of all participants. The study was approved by the human ethics research committees of the Royal Children’s Hospital Melbourne (human research ethics committee [HREC] authorization 33025) and Mercy Hospital for Women (HREC authorization R12/28).

Participants were visited in their home at a mean duration ($\pm$ SD) of 7 ± 4 days after randomization. Blood specimens were collected via a closed loop system into tubes containing sodium-heparin (S-monovette) and pretested for endotoxin. Blood samples were kept at room temperature, protected from direct sunlight, and stimulated in the laboratory for a mean duration of 4 hours $\pm$ 24 minutes after collection. Laboratory personnel were masked to the participants’ BCG vaccination status.

Whole-blood specimens, diluted 1:1 with Roswell Park Memorial Institute (RPMI) 1640 medium (Glutamax supplement and HEPES; Gibco), were added to preprepared stimulation assay strips (Microlon 600 Greiner-One 8-well strips). The assay strips were made using a validated robotic system, to maximize standardization of stimulant volume, and were stored at -80°C until addition of blood. The final stimulant concentrations were as follows: BCG Denmark, $75\text{ }\mu\text{g/mL}$; killed *Mycobacterium tuberculosis*, 1.0×10^6 colony-forming units (CFU)/mL; 7 heat-killed bacteria (*Escherichia coli*, 1.0×10^6 CFU/mL; *Haemophilus influenzae* type B, 1.0×10^6 CFU/mL; *Staphylococcus aureus*, 1.0×10^7 CFU/mL; group B streptococcus [GBS; *Streptococcus agalactiae*], 1.0×10^7 CFU/mL; *Streptococcus pneumoniae* serotype 15C 1.0×10^7 CFU/mL; and *Listeria monocytogenes*, 1.0×10^7 CFU/mL), 1 fungus (*Candida albicans*, 1.0×10^6 CFU/mL), and 4 TLR ligands (lipopolysaccharide [LPS], 100 ng/mL ; resiquimod [R848], $3.5\text{ }\mu\text{g/mL}$; peptidoglycan [PEPG], $10\text{ }\mu\text{g/mL}$; and (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride [Pam3CSK4], $1\text{ }\mu\text{g/mL}$; all from InvivoGen), and RPMI 1640 medium. Pathogens were clinical isolates from children with invasive disease at the Royal Children’s Hospital Melbourne and were killed by heat treatment at 70°C for 2 hours. Sterility was confirmed by the absence of growth in culture medium. Concentrations of all stimulants

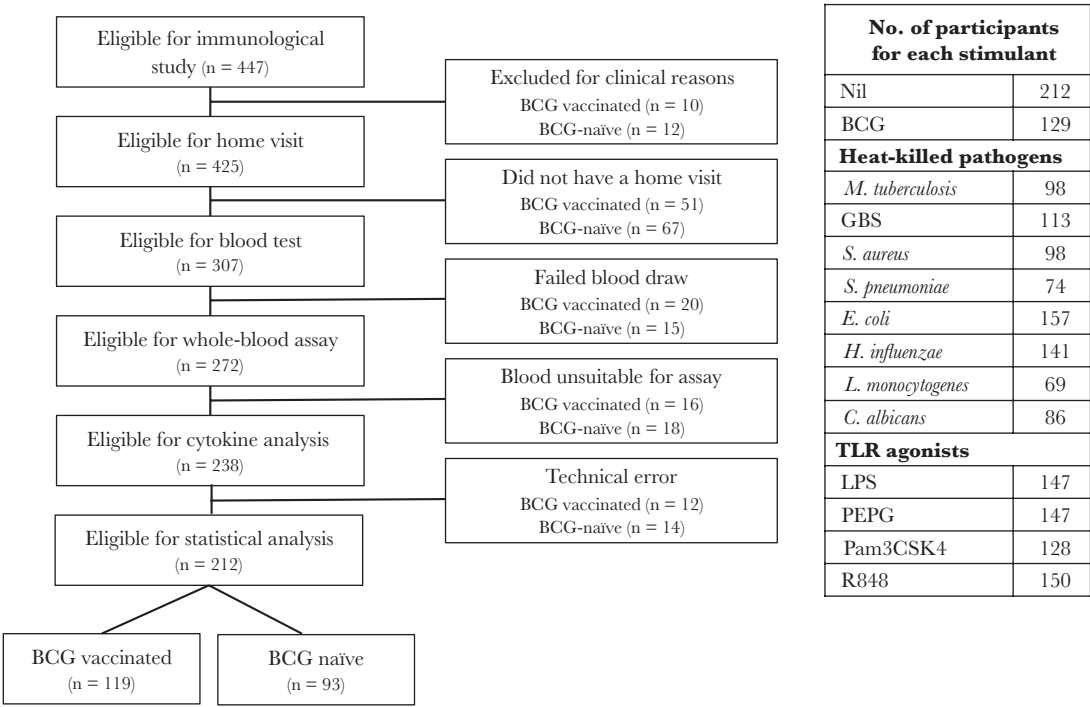


Figure 1. Flow chart of eligible participants and relevant exclusions. Table shows number of individual stimulations done for each antigen. *C. albicans*, *Candida albicans*; GBS, group B streptococcus; *E. coli*, *Escherichia coli*; *H. influenzae*, *Haemophilus influenzae* type B; *L. monocytogenes*, *Listeria monocytogenes*; LPS, lipopolysaccharide; *M. tuberculosis*, *Mycobacterium tuberculosis*; Pam3CSK4, (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride; PEPG, peptidoglycan; R848, resiquimod; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae* serotype 15C.

were optimized in preliminary experiments (data not shown). The stimulation assay was incubated at 37°C (5% CO₂ in air) for a mean duration ($\pm$ SD) of 20 $\pm$ 2 hours.

Supernatants were analyzed in batches using Bio-Rad human cytokine kits following the manufacturer's instructions. The mean fluorescence intensity was read for each cytokine, using an xMAP Luminex 200 Bioanalyzer. Each sample was analyzed in 2 separate kits at different dilutions to account for the anticipated dynamic range of the cytokine results indicated by preliminary experiments (data not shown): (1) 1:100 dilution (7-plex): interleukin 1 β (IL-1 β), IL-1 receptor antagonist (IL-1RA), interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1; also known as monocyte chemotactic and activating factor and CCL2), macrophage inflammatory protein 1 α /CCL3 (MIP-1 α), and MIP-1 β (CCL4); and (2) 1:4 dilution (5-plex): interferon γ (IFN- γ), interleukin 10 (IL-10), tumor necrosis factor α (TNF- α), macrophage migration inhibitory factor (MIF), and monokine induced by IFN- γ /CXCL9 (MIG).

Statistical analysis was done using Stata, version 13.1. Cytokine levels that were below the lower limit of detection were assigned a value of half the lowest detectable value (either the lowest standard on the curve or the lowest extrapolated value below). There were no instances when cytokine results were greater than the upper limit of detection. Prior to analysis, cytokine data were log transformed.

The effect of (1) BCG vaccination, (2) reported maternal BCG vaccination status, and (3) timing of BCG vaccination (early, <48 hours after birth; late, $\geq$ 48 hours after birth) on cytokine production in response to each stimulant was investigated using logistic regression, with the log-transformed value of the unstimulated (RPMI 1640 medium only) cytokine values as a covariate. Results were expressed as geometric mean ratios (GMRs) with 95% CIs. Because IFN- γ values were not normally distributed, IFN- γ production was also analyzed using nonparametric methods (ie, quantile regression). One participant was an extreme outlier for all cytokines in unstimulated samples, but use of the unstimulated value as a covariate successfully corrected for this in stimulated cytokine results; because this could not be done for the analysis of unstimulated cytokine results, this participant was excluded in that analysis. Results of cytokine/stimulant pairs are presented as GMRs (95% CIs) without emphasis on tests of statistical significance [17]. Subgroup effects (ie, sex, mode of delivery, and maternal BCG vaccination status) were investigated where there was statistical evidence of an interaction between BCG vaccination and the subgroup variable. Sensitivity analyses were performed to investigate the effect of excluding values assigned at the lower end of the standard curve and values extrapolated by xMAP software.

RESULTS

Demographic features are outlined in Table 1. There was a high degree of standardization in the laboratory procedures (Supplementary Table 1). Our data exhibited the marked

interindividual variability in cytokine responses in both stimulated and unstimulated samples, characteristic of cytokine data (Supplementary Figure 1).

In unstimulated samples (ie, those exposed to RPMI 1640 medium), BCG-vaccinated infants showed increased production of IL-6, IL-1RA, and IL-1 β , but the evidence that this was a real effect was weak for IL-1RA and IL-1 β (Table 2). For TLR agonist responses, chemokine and cytokine production was unchanged by BCG in response to Pam3CSK4 and LPS. However, responses to stimulation with the TLR ligands PEPG and R848 were decreased for most cytokines in BCG-vaccinated infants as compared to BCG-naïve. This effect was strongest for the cytokines IL-6 and IL-10 and the chemokines MIP-1 α , MIP-1 β , and MCP-1 (Figure 2 and Supplementary Tables 2 and 3).

In response to stimulation with BCG, *M. tuberculosis* and the heterologous bacteria *S. aureus* and GBS, there was no difference in cytokine production between BCG-vaccinated and BCG-naïve infants. Conversely, responses to stimulation with *S. pneumoniae*, *E. coli*, *H. influenzae*, and particularly the intracellular organisms *L. monocytogenes* and *C. albicans* were decreased for most cytokines in BCG-vaccinated infants as compared to BCG-naïve infants (Figure 2 and Supplementary Tables 2 and 3). The chemokines and cytokines for which production was most consistently decreased were IL-1RA, MCP-1, and MIP-1 β .

Sex independently influenced cytokine responses in our study (data not shown). There was a significant interaction between sex and BCG vaccination for production of MIF in response to stimulation with *L. monocytogenes*, *H. influenzae*, GBS, *M. tuberculosis*, and R848 (Supplementary Table 4). Subset analyses of males and females separately showed that there were generally lower MIF responses in BCG-vaccinated males, compared with BCG-naïve males, and higher responses in BCG-naïve females, compared with BCG-naïve females. BCG-vaccinated females had higher MIF production in response to stimulation with GBS, whereas BCG-vaccinated males had lower MIF production in response to stimulation with the intracellular stimulants R848, *L. monocytogenes*, and *M. tuberculosis* (Figure 3) as compared to BCG-naïve controls of the same sex.

Mode of delivery independently influenced cytokine responses in our population (data not shown), but there was minimal evidence of an interaction between BCG vaccination and mode of delivery on cytokine responses (Supplementary Table 4). Despite randomization of participants to BCG vaccination, attrition from the immunological study led to differences in the distribution of sex and mode of delivery between the 2 groups (Table 1). A multivariate regression model including these 2 variables and the log-transformed unstimulated cytokine yielded the same results as the primary analysis and excluded confounding as a contributor to the primary results.

Table 1. Baseline Characteristics of Study Participants

Characteristic	BCG-vaccinated, No. (%)	BCG-naïve No. (%)	Overall, No. (%)
Overall	119 (56.1)	93 (43.9)	212 (100)
Sex			
Male	58 (48.7)	43 (46.2)	101 (47.6)
Female	61 (51.3)	50 (53.8)	111 (52.3)
Gestational age, wk, mean $\pm$ SD	39.3 $\pm$ 1.5	39.2 $\pm$ 1.3	39.3 $\pm$ 0.1
Birth weight, g, mean $\pm$ SD	3388 $\pm$ 487.3	3333 $\pm$ 492.7	3364 $\pm$ 489.3
Mode of delivery			
Vaginal birth	64 (53.8)	62 (66.7)	126 (59.4)
Cesarean section	55 (46.2)	31 (33.3)	86 (40.6)
Last trimester vaccination ^a			
Yes	59 (49.6)	43 (46.2)	102 (48.1)
No	58 (48.7)	49 (52.8)	107 (50.5)
Not reported	2 (1.7)	1 (1.1)	3 (1.4)
Feeding			
Breast feeding	82 (69.2)	67 (71.7)	149 (70.2)
Formula feeding	3 (2.4)	7 (7.6)	10 (4.8)
Combination	29 (24.2)	18 (19.6)	47 (22.2)
Not reported	5 (4.2)	1 (1.1)	6 (2.8)
Infant vitamin D supplementation			
Yes	64 (53.4)	48 (51.8)	112 (52.8)
No	55 (46.6)	45 (48.2)	100 (47.2)
Day of BCG vaccination			
Mean $\pm$ SD	2.1 $\pm$ 1.9	...	...
Median (IQR)	2 (0–6)	...	...
Interval from randomization to blood collection, d, mean $\pm$ SD	6.9 $\pm$ 2.2	...	...
Timing of hepatitis B vaccination in relation to BCG vaccination, h			
≤ 24	82 (69.0)	...	...
24–48	12 (10.1)	...	...
> 48	17 (14.3)	...	...
Not recorded	8 (6.3)	...	...
Maternal BCG vaccinations status			
Yes	24 (20.2)	31 (33.3)	55 (25.9)
No	90 (75.6)	54 (58.1)	144 (67.9)
Not reported	5 (4.2)	8 (8.6)	13 (6.2)

Data are no. (%) of participants, unless otherwise indicated.

Abbreviation: IQR, interquartile range.

^aBoostrix (diphtheria-tetanus-acellular pertussis) or influenza vaccine in the last trimester, in line with local recommendations.

Infants whose mothers had a history of BCG vaccination had increased production of IL-1 β , IL-1RA, IL-6, and MIP-1 α in response to Pam3CSK4, IL-6, and MIP-1 α in response to *H. influenzae* and increased production of MIP-1 β in response to PEPG. Conversely, infants whose mothers were not vaccinated with BCG had higher production of TNF- α and IL-1 β in response to R848 and higher IL-10 production in response to GBS, independent of infant BCG vaccination status (Supplementary Table 2).

There was evidence of an interaction between maternal BCG vaccination status and infant BCG vaccination status for 5 cytokine/stimulant pairs (IFN- γ /*S. pneumoniae*, IL-1 β /BCG, MIP-1 α /PEPG, MIP-1 α /Pam3CSK4, and MIG/*H. influenzae*; Supplementary Table 4). Separate analyses in infants of mothers who were vaccinated with BCG and those whose mothers were not vaccinated with BCG showed that in the former, BCG-vaccinated infants had higher IFN- γ production in response

to stimulation with *S. pneumoniae* and MIP-1 α in response to PEPG. Conversely for BCG-vaccinated infants whose mothers were not vaccinated with BCG, MIP-1 α expression was increased in response to Pam3CSK4 (Figure 4).

Infants were categorized into those who were vaccinated with BCG early (< 48 hours after birth) and those who were vaccinated late (≥ 48 hours after birth; Supplementary Figure 2A and 2B). The median interval between BCG vaccination and blood sampling was similar between both groups (7 days [range, 0–11 days] in the BCG-vaccinated group and 8 days [range, 3–11 days] in the BCG-naïve group; Supplementary Figure 2C).

Compared with those in the late BCG vaccination group, infants in the early BCG vaccination group had significantly higher cytokine and chemokine production following stimulation with all stimulants except for BCG and *M. tuberculosis* (Supplementary Table 5).

Table 2. Geometric Mean Ratio (GMR) for Unstimulated Cytokines

Cytokine	GMR (95% CI)	P
IFN- γ	1.0 (.8–1.2)	.85
TNF- α	1.0 (.9–1.2)	.80
IL-1 β	1.6 (.8–3.0)	.18
IL-6	2.0 (1.1–3.7)	.03
MIF	1.0 (.9–1.2)	.90
MIG	0.9 (.8–1.1)	.42
MCP-1	1.2 (.8–1.6)	.41
IL-8	1.0 (.4–2.1)	.94
MIP-1 α	0.8 (.5–1.4)	.50
MIP-1 β	1.0 (.7–1.4)	1.00
IL-1RA	1.9 (.9–3.8)	.08
IL-10	1.0 (1.0–1.1)	.55

Data are for Roswell Park Memorial Institute 1640 (RPMI) medium stimulation. Univariate analysis was for the effect of BCG vaccination.

Abbreviations: CI, confidence interval; IFN- γ , interferon γ ; IL-1RA, interleukin 1 receptor antagonist; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; MCP-1, monocyte chemoattractant protein 1/CCL2; MIF, macrophage migration inhibitory factor; MIG, monokine induced by interferon γ (CXCL9); MIP-1 α/β , macrophage inflammatory protein 1 CCL3/4; TNF- α , tumor necrosis factor α .

To determine how the timing of BCG vaccination influenced the results of the primary analysis, we compared cytokine production in the early BCG-vaccinated group to that in the BCG-naïve group and production in the late BCG-vaccinated group to that in the BCG-naïve group (Supplementary Table 5). The influence of BCG vaccination on cytokine production (ie, that overall cytokine production was decreased in BCG-vaccinated infants following heterologous stimulation) was more marked in the late BCG-vaccinated group than in the early BCG-vaccinated group when each was compared to the BCG-naïve group (Figure 5).

Sensitivity analyses were performed for the primary outcome—the effect of BCG vaccination on cytokine responses—to investigate the influence of (1) the assigned values at the lower end of the standard curve and (2) the values extrapolated by Luminex xMAP software at both the upper and lower ends of the standard curve (Supplementary Table 6). Overall, the results of the primary analysis were preserved across sensitivity analyses. Notable differences were seen only for production of IL-1RA in response to R848, MIP-1 β in response to R848, and IFN- γ in response to several stimulants, including *M. tuberculosis* (Supplementary Table 6).

DISCUSSION

This study adds to the immunological evidence for the heterologous effects of BCG vaccination by finding differential cytokine production between BCG-vaccinated infants and BCG-naïve infants following in vitro stimulation with heterologous stimulants. This is the first study to investigate the heterologous effects of BCG in the first week of life, the time from which a reduction in all-cause mortality associated with BCG-vaccination is reported [1].

The pattern of cytokine production observed in our study was consistent with findings of previous studies in neonates [18, 19]. IL-10 production was high relative to that of IFN- γ for all stimulants [19]. IL-6 production was relatively high as compared to that for proinflammatory cytokines, such as TNF- α and IL-1 β [20]. In addition, there was robust chemokine production in response to TLR ligand stimulation, particularly R848 [21]. These patterns were observed despite considerable interindividual variability, both in unstimulated samples and following stimulation [22].

In our study, compared with BCG-naïve infants, BCG-vaccinated infants had reduced production of multiple cytokines and chemokines in response to stimulation with the TLR ligands PEPG (TLR2/[Nod]-like receptor 1/2) and R848 (TLR7/8). PEPG and R848 are potent activators of neonatal innate immunity when compared to Pam3CSK4 (TLR2) and LPS (TLR4) [23, 24]. R848 induces neonatal antigen-presenting cells, leading to T-helper cell type 1 polarization and production of interleukin 12p70 and TNF- α at levels equivalent to those for adults [24]. Excess stimulation of TLR7/8 with R848 is associated with neutrophil-mediated lung injury following RSV infection and an increased incidence of viral-associated wheeze in mouse models [25, 26]. PEPG has endotoxic properties and potentiates LPS signaling, augmenting its pathogenicity in sepsis syndromes [27–29]. The observed differential production of MCP-1 following stimulation with several heterologous bacteria between BCG-vaccinated and BCG-naïve infants is interesting in light of MCP-1's pivotal role in the control of mycobacterial disease. Elevated levels of MCP-1 are associated with proinflammatory diseases, including asthma, multiple sclerosis, and malignancy [30].

The modified neonatal cytokine profile induced by BCG vaccination in our study, characterized by reduced antiinflammatory cytokine (IL-10 and IL-1RA) and IL-6 responses following stimulation with heterologous bacteria, suggests a proinflammatory bias in BCG-vaccinated infants in the context of bacterial challenge. A concomitant downregulation in production of several chemokines, including MCP-1, could reduce leukocyte recruitment and associated tissue damage during infection. Overall, these effects might result in a less severe sepsis response and contribute to the reduction in mortality reported in BCG-vaccinated infants in high-mortality settings.

Jensen et al also investigated the immunological nonspecific effects of BCG by measuring cytokine responses in whole-blood specimens [15]. In low-birth-weight infants in Guinea-Bissau, compared with BCG-naïve infants, BCG-vaccinated infants had increased IL-1 β and TNF- α production in unstimulated samples. Consistent with this, we found increased IL-6 and IL-1RA (but not TNF- α) production in unstimulated samples. Also consistent, we found no effect of BCG vaccination on cytokine production following stimulation with LPS. In Jensen et al's study, BCG-vaccinated infants had increased IL-1 β , IL-6, and TNF- α

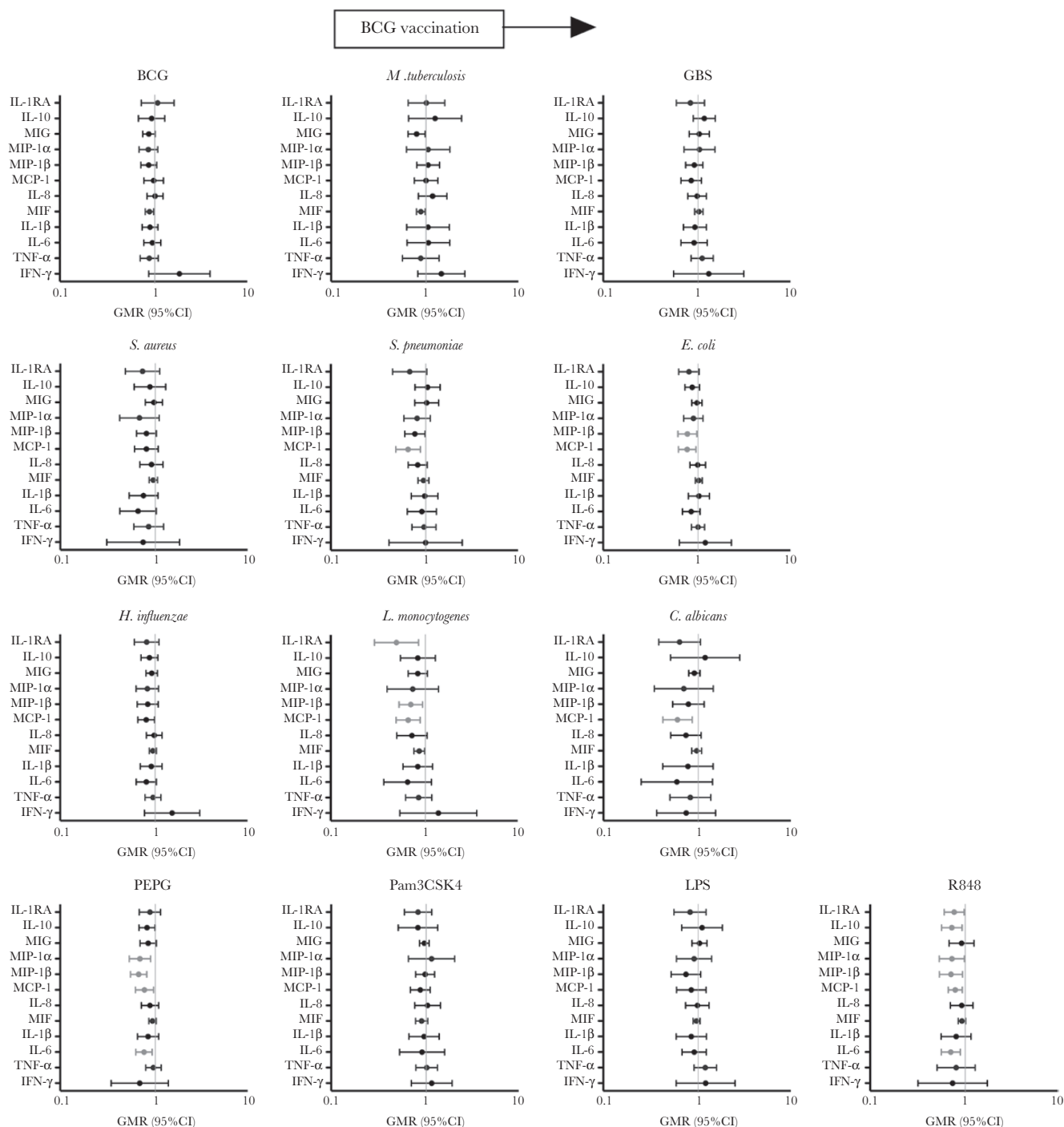


Figure 2. The effect of BCG vaccination on cytokine expression. Significant results $P < .05$ are depicted in pale gray. A geometric mean ratio (GMR) of >1.0 indicates cytokine levels were higher in BCG-vaccinated infants, compared with BCG-naïve infants. *C. albicans*, *Candida albicans*; CI, confidence interval; *E. coli*, *Escherichia coli*; GBS, group B streptococcus; *H. influenzae*, *Haemophilus influenzae* type B; IFN- γ , interferon γ ; IL-1RA, interleukin 1 receptor antagonist; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; *L. monocytogenes*, *Listeria monocytogenes*; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1/CCL2; MIF, macrophage migration inhibitory factor; MIG, monokine induced by interferon γ /CXCL9; MIP-1 α/β , macrophage inflammatory protein 1 CCL3/CCL4; *M. tuberculosis*, *Mycobacterium tuberculosis*; Pam3CSK4, (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys40H, trihydrochloride; PEPG, peptidoglycan; R848, resiquimod; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae* serotype 15C; TNF- α , tumor necrosis factor α .

production in response to stimulation with Pam3CSK4, whereas in our study BCG vaccination had no effect on cytokine responses to Pam3CSK4. Several factors that influence cytokine production differed between the two studies. These include ethnicity,

geography [31], mean age at sampling (30 days in Guinea-Bissau vs 8.9 days in our study) [32], maternal HIV status [33], weight, gestational age, underlying health, hepatitis B vaccination status, maternal BCG vaccination status, blood sampling method, and

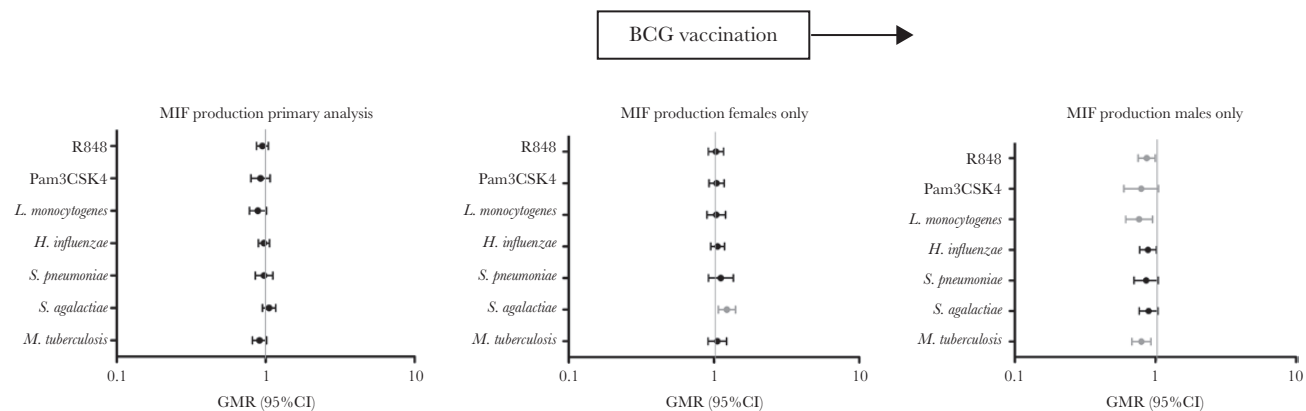


Figure 3. Interaction and subgroup analysis for the effect of sex on BCG vaccination. Macrophage migration inhibitory factor (MIF) production for cytokine/stimulant pairs where a significant interaction between sex and BCG vaccination was seen are shown for all participants (left), BCG-vaccinated females versus BCG-naïve females (middle), and BCG-vaccinated males versus BCG-naïve males. CI, confidence interval; GMR, geometric mean ratio; *H. influenzae*, *Haemophilus influenzae* type B; *L. monocytogenes*, *Listeria monocytogenes*; *M. tuberculosis*, *Mycobacterium tuberculosis*; Pam3CSK4, (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys40H, trihydrochloride; R848, resiquimod; *S. pneumoniae*, *Streptococcus pneumoniae* serotype 15C.

anticoagulant. Jensen et al's conclusion that BCG vaccination results in a stronger proinflammatory profile is consistent with our findings, albeit through different mechanisms.

Kleinnijenhuis et al also found a proinflammatory response to heterologous bacteria induced by BCG vaccination. In this study, in adults, increased IFN- γ , TNF- α and IL-1 β production in PBMCs was observed following stimulation with heat-killed *M. tuberculosis*, *S. aureus* and *C. albicans* [16]. More recently, a similar study in infants born in the United Kingdom showed increased IL-6 production in PBMCs stimulated with *C. albicans*, *S. aureus*, and Pam3CSK4 [34]. The authors' attributed these changes to epigenetic reprogramming that resulted in so-called trained immunity or innate immune memory.

Our finding that maternal BCG vaccination influenced the production of cytokines in infants is interesting. Latent maternal *M. tuberculosis* infection is associated with increased production of proinflammatory cytokines in response to mycobacterial

stimulants in BCG-vaccinated infants [35]. Moreover, in a recently reported trial from Denmark, BCG-vaccinated infants had a decreased risk of infectious illnesses requiring hospitalization during the first 3 months of life if their mother had a history of BCG vaccination [36]. Potential mechanisms for the potentiating effect of maternal BCG vaccination include transplacentally acquired maternal antibodies [37] and trans-generational epigenetic changes in immune responses, such as has been reported in lower organisms [38, 39]. Understanding how maternal and infant mycobacterial exposures influence the neonatal response to BCG warrants further investigation.

It is well recognized that vaccine responses are affected by sex [40]. Although BCG vaccination alone did not affect the production of MIF in our study, there was a significant interaction between BCG vaccination and sex for MIF production in response to several heterologous antigens. MIF is produced both constitutively and in response to bacterial infection or

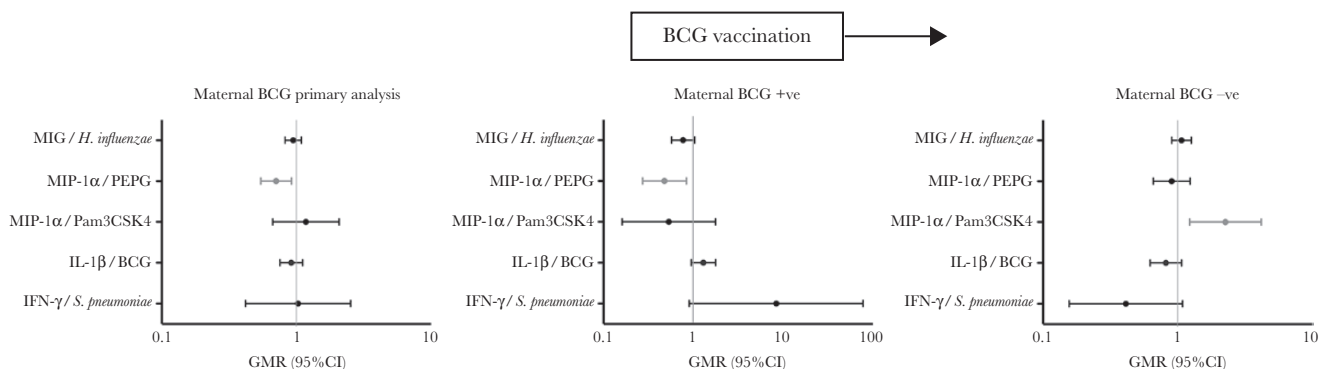


Figure 4. Interaction and subgroup analysis for the effect of maternal BCG vaccination status on neonatal BCG vaccination. Cytokine responses for cytokine/stimulant pairs where a significant interaction between maternal BCG vaccination status and neonatal BCG vaccination was seen are shown for all participants (left), in participants whose mothers were vaccinated with BCG (BCG-vaccinated infants vs BCG-naïve infants; middle), and participants whose mothers were not vaccinated with BCG (BCG-vaccinated infants vs BCG-naïve infants; right). CI, confidence interval; GMR, geometric mean ratio; *H. influenzae*, *Haemophilus influenzae* type B; IFN- γ , interferon γ ; IL-1 β , interleukin 1 β ; MIG, monokine induced by interferon γ /CXCL9; MIP-1 α , macrophage inflammatory protein 1 α CCL3; Pam3CSK4, (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys40H, trihydrochloride; PEPG, peptidoglycan; *S. pneumoniae*, *Streptococcus pneumoniae* serotype 15C.

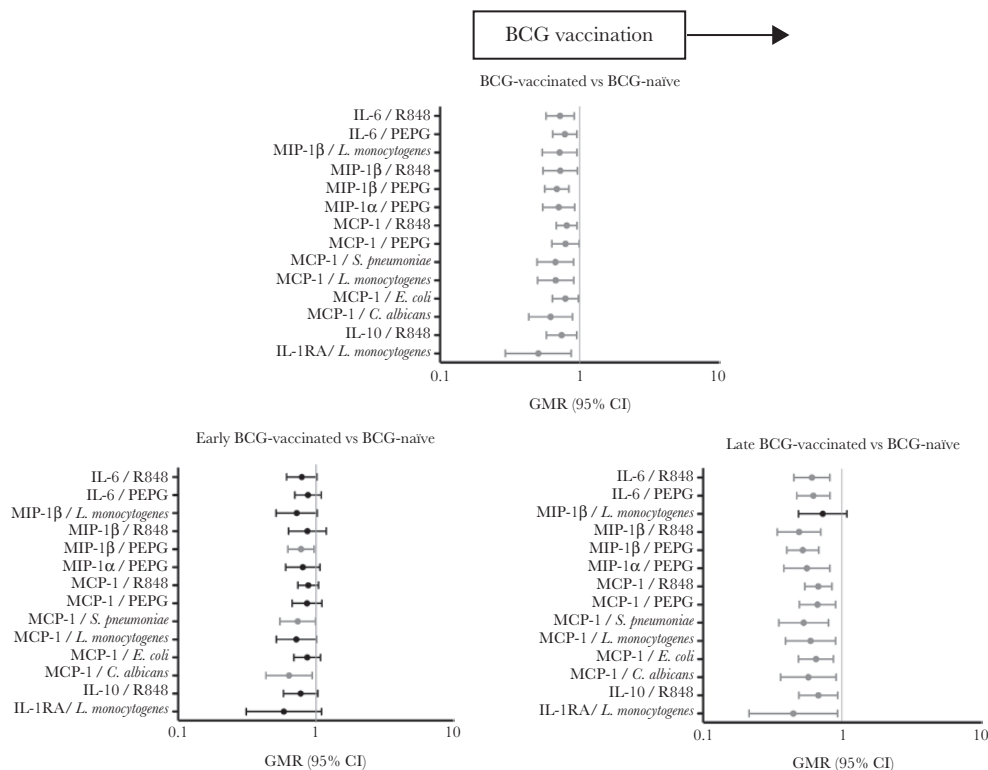


Figure 5. Effect of the timing of BCG on the overall effect of BCG shown by the primary analysis. Cytokine responses for cytokine/stimulant pairs where a significant effect of BCG vaccination was seen are shown for all participants (top), for infants who received BCG < 48 hours of birth (early) vs controls (bottom left), for participants who received BCG $\geq$ 48 hours from birth (late) vs controls (bottom right). *C. albicans*, *Candida albicans*; CI, confidence interval; GMR, geometric mean ratio; *E. coli*, *Escherichia coli*; IL-1RA, interleukin 1 receptor antagonist; IL-10, interleukin 10; *L. monocytogenes*, *Listeria monocytogenes*; MCP-1, monocyte chemoattractant protein 1/CCL2; MIP-1 α/β , macrophage inflammatory protein 1 (CCL3/CCL4); PEPG, peptidoglycan; R848, resiquimod; *S. pneumoniae*, *Streptococcus pneumoniae* serotype 15C.

stress [41]. MIF has been implicated in severe sepsis, malaria, and chronic inflammatory diseases [42, 43]. The high baseline MIF levels among infants in our study are consistent with previous findings [44]. Given its pivotal role in the control of sepsis, the sex-differential effect of BCG vaccination on MIF production in our study is noteworthy.

The effect of the timing of BCG vaccination on the vaccine response is complex. In our study, when compared to late BCG vaccination, early BCG vaccination was associated with increased production of multiple cytokines in response to most stimulants. We had inadequate power for subgroup analysis of the effect of age on BCG vaccination, and instead we explored the influence of timing of BCG vaccination on the results of our primary analysis. We found that the effect of BCG was more pronounced with older age at vaccination. Interpretation of this finding is difficult because the analysis is potentially confounded by the older age of infants at the time of blood sampling. Furthermore, all infants in the late BCG vaccination group received hepatitis B vaccination prior to BCG vaccination, while some of those in the early BCG vaccination group received hepatitis B vaccination on the same day as or after BCG vaccination.

The strengths of our study include our ability to randomize term infants, because BCG is not routinely given in Australia, and

the early timing of our blood sampling. Further strengths are the highly standardized and controlled protocols that were used for blood sample collection, handling of samples and laboratory assays [45], as well as the consideration given to the effect of assigned values outside the limit of detection of the assay and values that have been extrapolated by the xMAP software. There is no agreement on the method for standardizing or reporting these parameters; methods described in the literature are highly variable. The findings in our study were preserved across sensitivity analyses, which confirmed the validity of our use of assigned variables. Finally, our study, like others, found that in neonates certain cytokines have high unstimulated (ie, baseline) levels, while others remain largely undetectable in the absence of stimulation [44]. To account for the variability between individuals in unstimulated levels of immunological markers [22], we included the unstimulated value for each cytokine as a covariate in the primary analysis.

A potential limitation is the coadministration of hepatitis B vaccine as part of the Australian immunization schedule. The results of our study should be interpreted as BCG vaccination and hepatitis B vaccination, compared with hepatitis B vaccination alone. BCG has been associated with effects on hepatitis B virus antibody production in studies in the Gambia [46] and Australia [47] but not in South Africa [48]. The effect of hepatitis B vaccines on

innate immunity has not been well studied [49], and there have been no studies investigating potential interactions with BCG. In our study, the vast majority of participants randomized to BCG vaccination received BCG and hepatitis B vaccines within 48 hours of each other. We were therefore unable to stratify our results by the timing of hepatitis B vaccination. A further limitation is the possibility of spurious statistically significant results arising from multiple subset analyses with reduced sample size. To mitigate this possibility, we restricted subset analyses to cytokine/stimulant pairs for which there was an interaction of BCG with the covariate of interest. In addition, to aid interpretation and avoid undue emphasis on tests of statistical significance or, conversely, to avoid discarding results that were not significant owing to type 2 error, we presented all our analyses with confidence intervals [50].

Based on our data, future studies on the innate immune response to BCG should focus on interrogation of chemokine-producing cells and pathways activated by R848 and PEPG, including TLR7/8, TLR2, RIG, and NOD. Furthermore, studies in high-mortality settings would be required to confirm any association between patterns of innate immune modulation in vitro and clinical outcomes following infection.

In conclusion, our study provides evidence of differential cytokine responses to heterologous antigens between BCG-vaccinated and BCG-naïve term infants. The responses can be broadly described as inhibition of antiinflammatory cytokine production, as well as downregulation of chemokine responses. Both sex and maternal BCG vaccination status interacted with BCG vaccination. This study supports the concept that BCG modifies the neonatal innate immune response.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis* **2011**; 204:245–52.
2. Shann F. The nonspecific effects of vaccines and the expanded program on immunization. *J Infect Dis* **2011**; 204:182–4.
3. Pollard AJ, Finn A, Curtis N. Non-specific effects of vaccines: plausible and potentially important, but implications uncertain. *Arch Dis Child* **2017**; 102:1077–81.
4. Kristensen I, Aaby P, Jensen H. Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa. *BMJ (Clinical research ed)* **2000**; 321:1435–8.
5. Higgins JP, Soares-Weiser K, Lopez-Lopez JA, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. *BMJ (Clinical research ed)* **2016**; 355:i5170.
6. Arnoldussen DL, Linehan M, Sheikh A. BCG vaccination and allergy: a systematic review and meta-analysis. *J Allergy Clin Immunol* **2011**; 127:246–53, 53 e1–21.
7. Freyne B, Curtis N. Does neonatal BCG vaccination prevent allergic disease in later life? *Arch Dis Child* **2014**; 99:182–4.
8. Hollm-Delgado MG, Stuart EA, Black RE. Acute lower respiratory infection among Bacille Calmette-Guérin (BCG)-vaccinated children. *Pediatrics* **2014**; 133:e73–81.
9. Aaby P, Jensen H, Rodrigues A, et al. Divergent female-male mortality ratios associated with different routine vaccinations among female-male twin pairs. *Int J Epidemiol* **2004**; 33: 367–73.
10. Shann F. The non-specific effects of vaccines. *Arch Dis Child* **2010**; 95:662–7.
11. Mawa PA, Webb EL, Filali-Mouhim A, et al. Maternal BCG scar is associated with increased infant proinflammatory immune responses. *Vaccine* **2017**; 35:273–82.
12. Kandasamy R, Voysey M, McQuaid F, et al. Non-specific immunological effects of selected routine childhood immunisations: systematic review. *BMJ (Clinical research ed)* **2016**; 355:i5225.
13. Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases. *Trans R Soc Trop Med Hyg* **2015**; 109:52–61.
14. Freyne B, Marchant A, Curtis N. BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms. *Trans R Soc Trop Med Hyg* **2015**; 109:46–51.
15. Jensen KJ, Larsen N, Biering-Sørensen S, et al. Heterologous immunological effects of early BCG vaccination in low-birth-weight infants in Guinea-Bissau: a randomized-controlled trial. *J Infect Dis* **2015**; 211:956–67.
16. Kleinnijenhuis J, Quintin J, Preijers F, et al. Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection

- from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA* **2012**; 109:17537–42.
17. Wasserstein RL, Lazar NA. The ASA's statement on p-values: context, process, and purpose. *J Anaesthesiol Clin Pharmacol* **2016**; 32:421–3.
 18. Kollmann TR, Crabtree J, Rein-Weston A, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. *J Immunol* **2009**; 183:7150–60.
 19. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly. *Immunity* **2012**; 37:771–83.
 20. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* **2007**; 7:379–90.
 21. van Haren SD, Ganapathi L, Bergelson I, et al. In vitro cytokine induction by TLR-activating vaccine adjuvants in human blood varies by age and adjuvant. *Cytokine* **2016**; 83:99–109.
 22. Li Y, Oosting M, Deelen P, et al. Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi. *Nat Med* **2016**; 22:952–60.
 23. Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. *Blood* **2006**; 108:1284–90.
 24. Levy O, Zarembek KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF- α induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J Immunol* **2004**; 173:4627–34.
 25. Johnson TR, Rao S, Seder RA, Chen M, Graham BS. TLR9 agonist, but not TLR7/8, functions as an adjuvant to diminish FI-RSV vaccine-enhanced disease, while either agonist used as therapy during primary RSV infection increases disease severity. *Vaccine* **2009**; 27:3045–52.
 26. Tang FS, Van Ly D, Spann K, et al. Differential neutrophil activation in viral infections: Enhanced TLR-7/8-mediated CXCL8 release in asthma. *Respirology* **2016**; 21:172–9.
 27. Hadley JS, Wang JE, Foster SJ, Thiernemann C, Hinds CJ. Peptidoglycan of *Staphylococcus aureus* upregulates monocyte expression of CD14, Toll-like receptor 2 (TLR2), and TLR4 in human blood: possible implications for priming of lipopolysaccharide signaling. *Infect Immun* **2005**; 73:7613–9.
 28. Wang JE, Dahle MK, McDonald M, Foster SJ, Aasen AO, Thiernemann C. Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock* **2003**; 20:402–14.
 29. Wang JE, Jørgensen PF, Ellingsen EA, et al. Peptidoglycan primes for LPS-induced release of proinflammatory cytokines in whole human blood. *Shock* **2001**; 16:178–82.
 30. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* **2009**; 29:313–26.
 31. Smolen KK, Ruck CE, Fortuno ES 3rd, et al. Pattern recognition receptor-mediated cytokine response in infants across 4 continents. *J Allergy Clin Immunol* **2014**; 133:818–26 e4.
 32. Levy O, Wynn JL. A prime time for trained immunity: innate immune memory in newborns and infants. *Neonatology* **2014**; 105:136–41.
 33. Reikie BA, Adams RC, Leliggowicz A, et al. Altered innate immune development in HIV-exposed uninfected infants. *J Acquir Immune Defic Syndr* **2014**; 66:245–55.
 34. Smith SG, Kleinnijenhuis J, Netea MG, Dockrell HM. Whole blood profiling of bacillus calmette-guérin-induced trained innate immunity in infants identifies epidermal growth factor, IL-6, platelet-derived growth factor-AB/BB, and natural killer cell activation. *Front Immunol* **2017**; 8:644.
 35. Mawa PA, Nkurunungi G, Egesa M, et al. The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to bacille Calmette-Guérin immunization. *Philos Trans R Soc Lond B Biol Sci* **2015**; 370.
 36. Stensballe LG, Sørup S, Aaby P, et al. BCG vaccination at birth and early childhood hospitalisation: a randomised clinical multicentre trial. *Arch Dis Child* **2017**; 102:224–31.
 37. Costello AM, Kumar A, Narayan V, et al. Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis? *Trans R Soc Trop Med Hyg* **1992**; 86:686–92.
 38. Beemelmans A, Roth O. Grandparental immune priming in the pipefish *Syngnathus typhle*. *BMC Evol Biol* **2017**; 17:44.
 39. Green TJ, Helbig K, Speck P, Raftos DA. Primed for success: Oyster parents treated with poly(I:C) produce offspring with enhanced protection against Ostreid herpesvirus type I infection. *Mol Immunol* **2016**; 78:113–20.
 40. Flanagan KL, Klein SL, Skakkebaek NE, et al. Sex differences in the vaccine-specific and non-targeted effects of vaccines. *Vaccine* **2011**; 29:2349–54.
 41. Calandra T. Macrophage migration inhibitory factor and host innate immune responses to microbes. *Scand J Infect Dis* **2003**; 35:573–6.
 42. Clark I, Awburn M. Migration inhibitory factor in the cerebral and systemic endothelium in sepsis and malaria. *Crit Care Med* **2002**; 30:S263–7.
 43. Renner P, Roger T, Calandra T. Macrophage migration inhibitory factor: gene polymorphisms and susceptibility to inflammatory diseases. *Clin Infect Dis* **2005**; 41(Suppl 7):S513–9.

44. Roger T, Schneider A, Weier M, et al. High expression levels of macrophage migration inhibitory factor sustain the innate immune responses of neonates. *Proc Natl Acad Sci USA* **2016**; 113:E997–1005.
45. Blimkie D, Fortuno ES 3rd, Yan H, et al. Variables to be controlled in the assessment of blood innate immune responses to Toll-like receptor stimulation. *J Immunol Methods* **2011**; 366:89–99.
46. Ota MO, Vekemans J, Schlegel-Haueter SE, et al. Influence of *Mycobacterium bovis* bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. *J Immunol* **2002**; 168:919–25.
47. Ritz N, Mui M, Balloch A, Curtis N. Non-specific effect of Bacille Calmette-Guérin vaccine on the immune response to routine immunisations. *Vaccine* **2013**; 31:3098–103.
48. Hesselink AC, Blakney AK, Jones CE, et al. Delayed BCG immunization does not alter antibody responses to EPI vaccines in HIV-exposed and -unexposed South African infants. *Vaccine* **2016**; 34:3702–9.
49. Said ZN, Abdelwahab KS. Induced immunity against hepatitis B virus. *World J Hepatol* **2015**; 7:1660–70.
50. Halsey LG, Curran-Everett D, Vowler SL, Drummond GB. The fickle P value generates irreproducible results. *Nat Methods* **2015**; 12:179–85.

The effect of neonatal BCG vaccination on cytokine responses to heterologous antigens in a randomised controlled trial

Supplementary Data

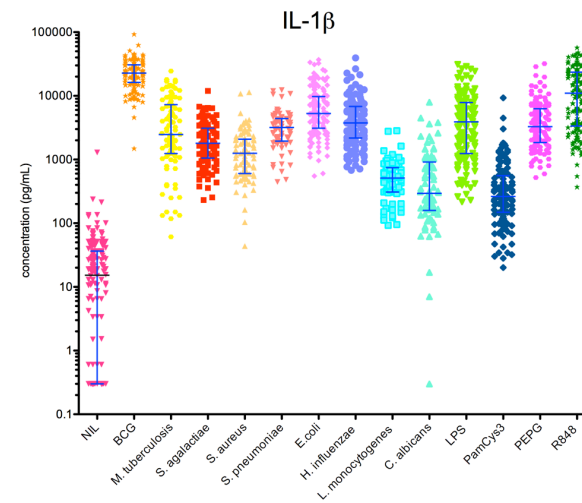
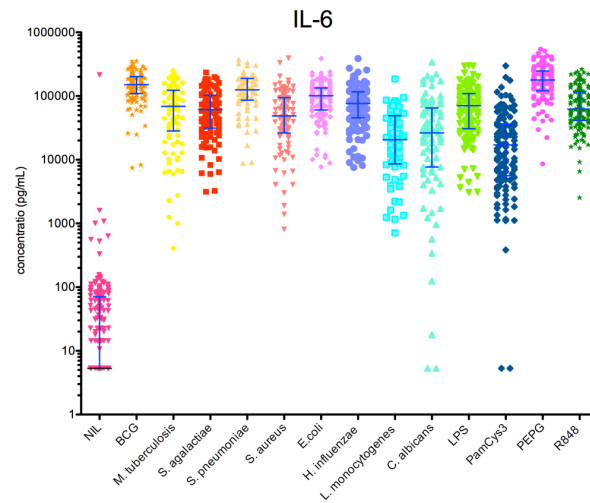
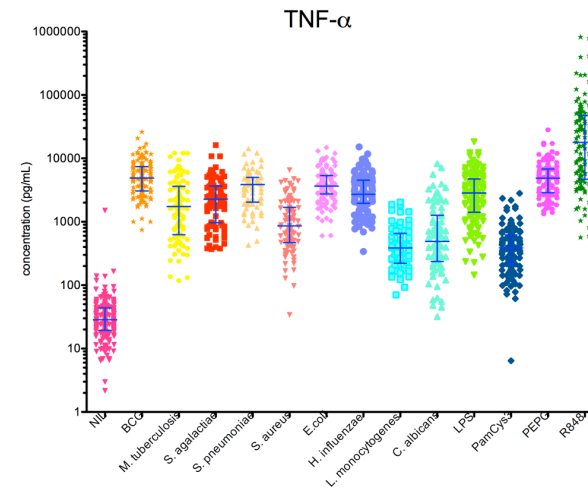
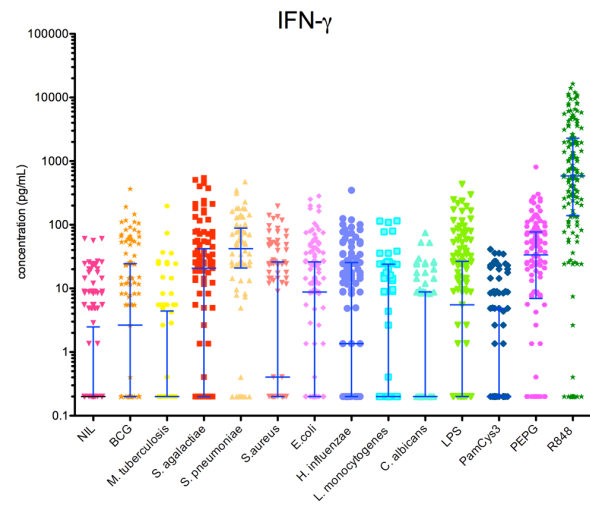


Fig 1, Panel A: Levels of pro-inflammatory cytokines (IFN γ , TNF α , IL-6, IL1 β) induced by stimulation with 9 killed pathogens, 4 TLR agonists and RPMI. Error bar represent median and IQR. Nil - RPMI, BCG - Bacille Calmette Guerin, LPS - Lipopolysaccharide, PamCys3- (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, PEPG - peptidoglycan, R848 - resiquimod.

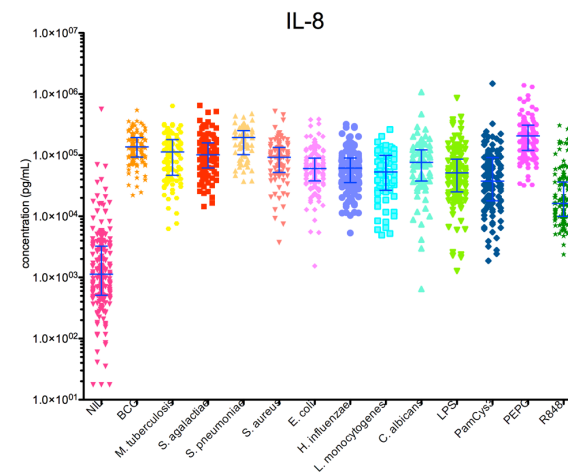
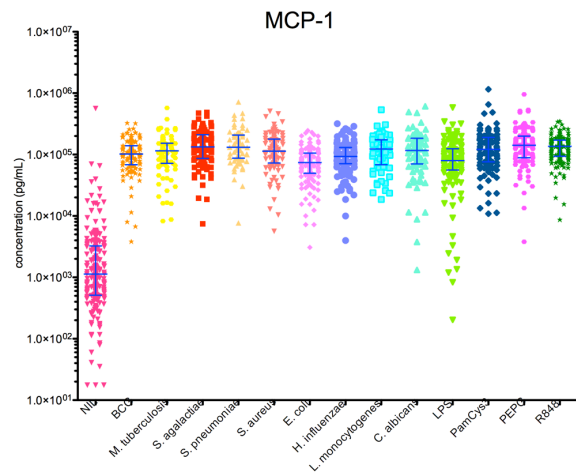
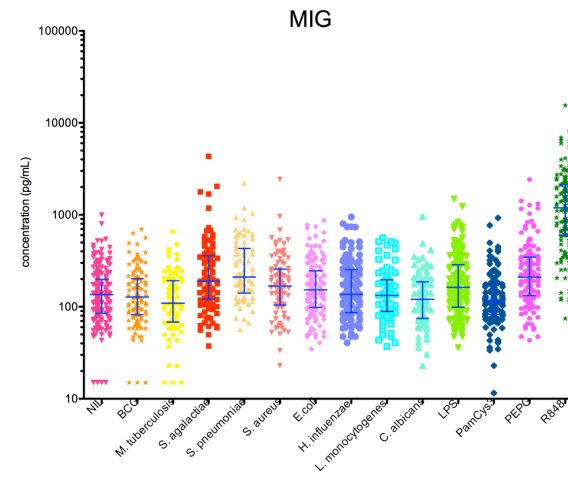
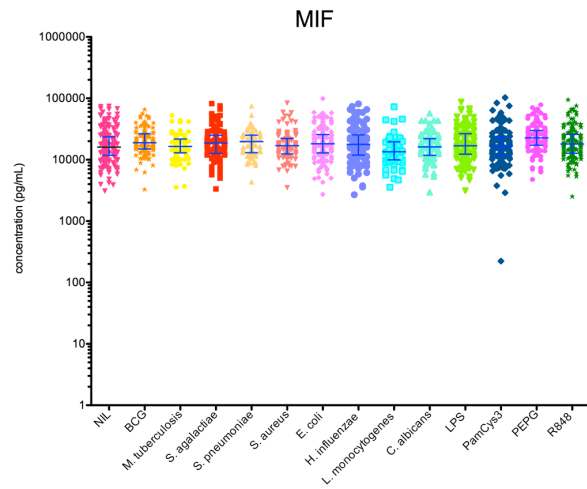


Fig 1, Panel B: Levels of chemokines (MIF, MIG, MCP-1, IL-8) induced by stimulation with 9 killed pathogens, 4 TLR agonists and RPMI.
 Error bar represent median and IQR. Nil - RPMI, BCG - Bacille Calmette Guerin, LPS - Lipopolysaccharide,
 PamCys3- (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, PEPG - peptidoglycan, R848 - resiquimod.

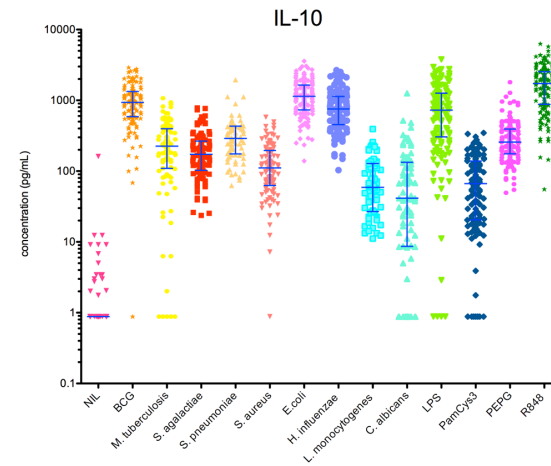
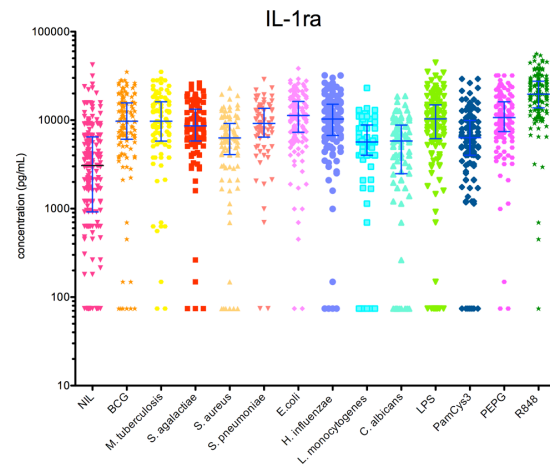
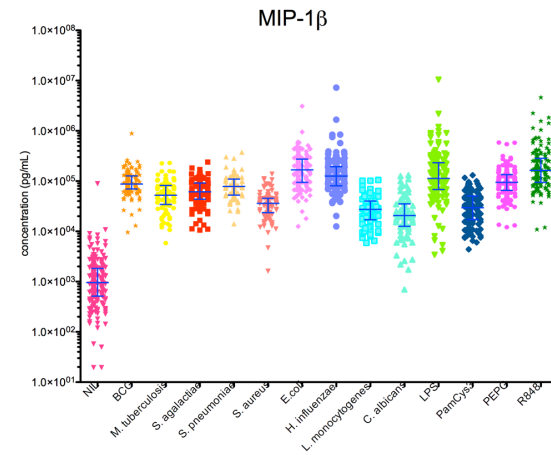
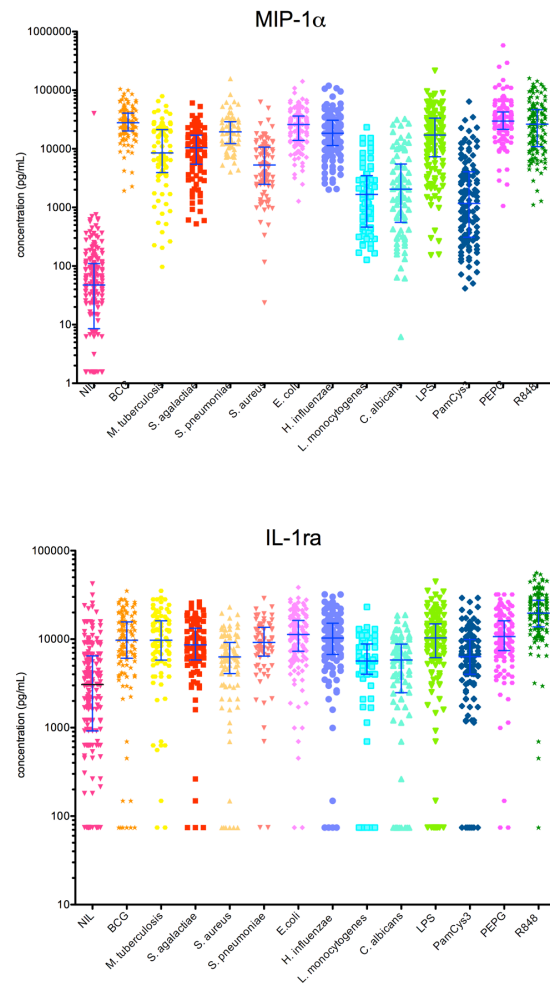


Fig 1, Panel C: Levels of chemokines (MIP-1 α , MIP-1 β) and anti-inflammatory cytokines IL-1ra and IL10 induced by stimulation with 9 killed pathogens, 4 TLR agonists and RPMI. Error bar represent median and IQR. Nil - RPMI, BCG - Bacille Calmette Guerin, LPS - Liopolysaccarhide, PamCys3- (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, PEPG - peptidoglycan, R848 - resiquimod.

		BCG-	Not BCG-	Total
		vaccinated	vaccinated	
All	n (%)	119 (56.1)	93 (43.9)	212
Age at blood collection (days)	Mean (SD)	9.1 (2.9)	8.6 (2.5)	8.9 (2.8)
Volume of blood (mL)	Mean (SD)	2.8 (1.6)	2.9 (1.6)	2.9 (1.6)
Time between blood collection and stimulation (hours)	Mean (SD)	4.0 (0.3)	4.1 (0.3)	4.0 (0.3)
Within 4 hours $\pm$ 24 minutes	n (%)	74 (62.2)	69 (74.2)	143 (67.5)
Incubation duration (hours)	Mean (SD)	20.5 (0.8)	20.3 (0.8)	20.4 (0.8)
Within 20 $\pm$ 2 hours	n (%)	115 (96.6)	92 (99.2)	207 (97.6)

Supplementary Table 1: Standardisation of blood collection, handling and laboratory methods.

Cytokine	Stim	% In Range	Total (n)	GMR	BCG 95%CI		p value	GMR	Maternal BCG 95%CI		p value
					LCI	UCI			LCI	UCI	
MIP-1 β	BCG	88.4	129	0.9	0.7	1.1	0.23	1.1	0.9	1.4	0.42
	MTBK	95.9	98	1.1	0.8	1.4	0.55	1.2	0.9	1.6	0.28
	LPS	68.0	147	0.8	0.5	1.1	0.14	0.9	0.6	1.4	0.73
	PEPG	81.6	147	0.7	0.6	0.8	0.00	1.2	1.0	1.6	0.05
	Pam3	99.2	128	1.0	0.8	1.3	0.96	1.2	0.9	1.6	0.16
	R848	63.3	150	0.7	0.5	1.0	0.03	0.9	0.7	1.3	0.58
	SA	100.0	98	0.8	0.7	1.1	0.14	1.2	0.9	1.5	0.25
	SP	91.9	74	0.8	0.6	1.0	0.07	1.3	1.0	1.7	0.06
	GBS	96.5	113	0.9	0.8	1.2	0.57	1.0	0.8	1.3	0.98
	EC	59.2	157	0.8	0.6	1.0	0.05	1.1	0.8	1.4	0.66
	HI	71.6	141	0.9	0.7	1.1	0.25	1.1	0.9	1.5	0.30
	LM	100.0	69	0.7	0.5	1.0	0.03	1.0	0.7	1.4	0.95
	CA	100.0	86	0.8	0.5	1.2	0.28	1.1	0.7	1.7	0.68
MCP-1	BCG	100.0	129	1.0	0.8	1.3	0.98	1.0	0.8	1.4	0.77
	MTBK	100.0	98	1.0	0.8	1.4	0.82	1.0	0.7	1.4	0.95
	LPS	100.0	147	0.9	0.6	1.3	0.45	1.3	0.8	1.9	0.27
	PEPG	99.3	147	0.8	0.6	1.0	0.04	1.2	0.9	1.6	0.14
	Pam3	99.2	128	0.9	0.7	1.1	0.36	1.2	0.9	1.5	0.30
	R848	98.0	150	0.8	0.7	1.0	0.01	1.1	0.9	1.4	0.29
	SA	99.0	98	0.8	0.6	1.1	0.20	1.1	0.8	1.6	0.47
	SP	98.6	74	0.7	0.5	0.9	0.01	1.2	0.8	1.6	0.37
	GBS	100.0	113	0.9	0.7	1.1	0.28	1.0	0.7	1.3	0.91
	EC	100.0	157	0.8	0.6	1.0	0.03	1.1	0.8	1.4	0.52
	HI	100.0	141	0.8	0.7	1.0	0.07	1.1	0.8	1.3	0.63
	LM	100.0	69	0.7	0.5	0.9	0.01	1.0	0.7	1.3	0.80
	CA	100.0	86	0.6	0.4	0.9	0.01	1.3	0.8	1.9	0.28
IL-8	BCG	100.0	129	1.0	0.9	1.3	0.70	1.1	0.9	1.4	0.38
	MTBK	100.0	98	1.2	0.9	1.7	0.27	1.2	0.8	1.8	0.26
	LPS	100.0	147	1.0	0.8	1.3	0.94	1.1	0.8	1.5	0.52
	PEPG	96.6	147	0.9	0.7	1.1	0.37	1.1	0.9	1.4	0.40
	Pam3	99.0	128	1.1	0.8	1.5	0.68	1.3	0.9	1.8	0.19
	R848	100.0	150	0.9	0.7	1.2	0.70	0.9	0.7	1.2	0.58
	SA	100.0	98	0.9	0.7	1.3	0.67	1.0	0.8	1.4	0.77
	SP	100.0	74	0.8	0.7	1.1	0.17	1.0	0.8	1.3	0.87
	GBS	100.0	113	1.0	0.8	1.3	0.95	1.0	0.7	1.2	0.79
	EC	100.0	157	1.0	0.8	1.2	0.81	1.2	0.9	1.4	0.16
	HI	100.0	141	1.0	0.8	1.2	0.92	1.1	0.9	1.4	0.30
	LM	100.0	69	0.7	0.5	1.1	0.11	0.9	0.6	1.4	0.61
	CA	99.8	86	0.8	0.5	1.1	0.15	1.2	0.8	1.9	0.37
MIF	BCG	100.0	129	0.9	0.8	1.0	0.06	1.0	0.9	1.1	0.79
	MTBK	100.0	98	0.9	0.8	1.0	0.09	1.0	0.9	1.2	0.67
	LPS	100.0	147	1.0	0.9	1.1	0.81	1.0	0.9	1.1	0.44
	PEPG	100.0	147	1.0	0.9	1.1	0.42	1.0	0.9	1.1	0.98
	Pam3	97.7	128	0.9	0.8	1.1	0.29	1.0	0.8	1.1	0.62
	R848	100.0	150	1.0	0.9	1.0	0.28	1.0	0.9	1.1	0.81
	SA	100.0	98	1.0	0.9	1.1	0.76	1.0	0.9	1.1	0.62
	SP	100.0	74	1.0	0.9	1.1	0.72	1.0	0.9	1.2	0.69
	GBS	100.0	113	1.1	0.9	1.2	0.32	1.0	0.9	1.1	0.97
	EC	100.0	157	1.1	1.0	1.1	0.25	1.0	0.9	1.1	0.64
	HI	100.0	141	1.0	0.9	1.1	0.56	1.0	0.9	1.1	0.44
	LM	100.0	69	0.9	0.8	1.0	0.07	1.0	0.9	1.2	0.61
	CA	100.0	86	1.0	0.9	1.1	0.88	1.0	0.9	1.1	0.76

Supplementary data Table 2 p1.

Cytokine	Stim	% In Range	Total (n)	BCG				Maternal BCG			
				GMR	95%CI		p value	GMR	95%CI		p value
					LCI	UCI			LCI	UCI	
IL-1ra	BCG	93.8	129	1.1	0.7	1.7	0.62	1.0	0.7	1.6	0.95
	MTBK	95.9	98	1.0	0.7	1.6	0.85	1.3	0.8	2.0	0.34
	LPS	93.2	147	0.8	0.6	1.3	0.40	1.3	0.8	2.0	0.32
	PEPG	98.0	147	0.9	0.7	1.2	0.46	1.0	0.7	1.3	0.98
	Pam3	91.4	128	0.8	0.6	1.2	0.32	1.5	1.0	2.2	0.03
	R848	99.3	150	0.8	0.6	1.0	0.06	1.3	1.0	1.7	0.08
	SA	92.9	98	0.8	0.5	1.2	0.19	1.5	0.9	2.4	0.09
	SP	97.3	74	0.7	0.5	1.1	0.09	1.3	0.8	2.1	0.26
	GBS	95.6	113	0.9	0.6	1.2	0.37	1.1	0.8	1.7	0.49
	EC	98.1	157	0.8	0.6	1.1	0.13	1.1	0.8	1.4	0.59
	HI	96.5	141	0.8	0.6	1.1	0.25	1.2	0.9	1.7	0.23
	LM	89.9	69	0.5	0.3	0.9	0.01	1.5	0.8	2.8	0.18
	CA	84.9	86	0.7	0.4	1.1	0.10	1.3	0.7	2.2	0.43
IL-10	BCG	98.4	129	1.0	0.7	1.3	0.77	1.0	0.7	1.4	0.81
	MTBK	90.8	98	1.3	0.7	2.5	0.44	0.9	0.5	1.8	0.84
	LPS	95.9	147	1.1	0.7	1.9	0.62	0.6	0.3	1.0	0.06
	PEPG	99.3	147	0.8	0.7	1.0	0.09	0.9	0.7	1.1	0.30
	Pam3	78.1	128	0.8	0.5	1.4	0.49	1.1	0.6	1.8	0.81
	R848	100.0	150	0.7	0.6	1.0	0.02	0.8	0.6	1.0	0.08
	SA	94.9	98	0.9	0.6	1.3	0.61	1.0	0.6	1.5	0.88
	SP	100.0	74	1.1	0.8	1.5	0.62	0.9	0.6	1.2	0.38
	GBS	100.0	113	1.2	0.9	1.6	0.18	0.7	0.6	1.0	0.05
	EC	100.0	157	0.9	0.7	1.1	0.23	0.9	0.7	1.1	0.22
	HI	100.0	141	0.9	0.7	1.1	0.31	0.9	0.7	1.1	0.22
	LM	82.6	69	0.9	0.6	1.3	0.47	0.8	0.5	1.3	0.45
	CA	64.0	86	1.2	0.5	2.9	0.64	1.0	0.4	2.7	0.93
MIG	BCG	97.7	129	0.9	0.8	1.0	0.14	1.1	1.0	1.4	0.11
	MTBK	96.9	98	0.8	0.7	1.0	0.06	1.0	0.8	1.3	0.80
	LPS	99.3	147	1.1	0.9	1.3	0.50	0.9	0.8	1.2	0.60
	PEPG	100.0	147	0.9	0.7	1.1	0.17	1.1	0.9	1.4	0.49
	Pam3	98.4	128	1.0	0.9	1.1	0.81	0.9	0.8	1.1	0.23
	R848	100.0	150	0.9	0.7	1.3	0.70	1.1	0.8	1.5	0.65
	SA	100.0	98	1.0	0.8	1.2	0.99	1.0	0.8	1.2	0.85
	SP	100.0	74	1.1	0.8	1.4	0.71	1.1	0.8	1.5	0.64
	GBS	100.0	113	1.1	0.8	1.4	0.63	1.0	0.8	1.3	0.90
	EC	100.0	157	1.0	0.9	1.1	0.94	1.0	0.9	1.2	0.67
	HI	100.0	141	0.9	0.8	1.1	0.45	1.1	0.9	1.2	0.48
	LM	100.0	69	0.9	0.7	1.1	0.19	1.1	0.8	1.4	0.63
	CA	98.8	86	0.9	0.8	1.1	0.34	1.0	0.9	1.2	0.92
MIP-1α	BCG	91.5	129	0.9	0.7	1.1	0.28	1.1	0.8	1.4	0.69
	MTBK	98.0	98	1.1	0.6	1.9	0.76	1.2	0.6	2.2	0.60
	LPS	91.8	147	0.9	0.6	1.4	0.75	1.0	0.6	1.6	0.99
	PEPG	84.4	147	0.7	0.5	0.9	0.01	1.3	1.0	1.8	0.06
	Pam3	99.2	128	1.2	0.7	2.1	0.57	2.3	1.3	4.3	0.01
	R848	84.0	150	0.7	0.5	1.0	0.06	0.9	0.6	1.3	0.64
	SA	98.0	98	0.7	0.4	1.1	0.15	1.7	1.0	2.8	0.07
	SP	95.9	74	0.8	0.6	1.2	0.27	1.3	0.9	1.8	0.17
	GBS	98.2	113	1.1	0.7	1.6	0.73	1.4	0.9	2.0	0.15
	EC	91.1	157	0.9	0.7	1.2	0.50	1.2	0.9	1.5	0.24
	HI	95.0	141	0.9	0.6	1.1	0.25	1.4	1.0	1.8	0.04
	LM	100.0	69	0.8	0.4	1.4	0.38	1.1	0.5	2.1	0.89
	CA	98.8	86	0.7	0.3	1.5	0.38	1.2	0.5	2.8	0.60

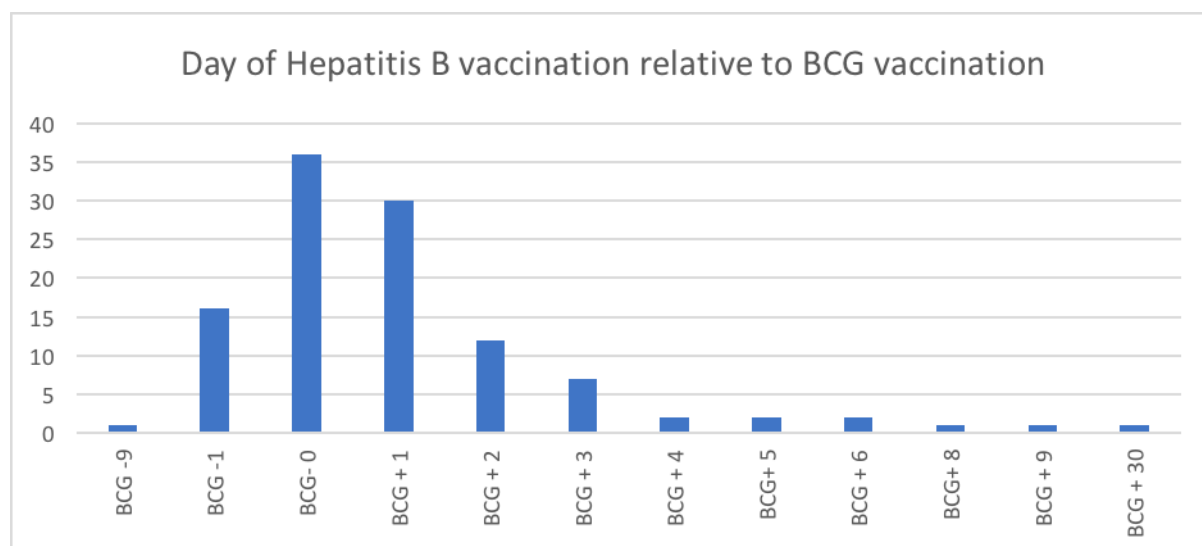
Supplementary data Table 2 p2.

Cytokine	Stim	% In Range	Total (n)	BCG				Maternal BCG			
				GMR	95%CI		p value	GMR	95%CI		p value
IL-1 β	BCG	99.2	129	0.9	0.8	1.1	0.38	1.0	0.8	1.2	0.89
	MTBK	99.0	98	1.1	0.6	1.8	0.76	1.1	0.6	2.0	0.75
	LPS	98.6	147	0.9	0.6	1.3	0.46	0.7	0.5	1.1	0.11
	PEPG	98.0	147	0.9	0.7	1.1	0.27	1.1	0.8	1.4	0.58
	Pam3	91.4	128	1.0	0.7	1.4	0.91	1.5	1.0	2.2	0.04
	R848	100.0	150	0.8	0.6	1.2	0.30	0.6	0.4	1.0	0.03
	SA	96.9	98	0.8	0.5	1.1	0.16	1.4	0.9	2.0	0.12
	SP	100.0	74	1.0	0.7	1.4	0.98	1.1	0.8	1.5	0.68
	GBS	96.5	113	1.0	0.7	1.3	0.75	1.1	0.8	1.5	0.58
	EC	95.5	157	1.1	0.8	1.4	0.68	1.2	0.9	1.6	0.27
	HI	96.5	141	0.9	0.7	1.2	0.63	1.2	0.9	1.7	0.13
	LM	100.0	69	0.9	0.6	1.2	0.40	0.9	0.6	1.3	0.43
	CA	94.2	86	0.8	0.4	1.5	0.48	1.2	0.6	2.4	0.60
IL-6	BCG	100.0	129	1.0	0.8	1.2	0.77	1.0	0.8	1.3	0.87
	MTBK	100.0	98	1.1	0.6	1.8	0.74	1.1	0.6	2.0	0.70
	LPS	100.0	147	0.9	0.7	1.3	0.66	1.0	0.7	1.4	0.96
	PEPG	100.0	147	0.8	0.6	1.0	0.02	1.2	0.9	1.5	0.17
	Pam3	98.4	128	0.9	0.5	1.6	0.80	2.2	1.2	3.9	0.01
	R848	100.0	150	0.7	0.6	0.9	0.01	0.9	0.7	1.1	0.26
	SA	100.0	98	0.7	0.4	1.1	0.09	1.5	0.9	2.4	0.13
	SP	100.0	74	0.9	0.7	1.3	0.73	1.2	0.8	1.8	0.35
	GBS	100.0	113	0.9	0.7	1.3	0.69	1.2	0.8	1.6	0.39
	EC	100.0	157	0.9	0.7	1.1	0.22	1.2	1.0	1.5	0.10
	HI	100.0	141	0.8	0.6	1.1	0.14	1.3	1.0	1.8	0.03
	LM	100.0	69	0.7	0.4	1.2	0.17	1.2	0.6	2.3	0.59
	CA	95.3	86	0.6	0.3	1.5	0.26	1.8	0.7	4.7	0.25
TNF- α	BCG	100.0	129	0.9	0.7	1.1	0.35	1.0	0.8	1.3	0.83
	MTBK	100.0	98	0.9	0.6	1.4	0.67	1.4	0.9	2.3	0.18
	LPS	100.0	147	1.2	0.9	1.6	0.14	0.8	0.6	1.1	0.19
	PEPG	99.3	147	1.0	0.8	1.2	0.89	1.2	1.0	1.5	0.10
	Pam3	99.2	128	1.0	0.8	1.4	0.74	0.9	0.7	1.2	0.43
	R848	80.0	150	0.8	0.5	1.3	0.42	0.5	0.3	0.9	0.01
	SA	100.0	98	0.9	0.6	1.3	0.49	1.2	0.8	1.7	0.47
	SP	100.0	74	1.0	0.7	1.3	0.94	1.1	0.8	1.5	0.68
	GBS	100.0	113	1.1	0.9	1.5	0.34	1.0	0.8	1.4	0.78
	EC	99.4	157	1.0	0.9	1.2	0.71	1.0	0.8	1.2	0.71
	HI	100.0	141	1.0	0.8	1.2	0.83	1.1	0.9	1.4	0.33
	LM	100.0	69	0.9	0.6	1.2	0.41	1.0	0.7	1.4	0.98
	CA	100.0	86	0.8	0.5	1.4	0.52	0.9	0.5	1.6	0.69
IFN- γ	BCG	20.2	129	1.9	0.9	4.0	0.10	1.3	0.6	3.1	0.49
	MTBK	7.1	98	1.5	0.8	2.7	0.16	1.3	0.6	2.5	0.49
	LPS	29.9	147	1.2	0.6	2.6	0.56	0.7	0.3	1.6	0.37
	PEPG	60.3	147	0.7	0.3	1.4	0.33	0.9	0.4	2.0	0.84
	Pam3	7.8	128	1.2	0.7	2.0	0.51	1.0	0.6	1.8	0.96
	R848	88.7	150	0.8	0.3	1.8	0.52	0.7	0.3	1.7	0.41
	SA	26.5	98	0.8	0.3	1.9	0.56	1.5	0.5	3.9	0.45
	SP	59.5	74	1.0	0.4	2.5	0.95	0.6	0.2	1.7	0.38
	GBS	40.7	113	1.3	0.6	3.2	0.50	1.8	0.7	4.5	0.21
	EC	29.9	157	1.2	0.7	2.3	0.51	0.9	0.5	1.8	0.80
	HI	22.7	141	1.6	0.8	3.1	0.19	0.9	0.4	1.8	0.69
	LM	15.9	69	1.4	0.5	3.7	0.47	0.6	0.2	1.7	0.33
	CA	5.8	86	0.8	0.4	1.6	0.47	0.7	0.3	1.5	0.34

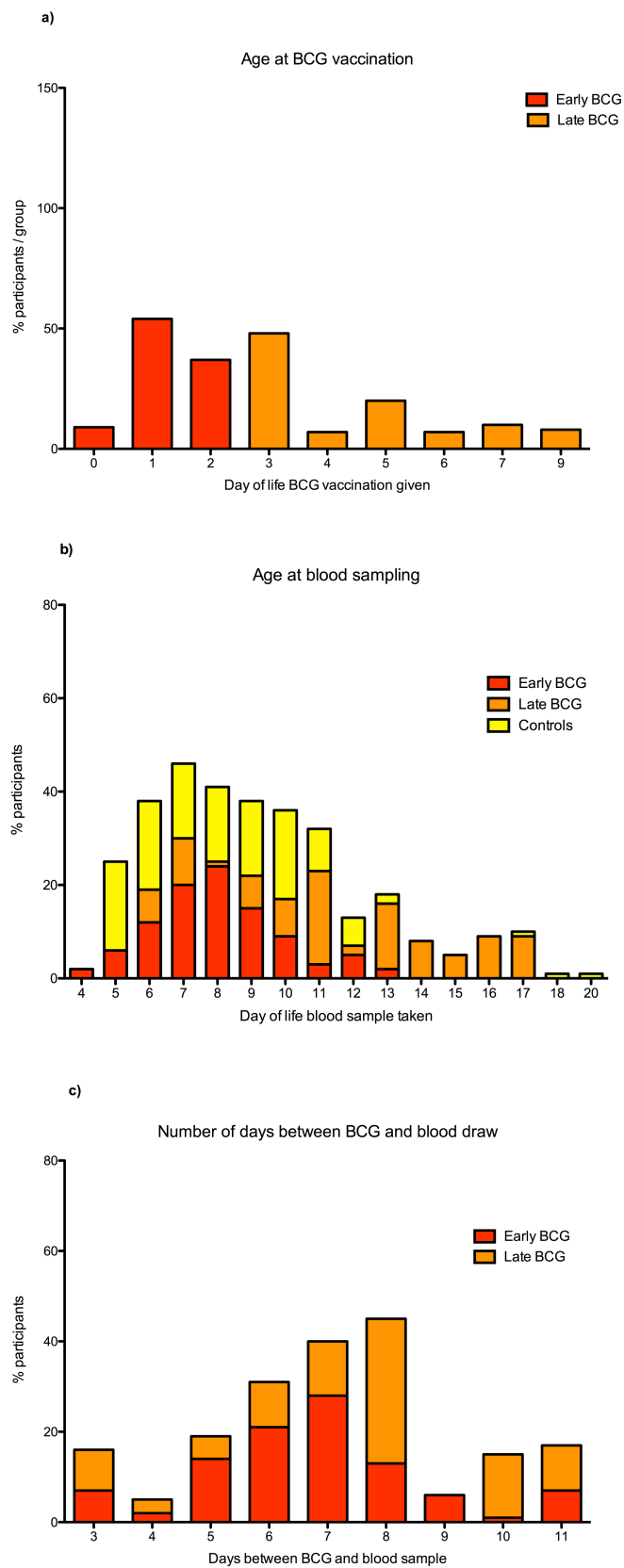
Supplementary data Table 2 p3.

Cytokine	Stimulant	Total (n)	Sex p value	MOD p value	Maternal BCG p value		Cytokine	Stimulant	Total (n)	Sex p value	MOD p value	Maternal BCG p value
IL-1ra	BCG	129	0.65	0.88	0.83		IL-8	BCG	129	0.19	0.95	0.38
	MTBK	98	0.54	0.68	0.27			MTBK	98	0.69	0.78	0.53
	LPS	147	0.45	0.13	0.53			LPS	147	0.42	0.40	0.42
	PEPG	147	0.17	0.41	0.60			PEPG	147	0.93	0.84	0.95
	Pam3	128	0.63	0.14	0.18			Pam3	128	0.58	0.89	0.34
	R848	150	0.74	0.69	0.91			R848	150	0.40	0.70	0.51
	SA	98	0.97	0.08	0.29			SA	98	0.26	0.94	0.83
	SP	74	0.97	0.32	0.18			SP	74	0.49	0.94	0.34
	GBS	113	0.74	0.82	0.25			GBS	113	0.34	0.54	0.69
	EC	157	0.99	0.77	0.60			EC	157	0.98	0.39	0.90
	HI	141	0.27	0.52	0.10			HI	141	0.23	0.95	0.73
	LM	69	0.99	0.30	0.47			LM	69	0.42	0.87	0.97
	CA	86	0.43	0.29	0.82			CA	86	0.64	0.48	0.39
IL-10	BCG	129	0.08	0.77	0.72		MIF	BCG	129	0.97	0.69	0.79
	MTBK	98	0.82	0.47	0.49			MTBK	98	0.01	0.36	0.56
	LPS	147	0.80	0.07	0.49			LPS	147	0.53	0.49	0.81
	PEPG	147	0.25	0.14	0.64			PEPG	147	0.76	0.78	0.50
	Pam3	128	0.98	0.18	0.06			Pam3	128	0.06	0.03	0.20
	R848	150	0.86	0.49	0.28			R848	150	0.06	0.27	0.92
	SA	98	0.09	0.87	0.57			SA	98	0.19	0.39	0.60
	SP	74	0.17	0.73	0.37			SP	74	0.06	0.53	0.88
	GBS	113	0.90	0.17	0.12			GBS	113	0.00	0.21	0.98
	EC	157	0.76	0.35	0.39			EC	157	0.10	0.13	0.70
	HI	141	0.83	0.56	0.53			HI	141	0.04	0.45	0.80
	LM	69	0.35	0.31	0.23			LM	69	0.03	0.24	0.59
	CA	86	0.79	0.08	0.40			CA	86	0.10	0.98	0.95
MIG	BCG	129	0.77	0.87	0.64		IL-1β	BCG	129	0.51	0.64	0.04
	MTBK	98	0.65	0.47	0.77			MTBK	98	0.12	0.79	0.35
	LPS	147	0.88	0.29	0.82			LPS	147	0.33	0.73	0.72
	PEPG	147	0.30	0.41	0.50			PEPG	147	0.15	0.92	0.51
	Pam3	128	0.08	0.02	0.29			Pam3	128	0.54	0.42	0.35
	R848	150	0.18	0.94	0.71			R848	150	0.68	0.50	0.48
	SA	98	0.06	0.20	0.50			SA	98	0.71	0.21	0.55
	SP	74	0.64	0.45	0.45			SP	74	0.79	0.67	0.60
	GBS	113	0.74	0.52	0.07			GBS	113	0.52	0.92	0.97
	EC	157	0.10	0.22	0.16			EC	157	0.57	0.32	0.74
	HI	141	0.22	0.41	0.04			HI	141	0.91	0.80	0.72
	LM	69	0.40	0.93	0.53			LM	69	0.83	0.97	0.49
	CA	86	0.45	0.46	0.84			CA	86	0.81	0.91	0.71
MIP-1α	BCG	129	0.42	0.79	0.11		IL-6	BCG	129	0.44	0.50	0.88
	MTBK	98	0.28	0.55	0.32			MTBK	98	0.29	0.90	0.19
	LPS	147	0.85	0.23	0.59			LPS	147	0.98	0.32	0.67
	PEPG	147	0.48	0.63	0.02			PEPG	147	0.79	0.51	0.31
	Pam3	128	0.10	0.95	0.01			Pam3	128	0.42	0.84	0.14
	R848	150	0.91	0.45	0.34			R848	150	0.99	0.41	0.72
	SA	98	0.84	0.55	0.60			SA	98	0.46	0.50	0.69
	SP	74	0.55	0.33	0.50			SP	74	0.59	0.79	0.94
	GBS	113	0.50	0.28	0.34			GBS	113	0.91	0.76	0.76
	EC	157	0.74	0.41	0.33			EC	157	0.64	0.75	0.36
	HI	141	0.31	0.86	0.12			HI	141	0.24	0.86	0.73
	LM	69	0.56	0.27	0.42			LM	69	0.47	0.79	0.92
	CA	86	0.89	0.24	0.94			CA	86	0.43	0.75	0.70
MIP-1β	BCG	129	0.48	0.59	0.53		TNF-α	BCG	129	0.90	0.06	0.15
	MTBK	98	0.46	0.22	0.21			MTBK	98	0.75	0.07	0.55
	LPS	147	0.71	0.04	0.59			LPS	147	0.96	0.04	0.61
	PEPG	147	0.79	0.05	0.13			PEPG	147	0.78	0.40	0.18
	Pam3	128	0.40	0.31	0.07			Pam3	128	0.28	0.73	0.13
	R848	150	0.88	0.34	0.98			R848	150	0.68	0.50	0.90
	SA	98	0.52	0.58	0.37			SA	98	0.96	0.80	0.99
	SP	74	0.61	0.12	0.41			SP	74	0.66	0.14	0.40
	GBS	113	0.42	0.15	0.33			GBS	113	0.08	0.16	0.74
	EC	157	0.98	0.04	0.75			EC	157	0.52	0.14	0.53
	HI	141	0.82	0.28	0.62			HI	141	0.52	0.44	0.79
	LM	69	0.21	0.11	0.29			LM	69	1.00	0.46	0.15
	CA	86	0.46	0.14	0.93			CA	86	0.89	0.13	0.47
MCP-1	BCG	129	0.33	0.99	0.44		IFN-γ	BCG	129	0.98	0.29	0.08
	MTBK	98	0.80	0.80	0.97			MTBK	98	0.71	0.10	0.09
	LPS	147	0.69	0.14	0.50			LPS	147	0.84	0.32	0.33
	PEPG	147	0.19	0.75	0.41			PEPG	147	0.40	0.97	0.20
	Pam3	128	0.39	0.78	0.39			Pam3	128	0.37	0.17	0.65
	R848	150	0.69	0.66	0.57			R848	150	0.70	0.76	0.62
	SA	98	0.46	0.85	0.58			SA	98	0.43	0.11	0.10
	SP	74	0.29	0.81	0.22			SP	74	0.24	0.78	0.00
	GBS	113	0.61	0.51	0.49			GBS	113	0.27	0.87	0.16
	EC	157	0.42	0.75	0.71			EC	157	0.94	0.85	0.40
	HI	141	0.18	0.44	0.86			HI	141	0.22	0.59	0.46
	LM	69	0.52	0.62	0.42			LM	69	0.19	0.35	0.69
	CA	86	0.29	0.39	0.22			CA	86	0.17	0.27	0.34

Supplementary Table 3



Supplementary Figure 2



Supplementary Figure 3

Stimulant	Coefficient (95% CI)	p-value
BCG	0 (-1.41 - 1.41)	1.0
MTBK	0 (-3.59 - 3.59)	1.0
GBS (Group-B-Streptococcus)	.76 (-.875-2.39)	.36
<i>S. aureus</i>	0 (-1.63 – 1.63)	1.0
<i>S. pneumoniae</i>	.109 (-5.6 - .808)	.76
<i>E. coli</i>	0 (-1.29 – 1.29)	1.0
<i>H. influenzae</i>	0 (-1.39 – 1.39)	1.0
<i>L. monocytogenes</i>	0 (-1.36 – 1.36)	1.0
<i>C. albicans</i>	0 (-3.84 – 3.84)	1.0
PEPG	-.61 (-1.36 - .15)	0.12
Pam3CYSK4	0 (-0.48 – 0.48)	1.0
LPS	0 (-1.37 – 1.37)	1.0
R848	-.11 (-.97 - .74)	.79

Supplementary Table 4

Cytokine	Stimulant	Total (n)	Early (n)	Late (n)	Controls (n)	BCG			Late v Early			Early v controls			Late v controls		
						GMR	95%CI	p value	GMR	95%CI	p value	GMR	95%CI	p value	GMR	95%CI	p value
						LCI	UCI		LCI	UCI		LCI	UCI		LCI	UCI	
IL-1ra	BCG	129	51	19	59	1.1	0.7	1.62	0.7	0.4	1.2	0.18	1.3	0.8	1.9	0.30	
	MTBK	98	37	17	44	1.0	0.7	1.6	0.85	0.7	0.4	1.3	0.26	1.3	0.8	2.0	
	LPS	147	54	26	67	0.8	0.6	1.3	0.40	0.6	0.4	1.0	0.07	1.0	0.7	1.6	
	PEPG	147	53	26	67	0.9	0.7	1.2	0.46	0.8	0.6	1.0	0.08	0.9	0.7	1.1	
	Pam3	128	49	21	58	0.8	0.6	1.2	0.32	1.0	0.7	1.7	0.86	0.9	0.6	1.2	
	RS48	150	57	27	66	0.8	0.6	1.0	0.06	0.8	0.6	1.2	0.28	0.8	0.6	1.1	
	SA	98	36	16	46	0.8	0.5	1.2	0.19	0.8	0.4	1.5	0.49	0.8	0.5	1.3	
	SP	74	27	13	34	0.7	0.5	1.1	0.09	0.6	0.3	1.0	0.06	0.9	0.6	1.4	
	GBS	113	43	19	51	0.9	0.6	1.2	0.37	0.8	0.5	1.3	0.30	1.0	0.7	1.4	
	EC	157	62	28	67	0.8	0.6	1.1	0.13	0.7	0.5	1.0	0.05	0.9	0.7	1.2	
	HI	141	56	22	63	0.8	0.6	1.1	0.25	0.8	0.5	1.2	0.23	0.9	0.7	1.2	
	LM	69	23	12	33	0.5	0.3	0.9	0.01	0.9	0.4	2.0	0.79	0.6	0.3	1.1	
	CA	86	32	15	39	0.7	0.4	1.1	0.10	1.0	0.5	2.1	0.97	0.7	0.4	1.2	
	IL-10	BCG	129	51	19	59	1.0	0.7	1.3	0.77	0.7	0.5	1.1	0.15	1.0	0.7	1.5
MTBK		98	37	17	44	1.3	0.7	2.5	0.44	1.3	0.6	3.0	0.52	1.2	0.5	2.6	
LPS		147	54	26	67	1.1	0.7	1.9	0.62	0.6	0.3	1.1	0.08	1.4	0.8	2.4	
PEPG		147	53	26	67	0.8	0.7	1.0	0.09	0.7	0.5	0.9	0.00	1.0	0.8	1.2	
Pam3		128	49	21	58	0.8	0.5	1.4	0.49	1.3	0.6	2.8	0.52	0.8	0.5	1.4	
RS48		150	57	27	66	0.7	0.6	1.0	0.02	0.8	0.6	1.2	0.38	0.8	0.6	1.0	
SA		98	36	16	46	0.9	0.6	1.1	0.61	0.7	0.4	1.2	0.18	1.0	0.7	1.5	
SP		74	27	13	34	1.1	0.8	1.5	0.62	0.5	0.3	0.7	0.00	1.3	1.0	1.9	
GBS		113	43	19	51	1.2	0.9	1.6	0.18	0.8	0.5	1.1	0.17	1.3	1.0	1.8	
EC		157	62	28	67	0.9	0.7	1.1	0.23	0.7	0.5	0.9	0.00	1.0	0.8	1.2	
HI		141	56	22	63	0.9	0.7	1.1	0.31	0.7	0.5	1.0	0.03	1.0	0.8	1.2	
LM		69	23	12	33	0.9	0.6	1.3	0.47	0.8	0.5	1.5	0.58	0.9	0.5	1.5	
CA		86	32	15	39	1.2	0.5	2.9	0.64	0.7	0.2	2.4	0.58	1.4	0.5	3.6	
MIG		BCG	129	51	19	59	0.9	0.8	1.0	0.14	1.0	0.8	1.2	0.98	0.9	0.8	1.0
	MTBK	98	37	17	44	0.8	0.7	1.0	0.06	1.0	0.7	1.4	0.94	0.8	0.6	1.1	
	LPS	147	54	26	67	1.1	0.9	1.3	0.50	0.8	0.6	1.1	0.20	1.1	0.9	1.4	
	PEPG	147	53	26	67	0.9	0.7	1.1	0.17	1.1	0.8	1.4	0.51	0.8	0.7	1.1	
	Pam3	128	49	21	58	1.0	0.9	1.1	0.81	1.1	0.9	1.4	0.34	1.0	0.8	1.1	
	RS48	150	57	27	66	0.9	0.7	1.3	0.70	0.9	0.6	1.5	0.74	1.0	0.7	1.4	
	SA	98	36	16	46	1.0	0.8	1.2	0.99	1.1	0.8	1.6	0.41	1.0	0.8	1.2	
	SP	74	27	13	34	1.1	0.8	1.4	0.71	0.7	0.5	1.1	0.15	1.2	0.9	1.6	
	GBS	113	43	19	51	1.1	0.8	1.4	0.63	0.9	0.6	1.3	0.65	1.1	0.8	1.4	
	EC	157	62	28	67	1.0	0.9	1.1	0.94	0.9	0.8	1.1	0.50	1.0	0.9	1.2	
	HI	141	56	22	63	0.9	0.8	1.1	0.45	1.0	0.8	1.2	0.86	1.0	0.8	1.1	
	LM	69	23	12	33	0.9	0.7	1.1	0.19	0.9	0.7	1.3	0.73	0.9	0.7	1.1	
	CA	86	32	15	39	0.9	0.8	1.1	0.34	1.1	0.9	1.4	0.19	0.9	0.8	1.1	
	MIP-1a	BCG	129	51	19	59	0.9	0.7	1.1	0.28	0.9	0.6	1.3	0.45	0.9	0.7	1.2
MTBK		98	37	17	44	1.1	0.6	1.9	0.76	1.0	0.5	2.2	0.93	1.1	0.6	2.0	
LPS		147	54	26	67	0.9	0.6	1.4	0.75	0.5	0.3	0.9	0.02	1.2	0.7	1.9	
PEPG		147	53	26	67	0.7	0.5	1.0	0.01	0.7	0.5	1.0	0.01	0.8	0.6	1.1	
Pam3		128	49	21	58	1.2	0.7	2.1	0.57	1.1	0.5	2.2	0.86	1.2	0.6	2.2	
RS48		150	57	27	66	0.7	0.5	1.0	0.06	0.6	0.4	1.0	0.04	0.9	0.6	1.2	
SA		98	36	16	46	0.7	0.4	1.1	0.15	0.9	0.4	1.7	0.63	0.7	0.4	1.2	
SP		74	27	13	34	0.8	0.6	1.2	0.27	0.8	0.5	1.2	0.26	0.9	0.6	1.3	
GBS		113	43	19	51	1.1	0.7	1.6	0.73	0.7	0.4	1.2	0.19	1.2	0.8	1.4	
EC		157	62	28	67	0.9	0.7	1.2	0.50	0.7	0.5	1.0	0.07	1.0	0.8	1.3	
HI		141	56	22	63	0.9	0.6	1.1	0.25	0.7	0.5	1.1	0.11	0.9	0.7	1.3	
LM		69	23	12	33	0.8	0.4	1.4	0.38	1.2	0.5	3.2	0.65	0.7	0.3	1.4	
CA		86	32	15	39	0.7	0.3	1.5	0.38	0.9	0.3	2.6	0.83	0.7	0.3	1.7	
MIP-1β		BCG	129	51	19	59	0.9	0.7	1.1	0.23	0.8	0.6	1.0	0.06	1.0	0.8	1.2
	MTBK	98	37	17	44	1.1	0.8	1.4	0.55	0.9	0.6	1.3	0.55	1.1	0.8	1.6	
	LPS	147	54	26	67	0.8	0.5	1.1	0.14	0.6	0.4	0.8	0.01	0.9	0.6	1.4	
	PEPG	147	53	26	67	0.7	0.6	0.8	0.00	0.7	0.5	0.9	0.01	0.8	0.6	1.0	
	Pam3	128	49	21	58	1.0	0.8	1.3	0.96	0.9	0.6	1.2	0.45	1.0	0.8	1.4	
	RS48	150	57	27	66	0.7	0.5	1.0	0.03	0.6	0.4	1.0	0.01	0.8	0.6	1.2	
	SA	98	36	16	46	0.7	0.4	1.1	0.15	0.9	0.4	1.7	0.63	0.7	0.4	1.2	
	SP	74	27	13	34	0.8	0.6	1.2	0.27	0.8	0.5	1.2	0.26	0.9	0.6	1.3	
	GBS	113	43	19	51	0.9	0.8	1.2	0.57	0.7	0.5	0.9	0.01	1.1	0.8	1.3	
	EC	157	62	28	67	0.8	0.6	1.0	0.05	0.7	0.5	0.9	0.01	0.9	0.7	1.2	
	HI	141	56	22	63	0.9	0.7	1.1	0.25	0.6	0.4	0.9	0.01	1.0	0.7	1.3	
	LM	69	23	12	33	0.7	0.5	1.0	0.03	1.0	0.7	1.5	0.99	0.7	0.5	1.0	
	CA	86	32	15	39	0.8	0.5	1.2	0.28	0.8	0.4	1.3	0.34	0.9	0.6	1.4	
	MCP-1	BCG	129	51	19	59	1.0	0.8	1.3	0.98	0.7	0.5	1.1	0.11	1.1	0.8	1.4
MTBK		98	37	17	44	1.0	0.8	1.4	0.82	0.8	0.5	1.2	0.28	1.1	0.8	1.5	
LPS		147	54	26	67	0.9	0.6	1.3	0.45	0.7	0.4	1.0	0.06	1.0	0.7	1.5	
PEPG		147	53	26	67	0.8	0.6	1.0	0.04	0.8	0.5	1.1	0.12	0.9	0.7	1.1	
Pam3		128	49	21	58	0.9	0.7	1.1	0.36	0.9	0.6	1.2	0.37	0.9	0.7	1.2	
RS48		150	57	27	66	0.8	0.7	1.0	0.01	0.8	0.6	1.0	0.05	0.9	0.7	1.0	
SA		98	36	16	46	0.8	0.6	1.1	0.20	0.8	0.5	1.2	0.27	0.9	0.6	1.2	
SP		74	27	13	34	0.7	0.5	0.9	0.01	0.7	0.4	1.1	0.11	0.7	0.5	1.0	
GBS		113	43	19	51	0.9	0.7	1.1	0.28	0.7	0.5	1.1	0.09	1.0	0.7	1.2	
EC		157	62	28	67	0.8	0.6	1.0	0.03	0.7	0.5	1.0	0.06	0.9	0.7	1.1	
HI		141	56	22	63	0.8	0.7	1.0	0.07	0.8	0.6	1.2	0.30	0.9	0.7	1.1	
LM		69	23	12	33	0.7	0.5	0.9	0.01	0.8	0.5	1.2	0.29	0.7	0.5	1.0	

Cytokine	Stimulant	DS1 (n)	Dataset 1			p value	Dataset 2			p value	Dataset 3			p value		
			GMR	95%CI	LCI UCI		DS2 (n)	GMR	95%CI		LCI UCI	DS3 (n)	GMR		95%CI	LCI UCI
IL-1ra	BCG	129	1.1	0.7	1.7	0.62	123	1.3	0.8	2.1	0.26	121	1.0	0.8	1.4	0.72
	MTBK	98	1.0	0.7	1.6	0.85	96	1.2	0.8	2.0	0.39	94	1.4	1.0	1.9	0.06
	LPS	147	0.8	0.6	1.3	0.40	138	0.9	0.6	1.5	0.75	137	0.9	0.7	1.1	0.22
	PEPG	146	0.9	0.7	1.2	0.46	145	1.0	0.7	1.3	0.79	144	0.9	0.7	1.1	0.19
	Pam3	128	0.8	0.6	1.2	0.32	121	0.9	0.6	1.4	0.73	117	1.0	0.8	1.3	0.89
	R848	150	0.8	0.6	1.0	0.06	149	0.8	0.6	1.0	0.11	149	0.8	0.6	0.9	0.01
	SA	98	0.8	0.5	1.2	0.19	92	0.8	0.5	1.4	0.47	91	0.9	0.7	1.2	0.35
	SP	74	0.7	0.5	1.1	0.09	72	0.8	0.5	1.3	0.39	72	0.8	0.6	1.1	0.15
	GBS	113	0.9	0.6	1.2	0.37	110	0.9	0.6	1.4	0.71	108	0.9	0.7	1.2	0.40
	EC	157	0.8	0.6	1.1	0.13	155	0.9	0.7	1.2	0.41	154	0.9	0.7	1.1	0.17
	HI	141	0.8	0.6	1.1	0.25	137	0.9	0.6	1.3	0.56	136	0.9	0.7	1.1	0.43
	LM	69	0.5	0.3	0.9	0.01	63	0.6	0.3	1.2	0.16	62	0.7	0.5	1.0	0.04
	CA	86	0.7	0.4	1.1	0.10	76	0.8	0.4	1.6	0.56	73	1.0	0.7	1.3	0.79
	IL-10	BCG	129	1.0	0.7	1.3	0.77	128	0.9	0.7	1.3	0.72	127	0.8	0.6	1.1
MTBK		98	1.3	0.7	2.5	0.44	93	1.3	0.7	2.5	0.44	89	0.9	0.6	1.3	0.63
LPS		147	1.1	0.7	1.9	0.62	143	1.1	0.7	1.8	0.67	141	0.9	0.7	1.3	0.72
PEPG		146	0.8	0.7	1.0	0.09	147	0.8	0.7	1.0	0.07	145	0.8	0.7	1.0	0.08
Pam3		128	0.8	0.5	1.4	0.49	128	0.8	0.5	1.3	0.41	100	0.9	0.6	1.2	0.40
R848		150	0.7	0.6	1.0	0.02	150	0.7	0.6	0.9	0.02	150	0.7	0.6	1.0	0.02
SA		98	0.9	0.6	1.3	0.61	98	0.9	0.6	1.3	0.56	93	0.9	0.7	1.2	0.40
SP		74	1.1	0.8	1.5	0.62	74	1.1	0.8	1.5	0.60	74	1.1	0.8	1.5	0.60
GBS		113	1.2	0.9	1.6	0.18	113	1.2	0.9	1.6	0.24	113	1.2	0.9	1.6	0.24
EC		157	0.9	0.7	1.1	0.23	157	0.9	0.7	1.1	0.18	157	0.9	0.7	1.1	0.26
HI		141	0.9	0.7	1.1	0.31	141	0.9	0.7	1.1	0.24	141	0.9	0.7	1.1	0.21
LM		69	0.9	0.6	1.3	0.47	69	0.9	0.6	1.3	0.47	57	0.8	0.5	1.2	0.24
CA		86	1.2	0.5	2.9	0.64	71	1.2	0.5	2.7	0.73	55	1.1	0.7	1.8	0.71
MIG		BCG	129	0.9	0.8	1.0	0.14	129	0.8	0.7	1.1	0.15	126	0.8	0.6	1.0
	MTBK	98	0.8	0.7	1.0	0.06	98	0.8	0.6	1.1	0.23	95	0.9	0.6	1.1	0.25
	LPS	147	1.1	0.9	1.3	0.50	147	0.9	0.7	1.2	0.63	146	1.0	0.8	1.2	0.79
	PEPG	146	0.9	0.7	1.1	0.17	146	0.8	0.6	1.0	0.05	146	0.8	0.6	1.0	0.05
	Pam3	128	1.0	0.9	1.1	0.81	127	0.9	0.7	1.1	0.17	126	0.9	0.8	1.2	0.65
	R848	150	0.9	0.7	1.3	0.70	150	0.9	0.6	1.2	0.43	150	0.9	0.7	1.2	0.47
	SA	98	1.0	0.8	1.2	0.99	98	0.9	0.7	1.2	0.50	98	0.9	0.7	1.2	0.50
	SP	74	1.1	0.8	1.4	0.71	74	1.0	0.7	1.5	0.84	74	1.0	0.7	1.5	0.84
	GBS	113	1.1	0.8	1.4	0.63	113	1.0	0.7	1.3	0.83	113	1.0	0.7	1.3	0.83
	EC	157	1.0	0.9	1.1	0.94	157	0.9	0.7	1.1	0.18	157	0.9	0.7	1.1	0.34
	HI	141	0.9	0.8	1.1	0.45	141	0.8	0.7	1.0	0.09	141	0.8	0.7	1.0	0.10
	LM	69	0.9	0.7	1.1	0.19	69	0.8	0.6	1.2	0.31	69	0.8	0.6	1.2	0.31
	CA	86	0.9	0.8	1.1	0.34	86	0.8	0.6	1.1	0.24	85	0.9	0.7	1.1	0.31
	MIP-1α	BCG	129	0.9	0.7	1.1	0.28	129	0.9	0.7	1.1	0.29	118	0.9	0.7	1.1
MTBK		98	1.1	0.6	1.9	0.76	98	1.1	0.6	1.9	0.70	96	1.2	0.7	2.1	0.44
LPS		147	0.9	0.6	1.4	0.75	147	1.0	0.6	1.5	0.86	135	1.1	0.7	1.7	0.65
PEPG		146	0.7	0.5	0.9	0.01	147	0.7	0.5	0.9	0.01	124	0.8	0.6	1.0	0.06
Pam3		128	1.2	0.7	2.1	0.57	128	1.2	0.7	2.1	0.57	127	1.2	0.7	2.2	0.44
R848		150	0.7	0.5	1.0	0.06	150	0.8	0.6	1.1	0.13	126	0.8	0.6	1.1	0.13
SA		98	0.7	0.4	1.1	0.15	98	0.7	0.4	1.1	0.15	96	0.7	0.4	1.0	0.05
SP		74	0.8	0.6	1.2	0.27	74	0.8	0.6	1.2	0.28	71	0.9	0.7	1.2	0.49
GBS		113	1.1	0.7	1.6	0.73	113	1.1	0.7	1.6	0.65	111	1.1	0.8	1.6	0.63
EC		157	0.9	0.7	1.2	0.50	157	0.9	0.7	1.2	0.48	143	0.9	0.7	1.2	0.61
HI		141	0.9	0.6	1.1	0.25	141	0.9	0.6	1.1	0.24	113	0.9	0.7	1.2	0.50
LM		69	0.8	0.4	1.4	0.38	69	0.8	0.4	1.4	0.39	69	0.8	0.4	1.4	0.39
CA		86	0.7	0.3	1.5	0.38	86	0.7	0.3	1.5	0.36	85	0.8	0.4	1.6	0.53
MIP-1β		BCG	129	0.9	0.7	1.1	0.23	129	0.9	0.7	1.1	0.23	114	0.9	0.8	1.1
	MTBK	98	1.1	0.8	1.4	0.55	98	1.1	0.8	1.4	0.54	94	1.1	0.8	1.4	0.69
	LPS	147	0.8	0.5	1.1	0.14	147	0.7	0.5	1.1	0.11	100	0.9	0.5	1.4	0.52
	PEPG	146	0.7	0.6	0.8	0.00	147	0.7	0.6	0.8	0.00	120	0.8	0.6	0.9	0.01
	Pam3	128	1.0	0.8	1.3	0.96	128	1.0	0.7	1.3	0.81	127	1.0	0.7	1.3	0.84
	R848	150	0.7	0.5	1.0	0.03	150	0.7	0.5	1.0	0.02	95	0.8	0.6	1.1	0.10
	SA	98	0.8	0.7	1.1	0.14	98	0.8	0.6	1.0	0.08	98	0.8	0.6	1.0	0.08
	SP	74	0.8	0.6	1.0	0.07	74	0.8	0.6	1.0	0.11	68	0.9	0.7	1.1	0.19
	GBS	113	0.9	0.8	1.2	0.57	113	0.9	0.7	1.1	0.42	109	0.9	0.8	1.2	0.62
	EC	157	0.8	0.6	1.0	0.05	157	0.8	0.6	1.0	0.05	93	1.0	0.7	1.3	0.72
	HI	141	0.9	0.7	1.1	0.25	141	0.8	0.6	1.1	0.21	101	0.9	0.7	1.1	0.25
	LM	69	0.7	0.5	1.0	0.03	69	0.7	0.5	1.0	0.07	69	0.7	0.5	1.0	0.07
	CA	86	0.8	0.5	1.2	0.28	86	0.8	0.5	1.2	0.19	86	0.8	0.5	1.2	0.19
	MCP-1	BCG	129	1.0	0.8	1.3	0.98	129	1.0	0.8	1.3	0.96	129	1.0	0.8	1.3
MTBK		98	1.0	0.8	1.4	0.82	98	1.1	0.8	1.4	0.67	98	1.1	0.8	1.4	0.67
LPS		146	0.9	0.6	1.3	0.45	147	0.9	0.6	1.3	0.53	147	0.9	0.6	1.3	0.52
PEPG		147	0.8	0.6	1.0	0.04	147	0.8	0.6	1.0	0.04	146	0.8	0.7	1.0	0.07
Pam3		128	0.9	0.7	1.1	0.36	128	0.9	0.7	1.2	0.46	127	1.0	0.7	1.2	0.77
R848		150	0.8	0.7	1.0	0.01	150	0.8	0.7	1.0	0.02	147	0.8	0.7	1.0	0.05
SA		98	0.8	0.6	1.1	0.20	98	0.9	0.6	1.2	0.39	97	0.9	0.6	1.2	0.33
SP		74	0.7	0.5	0.9	0.01	74	0.7	0.5	1.0	0.04	73	0.7	0.5	0.9	0.02
GBS		113	0.9	0.7	1.1	0.28	113	0.9	0.7	1.1	0.35	113	0.9	0.7	1.1	0.35
EC		157	0.8	0.6	1.0	0.03	157	0.8	0.6	1.0	0.04	157	0.8	0.7	1.0	0.06
HI		141	0.8	0.7	1.0	0.07	141	0.8	0.7	1.0	0.08	141	0.8	0.7	1.0	0.07
LM		69	0.7	0.5	0.9	0.01	69	0.8	0.6	1.1	0.15	69	0.8	0.6	1.1	0.15
CA		86	0.6	0.4	0.9	0.01	86	0.7	0.4	1.0	0.06	86	0.7	0.4	1.0	0.06
IL-8		BCG	129	1.0	0.9	1.3	0.70	129	1.0	0.9	1.3	0.70	129	1.0	0.9	1.3
	MTBK	98	1.2	0.9	1.7	0.27	98	1.2	0.9	1.8	0.24					

Supplementary data Table 2: Univariate analysis of the effect of BCG vaccination and maternal BCG vaccination on neonatal cytokine response to toll like receptor ligands and a selection of heterologous antigens. Total (n) refers to the number of stimulations included in the analysis. % In range refers to the % that fell within the detectable limit of the assay. Those outside were all below the LLD and were assigned a value. GMR= Geometric Means Ratio. Statistically significant values ($p < 0.05$) are depicted in red. GMR > 1.0 indicates the effect was stronger in BCG vaccinated subjects and infants whose mothers' were BCG-vaccinated. BCG= Bacille Calmette Guerin, MTBK= Killed Mycobacterium Tuberculosis, LPS = Lipopolysaccharide, PEPG = peptidoglycan, PamCys3=(S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, R848= resiquimod, SA= *S. aureus*, SP= *S. pneumoniae*, GBS= Group B Streptococcus, EC= *E. coli*, HI= *H. influenzae*, LM= *L. monocytogenes*, CA= *C. albicans*.

Supplementary data Table 3: Interaction analyses looking for a differential effect on cytokine expression following BCG vaccination between (p-values) (i) boys and girls (Sex) (ii) infants born by vaginal delivery compared with infants born by Cesarean section (MOD=Mode of delivery) (iii) infants whose mothers are BCG vaccinated compared with infants whose mothers are non BCG vaccinated (Maternal BCG). Total (n) refers to the number of stimulations included in the analysis. Significant p-values are depicted in red ($p \leq 0.05$). BCG= Bacille Calmette Guerin, MTBK= Killed Mycobacterium Tuberculosis, LPS = Lipopolysaccharide, PEPG = peptidoglycan, PamCys3=(S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, R848= resiquimod, SA= *S. aureus*, SP= *S. pneumoniae*, EC= *E. coli*, HI= *H. influenzae*, LM= *L. monocytogenes*, CA= *C. albicans*, GBS= Group B Streptococcus.

Supplementary Figure 2: The relationship between the timing of administration of Hepatitis B and BCG vaccination.

Supplementary Figure 3: Relationship between timing of randomisation (early BCG = randomisation < 48 hours, late BCG= randomisation > 48 hours) and a) age at BCG vaccination, b) age at blood sample and c) time to blood sample.

Supplementary Table 4: Quantile regression of the effect of BCG vaccination on IFN- γ production following stimulation with heterologous bacteria and toll-like receptor ligands. The coefficient is the modelled difference in the medians between groups. BCG= Bacille Calmette Guerin, MTBK= Killed Mycobacterium Tuberculosis, GBS= Group B Streptococcus, LPS = Lipopolysaccharide, PEPG = peptidoglycan, PamCys3=(S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, R848= resiquimod,

Supplementary data Table 5: Univariate analysis of the effect of BCG vaccination based on the timing of randomisation. Early BCG = randomised < 48 hours of life, Late BCG vaccination > 48 hours of life on neonatal cytokine response to toll like receptor ligands and a selection of heterologous antigens. BCG= Bacille Calmette Guerin, MTBK= Killed Mycobacterium Tuberculosis, LPS = Lipopolysaccharide, PEPG = peptidoglycan, PamCys3=(S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-

Lys4OH, trihydrochloride, R848= resiquimod, SA= *S. aureus*, SP= *S. pneumoniae*, GBS= Group B Streptococcus, EC= *E. coli*, HI= *H. influenzae*, LM= *L. monocytogenes*, CA= *C. albicans*.

Supplementary data Table 6: Sensitivity analyses for the effect of BCG vaccination.

Dataset 1= Complete dataset. Dataset 2= Complete dataset following removal of values assigned at the lower limit of detection. Dataset 3= Complete dataset following removal of values assigned at the lower limit of detection and values extrapolated by the Luminex Xmap software at both the upper and lower ends of the curve. Total (n) refers to the number of stimulations included in the analysis. GMR= Geometric Means Ratio. Statistically significant values ($p < 0.05$) are depicted in red, those approaching significance ($p < 0.07$) are shown in orange to illustrate where trends are maintained. GMR > 1.0 indicates the effect was stronger in BCG vaccinated subjects.

3.5 CONCLUSION

This study shows that, compared to BCG-naïve neonates, BCG-vaccinated neonates have distinct heterologous cytokine responses which are modified by sex and maternal BCG status. By using an assay with a wide variety of clinically relevant pathogens and innate ligands, and by measuring multiple cytokines, a hypothetical model as to how BCG vaccine might result in improved neonatal outcomes during infection was generated (Figure 3.2). The large sample size made analysis of effect modifiers possible.

An increase in baseline levels of IL-6 following BCG vaccination might 'prime' the infant immune system to deal with subsequent infection. Following stimulation with heterologous pathogens and innate immune ligands, BCG-vaccinated infants had lower production of IL-6, IL-1Ra, IL-10, MCP-1, MIP-1 α and MIP-1 β than BCG-naïve infants. This may reflect effective 'training' of the immune system resulting in a more controlled inflammatory response during *in vitro* stimulation. Infants of BCG-vaccinated mothers, who themselves were BCG-vaccinated, had higher pro-inflammatory (IL-1 β , IFN- γ) responses than BCG-vaccinated infants whose mothers were unvaccinated. Females who were BCG-vaccinated had increased heterologous production of MIF compared to BCG-vaccinated boys. Figure 3.2 shows where these effects might modify the main BCG vaccine effect.

In the absence of clinical correlates of protection, this model requires further validation. These results indicate that BCG vaccine results in differential cytokine responses following signalling through Toll-like receptors at the cell-surface (1/2) and intracellularly (7/8) or possibly Rig-like receptors (RLR) and Nod-like receptors (NLR) all of which are stimulated by PEPG or R848. Furthermore, the effect of BCG vaccine on heterologous chemokine responses (MCP-1, MIP-1 α , MIP-1 β) suggests an effect on monocytes and dendritic cells. Future research should focus on these pathways and cell-types, which is reflected in the results presented in chapter 5.

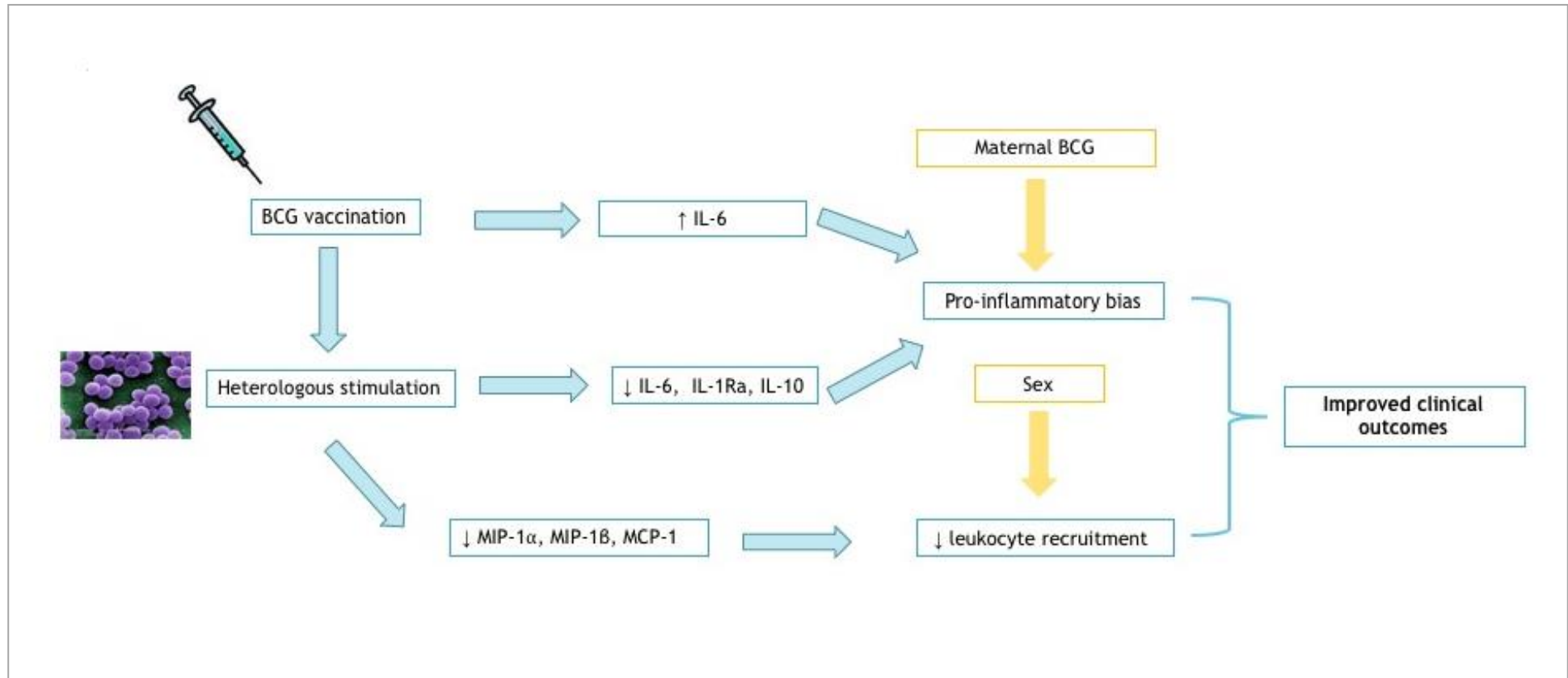


Figure 3.4 The effect of neonatal BCG vaccine on heterologous cytokine responses in 7-day old infants (MIS BAIR)

**Chapter 4 NEONATAL BCG VACCINATION REDUCES INTERFERON-
GAMMA RESPONSIVENESS TO HETEROLOGOUS PATHOGENS
IN INFANTS FROM A RANDOMISED CONTROLLED TRIAL**

4.1 INTRODUCTION

This chapter presents the analysis of cytokine production following stimulation with heterologous bacterial pathogens and innate immune ligands in infants 7 months post randomisation in the MIS BAIR study. Similar to the published work in chapter 3 these infants were randomised to either BCG vaccine or no BCG vaccine at birth. This manuscript has been submitted for peer review in the *Journal of Infectious Diseases*, and is included here as a 'publication in process'.

4.1.1 CHOICE OF TIME POINT, STIMULANT AND CYTOKINES

The 7-month time point was chosen for two reasons. Firstly, to assess the persistence of BCG-induced heterologous cytokine responses beyond the neonatal period. Secondly, to assess the nature of BCG-induced heterologous responses at a time point after administration of routine vaccinations at two and four months of age as per the Australian Immunisation schedule (hepatitis B, diphtheria-tetanus-acellular pertussis, *Haemophilus influenza* type B, Inactivated poliovirus (IPV), 13-valent conjugate pneumococcal vaccine and rotavirus. Group B streptococcus (*S. agalactiae*) was replaced with Group A streptococcus (*S. pyogenes*) in this study, as the former is rarely a pathogen outside the neonatal period. IP-10 (CXCL10) was added to the multiplex assay following the publication of new evidence that along with MIG (CXCL9), it is an important marker of trained immunity following BCG vaccination [190].

4.2 PUBLICATION IN PROGRESS RESULTS PAPER

Neonatal BCG vaccination reduces interferon gamma responsiveness to heterologous pathogens in infants from a randomised controlled trial

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Summary

Neonatal BCG vaccination results in a decreased proportion of infants mounting an IFN- γ response to heterologous stimulation at 7 months of age. Both sex and maternal BCG vaccination status influenced the effect of BCG vaccination on heterologous cytokine responses.

Abstract

Background. Bacille Calmette-Guérin (BCG) vaccination has beneficial non-specific (heterologous) effects that protect against non-mycobacterial infections. We have previously reported that BCG vaccination at birth alters *in vitro* cytokine responses to heterologous stimulants in the neonatal period. This study investigated heterologous responses in 167 infants in the same trial seven months after randomisation.

Methods. A whole blood assay was used to interrogate *in vitro* cytokine responses to heterologous stimulants (killed pathogens) and Toll-like receptor (TLR) ligands.

Results. Compared to BCG-naïve infants, BCG-vaccinated infants had increased production of MIG and IFN- γ in response to mycobacterial stimulation and decreased production of IFN- γ in response to heterologous stimulation. Reduced IFN- γ responses to heterologous stimulants and TLR ligands were attributable to a decrease in the proportion of infants who mounted a detectable IFN- γ response. BCG-vaccinated infants also had increased production of MIG and IL-8, and decreased production of IL-10, MIP-1 α and MIP-1 β , the pattern of which varied by stimulant. IL-1Ra responses following TLR1/2 (Pam3CYSK4) stimulation were increased in BCG-vaccinated infants. Both sex and maternal BCG vaccination status influenced the effect of neonatal BCG vaccination.

Conclusions. BCG vaccination leads to changes in IFN- γ responsiveness to heterologous stimulation. BCG-induced changes in other cytokine responses to heterologous stimulation varies by pathogen.

Introduction

Bacille Calmette-Guérin (BCG) vaccination at birth is associated with decreased mortality in infants [1, 2]. This is proposed to be due to heterologous ('non-specific') protection against early-life infections, an effect presumed to result from the immunomodulatory properties of BCG [3, 4]. The beneficial non-specific effects of BCG vaccine persist beyond the neonatal period. BCG vaccination is associated with reduced incidence of lower respiratory tract infections in childhood and sepsis in the first year of life [5, 6] and protection against eczema in infants with an atopic disposition [7]. Maternal BCG, delivery method and gestational age have been reported to modify the non-specific effects of BCG [8, 9].

The mechanisms underlying the beneficial heterologous effects of BCG vaccination remain unclear [10]. We have previously shown that *in vitro* cytokine responses to heterologous stimulants differs between BCG-vaccinated and BCG-naïve neonates [11]. The longer term heterologous immunological effects induced by neonatal BCG are poorly defined but are likely to be different. Maturational changes mean the immune response in infancy is different to that of neonates [12-14]. In addition, a central hypothesis in the literature on the non-specific effects of vaccination is that live vaccines confer protection against all-cause mortality, which is negated by subsequent non-live vaccines in the first six months of life [15]. In the current study, we report the effect of neonatal BCG vaccination on heterologous cytokine responses in infants from a randomised controlled trial at seven months of age.

Methods

Participants were a subset of infants recruited from The Melbourne Infant Study: BCG for the prevention of allergy and infection (MIS BAIR) in which neonates were randomised to vaccination with BCG-Denmark 0.05 mL intradermally or no BCG vaccination (Clinical trials registration NCT01906853). The inclusion and exclusion criteria for MIS BAIR are described elsewhere [16]. MIS BAIR participants were invited to attend a 7-month study visit to provide a blood sample. All participants with a blood sample from December 2014 to April 2016 were recruited to this sub-study. Inclusion criteria for cytokine analysis were: (i) sufficient blood for all stimulants or (ii) participant provided sample in previous study [11]. Exclusion criteria were: (i) fever in the previous 24 hours; (ii) chronic illness (iii) less than two doses of the routine scheduled vaccines; (iv) any vaccination within the last 7 days; and (iv) any blood products since birth (Figure 1).

Venous blood was collected and *in vitro* stimulation assays were done as described previously [17]. Briefly, whole blood, diluted 1:1 with RPMI-1640 medium (GlutaMAX™ Supplement, HEPES, Gibco, Life Technologies), was stimulated at 37°C (5% CO₂: air) for 20 (± 2) hours with RPMI alone (unstimulated control), BCG-Denmark (Serum Statens Institute, Denmark) 75 µg/mL, killed *Mycobacterium tuberculosis* 1.0 x 10⁶ CFU/mL, *Escherichia coli* 1.0 x 10⁶ CFU/mL, *Haemophilus influenzae* type B 1.0 x 10⁶ CFU/mL, *Staphylococcus aureus* 1.0 x 10⁷ CFU/mL, *Streptococcus pyogenes* (GAS) 1.0 x 10⁷ CFU/mL, *Streptococcus pneumoniae* serotype 15C (non-vaccine serotype) 1.0 x 10⁷ CFU/mL, *Listeria monocytogenes* 1.0 x 10⁷ CFU/mL, *Candida albicans* 1.0 x 10⁶ CFU/mL, and the Toll-like receptor (TLR) ligands lipopolysaccharide (LPS; TLR 4 agonist) 100 ng/mL, resiquimod (R848; TLR 7/8) 3.5 µg/mL, peptidoglycan (PEPG; TLR 2/4) 10 µg/mL and (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-

Lys4OH, trihydrochloride (Pam3CSK4; TLR 1/2) 1 µg/mL; all from InvivoGen. When insufficient blood was available for all stimulations, a pre-determined priority order was used. The following cytokines were measured in supernatants using an xMAP Luminex 200 Analyser as described previously: IFN-γ, TNF-α, IL-6, IL-1β, MIF, IL-8 (CXCL8), MIG (CXCL9), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), IL-10, IL-1Ra [17]. Study staff were blinded to the BCG-vaccination status of participants.

Statistical analysis was done using Stata version 13.1. Cytokine results below the lower limit of detection (LLD) were assigned a value of half the lowest detectable value. There were 14 values above the upper limit of detection (ULD); these were excluded from the analysis. Proportions of detectable values for each cytokine are shown in Supplementary Table 1.

Prior to analysis, all cytokine data were log transformed. Regression analyses, with the log transformed value of the unstimulated cytokine values as a covariate, was used to determine the effect of (i) BCG vaccination, (ii) maternal BCG vaccination status and (iii) infant's sex on cytokine production in response to each stimulant. This approach was used in all regression analyses to account for variability in unstimulated samples and avoids the need for nil subtraction. For cytokine/stimulant pairs with normally distributed data (Supplementary Figure 1), linear regression was used and results presented as geometric mean ratios (GMR) with 95% confidence intervals. For cytokine/stimulant pairs with non-normally distributed data (Supplementary Figure 1), quantile regression was used and results presented as differences in the medians with 95% confidence intervals.

Subgroup analysis (sex and maternal BCG vaccination status) was done when there was statistical evidence of an interaction between BCG vaccination and the subgroup variable, as

determined by interaction analysis (Supplementary Table 4). Given the smaller numbers of participants in sub-group analyses, quantile regression was used, with bootstrapping as required.

Multivariable analysis was done to assess the effect of sex, delivery method, age at infant BCG vaccination (less than or greater than 48 hours of life), maternal BCG vaccination status, age at blood sample and the number of doses of routine vaccinations received.

Multivariable analyses, with the unstimulated values as a covariate, were done using linear or quantile regression as per the corresponding cytokine/stimulant pair in the primary univariate analysis.

For cytokines that showed a dichotomous response, participants were classified according to whether cytokine concentrations in the stimulated supernatants were above ('responders') or below ('non-responders') the LLD. Fisher's exact test was used to compare the proportion of 'responders' between BCG-vaccinated and BCG-naïve participants. To assess the contribution of this observation to the results of the primary analysis, a sensitivity analyses was done for the effect of BCG vaccination when 'non-responders' were excluded.

The study was approved by the research ethics committees of the Royal Children's Hospital (HREC 33025) and the Mercy Hospital for Women (HREC R12/28).

Results

Demographic features of participants were similar between BCG-vaccinated and BCG-naïve participants (Table 1). In unstimulated samples, BCG-vaccinated infants had lower overall cytokine concentrations (particularly IL-10, MIF, IP-10 and IL-8) compared with BCG-naïve

infants (Table 2). In response to all stimulants, there was marked inter-individual variability in cytokine production (Supplementary Figure 1). For IFN- γ , IP-10, IL-1Ra and IL-1 β , there was evidence of dichotomous responses, i.e. participants who did or did not mount a measurable cytokine response following stimulation.

In response to the 'specific' mycobacterial antigens BCG and *M. tuberculosis*, BCG-vaccinated infants had higher production of IFN- γ and MIG compared to BCG-naïve infants, and also higher TNF- α and IL-6 following stimulation with BCG (Figure 2, Supplementary Table 1). In response to heterologous stimulation with the Gram-positive bacteria *S. pyogenes* and *S. aureus*, BCG-vaccinated infants had differential production of chemokines including MIF, MIG and MIP-1 β . Chemokine production in response to stimulation with the intracellular pathogens (*L. monocytogenes* and *C. albicans*) was also altered in BCG-vaccinated infants (Figure 2, Supplementary Table 1). In response to stimulation with the Gram-negative bacteria *E. coli* and *H. influenzae*, and the TLR agonist LPS, BCG-vaccinated infants had decreased IFN- γ responses and increased IL-8 response compared to BCG-naïve infants. In response to stimulation with TLR agonists Pam3CYSK4 (TLR1/2) and PEPG (TLR2), BCG-vaccinated infants had increased IL-1Ra production and decreased IFN- γ production respectively (Figure 2, Supplementary Table 1).

There was a dichotomous IFN- γ response to all stimulants aside from R848, with a distinct separation between 'responders' and 'non-responders'. For the 'specific' mycobacterial antigens, BCG and *M. tuberculosis*, there was a higher proportion of IFN- γ responders in the BCG-vaccinated group compared to the BCG-naïve group (Figure 3, Supplementary Table 2). Conversely, for the heterologous stimulants, the proportion of IFN- γ responders was significantly lower in BCG-vaccinated infants for all stimulants with the exception of *S.*

pneumoniae (Figure 3, Supplementary Table 2). IP-10, IL-1Ra and IL-1 β responses showed a similar pattern; however, differences in the proportion of responders were not statistically significant between BCG-vaccinated and BCG-naïve infants (Supplementary Table 2).

A sensitivity analysis done after removal of all non-responders showed no significant difference in IFN- γ responses to heterologous stimulants between BCG-vaccinated and BCG-naïve infants, revealing that the observed difference in IFN- γ GMRs was attributable to the change in proportion of ‘responders’ (Supplementary Table 3). In contrast, the difference in the specific IFN- γ response to *in vitro* BCG stimulation between BCG-vaccinated and BCG-naïve infants was still observed.

Infants’ sex also independently influenced cytokine responses. Compared with girls, boys had increased production of both MCP-1 and MIP-1 β in response to almost all stimulants (Supplementary Table 1 & Supplementary Figure 2). Boys also had increased production of IFN- γ in response to stimulation with BCG, *E. coli*, *H. influenzae* and PEPG, and TNF- α in response to most stimulants, particularly *E. coli*, *H. influenzae*, LPS and R848 (Supplementary Table 1 & Supplementary Figure 2). MIG production was also higher in boys following stimulation with *E. coli*. Conversely, boys had decreased production of IL-1Ra in response to *C. albicans* and IL-1 β in response to *L. monocytogenes*, and in response to Pam3CYSK4 (Supplementary Table 1 & Supplementary Figure 2).

A significant interaction between sex and infant BCG vaccination was seen for multiple cytokine/stimulant pairs but most consistently for production of IL-6 and IL-1 β (Supplementary Table 4). Subgroup analyses of the effect of BCG-vaccination in boys and girls separately showed that, compared to BCG-naïve girls, BCG-vaccinated girls had

increased pro-inflammatory cytokine production: (a) IL-6 in response to *E. coli*, *H. influenzae*, LPS and R848 and (b) IL-1 β in response to *S. pneumoniae* (Figure 4, panel a and b), and an increase in the anti-inflammatory cytokine IL-1Ra in response to Pam3CYSK4 (Figure 4, panel c). Overall, compared to BCG-naïve boys, BCG-vaccinated boys had lower cytokine production for all cytokine/stimulant pairs when there was an interaction between infant BCG vaccination and sex. This was statistically significant for IL-1 β /*E. coli*, IFN- γ /Pam3CYSK4 and TNF- α /*C. albicans* responses (Figure 4, panel a-c).

Maternal BCG vaccination status independently influenced cytokine responses to heterologous stimulants and TLR ligands. Compared to infants of mothers who had not had BCG vaccination, infants of mothers who were BCG-vaccinated had increased IFN- γ production in response to stimulation with *M. tuberculosis*, decreased IFN- γ in response to PEPG (TLR2/4); decreased TNF- α in response to BCG, *E. coli*, *H. influenzae*, PEPG (TLR2/4), LPS (TLR4) and R848 (TLR7/8); decreased IL-1Ra in response to *H. influenzae*; decreased MCP-1 in response to *S. pneumoniae* and *S. pyogenes*, decreased IP-10 in response to *L. monocytogenes*, decreased MIP-1 β in response to *E. coli* and *S. pneumoniae* (Supplementary Table 1 and Supplementary Figure 3).

A significant interaction between maternal BCG vaccination and infant BCG vaccination was seen for MIG/*E. coli*, IL-10/BCG and IFN- γ /*S. pneumoniae* cytokine/stimulant pairs (Supplementary Table 4). Separate analyses of the effect of BCG in infants of BCG-vaccinated mothers and infants of BCG-naïve mothers showed that in the former there was lower production of MIG in response to *E. coli* and IFN- γ in response to *S. pneumoniae*, whereas, BCG vaccination had no effect on this response in infants of BCG-naïve mothers

(Figure 4, panel d). Conversely, infants of BCG-vaccinated mothers had higher production of IL-10 in response to stimulation with BCG (Figure 4, panel d).

Multivariable analyses were done to evaluate the potential confounding effect of sex, maternal BCG vaccination status, mode of delivery, age at BCG vaccination, age at blood draw, and number of routine vaccination doses received (Supplementary Table 5). Covariate analysis with these variables did not reveal any meaningful effect on the primary analysis, thus excluding confounding by these variables.

Discussion

We previously reported that BCG vaccination at birth led to decreased production of IL-1 α , IL-6, MCP-1, MIP-1 α and MIP-1 β in response to heterologous stimulants and TLR ligands in the neonatal period (7 days post after randomisation) [17]. This study extends these findings to show that BCG-induced changes in cytokine expression following heterologous stimulation are detectable at 7 months of age, characterised by decreased IFN- γ responsiveness in BCG-vaccinated infants.

Our finding that BCG vaccination was associated with an increase in IFN- γ and pro-inflammatory cytokines (IL-6, IL-1 β and TNF- α) in response to mycobacterial antigens (BCG and *M. tuberculosis*) is consistent with prior studies [13, 14, 18-20]. Furthermore, our finding that two-thirds of BCG-naïve infants produced IFN- γ in response to *in vitro* stimulation with BCG supports our previous finding of a role for NK cells and unconventional T-cells in early IFN- γ responses to BCG [21]. Our main finding that neonatal BCG vaccination is associated with a decrease in the proportion of infants who produce IFN- γ in response to heterologous stimulants has not previously been reported.

Nissen *et al.* reported that, in Danish infants at 12 months of age, BCG vaccination did not lead to differential IFN- γ production either in response to *ex vivo* heterologous bacterial (*E. coli*, *S. pneumoniae*, *C. albicans*) or LPS stimulation [22]. However, in that study approximately half of the BCG-vaccinated participants did not have a specific IFN- γ response to *in vitro* BCG stimulation. Smith *et al.* also assessed the effects of BCG on *in vitro* heterologous cytokine responses in infants, although BCG was given at 6 weeks of age [23]. As in our study, BCG-vaccinated infants had an increase in *M. tuberculosis* lysate-induced IFN- γ response. Although there was no significant difference between BCG-vaccinated and BCG-naïve infants in IFN- γ production following *E. coli*, *S. aureus*, *C. albicans*, LPS or Pam3CYSK4 stimulation, fold-change analysis showed decreased LPS-induced IFN- γ responses in BCG-vaccinated infants, an effect which did not reach statistical significance given the small sample size.

Compared to BCG-naïve infants, BCG-vaccinated infants in our study showed a distinctive pattern of differential chemokine production which varied between biologically-related stimulants. Specifically, in response to mycobacterial, Gram-positive and intracellular heterologous stimulants, chemokine production in BCG-vaccinated infants was characterised by increased production of MIG and a concomitant decrease in MIP-1 α and MIP-1 β . MIG is produced by activated monocytes and is the primary chemoattractant for T-helper type 1 cells, which are central to the IFN- γ -dependent pro-inflammatory response to intracellular bacteria and parasites, and has been recently reported to represent a marker of trained immunity after BCG vaccination [24]. In response to Gram-negative bacteria and LPS, BCG-vaccinated infants had increased IL-8 production. IL-8 is central in the innate response to bacterial infection by promoting neutrophil chemotaxis and macrophage activation. Smith *et*

al reported that, as in our study, BCG-vaccinated infants had significantly higher IL-8 production following stimulation with LPS compared to BCG-naïve infants [23]. It remains to be seen whether variable ‘pathogen-specific’ *in vitro* patterns of innate immune response following BCG-vaccination correlate with clinical protection.

For the majority of heterologous stimulants, IFN- γ responses in our study were characterised by a significantly lower proportion of IFN- γ responders in the BCG-vaccinated group, which was responsible for the lower IFN- γ GMR in these infants compared to BCG-naïve infants. Variable production of IFN- γ between individuals and between populations is well described [13, 20, 25]. This variability in individual IFN- γ responsiveness (which may be genetically or environmentally determined [26, 27]) is a potential modifier of the response to BCG. In a group of BCG-naïve, TST negative, adult volunteers in the Netherlands who received BCG vaccination, ‘non-responders’ were classified as individuals with no IFN- γ response to PPD and expression of CD8⁺ regulatory T-cells which persisted up to one year post-vaccination [28]. Fletcher *et al.* reported differential immune responses following BCG vaccination in infants characterised by differences in monocyte:T-cell ratios and alternatively activated macrophage phenotype (M2) [29]. More recently, Walk *et al* reported two distinct patterns of immune response to human malaria challenge in BCG-vaccinated volunteers. A subgroup of volunteers with strong monocyte and lymphocyte activation were also characterised by increased IFN- γ , granzyme-B production and CRP production [30]. This study is the first to report significant changes in IFN- γ responsiveness in the context of BCG-induced heterologous immunity in infants.

IP-10, IL-1Ra and IL-1 β were the only other cytokines which had a subgroup of non-responders. This is notable in light of the functional relationship of these cytokines to IFN- γ

and their role in the control of inflammation and pathogen persistence following mycobacterial infection [31, 32]. The IL-1 signalling axis is central in mediating the response to BCG vaccination in the setting of both mycobacterial and heterologous infections, and IL-1 β is a crucial component for the induction of trained immunity[24, 33-35]. However, in our study the proportion of IL-1Ra and IL-1 β responders was not significantly different between BCG-vaccinated and BCG-naïve infants. Moreover, sensitivity analysis confirmed that increased IL-1Ra responses following stimulation of TLR 1/2 (Pam3CYSK4) seen in BCG-vaccinated infants was unaffected by removal of ‘non-responders’ from the analysis.

In our previous study, we found that infant sex influenced the effect of BCG vaccination on the production of MIF following heterologous stimulation [17]. In the present study, we observed enhanced *E. coli*, *H. influenzae*, LPS and R848-induced production of the macrophage derived pro-inflammatory cytokines IL-6 and IL-1 β in BCG-vaccinated girls (in contrast to boys). This is interesting in light of the suggestion that beyond the neonatal period, the beneficial effects of BCG vaccination on mortality is stronger in girls [36].

Maternal BCG vaccination status is associated with increased BCG scar formation and decreased all-cause mortality in BCG-vaccinated infants [8]. Mawa *et al.* reported altered cytokine responses to TLR ligands, including LPS, in the cord blood of infants of BCG-vaccinated mothers [37]. Our finding, that infants of BCG-vaccinated mothers had altered cytokine production following heterologous stimulation, independent of infant BCG vaccination status, raises the possibility that maternal BCG vaccination ‘primes’ the response to infant BCG vaccination. Further, an interaction between maternal and infant BCG vaccination on IFN- γ production in response to *S. pneumoniae* was also found in our previous study; however maternal BCG had the opposite effect at this time point [17]. This finding is

particularly intriguing in light of the finding that BCG vaccine protects infants against respiratory infections only in infants whose mothers were also BCG-vaccinated [9, 38].

There is longstanding interest in the variability of BCG vaccine efficacy against tuberculosis infection in different geographic locations. To date, clinically significant heterologous effects of BCG have mainly been documented in African populations [36]. Following BCG vaccination, infant cytokine responses to mycobacteria differ between African and European populations, however it remains uncertain whether this is due to genetic or environmental influences [13, 14, 39]. In this study, the majority of participants were Caucasian precluding subgroup analysis by ethnicity. Exposure to environmental mycobacteria and chronic helminth infection has been associated with altered BCG-induced IFN- γ production in some populations [39]. It is unlikely that exposure to infection was a confounder in our study as proxy measures of exposure to common childhood infections (attendance at childcare and number of children under five years in the household) were similar between groups.

The strengths of this study include its large sample size and the use of samples from participants from a randomised controlled trial. The use of a large number of stimulants and multiplex cytokine analysis enabled a comprehensive interrogation of the immune response. In addition, we used multivariable analysis to identify potential confounders and a highly standardised laboratory protocol to reduce variation. Furthermore, we used the unstimulated sample as a co-variate to control for inter-individual variability in cytokine responses. A sensitivity analysis, excluding the use of unstimulated samples as a co-variate in samples where there was an independent effect on the constitutive expression of a cytokine, showed this did not lead to overcorrection (data not shown). Our study has several limitations which are common to research of this type. Firstly, for some cytokine/stimulant pairs, there were a

lower numbers of participants from whom samples were available. However, this is a large study and our statistical analysis accounted for the varying distributions of individual cytokine/stimulant pair data. Secondly, while we tested for several potential confounders selected due to their biological plausibility and previously published data, it is possible there are others that may have influenced our findings. Thirdly, there was a risk of type 1 error due to multiple comparisons. Statistical correction for multiple comparisons was not done as this was an exploratory analysis that aimed to find consistent patterns in BCG-induced alterations in cytokine responses. In addition, we have shown all analyses in their entirety to aid interpretation of the results in context. Finally, routine vaccines from the Australian immunisation schedule differ to those administered in low and middle-income countries.

In summary, at 7 months of age, following neonatal BCG vaccination, infants had lower IFN- γ responses to heterologous stimulants and TLR ligands compared with BCG-naïve infants. This results from a reduction in the proportion of infants who responded by producing IFN- γ rather than a reduced level of IFN- γ production by all participants. This study is the first to report divergent IFN- γ responses between mycobacterial and heterologous stimulation following BCG-vaccination. BCG-vaccination is associated with variable patterns of chemokine response depending on the stimulating pathogen. There was a significant interaction between both sex and maternal BCG vaccination and neonatal BCG vaccination. Relating the immunological effects of BCG observed in *in vitro* studies with clinical outcome should be a priority for future research.

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Conflict of interest

The authors state that they have no conflict of interest.

References

1. Higgins JP, Soares-Weiser K, Lopez-Lopez JA, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. *BMJ* **2016**; 355:i5170.
2. Pollard AJ, Finn A, Curtis N. Non-specific effects of vaccines: plausible and potentially important, but implications uncertain. *Archives of disease in childhood* **2017**; 102:1077-81.
3. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis* **2011**; 204:245-52.
4. Biering-Sorensen S, Aaby P, Napirna BM, et al. Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. *Pediatr Infect Dis J* **2012**; 31:306-8.
5. Hollm-Delgado MG, Stuart EA, Black RE. Acute lower respiratory infection among Bacille Calmette-Guerin (BCG)-vaccinated children. *Pediatrics* **2014**; 133:e73-81.
6. de Castro MJ, Pardo-Seco J, Martinon-Torres F. Nonspecific (Heterologous) Protection of Neonatal BCG Vaccination Against Hospitalization Due to Respiratory Infection and Sepsis. *Clin Infect Dis* **2015**; 60:1611-9.
7. Thostesen LM, Kjaergaard J, Pihl GT, et al. Neonatal BCG vaccination and atopic dermatitis before 13 months of age: A randomized clinical trial. *Allergy* **2018**; 73:498-504.
8. Berendsen MLT, Oland CB, Bles P, et al. Maternal Priming: Bacillus Calmette-Guerin (BCG) Vaccine Scarring in Mothers Enhances the Survival of Their Child With a BCG Vaccine Scar. *J Pediatric Infect Dis Soc* **2019**.
9. Kjaergaard J, Birk NM, Nissen TN, et al. Nonspecific effect of BCG vaccination at birth on early childhood infections: a randomized, clinical multicenter trial. *Pediatr Res* **2016**; 80:681-5.
10. Kandasamy R, Voysey M, McQuaid F, et al. Non-specific immunological effects of selected routine childhood immunisations: systematic review. *BMJ* **2016**; 355:i5225.
11. Freyne B, Donath S, Germano S, et al. Neonatal BCG Vaccination Influences Cytokine Responses to Toll-like Receptor Ligands and Heterologous Antigens. *J Infect Dis* **2018**; 217:1798-808.
12. Kleinnijenhuis J, Quintin J, Preijers F, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J Innate Immun* **2014**; 6:152-8.
13. Lalor MK, Floyd S, Gorak-Stolinska P, et al. BCG vaccination induces different cytokine profiles following infant BCG vaccination in the UK and Malawi. *J Infect Dis* **2011**; 204:1075-85.
14. Lalor MK, Smith SG, Floyd S, et al. Complex cytokine profiles induced by BCG vaccination in UK infants. *Vaccine* **2010**; 28:1635-41.
15. Flanagan KL, van Crevel R, Curtis N, Shann F, Levy O. Heterologous ("nonspecific") and sex-differential effects of vaccines: epidemiology, clinical trials, and emerging immunologic mechanisms. *Clin Infect Dis* **2013**; 57:283-9.
16. Messina NL, Gardiner K, Donath S, et al. Study protocol for the Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR), a randomised controlled trial to determine the non-specific effects of neonatal BCG vaccination in a low-mortality setting. *BMJ Open* **2019**; 9:e032844. doi: 10.1136/bmjopen-2019-.
17. Freyne B, Donath S, Germano S, et al. Neonatal BCG vaccination influences cytokine responses to Toll-like receptor ligands and heterologous antigens. *J Infect Dis* **2018**.

18. Burl S, Adetifa UJ, Cox M, et al. Delaying bacillus Calmette-Guerin vaccination from birth to 4 1/2 months of age reduces postvaccination Th1 and IL-17 responses but leads to comparable mycobacterial responses at 9 months of age. *J Immunol* **2010**; 185:2620-8.
19. Corral-Fernandez NE, Cortez-Espinosa N, Salgado-Bustamante M, et al. Induction of transcription factors, miRNAs and cytokines involved in T lymphocyte differentiation in BCG-vaccinated subjects. *Mol Immunol* **2016**; 77:44-51.
20. van den Biggelaar AH, Prescott SL, Roponen M, et al. Neonatal innate cytokine responses to BCG controlling T-cell development vary between populations. *J Allergy Clin Immunol* **2009**; 124:544-50, 50.e1-2.
21. Zufferey C, Germano S, Dutta B, Ritz N, Curtis N. The contribution of non-conventional T cells and NK cells in the mycobacterial-specific IFN γ response in Bacille Calmette-Guerin (BCG)-immunized infants. *PLoS One* **2013**; 8:e77334.
22. Nissen TN, Birk NM, Blok BA, et al. Bacillus Calmette-Guerin vaccination at birth and in vitro cytokine responses to non-specific stimulation. A randomized clinical trial. *Eur J Clin Microbiol Infect Dis* **2018**; 37:29-41.
23. Smith SG, Kleinnijenhuis J, Netea MG, Dockrell HM. Whole Blood Profiling of Bacillus Calmette-Guerin-Induced Trained Innate Immunity in Infants Identifies Epidermal Growth Factor, IL-6, Platelet-Derived Growth Factor-AB/BB, and Natural Killer Cell Activation. *Front Immunol* **2017**; 8:644.
24. Joosten SA, van Meijgaarden KE, Arend SM, et al. Mycobacterial growth inhibition is associated with trained innate immunity. *J Clin Invest* **2018**; 128:1837-51.
25. Djuardi Y, Sartono E, Wibowo H, Supali T, Yazdanbakhsh M. A longitudinal study of BCG vaccination in early childhood: the development of innate and adaptive immune responses. *PLoS One* **2010**; 5:e14066.
26. Finan C, Ota MO, Marchant A, Newport MJ. Natural variation in immune responses to neonatal *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) Vaccination in a Cohort of Gambian infants. *PLoS One* **2008**; 3:e3485.
27. Newport MJ, Goetghebuer T, Weiss HA, Whittle H, Siegrist CA, Marchant A. Genetic regulation of immune responses to vaccines in early life. *Genes Immun* **2004**; 5:122-9.
28. Boer MC, Prins C, van Meijgaarden KE, van Dissel JT, Ottenhoff TH, Joosten SA. *Mycobacterium bovis* BCG Vaccination Induces Divergent Proinflammatory or Regulatory T Cell Responses in Adults. *Clin Vaccine Immunol* **2015**; 22:778-88.
29. Fletcher HA, Filali-Mouhim A, Nemes E, et al. Human newborn bacille Calmette-Guerin vaccination and risk of tuberculosis disease: a case-control study. *BMC Med* **2016**; 14:76.
30. Walk J, de Bree LCJ, Graumans W, et al. Outcomes of controlled human malaria infection after BCG vaccination. *Nat Commun* **2019**; 10:874.
31. Bohrer AC, Tocheny C, Assmann M, Ganusov VV, Mayer-Barber KD. Cutting Edge: IL-1R1 Mediates Host Resistance to *Mycobacterium tuberculosis* by Trans-Protection of Infected Cells. *J Immunol* **2018**; 201:1645-50.
32. Mayer-Barber KD, Andrade BB, Oland SD, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* **2014**; 511:99-103.
33. Arts RJW, Moorlag S, Novakovic B, et al. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host Microbe* **2018**; 23:89-100.e5.
34. Koeken V, Verrall AJ, Netea MG, Hill PC, van Crevel R. Trained innate immunity and resistance to *Mycobacterium tuberculosis* infection. *Clin Microbiol Infect* **2019**.
35. Moorlag S, Roring RJ, Joosten LAB, Netea MG. The role of the interleukin-1 family in trained immunity. *Immunol Rev* **2018**; 281:28-39.

36. Biering-Sorensen S, Jensen KJ, Monterio I, Ravn H, Aaby P, Benn CS. Rapid Protective Effects of Early BCG on Neonatal Mortality Among Low Birth Weight Boys: Observations From Randomized Trials. *J Infect Dis* **2018**; 217:759-66.
37. Mawa PA, Webb EL, Filali-Mouhim A, et al. Maternal BCG scar is associated with increased infant proinflammatory immune responses. *Vaccine* **2017**; 35:273-82.
38. Stensballe LG, Ravn H, Birk NM, et al. BCG Vaccination at Birth and Rate of Hospitalization for Infection Until 15 Months of Age in Danish Children: A Randomized Clinical Multicenter Trial. *Journal of the Pediatric Infectious Diseases Society* **2018**.
39. Weir RE, Black GF, Nazareth B, et al. The influence of previous exposure to environmental mycobacteria on the interferon-gamma response to bacille Calmette-Guerin vaccination in southern England and northern Malawi. *Clin Exp Immunol* **2006**; 146:390-9.

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Table & Figure Legends

Table 1: Demographic features of the study participants. Routine immunisations: Infanrix Hexa™ (hepatitis B, diphtheria-tetanus-pertussis, *H. influenzae* type B, inactivated poliovirus), Prevenar 13™ (13-valent pneumococcal vaccine), rotavirus (Rotarix™ or RotaTeq™).

Table 2: Univariate analysis for the effect of neonatal BCG vaccination versus no BCG vaccination on unstimulated cytokine levels. Results shown as GMR (95% CI) for normally-distributed data and difference in medians (95% CI) from non-parametric quantile regression for data which was not normally distributed.

Figure 1: Flow diagram showing participant inclusion. Table shows number of individual stimulations done for each stimulant.

Figure 2: The effect of neonatal BCG vaccination versus no BCG vaccination on cytokine responses to heterologous stimulants. Significant results $p < 0.05$ are depicted in black. GMR > 1.0 indicates cytokine levels were higher in BCG-vaccinated infants compared with BCG-naïve infants. Data which were not normally distributed were analysed using quantile regression and differences in medians (95%CI). As this only applied to IFN- γ , to aid interpretation, all IFN- γ analyses are displayed on stand-alone axes irrespective of the type of analysis done.

Figure 3: Proportion of BCG-vaccinated and BCG-naïve participants with production of IFN- γ in response to heterologous stimulation ('responders') (Fisher's exact test). Data for R848 not included as IFN- γ responses were above the LLD for all participants.

Figure 4: Subgroup analysis for the effect of infant sex and maternal BCG vaccination on *in vitro* cytokine responses. Panels a-c: IL-6 (a), IL-1 β (b), and remaining cytokine/stimulant pairs (c) with a significant interaction between sex and BCG vaccination. Geometric mean ratios (GMR) & 95% confidence limits (CI) are shown for the effect of infant BCG vaccination on (i) all study participants; (ii) in girls; (ii) in boys. Panel d): cytokine/stimulant pairs with a significant interaction between maternal BCG vaccination and BCG vaccination. GMR & 95%CI are shown for the effect of infant BCG vaccination on (i) all study participants; (ii) infants whose mothers were BCG-vaccinated; (iii) infants whose mothers were BCG-naïve. GMR > 1 indicates cytokine production is higher in BCG-vaccinated infants.

Figure 1

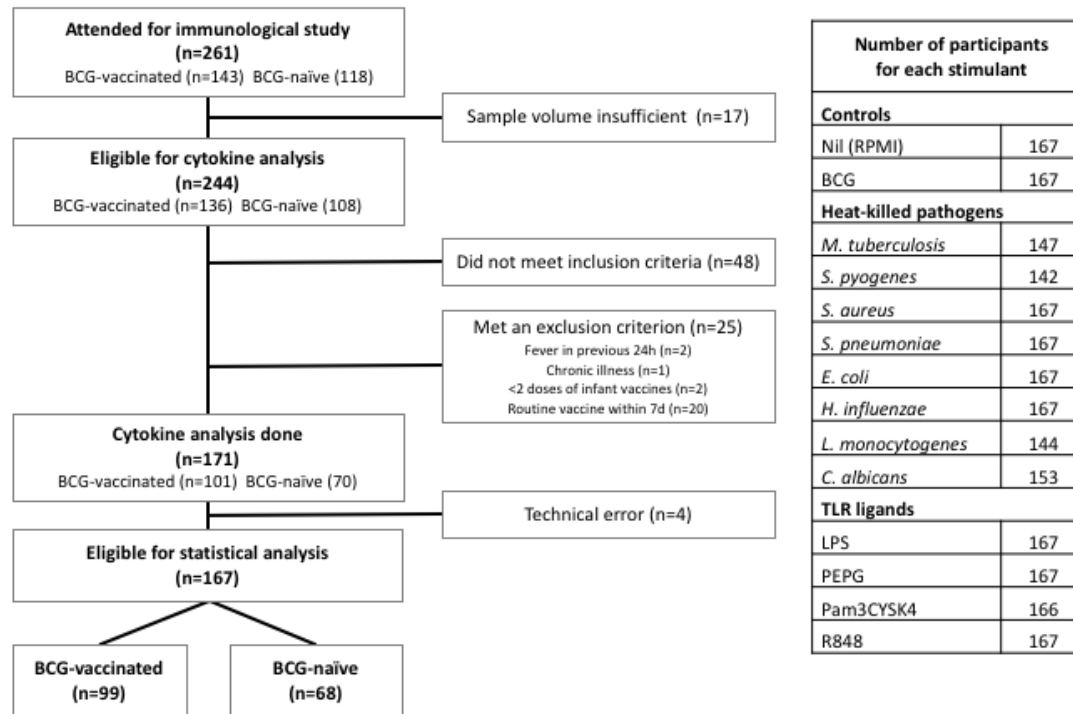


Figure 2

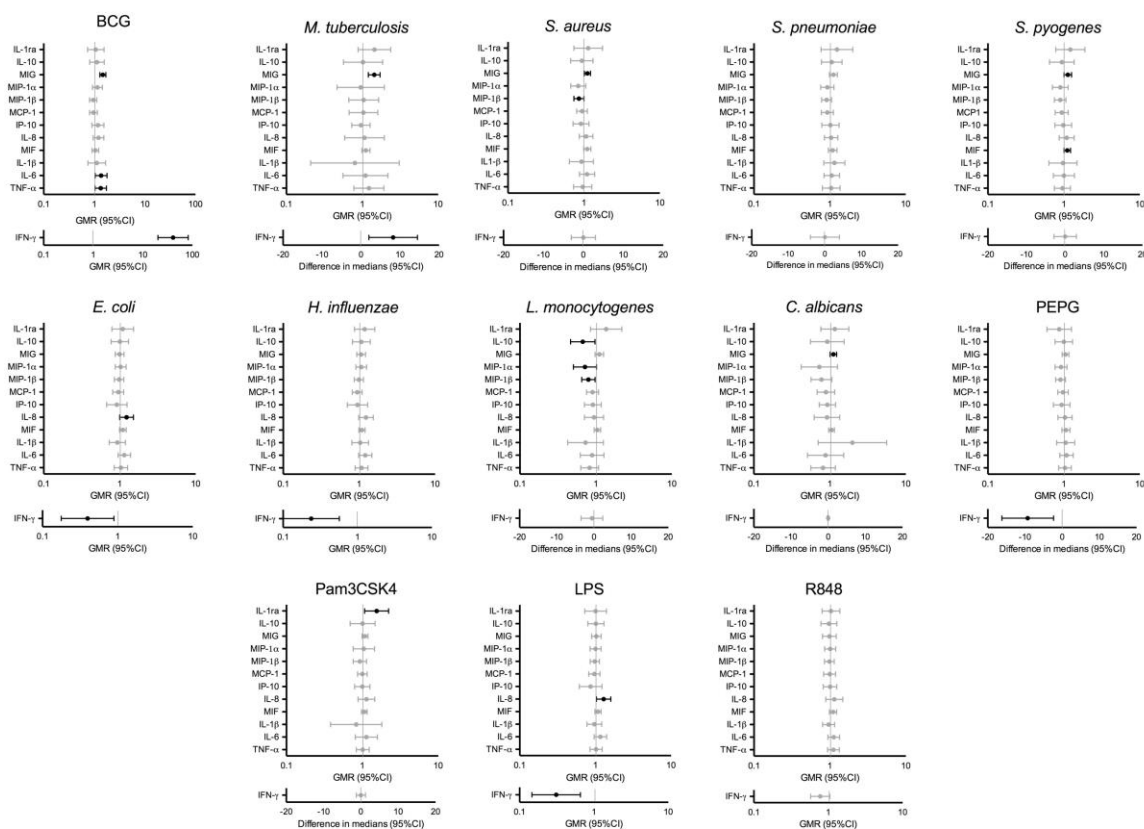


Figure 3

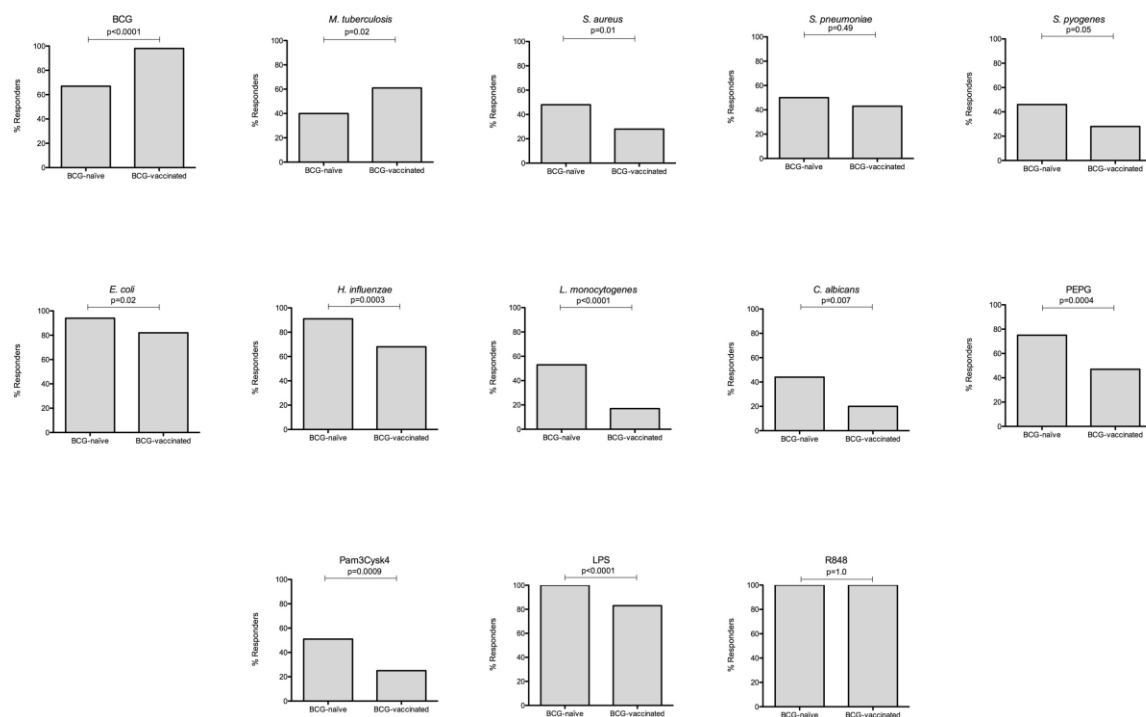
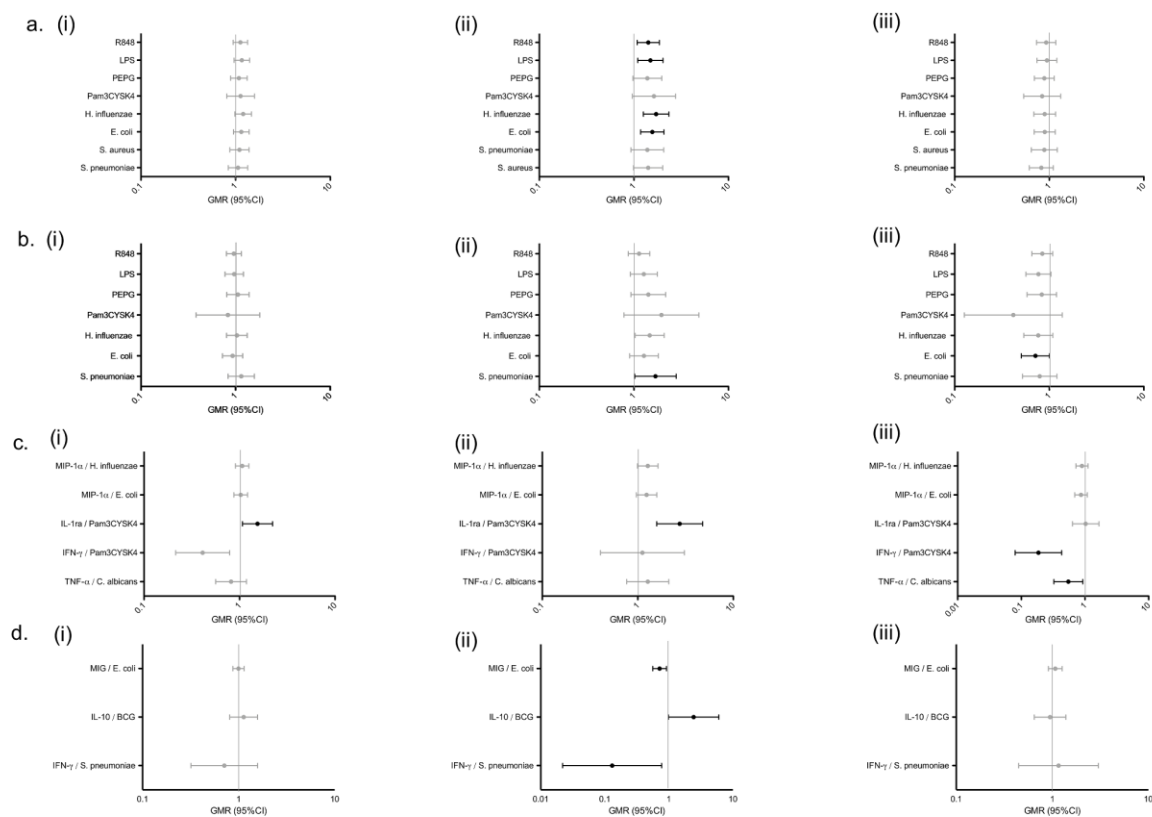


Figure 4



		BCG- vaccinated n (%)	BCG-naïve n (%)	Total n (%)
All		99 (59.3)	68 (40.7)	167 (100%)
Sex	Male	57 (57.6)	37 (54.4)	94 (56.3)
	Female	42 (42.4)	31 (45.6)	73 (43.7)
Ethnicity	Caucasian	79 (79.8)	57 (83.8)	136 (81.4)
	Asian	4 (4.0)	3 (4.4)	7 (4.3)
	Mixed ethnicity	4 (4.0)	2 (3.0)	6 (3.6)
	Middle Eastern	1 (1.0)	0 (0.0)	1 (0.5)
	Missing	11 (11.2)	6 (8.8)	17 (10.2)
Mode of delivery	Vaginal delivery	63 (63.6)	40 (58.8)	103 (61.7)
	Caesarean section	36 (36.4)	28 (41.2)	64 (38.3)
Maternal BCG vaccination	Yes	21 (21.2)	12 (17.7)	33 (19.8)
	No	73 (73.8)	51 (75.0)	124 (74.2)
	Unknown	5 (5.0)	5 (7.3)	10 (6.0)
Maternal education level	School leaver	15 (15.2)	8 (11.8)	23 (13.8)
	Trade certificate	12 (12.1)	8 (11.7)	20 (12.0)
	University degree	48 (48.5)	31 (45.6)	79 (47.3)
	University higher degree	21 (21.2)	18 (26.5)	39 (23.4)
	Other	3 (3.0)	3 (4.4)	6 (3.6)

Number of other children <5y of age in house	0	54 (54.6)	45 (66.2)	99 (59.3)
	1	36 (36.4)	20 (29.4)	56 (33.5)
	2	9 (9.1)	3 (4.4)	12 (7.2)
Participant attends childcare	Yes	7 (7.1)	5 (7.4)	12 (7.2)
	No	92 (92.9)	63 (92.6)	155 (92.8)
Day of life of BCG vaccination	Mean (SD)	2.0 (1.6)	-	-
	Median (IQR)	1 (0-6)	-	-
Age at 7-month blood sample (days)	Mean (SD)	201 (23.1)	200 (22.9)	201 (23.0)
	Median (IQR)	197 (184-223)	200 (182-218)	199 (182-221)
Interval between BCG vaccination and 7-month blood sample (days)	Mean (SD)	199.3 (23.2)	-	-
	Median (IQR)	196 (161-238)	-	-
Number of routine immunisation doses prior to 7-month blood sample	2	51 (51.5)	34 (50.0)	85 (50.9)
	3	47 (47.5)	33 (48.5)	80 (47.9)
	Not recorded	1 (1.0)	1 (1.5)	2 (1.2)
BCG vaccine batch	1	5 (5.1)	-	-
	2	85 (85.9)	-	-
	3	9 (9.1)	-	-

Table 1

Cytokine	GMR (95% CI)	p-value
IL-10	0.59 (0.43 – 0.81)	0.001
TNF- α	0.80 (0.60 – 1.08)	0.14
IL-6	0.90 (0.65 – 1.25)	0.53
MIF	0.78 (0.62 – 0.99)	0.04
MIG	0.79 (0.60 – 1.08)	0.14
IP-10	0.64 (0.49 – 0.86)	0.003
IL-8	0.61 (0.41 – 0.93)	0.02
MIP-1 α	0.65 (0.41 – 1.03)	0.06
MIP-1 β	0.81 (0.57 – 1.17)	0.26
IL-1Ra	0.87 (0.52 – 1.46)	0.59
Cytokine	Difference in the medians (95% CI)	p-value
IFN- γ	1.39x10 ⁻¹⁷ (-1.57 – 1.57)	1
IL-1 β	6.94 x10 ⁻¹⁸ (-5.14 – 5.14)	1
MCP-1	562.05 (-675.46 – 1799.56)	0.37

Table 2

Neonatal BCG vaccination reduces interferon gamma responsiveness to heterologous pathogens in infants from a randomised controlled trial

Supplementary Figure 1

Cytokine and chemokine responses to killed heterologous pathogens and Toll-like receptor (TLR) ligands at 7 months of age for all participants.

Error bars are median (IQR) for each cytokine/stimulant combination.

Supplementary Figure 2 and Figure 3

The effect of sex (Figure 2) and maternal BCG vaccination (Figure 3) on cytokine expression.

GMR > 1.0 indicates cytokine levels were higher in boys compared with girls (Figure 2) or in infants whose mothers were BCG-vaccinated compared with infants whose mothers were BCG-naïve (Figure 3). Significant results ($p < 0.05$) are depicted in red.

Supplementary Table 1

Univariate analysis of the effect of BCG vaccination, maternal BCG vaccination and sex on neonatal cytokine response to Toll-like receptor ligands and heterologous pathogens.

% In range refers to the proportion of values that fell within the detectable limit of the assay and were available for statistical analysis. Statistically significant values ($p < 0.05$) are depicted in red. GMR > 1.0 indicates the effect was stronger in BCG vaccinated subjects, infants whose mothers' were BCG-vaccinated and boys. Logistic regression was done with the log-transformed value of the RPMI (nil) cytokine values as a covariate.

* IFN- γ stimulations that were not normally distributed were analysed using quantile regression and the results are depicted as DOM (95% CI).

Supplementary Table 2

Proportion of participants with detectable cytokine production.

Proportion of BCG-naïve and BCG-vaccinated participants with production of IFN- γ , IP-10, IL-1Ra and IL-1 β above the lower limit of detection ('responders') in response to heterologous stimulation.

Supplementary Table 3

Sensitivity analysis for the effect of non-responders.

Sensitivity analysis for the effect of BCG vaccination on the production of IFN- γ , IP-10, IL-1 β and IL-1Ra after removal of non-responders (all values below the lower limit of detection of the assay) from each stimulation. GMR>1 indicates responses were higher in BCG-vaccinated subjects.

* IFN- γ stimulations that were not normally distributed were analysed using quantile regression and the results are depicted as DOM (95% CI).

Supplementary Table 4

Interaction analysis between infant BCG vaccination and (i) maternal BCG vaccination status and (ii) sex on neonatal cytokine response to Toll-like receptor ligands and heterologous pathogens.

Statistically significant interactions ($p<0.05$) are depicted in red, no further results are shown as significant interactions were used as a marker of the appropriateness for further sub-group analysis. Logistic regression was done with the log-transformed value of the RPMI (nil) cytokine values as a covariate. In the absence of a significant interaction no further association is explored.

Supplementary Table 5

Multivariate analysis of the effect of BCG.

Effect of unstimulated (RPMI) value and individual demographic variables of interest (age at BCG vaccine, age at blood draw, mode of delivery, maternal BCG-vaccination status, sex, number of routine immunisations received) were analysed. Results are depicted as GMR (95% CI) or DOM* (95%CI) and statistically significant results ($p<0.05$) are marked in red. Logistic regression was done with the log-transformed value of the RPMI (nil) cytokine values as a covariate. The univariate analysis for the effect of BCG vaccination alone is shown in the left-hand column to facilitate comparison with other analyses.

Abbreviations

BCG = Bacille Calmette-Guérin, MTBK = killed *Mycobacterium tuberculosis*,

LPS = lipopolysaccharide, PEPG = peptidoglycan, Pam3CYSK4 = (S)-(2,3-

bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-

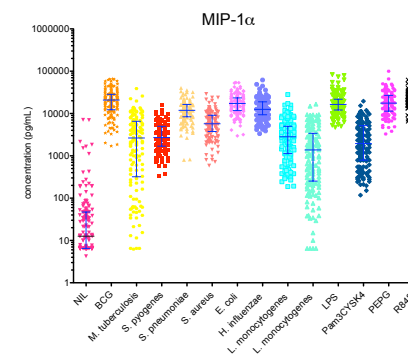
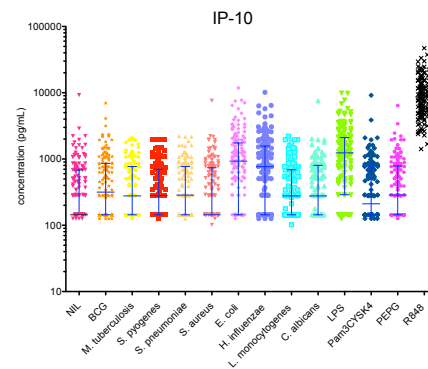
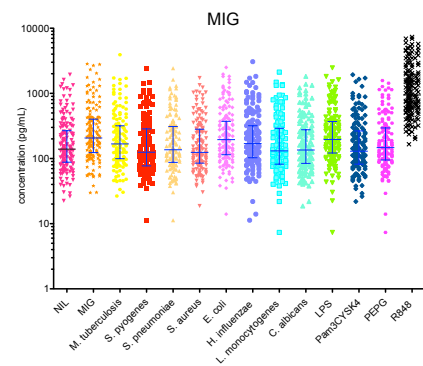
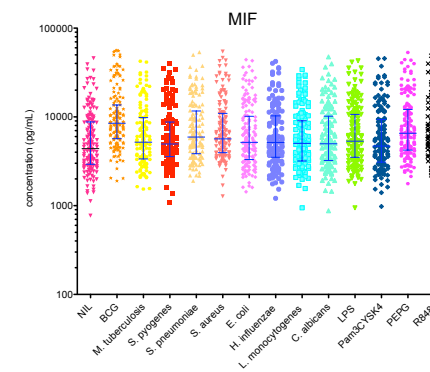
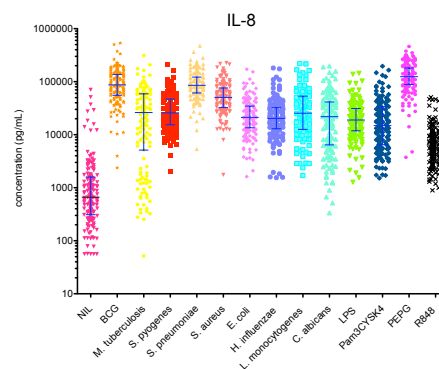
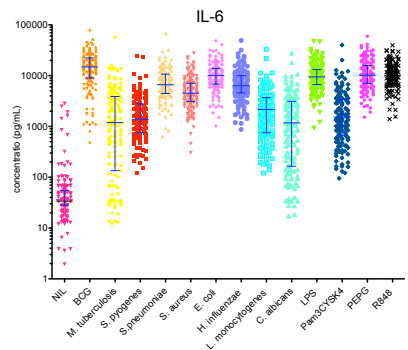
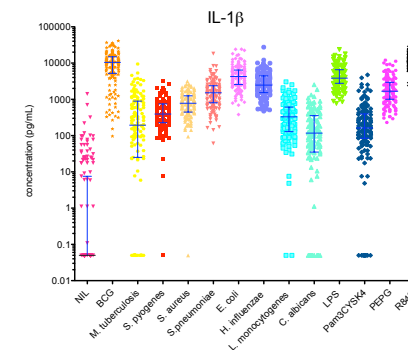
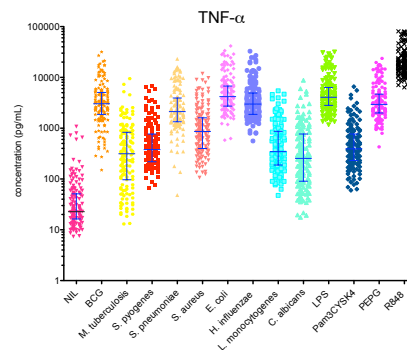
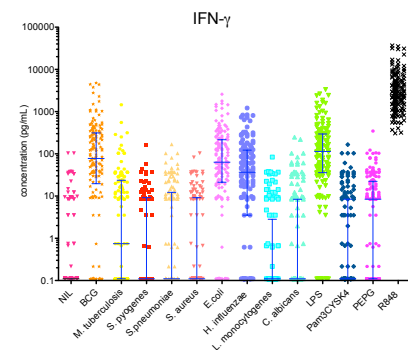
Lys4OH, trihydrochloride, R848 = resiquimod,

SA = *S. aureus*, SP = *S. pneumoniae*, GAS = *S. pyogenes*, EC = *E. coli*, HI = *H. influenzae*,

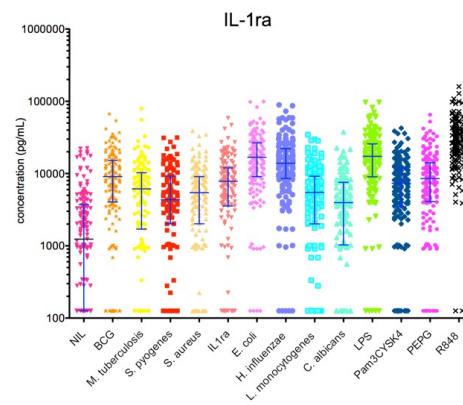
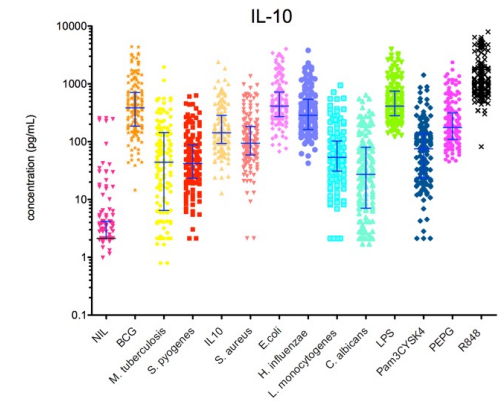
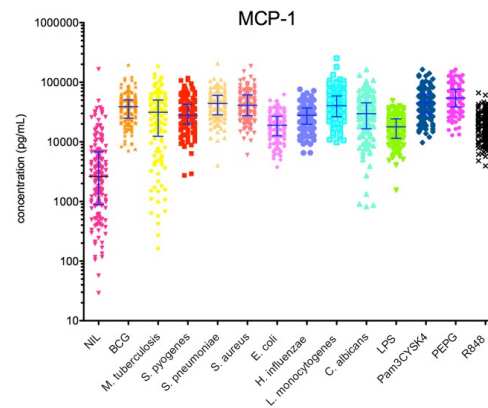
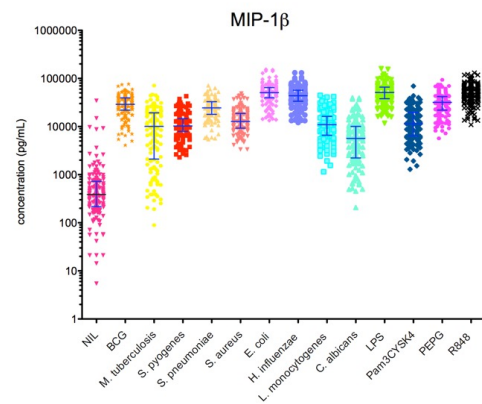
LM = *L. monocytogenes*, CA = *C. albicans*.

DOM = difference in the medians, GMR = geometric mean ratio, IQR = interquartile range,

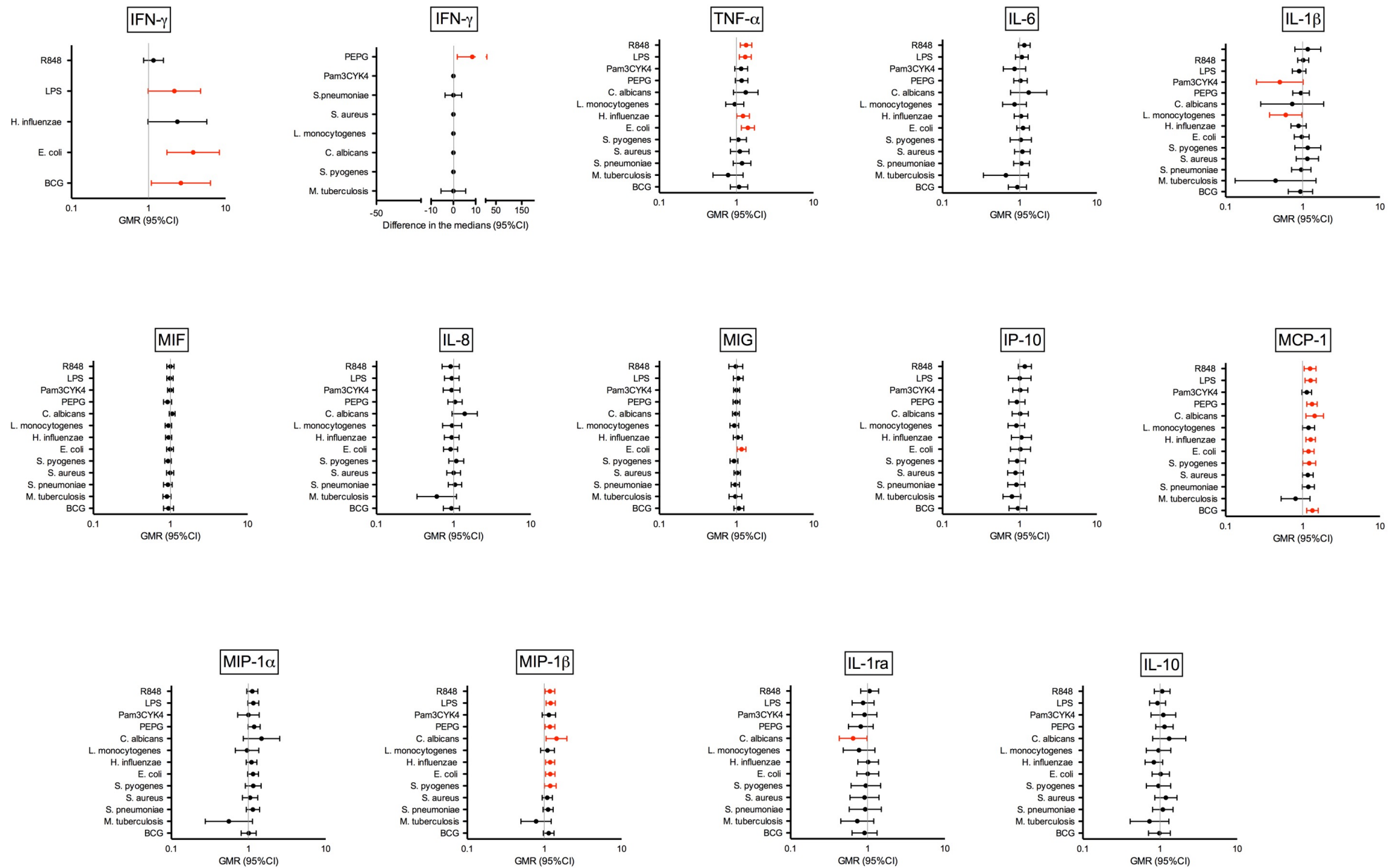
LCI = lower confidence interval, UCI = upper confidence interval.



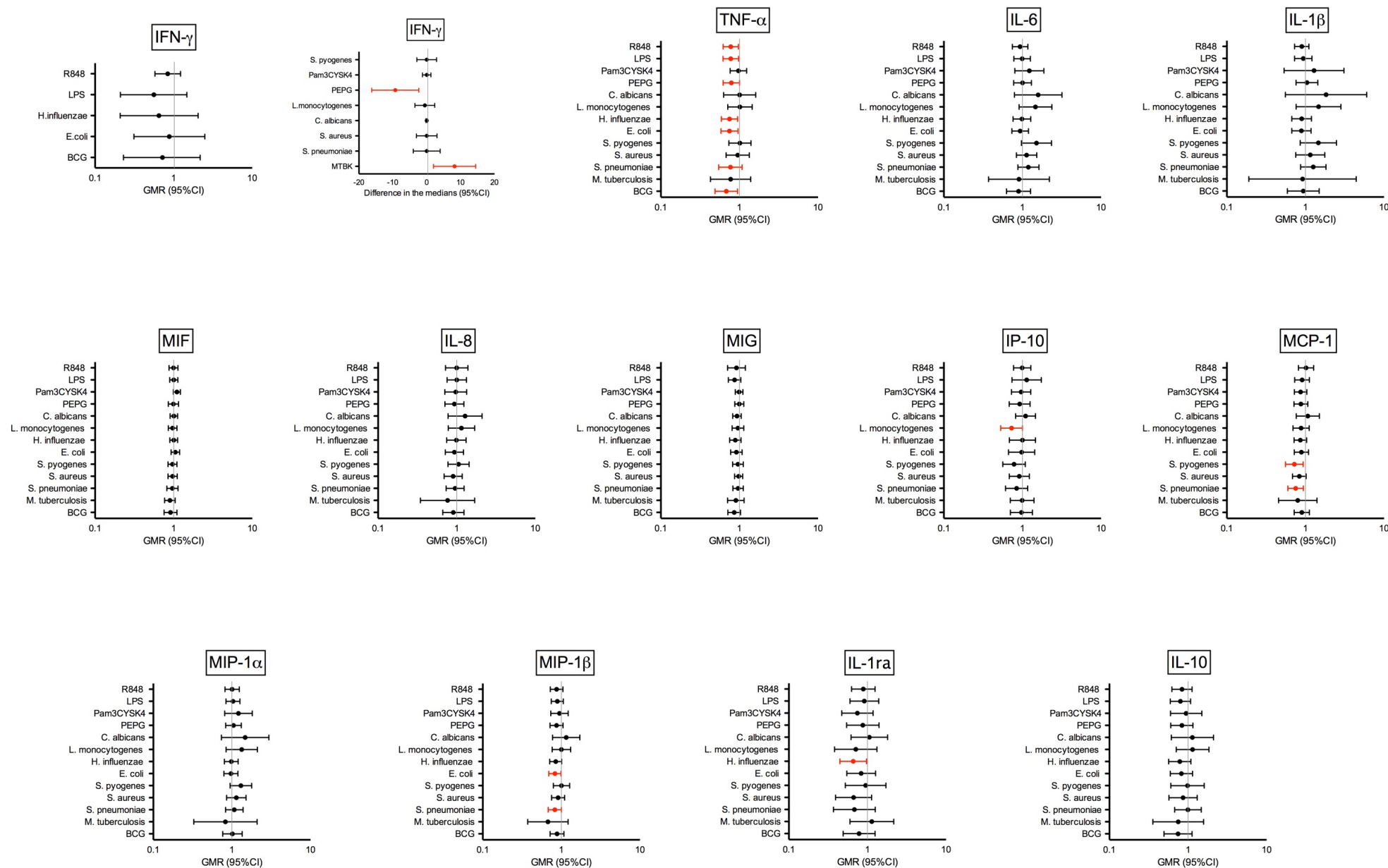
Supplementary Figure 1, p1



Supplementary Figure 1, p2



Supplementary Figure 2



Supplementary Figure 3

Cytokine	Stim	% in Range	Neonatal BCG				Maternal BCG				Sex			
			GMR	95%CI		p-value	GMR	95%CI		p-value	GMR	95%CI		p-value
				LCI	UCI			LCI	UCI			LCI	UCI	
IFN- γ	BCG	85.6	40.82	20.19	82.54	0.00	0.72	0.23	2.18	0.55	2.63	1.09	6.38	0.03
	MTBK*	53.1	8.22	1.99	14.45	0.01	8.22	1.99	14.45	0.01	-5.53	0.00	5.53	1.00
	SP*	45.9	0.00	-3.97	3.97	1.00	0.00	-3.97	3.97	1.00	1.66	0.75	3.65	0.21
	SA*	36.1	0.00	-3.02	3.02	1.00	0.00	-3.03	3.03	1.00	1.36	0.68	2.70	0.38
	GAS*	35.2	0.00	-2.90	2.90	1.00	0.00	-2.91	2.91	1.00	0.00	0.00	0.00	0.91
	EC	86.8	0.40	0.18	0.89	0.03	0.88	0.31	2.49	0.81	3.80	1.73	8.33	0.00
	HI	77.3	0.24	0.10	0.57	0.00	0.65	0.21	2.05	0.46	2.37	0.98	5.71	0.05
	LM*	30.6	-0.56	-3.48	2.36	0.71	-0.56	-3.48	2.36	0.71	0.00	0.00	0.00	1.00
	CA*	30.7	0.00	-0.23	0.23	1.00	0.00	-0.23	0.23	1.00	0.00	0.00	0.00	1.00
	PEPG*	58.7	-9.27	-16.23	-2.32	0.01	-9.27	-16.23	-2.32	0.01	4.58	8.50	12.42	0.00
	Pam3*	34.3	-1.23	0.00	1.23	1.00	0.00	-1.24	1.24	1.00	0.00	0.00	0.00	0.72
	LPS	90.4	0.31	0.15	0.66	0.00	0.56	0.21	1.48	0.24	-22.6	60.68	143.94	0.02
	R848	100.0	0.78	0.58	1.05	0.10	0.84	0.58	1.23	0.37	1.16	0.86	1.56	0.32
TNF- α	BCG	100.0	1.34	1.03	1.74	0.03	0.68	0.49	0.95	0.02	1.08	0.83	1.40	0.56
	MTBK	100.0	1.23	0.78	1.93	0.38	0.77	0.43	1.39	0.39	0.78	0.50	1.22	0.27
	SP	100.0	1.04	0.80	1.36	0.76	0.77	0.54	1.08	0.13	1.18	0.91	1.54	0.21
	SA	100.0	0.96	0.73	1.27	0.79	0.95	0.68	1.34	0.77	1.11	0.84	1.46	0.47
	GAS	100.0	0.96	0.75	1.22	0.72	1.01	0.73	1.40	0.93	1.06	0.83	1.35	0.64
	EC	100.0	1.04	0.85	1.27	0.73	0.75	0.58	0.96	0.02	1.41	1.16	1.71	0.00
	HI	100.0	1.08	0.89	1.31	0.46	0.75	0.59	0.95	0.02	1.22	1.01	1.47	0.04
	LM	100.0	0.83	0.63	1.09	0.17	1.02	0.71	1.45	0.93	0.95	0.73	1.25	0.71
	CA	100.0	0.81	0.56	1.18	0.28	1.01	0.63	1.61	0.98	1.32	0.92	1.90	0.14
	PEPG	100.0	1.04	0.86	1.25	0.72	0.79	0.62	1.00	0.05	1.17	0.97	1.41	0.10
	Pam3	100.0	1.01	0.83	1.23	0.90	0.97	0.76	1.24	0.81	1.15	0.95	1.40	0.15
	LPS	100.0	1.02	0.85	1.23	0.83	0.78	0.62	0.97	0.03	1.30	1.09	1.56	0.00
	R848	100.0	1.11	0.93	1.33	0.24	0.78	0.62	0.97	0.03	1.33	1.12	1.58	0.00
IL-6	BCG	100.0	1.36	1.04	1.78	0.03	0.89	0.63	1.27	0.52	0.93	0.71	1.22	0.62
	MTBK	83.7	1.10	0.56	2.18	0.78	0.90	0.37	2.20	0.82	0.66	0.34	1.30	0.23
	SP	100.0	1.06	0.84	1.34	0.63	1.19	0.87	1.62	0.27	1.06	0.84	1.34	0.64
	GAS	100.0	1.00	0.73	1.38	0.99	1.51	0.97	2.34	0.07	1.03	0.75	1.42	0.83
	EC	100.0	1.15	0.95	1.39	0.15	0.94	0.73	1.19	0.59	1.11	0.92	1.34	0.29
	HI	100.0	1.20	0.98	1.47	0.07	0.99	0.76	1.28	0.91	1.04	0.85	1.27	0.70
	LM	100.0	0.89	0.62	1.28	0.54	1.47	0.91	2.37	0.12	0.86	0.60	1.22	0.39
	CA	94.1	0.88	0.51	1.52	0.64	1.59	0.79	3.18	0.19	1.31	0.76	2.25	0.33
	PEPG	100.0	1.08	0.89	1.33	0.44	1.00	0.77	1.30	0.98	1.03	0.84	1.25	0.81
	Pam3	100.0	1.13	0.81	1.58	0.48	1.22	0.80	1.87	0.35	0.86	0.61	1.20	0.36
	LPS	100.0	1.16	0.96	1.41	0.12	0.99	0.78	1.26	0.94	1.07	0.88	1.29	0.50
	R848	100.0	1.12	0.94	1.34	0.19	0.93	0.74	1.17	0.55	1.15	0.96	1.37	0.12
IL-1 β	BCG	100.0	1.12	0.75	1.67	0.58	0.94	0.59	1.50	0.79	0.93	0.65	1.34	0.70
	MTBK	81.9	0.80	0.21	3.07	0.75	0.92	0.19	4.43	0.92	0.44	0.13	1.48	0.19
	SP	100.0	1.15	0.83	1.58	0.40	1.26	0.87	1.82	0.23	0.95	0.72	1.27	0.75
	SA	99.4	0.93	0.64	1.33	0.68	1.15	0.75	1.76	0.51	1.15	0.82	1.60	0.42
	GAS	100.0	0.97	0.64	1.49	0.90	1.46	0.86	2.48	0.16	1.16	0.79	1.71	0.45
	EC	100.0	0.93	0.73	1.19	0.56	0.89	0.67	1.18	0.41	0.97	0.77	1.21	0.77
	HI	100.0	1.03	0.80	1.33	0.80	0.90	0.67	1.19	0.45	0.88	0.70	1.11	0.28
	LM	98.6	0.73	0.43	1.26	0.25	1.47	0.76	2.83	0.25	0.60	0.37	0.97	0.04
	CA	85.6	1.98	0.70	5.60	0.19	1.83	0.56	6.02	0.32	0.73	0.28	1.86	0.51
	PEPG	100.0	1.06	0.81	1.39	0.68	1.04	0.76	1.44	0.79	0.95	0.74	1.21	0.67
	Pam3	93.4	0.83	0.38	1.80	0.64	1.29	0.53	3.10	0.57	0.50	0.25	1.01	0.05
	LPS	100.0	0.97	0.77	1.21	0.77	0.94	0.73	1.21	0.62	0.90	0.73	1.10	0.28
	R848	100.0	0.96	0.80	1.15	0.65	0.90	0.73	1.11	0.31	1.01	0.86	1.19	0.89

Supplementary Table 1, p1

Cytokine	Stim	% in Range	Neonatal BCG				Maternal BCG				Sex			
			GMR	95%CI		p-value	GMR	95%CI		p-value	GMR	95%CI		p-value
				LCI	UCI			LCI	UCI			LCI	UCI	
MIF	BCG	100.0	1.05	0.90	1.22	0.54	0.91	0.76	1.10	0.33	0.94	0.81	1.09	0.43
	MTBK	100.0	1.12	0.99	1.26	0.07	0.90	0.77	1.05	0.16	0.90	0.80	1.01	0.08
	SP	100.0	1.09	0.96	1.24	0.19	0.96	0.82	1.13	0.66	0.92	0.81	1.05	0.23
	SA	100.0	1.11	0.99	1.23	0.06	0.97	0.85	1.11	0.64	0.99	0.89	1.10	0.84
	GAS	100.0	1.11	1.01	1.22	0.04	0.97	0.85	1.10	0.60	0.93	0.84	1.02	0.14
	EC	100.0	1.10	0.99	1.22	0.06	1.05	0.93	1.19	0.44	0.99	0.89	1.09	0.78
	HI	100.0	1.09	0.99	1.20	0.07	1.00	0.89	1.12	0.97	0.94	0.86	1.04	0.22
	LM	100.0	1.05	0.95	1.15	0.35	0.97	0.86	1.10	0.63	0.94	0.85	1.03	0.19
	CA	100.0	1.05	0.97	1.15	0.24	1.01	0.90	1.12	0.92	1.05	0.96	1.15	0.26
	PEPG	100.0	1.07	0.94	1.20	0.31	0.99	0.86	1.15	0.92	0.92	0.81	1.03	0.15
	Pam3	100.0	1.06	0.98	1.16	0.16	1.10	0.99	1.22	0.07	1.00	0.92	1.09	0.97
	LPS	100.0	1.08	0.98	1.19	0.10	1.00	0.89	1.13	0.97	0.98	0.90	1.08	0.75
	R848	100.0	1.10	0.99	1.22	0.09	0.99	0.87	1.13	0.93	1.00	0.89	1.11	0.94
MIG	BCG	100.0	1.48	1.29	1.69	0.00	0.86	0.71	1.03	0.10	1.07	0.92	1.24	0.36
	MTBK	100.0	1.44	1.20	1.72	0.00	0.90	0.71	1.15	0.39	0.97	0.80	1.17	0.72
	SP	100.0	1.12	0.99	1.26	0.07	0.96	0.82	1.12	0.61	0.96	0.85	1.09	0.54
	SA	100.0	1.11	1.01	1.22	0.02	0.98	0.87	1.10	0.69	1.02	0.93	1.12	0.68
	GAS	100.0	1.13	1.01	1.26	0.04	0.96	0.82	1.12	0.59	0.93	0.82	1.04	0.20
	EC	100.0	1.00	0.87	1.14	0.96	0.91	0.77	1.08	0.30	1.16	1.02	1.33	0.03
	HI	100.0	1.06	0.93	1.21	0.36	0.89	0.75	1.05	0.16	1.04	0.91	1.18	0.59
	LM	99.3	1.11	0.98	1.27	0.11	0.96	0.81	1.14	0.62	0.94	0.82	1.07	0.32
	CA	100.0	1.11	1.01	1.23	0.03	0.93	0.82	1.05	0.26	0.98	0.89	1.08	0.70
	PEPG	99.4	1.05	0.95	1.17	0.34	1.00	0.87	1.13	0.94	1.00	0.90	1.11	0.98
	Pam3	100.0	1.08	0.99	1.19	0.07	0.99	0.89	1.11	0.85	1.00	0.92	1.10	0.95
	LPS	99.4	1.03	0.89	1.19	0.70	0.87	0.72	1.04	0.13	1.05	0.91	1.22	0.47
	R848	100.0	0.98	0.80	1.21	0.86	0.92	0.71	1.19	0.51	0.98	0.80	1.20	0.83
MCP-1	BCG	100.0	0.96	0.81	1.15	0.68	0.90	0.72	1.12	0.35	1.33	1.12	1.58	0.00
	MTBK	100.0	1.04	0.67	1.60	0.87	0.80	0.45	1.40	0.43	0.80	0.52	1.24	0.32
	SP	100.0	0.93	0.77	1.11	0.42	0.75	0.60	0.94	0.01	1.18	0.99	1.41	0.07
	SA	100.0	0.94	0.80	1.11	0.48	0.84	0.68	1.02	0.08	1.16	0.99	1.36	0.06
	GAS	100.0	0.94	0.77	1.14	0.52	0.72	0.56	0.94	0.01	1.21	1.00	1.47	0.05
	EC	100.0	0.96	0.81	1.13	0.60	0.89	0.72	1.09	0.27	1.19	1.01	1.40	0.04
	HI	100.0	0.94	0.81	1.09	0.44	0.86	0.72	1.03	0.11	1.26	1.10	1.46	0.00
	LM	100.0	0.91	0.76	1.09	0.29	0.88	0.69	1.12	0.29	1.19	0.99	1.42	0.06
	CA	100.0	0.89	0.68	1.16	0.38	1.07	0.76	1.51	0.68	1.42	1.10	1.85	0.01
	PEPG	100.0	0.97	0.83	1.14	0.75	0.88	0.72	1.07	0.19	1.31	1.13	1.53	0.00
	Pam3	100.0	1.00	0.86	1.16	0.99	0.87	0.72	1.04	0.13	1.13	0.97	1.30	0.18
	LPS	100.0	0.97	0.82	1.15	0.71	0.90	0.73	1.12	0.36	1.26	1.07	1.49	0.01
	R848	100.0	0.99	0.83	1.19	0.91	1.02	0.81	1.27	0.88	1.24	1.04	1.48	0.02
IL-8	BCG	100.0	1.21	0.95	1.54	0.12	0.91	0.66	1.24	0.54	0.94	0.74	1.19	0.58
	MTBK	99.3	1.07	0.59	1.96	0.82	0.77	0.35	1.70	0.51	0.60	0.34	1.09	0.09
	SP	100.0	1.04	0.85	1.27	0.70	0.95	0.73	1.24	0.73	1.04	0.85	1.27	0.70
	SA	100.0	1.07	0.87	1.31	0.53	0.90	0.69	1.18	0.44	1.00	0.82	1.23	0.99
	GAS	100.0	1.09	0.87	1.37	0.46	1.06	0.78	1.44	0.73	1.08	0.87	1.35	0.49
	EC	100.0	1.23	1.00	1.52	0.05	0.94	0.72	1.22	0.63	0.91	0.74	1.12	0.39
	HI	100.0	1.23	0.98	1.53	0.07	0.99	0.75	1.32	0.95	0.94	0.76	1.18	0.61
	LM	100.0	0.95	0.71	1.26	0.70	1.15	0.78	1.69	0.47	0.95	0.72	1.27	0.75
	CA	100.0	0.92	0.62	1.35	0.65	1.28	0.78	2.10	0.33	1.39	0.95	2.03	0.09
	PEPG	100.0	1.04	0.84	1.29	0.74	0.94	0.71	1.23	0.63	1.04	0.84	1.29	0.71
	Pam3	100.0	1.13	0.88	1.46	0.34	0.97	0.71	1.34	0.87	0.94	0.73	1.21	0.63
	LPS	100.0	1.29	1.03	1.60	0.02	1.00	0.76	1.33	0.98	0.95	0.76	1.18	0.62
	R848	100.0	1.14	0.88	1.48	0.31	1.00	0.72	1.39	0.99	0.91	0.71	1.18	0.49

Supplementary Table 1, p2

Cytokine	Stim	% in Range	Neonatal BCG				Maternal BCG				Sex			
			GMR	95%CI		p-value	GMR	95%CI		p-value	GMR	95%CI		p-value
				LCI	UCI			LCI	UCI			LCI	UCI	
IP-10	BCG	60.8	1.18	0.90	1.55	0.23	0.97	0.70	1.34	0.85	0.95	0.73	1.24	0.69
	MTBK	49.7	0.96	0.73	1.27	0.77	0.99	0.70	1.40	0.96	0.79	0.61	1.04	0.09
	SP	100.0	1.02	0.78	1.32	0.89	0.84	0.61	1.17	0.30	0.91	0.70	1.17	0.45
	SA	100.0	0.91	0.72	1.16	0.47	0.91	0.68	1.22	0.54	0.88	0.70	1.11	0.29
	GAS	51.4	0.98	0.76	1.27	0.89	0.78	0.56	1.09	0.14	0.93	0.72	1.19	0.55
	EC	74.8	0.92	0.67	1.25	0.58	0.98	0.67	1.43	0.90	1.02	0.76	1.38	0.88
	HI	65.9	0.95	0.70	1.29	0.74	1.00	0.68	1.46	1.00	1.05	0.78	1.42	0.73
	LM	52.1	0.91	0.71	1.18	0.48	0.73	0.53	1.00	0.05	0.91	0.71	1.16	0.43
	CA	51.0	0.93	0.72	1.19	0.56	1.10	0.82	1.47	0.53	1.02	0.80	1.30	0.89
	PEPG	100.0	0.94	0.73	1.20	0.60	0.92	0.68	1.25	0.60	0.92	0.72	1.17	0.50
	Pam3	100.0	1.00	0.79	1.26	0.98	0.96	0.73	1.27	0.79	1.02	0.81	1.27	0.87
	LPS	77.2	0.86	0.61	1.22	0.41	1.13	0.73	1.74	0.57	1.00	0.72	1.40	0.99
	R848	100.0	1.00	0.82	1.23	0.98	0.99	0.77	1.28	0.95	1.17	0.96	1.42	0.12
MIP1- α	BCG	100.0	1.15	0.92	1.44	0.21	1.02	0.76	1.35	0.92	1.00	0.80	1.26	0.97
	MTBK	100.0	0.95	0.47	1.96	0.90	0.83	0.33	2.09	0.69	0.56	0.28	1.12	0.10
	SP	100.0	0.93	0.76	1.13	0.45	1.07	0.83	1.39	0.58	1.14	0.94	1.39	0.19
	SA	100.0	0.84	0.67	1.06	0.13	1.14	0.86	1.51	0.37	1.05	0.83	1.32	0.69
	GAS	100.0	0.90	0.71	1.14	0.37	1.30	0.95	1.78	0.10	1.15	0.91	1.46	0.24
	EC	100.0	1.03	0.87	1.21	0.74	0.97	0.79	1.19	0.78	1.15	0.98	1.35	0.10
	HI	100.0	1.06	0.91	1.25	0.44	0.98	0.80	1.20	0.86	1.09	0.93	1.28	0.27
	LM	100.0	0.72	0.51	1.02	0.06	1.33	0.84	2.11	0.22	0.96	0.68	1.36	0.81
	CA	100.0	0.73	0.42	1.26	0.25	1.47	0.74	2.95	0.27	1.48	0.86	2.54	0.16
	PEPG	100.0	0.92	0.77	1.10	0.35	1.05	0.83	1.32	0.68	1.18	0.99	1.41	0.06
	Pam3	100.0	1.05	0.76	1.45	0.78	1.21	0.81	1.81	0.35	1.00	0.72	1.38	0.99
	LPS	100.0	1.00	0.85	1.19	0.96	1.03	0.84	1.26	0.76	1.15	0.98	1.36	0.09
	R848	100.0	1.01	0.85	1.19	0.94	1.01	0.82	1.24	0.95	1.12	0.95	1.32	0.18
MIP-1 β	BCG	100.0	0.95	0.81	1.11	0.51	0.88	0.72	1.08	0.22	1.14	0.97	1.33	0.12
	MTBK	100.0	1.05	0.66	1.65	0.84	0.67	0.37	1.22	0.19	0.78	0.50	1.23	0.28
	SP	100.0	0.91	0.78	1.05	0.20	0.83	0.68	1.00	0.05	1.12	0.96	1.30	0.15
	SA	100.0	0.86	0.74	1.00	0.05	0.91	0.75	1.10	0.32	1.09	0.93	1.27	0.27
	GAS	100.0	0.90	0.75	1.07	0.21	1.00	0.79	1.27	0.98	1.19	1.00	1.42	0.05
	EC	100.0	0.98	0.85	1.13	0.80	0.83	0.69	0.99	0.04	1.19	1.04	1.37	0.01
	HI	100.0	0.99	0.86	1.14	0.91	0.85	0.71	1.01	0.06	1.19	1.04	1.36	0.01
	LM	100.0	0.80	0.65	0.98	0.03	1.00	0.76	1.31	1.00	1.10	0.89	1.35	0.38
	CA	100.0	0.78	0.57	1.06	0.11	1.15	0.77	1.72	0.48	1.43	1.05	1.96	0.02
	PEPG	100.0	0.90	0.78	1.05	0.19	0.87	0.72	1.05	0.14	1.18	1.02	1.37	0.03
	Pam3	100.0	0.93	0.76	1.13	0.46	0.95	0.73	1.22	0.68	1.14	0.93	1.39	0.19
	LPS	100.0	0.98	0.85	1.13	0.78	0.89	0.74	1.06	0.19	1.20	1.05	1.38	0.01
	R848	100.0	0.98	0.85	1.14	0.82	0.87	0.72	1.05	0.14	1.18	1.02	1.36	0.03
IL-10	BCG	100.0	1.13	0.81	1.57	0.47	0.75	0.50	1.13	0.16	0.98	0.71	1.36	0.91
	MTBK	100.0	1.02	0.56	1.86	0.94	0.75	0.36	1.58	0.45	0.73	0.41	1.30	0.28
	SP	100.0	1.06	0.77	1.45	0.72	1.00	0.68	1.47	0.99	1.09	0.80	1.47	0.59
	SA	100.0	0.94	0.67	1.31	0.71	0.86	0.57	1.31	0.49	1.19	0.86	1.66	0.30
	GAS	100.0	0.94	0.65	1.37	0.75	0.99	0.60	1.61	0.95	0.96	0.66	1.38	0.81
	EC	100.0	1.00	0.77	1.31	0.97	0.82	0.60	1.14	0.24	1.02	0.79	1.32	0.87
	HI	100.0	1.07	0.82	1.40	0.63	0.79	0.57	1.09	0.14	0.83	0.64	1.08	0.17
	LM	100.0	0.67	0.46	0.97	0.04	1.14	0.71	1.84	0.58	0.96	0.66	1.38	0.81
	CA	100.0	0.92	0.56	1.54	0.76	1.14	0.61	2.11	0.68	1.31	0.80	2.15	0.28
	PEPG	100.0	1.00	0.77	1.30	0.99	0.84	0.60	1.16	0.28	1.14	0.88	1.48	0.31
	Pam3	100.0	1.01	0.69	1.47	0.97	0.95	0.60	1.50	0.82	1.10	0.76	1.60	0.59
	LPS	100.0	1.02	0.80	1.30	0.90	0.80	0.59	1.08	0.14	0.93	0.73	1.18	0.54
	R848	100.0	0.97	0.76	1.23	0.80	0.84	0.62	1.12	0.24	1.07	0.84	1.35	0.59
IL-1ra	BCG	91.7	1.07	0.73	1.56	0.73	0.78	0.49	1.25	0.30	0.91	0.63	1.32	0.62
	MTBK	83.7	1.45	0.88	2.38	0.14	1.14	0.60	2.16	0.69	0.73	0.45	1.20	0.21
	SP	87.0	1.24	0.77	2.01	0.38	0.68	0.37	1.26	0.22	0.93	0.57	1.50	0.76
	SA	84.8	1.14	0.74	1.76	0.55	0.67	0.39	1.13	0.13	0.91	0.59	1.40	0.66
	GAS	84.5	1.22	0.78	1.89	0.38	0.95	0.52	1.72	0.86	0.94	0.61	1.47	0.80
	EC	97.6	1.10	0.79	1.52	0.58	0.83	0.55	1.27	0.39	1.00	0.72	1.39	1.00
	HI	97.0	1.18	0.87	1.61	0.29	0.66	0.45	0.97	0.04	1.01	0.74	1.38	0.94
	LM	84.7	1.37	0.85	2.20	0.20	0.71	0.38	1.32	0.28	0.77	0.48	1.24	0.28
	CA	83.7	1.16	0.76	1.78	0.48	1.06	0.62	1.81	0.84	0.65	0.43	0.98	0.04
	PEPG	92.8	0.87	0.60	1.26	0.45	0.87	0.54	1.40	0.57	0.81	0.56	1.17	0.27
	Pam3	90.4	1.54	1.07	2.22	0.02	0.75	0.47	1.18	0.21	0.91	0.63	1.31	0.61
	LPS	97.0	1.01	0.72	1.40	0.97	0.91	0.60	1.39	0.67	0.87	0.63	1.21	0.41
	R848	98.8	1.03	0.79	1.35	0.82	0.89	0.63	1.25	0.49	1.06	0.81	1.38	0.69

Supplementary Table 1, p3

	IFN- γ			IP-10			IL-1Ra			IL-1 β		
Stimulant	BCG naïve	BCG vaccinated	p-value	BCG naïve	BCG vaccinated	p-value	BCG naïve	BCG vaccinated	p-value	BCG naïve	BCG vaccinated	p-value
BCG	46/68 (67.6)	97/99 (98.0)	<0.0001	40/69 (58.0)	59/99 (59.6)	0.87	64/69 (93.0)	91/99 (92.0)	1.00	68/68 (100.0)	99/99 (100.0)	-
<i>M. tuberculosis</i>	24/59 (40.7)	54/88 (61.4)	0.02	33/59 (55.9)	41/88 (46.6)	0.31	37/59 (62.7)	77/88 (87.5)	0.25	52/59 (88.1)	67/88 (76.1)	0.09
<i>E. coli</i>	64/68 (94.1)	81/99 (81.8)	0.02	55/68 (80.9)	71/99 (71.7)	0.21	66/68 (97.1)	97/99 (98.0)	1.00	68/68 (100.0)	99/99 (100.0)	-
<i>H. influenzae</i>	62/68 (91.2)	67/99 (67.7)	0.0003	40/68 (58.8)	61/99 (61.6)	0.25	66/68 (97.1)	96/99 (97.0)	1.00	68/68 (100.0)	99/99 (100.0)	-
<i>L. monocytogenes</i>	30/57 (52.6)	15/87 (17.2)	<0.0001	32/57 (56.1)	45/87 (51.7)	0.61	45/57 (78.9)	77/87 (88.5)	0.24	57/57 (100.0)	85/87 (97.7)	0.51
<i>C. albicans</i>	27/62 (43.5)	19/91 (20.9)	0.0071	35/62 (56.4)	42/91 (46.2)	0.25	50/62 (80.6)	78/91 (85.7)	0.50	52/62 (83.9)	79/91 (86.8)	0.64
<i>S. aureus</i>	32/66 (48.5)	26/92 (28.3)	0.01	35/66 (53.0)	40/90 (44.4)	0.33	56/66 (84.8)	80/92 (87.0)	0.81	66/66 (100.0)	91/92 (98.9)	1.00
<i>S. pneumoniae</i>	29/58 (50.0)	38/88 (43.2)	0.49	30/58 (51.7)	47/88 (53.4)	0.87	50/58 (86.2)	78/88 (88.6)	0.80	58/58 (100.0)	88/88 (100.0)	-
<i>S. pyogenes</i>	26/57 (45.6)	24/85 (28.2)	0.05	31/57 (54.4)	42/85 (49.4)	0.61	47/57 (82.5)	76/87 (87.4)	0.47	57/57 (100.0)	84/85 (98.8)	1.00
Pam3CYSK4	35/68 (51.5)	25/98 (25.5)	0.0009	36/68 (52.9)	47/98 (48.0)	0.53	59/68 (86.8)	91/98 (93.0)	0.28	66/68 (97.1)	89/98 (90.8)	0.20
PEPG	51/68 (75.0)	47/99 (47.5)	0.0004	41/69 (59.4)	53/99 (53.5)	0.42	66/68 (97.1)	90/99 (91.1)	0.20	68/68 (100.0)	99/99 (100.0)	-
LPS	68/68 (100.0)	83/99 (83.8)	<0.0001	56/68 (82.4)	73/99 (73.7)	0.56	66/68 (97.1)	96/99 (97.0)	1.00	68/68 (100.0)	99/99 (100.0)	-
R848	68/68 (100.0)	99/99 (100.0)	1.00	99/99 (100.0)	68/68 (100)	1.00	68/68 (100)	97/99 (98.0)	1.00	68/68 (100.0)	99/99 (100.0)	-

Supplementary Table 2

Cytokine	Stimulant	GMR	LCI	UCI	p-value	Cytokine	Stimulant	GMR	LCI	UCI	p-value
IFN- γ	BCG	6.466	2.780	15.042	0.00	IP-10	BCG	1.012	0.792	1.293	0.92
	MTBK*	1.749	0.646	4.734	0.26		MTBK	1.109	0.811	1.518	0.51
	SA*	1.000	0.427	2.343	1.00		SA	0.844	0.640	1.112	0.22
	SP*	1.442	0.560	3.714	0.43		SP	0.919	0.709	1.190	0.51
	GAS*	0.976	0.415	2.292	0.95		GAS	1.099	0.846	1.427	0.47
	EC	1.093	0.450	2.656	0.84		EC	1.087	0.790	1.496	0.60
	HI	1.053	0.460	2.414	0.90		HI	0.964	0.698	1.330	0.82
	LM*	1.000	0.186	5.373	1.00		LM	0.867	0.659	1.140	0.30
	CA*	1.000	0.430	2.325	1.00		CA	0.880	0.681	1.138	0.32
	Pam3*	1.000	0.435	2.300	1.00		Pam3	0.961	0.769	1.202	0.73
	PEPG*	0.582	0.262	1.294	0.18		PEPG	1.069	0.881	1.297	0.49
	LPS	0.950	0.361	2.499	0.92		LPS	1.086	0.758	1.556	0.65
	R848	1.206	0.702	2.070	0.49		R848	1.054	0.772	1.438	0.74
IL-1Ra	BCG	1.200	0.877	1.642	0.25	IL-1 β	BCG	1.917	0.083	44.026	0.65
	MTBK	1.217	0.874	1.694	0.24		MTBK	6.651	0.062	715.773	0.36
	SA	1.024	0.759	1.382	0.88		SA	1.935	0.515	7.270	0.29
	SP	1.121	0.847	1.482	0.42		SP	3.969	0.730	21.571	0.10
	GAS	1.102	0.791	1.534	0.56		GAS	2.816	0.393	20.153	0.25
	EC	1.205	0.861	1.685	0.27		EC	2.078	0.461	9.375	0.30
	HI	1.265	1.001	1.598	0.05		HI	5.020	0.860	29.299	0.07
	LM	1.048	0.756	1.454	0.77		LM	2.183	0.217	21.980	0.44
	CA	1.110	0.832	1.481	0.47		CA	3.395	0.879	13.115	0.07
	Pam3	1.349	1.063	1.711	0.01		Pam3	4.111	0.541	31.241	0.15
	PEPG	1.208	0.880	1.657	0.24		PEPG	1.803	0.268	12.139	0.50
	LPS	1.170	0.863	1.586	0.31		LPS	3.311	0.750	14.619	0.10
	R848	1.232	0.947	1.602	0.12		R848	1.509	0.388	5.875	0.51

Supplementary Table 3

Cytokine	Stimulant	Maternal BCG	Sex	Cytokine	Stimulant	Maternal BCG	Sex
IFN- γ	BCG	0.59	0.26	MCP-1	BCG	0.12	0.68
	MTBK	0.19	0.90		MTBK	0.56	0.44
	SA	0.11	0.07		SA	0.25	0.52
	SP	0.04	0.10		SP	0.26	0.62
	GAS	0.37	0.41		GAS	0.29	0.78
	EC	0.35	0.55		EC	0.11	0.67
	HI	0.28	0.66		HI	0.31	0.47
	LM	0.60	0.15		LM	0.66	0.65
	CA	0.56	0.42		CA	0.89	0.78
	Pam3	0.89	0.01		Pam3	0.90	0.37
	PEPG	0.28	0.94		PEPG	0.09	0.73
TNF- α	LPS	0.27	0.55	IL-8	LPS	0.61	0.73
	R848	0.30	0.89		R848	0.03	0.58
	BCG	0.18	0.24		BCG	0.16	0.68
	MTBK	0.47	0.44		MTBK	0.89	0.56
	SA	0.60	0.89		SA	0.47	0.57
	SP	0.89	0.28		SP	0.54	0.55
	GAS	0.85	0.99		GAS	0.95	0.87
	EC	0.63	0.37		EC	0.98	0.17
	HI	0.57	0.47		HI	0.88	0.33
	LM	0.86	0.40		LM	0.56	0.90
	CA	0.56	0.04		CA	0.62	0.56
IL-6	Pam3	0.71	0.73	IP-10	Pam3	0.64	0.63
	PEPG	0.21	0.68		PEPG	0.40	0.46
	LPS	0.26	0.32		LPS	0.41	0.48
	R848	0.14	0.62		R848	0.09	0.41
	BCG	0.39	0.75		BCG	0.22	0.76
	MTBK	0.87	0.85		MTBK	0.66	0.87
	SA	0.87	0.05		SA	0.37	0.46
	SP	0.69	0.04		SP	0.64	0.69
	GAS	0.67	0.31		GAS	0.43	0.95
	EC	0.47	0.00		EC	0.99	0.19
	HI	0.32	0.00		HI	0.69	0.72
IL-1 β	LM	0.24	0.15	MIP-1 α	LM	0.62	0.95
	CA	0.69	0.06		CA	0.62	0.30
	Pam3	0.45	0.06		Pam3	0.80	0.82
	PEPG	0.63	0.03		PEPG	0.06	0.59
	LPS	0.96	0.02		LPS	0.74	0.88
	R848	0.26	0.02		R848	0.74	0.59
	BCG	0.21	0.97		BCG	0.59	0.71
	MTBK	0.52	0.76		MTBK	0.84	0.85
	SA	0.70	0.79		SA	0.97	0.23
	SP	0.29	0.04		SP	0.58	0.08
	GAS	0.82	0.66		GAS	0.49	0.53
MIF	EC	0.40	0.01	MIP-1 β	EC	0.65	0.04
	HI	0.50	0.01		HI	0.33	0.04
	LM	0.99	0.18		LM	0.38	0.64
	CA	0.24	0.23		CA	0.46	0.14
	Pam3	0.93	0.05		Pam3	0.37	0.25
	PEPG	0.82	0.03		PEPG	0.77	0.34
	LPS	1.00	0.03		LPS	0.99	0.13
	R848	0.27	0.04		R848	0.10	0.32
	BCG	0.78	0.71		BCG	0.32	0.51
	MTBK	0.59	0.31		MTBK	0.59	0.30
	SA	0.34	0.61		SA	0.31	0.19
MIG	SP	0.13	0.13	IL-10	SP	0.93	0.26
	GAS	0.47	0.32		GAS	0.71	0.44
	EC	0.40	0.08		EC	0.88	0.17
	HI	0.94	0.62		HI	0.95	0.10
	LM	0.34	0.18		LM	0.75	0.30
	CA	0.87	0.06		CA	0.46	0.07
	Pam3	0.73	0.18		Pam3	0.90	0.45
	PEPG	0.26	0.08		PEPG	0.62	0.37
	LPS	0.66	0.24		LPS	0.66	0.34
	R848	0.99	0.42		R848	0.18	0.34
	BCG	0.35	0.74		BCG	0.05	0.92
IL-1ra	MTBK	0.39	0.95	IL-1ra	MTBK	0.69	0.64
	SA	0.74	0.91		SA	0.92	0.22
	SP	0.38	0.17		SP	0.66	0.16
	GAS	0.09	0.22		GAS	0.17	0.51
	EC	0.02	0.20		EC	0.86	0.08
	HI	0.13	0.70		HI	0.60	0.12
	LM	0.49	0.24		LM	0.65	0.34
	CA	0.29	0.19		CA	0.81	0.30
	Pam3	0.94	0.36		Pam3	0.66	0.33
	PEPG	0.63	0.65		PEPG	0.26	0.49
	LPS	0.30	0.50		LPS	0.11	0.20
IL-1ra	R848	0.36	0.38		R848	0.38	0.54
	BCG	0.20	0.25		LM	0.62	0.18
	MTBK	0.67	0.14		CA	0.61	0.43
	SA	0.30	0.25		Pam3	0.42	0.01
	SP	0.26	0.29		PEPG	0.28	0.58
	GAS	0.81	0.55		LPS	0.28	0.30
IL-1ra	EC	0.27	0.37		R848	0.33	0.93
	HI	0.07	0.39				

Supplementary Table 4

		Primary analysis (BCG)				Age at BCG vaccine				Age				Mode of Delivery				Maternal BCG				Sex				Immunisation (2 v 3)			
		95% CI				95% CI				95% CI				95% CI				95% CI				95% CI				95% CI			
cytokine	stimulant	GMR*	LCI	UCI	p-value	GMR*	LCI	UCI	p-value	GMR*	LCI	UCI	p-value	GMR*	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value
IFN-γ	BCG	40.8	20.2	82.5	0.00	53.6	19.4	148.2	0.00	59.7	21.9	162.6	0.00	39.1	18.9	81.0	0.00	38.1	18.3	78.9	0.00	38.3	18.7	78.6	0.00	38.0	18.3	78.8	0.00
	MTBK*	8.2	2.0	14.5	0.01	32.8	3.7	292.3	0.00	19.0	4.8	74.5	0.00	51.1	11.4	228.7	0.00	75.8	3.4	1694.9	0.01	75.8	16.5	347.6	0.00	70.0	15.5	315.5	0.00
	SA*	0.0	-3.0	3.0	1.00	1.0	0.8	1.3	1.00	0.3	0.1	1.0	0.05	1.0	0.8	1.2	1.00	1.0	0.6	1.7	1.00	1.0	0.8	1.2	1.00	1.0	0.8	1.2	1.00
	SP*	0.0	-4.0	4.0	1.00	1.0	0.1	6.7	1.00	0.4	0.1	1.4	0.16	1.0	0.2	4.6	1.00	1.0	0.4	2.4	1.00	1.0	0.2	4.4	1.00	1.0	0.3	3.6	1.00
	GAS*	0.4	0.2	0.9	0.02	0.2	0.1	0.6	0.00	0.3	0.1	1.0	0.05	1.0	0.7	1.4	0.91	1.0	0.2	4.6	1.00	1.0	0.8	1.3	1.00	1.0	0.8	1.3	1.00
	EC	0.4	0.2	0.9	0.03	0.2	0.0	0.5	0.00	0.4	0.1	1.5	0.17	0.4	0.2	0.9	0.03	0.4	0.2	0.9	0.03	0.4	0.2	0.9	0.03	0.4	0.2	0.9	0.03
	HI	0.2	0.1	0.6	0.00	0.0	0.0	0.2	0.00	0.2	0.1	0.9	0.04	0.2	0.1	0.6	0.00	0.2	0.1	0.6	0.00	0.2	0.1	0.6	0.00	0.2	0.1	0.6	0.00
	LM*	-0.6	-3.5	2.4	0.71	0.5	0.1	1.8	0.29	0.1	0.0	0.4	0.00	0.9	0.6	1.3	0.46	0.5	0.1	2.4	0.39	0.5	0.2	1.3	0.14	0.5	0.2	1.3	0.14
	CA*	0.0	-0.2	0.2	1.00	1.0	0.9	1.1	1.00	0.5	0.2	1.5	0.19	0.9	0.4	2.0	0.79	1.0	0.1	7.1	1.00	1.0	0.9	1.1	1.00	1.0	0.9	1.1	1.00
	PAM3*	-1.2	0.0	1.2	1.00	1.0	0.7	1.4	1.00	0.4	0.1	1.0	0.05	1.4	0.9	2.3	0.11	1.0	0.3	3.6	1.00	1.0	0.8	1.3	1.00	1.0	0.8	1.3	1.00
	PEPG*	-9.3	-16.2	-2.3	0.01	0.0	0.0	0.1	0.00	0.2	0.1	0.6	0.00	1.0	0.7	1.4	0.97	0.1	0.0	1.3	0.08	0.1	0.0	0.3	0.00	0.0	0.0	0.1	0.00
	LPS	0.3	0.1	0.7	0.00	0.1	0.0	0.4	0.00	0.3	0.1	1.1	0.08	0.3	0.1	0.7	0.00	0.3	0.1	0.7	0.00	0.3	0.1	0.7	0.00	0.3	0.1	0.7	0.00
	R848	0.8	0.6	1.1	0.10	0.6	0.4	0.9	0.01	0.7	0.4	1.1	0.08	0.8	0.6	1.1	0.10	0.8	0.6	1.0	0.09	0.8	0.6	1.0	0.09	0.8	0.6	1.0	0.09
IL-10	BCG	1.1	0.8	1.6	0.47	1.1	0.7	1.9	0.59	0.9	0.5	1.4	0.57	1.2	0.8	1.7	0.31	1.2	0.8	1.7	0.32	1.2	0.8	1.7	0.34	1.2	0.8	1.7	0.36
	MTBK	1.0	0.6	1.9	0.94	0.9	0.4	2.1	0.76	1.4	0.6	3.5	0.41	1.2	0.6	2.2	0.64	1.2	0.6	2.2	0.63	1.1	0.6	2.1	0.67	1.1	0.6	2.1	0.66
	SA	0.9	0.7	1.3	0.71	1.2	0.7	1.9	0.54	0.9	0.5	1.5	0.66	1.0	0.7	1.4	1.00	1.0	0.7	1.4	0.98	1.0	0.7	1.4	0.96	1.0	0.7	1.4	0.92
	SP	1.1	0.8	1.4	0.72	1.0	0.7	1.6	0.84	0.7	0.4	1.2	0.18	1.1	0.8	1.5	0.59	1.1	0.8	1.5	0.58	1.1	0.8	1.5	0.55	1.1	0.8	1.5	0.57
	GAS	0.9	0.6	1.4	0.75	1.1	0.7	1.9	0.66	0.7	0.4	1.3	0.28	1.0	0.7	1.5	0.92	1.0	0.7	1.5	0.99	1.0	0.7	1.5	1.00	1.0	0.7	1.5	0.99
	EC	1.0	0.8	1.3	0.97	0.9	0.6	1.3	0.55	0.9	0.6	1.4	0.65	1.0	0.8	1.3	0.99	1.0	0.8	1.3	0.93	1.0	0.8	1.3	0.99	1.0	0.8	1.3	1.00
	HI	1.1	0.8	1.4	0.63	1.0	0.7	1.5	1.00	0.9	0.6	1.4	0.71	1.1	0.8	1.4	0.54	1.1	0.8	1.5	0.46	1.1	0.8	1.4	0.57	1.1	0.8	1.5	0.52
	LM	0.7	0.5	1.0	0.04	0.7	0.4	1.2	0.21	0.5	0.3	1.0	0.04	0.7	0.5	1.0	0.07	0.7	0.5	1.1	0.09	0.7	0.5	1.1	0.10	0.7	0.5	1.1	0.09
	CA	0.9	0.6	1.5	0.76	1.4	0.7	2.9	0.35	0.9	0.4	2.0	0.74	1.0	0.6	1.6	0.86	1.0	0.6	1.6	0.87	1.0	0.6	1.7	0.98	1.0	0.6	1.6	0.90
	PAM3	1.0	0.7	1.5	0.97	0.8	0.5	1.4	0.47	0.8	0.4	1.6	0.55	1.1	0.7	1.6	0.74	1.1	0.7	1.6	0.75	1.1	0.7	1.6	0.69	1.1	0.7	1.6	0.76
	PEPG	1.0	0.8	1.3	0.99	0.9	0.6	1.3	0.43	0.8	0.5	1.2	0.27	1.0	0.7	1.3	0.87	1.0	0.7	1.3	0.92	1.0	0.7	1.3	0.92	1.0	0.7	1.3	0.85
	LPS	1.0	0.8	1.3	0.90	0.9	0.6	1.3	0.61	0.9	0.6	1.3	0.44	1.0	0.8	1.3	0.89	1.0	0.8	1.3	0.82	1.0	0.8	1.3	0.92	1.0	0.8	1.3	0.90
	R848	1.0	0.8	1.2	0.80	0.9	0.6	1.3	0.54	0.9	0.6	1.2	0.40	1.0	0.8	1.3	0.92	1.0	0.8	1.3	0.96	1.0	0.8	1.3	0.94	1.0	0.8	1.3	0.88
MIF	BCG	1.0	0.9	1.2	0.54	1.2	0.9	1.5	0.17	1.1	0.9	1.4	0.39	1.0	0.9	1.2	0.62	1.0	0.9	1.2	0.63	1.0	0.9	1.2	0.65	1.0	0.9	1.2	0.66
	MTBK	1.1	1.0	1.3	0.07	1.2	1.0	1.5	0.03	1.2	1.0	1.4	0.06	1.1	1.0	1.3	0.06	1.1	1.0	1.3	0.06	1.1	1.0	1.3	0.06	1.1	1.0	1.3	0.06
	SA	1.1	1.0	1.2	0.06	1.3	1.1	1.5	0.00	1.1	0.9	1.3	0.38	1.1	1.0	1.2	0.08	1.1	1.0	1.2	0.07	1.1	1.0	1.2	0.07	1.1	1.0	1.2	0.08
	SP	1.1	1.0	1.2	0.19	1.1	0.9	1.4	0.16	1.0	0.8	1.2	0.82	1.1	0.9	1.2	0.34	1.1	0.9	1.2	0.30	1.1	0.9	1.2	0.29	1.1	0.9	1.2	0.29
	GAS	1.1	1.0	1.2	0.04	1.2	1.0	1.4	0.03	1.1	1.0	1.3	0.17	1.1	1.0	1.2	0.07	1.1	1.0	1.2	0.05	1.1	1.0	1.2	0.05	1.1	1.0	1.2	0.05
	EC	1.1	1.0	1.2	0.06	1.2	1.1	1.4	0.01	1.1	1.0	1.3	0.15	1.1	1.0	1.2	0.09	1.1	1.0	1.2	0.08	1.1	1.0	1.2	0.08	1.1	1.0	1.2	0.08
	HI	1.1	1.0	1.2	0.07	1.2	1.1	1.4	0.01	1.2	1.0	1.3	0.04	1.1	1.0	1.2	0.07	1.1	1.0	1.2	0.06	1.1	1.0	1.2	0.06	1.1	1.0	1.2	0.06
	LM	1.0	1.0	1.2	0.35	1.1	1.0	1.3	0.08	1.1	0.9	1.3	0.34	1.0	0.9	1.2	0.40	1.1	0.9	1.2	0.34	1.1	0.9	1.2	0.34	1.1	0.9	1.2	0.33
	CA	1.1	1.0	1.2	0.24	1.2	1.1	1.4	0.00	1.1	1.0	1.3	0.08	1.1	1.0	1.2	0.24	1.1	1.0	1.2	0.22	1.1	1.0	1.2	0.21	1.1	1.0	1.2	0.22
	PAM3	1.1	1.0	1.2	0.16	1.1	1.0	1.3	0.06	1.1	1.0	1.3	0.15	1.1	1.0	1.2	0.15	1.1	1.0	1.2	0.17	1.1	1.0	1.2	0.15	1.1	1.0	1.2	0.16
	PEPG	1.1	0.9	1.2	0.31	1.1	0.9	1.3	0.30	1.0	0.9	1.2	0.70	1.0	0.9	1.2	0.57	1.0	0.9	1.2	0.56	1.0	0.9	1.2	0.59	1.0	0.9	1.2	0.56
	LPS	1.1	1.0	1.2	0.10	1.2	1.0	1.3	0.02	1.1	1.0	1.3	0.16	1.1	1.0	1.2	0.14	1.1	1.0	1.2	0.12	1.1	1.0	1.2	0.12	1.1	1.0	1.2	0.12
	R848	1.1	1.0	1.2	0.09	1.2	1.0	1.4	0.01	1.1	1.0	1.3	0.16	1.1	1.0	1.2	0.13	1.1	1.0	1.2	0.11	1.1	1.0	1.2	0.11	1.1	1.0	1.2	0.11
MIG	BCG	1.5	1.3	1.7	0.00	1.7	1.4	2.0	0.00	1.5	1.2	1.9	0.00	1.5	1.3	1.7	0.00	1.5	1.3	1.7	0.00	1.5	1.3	1.7	0.00	1.5	1.3	1.7	0.00
	MTBK	1.4	1.2	1.7	0.00	1.9	1.4	2.4	0.00	1.7	1.3	2.4	0.00	1.5	1.2	1.8	0.00	1.5	1.2	1.8	0.00	1.5	1.2	1.8	0.00	1.5	1.2	1.8	0.00
	SA	1.1	1.0	1.2	0.02	1.3	1.1	1.4	0.00	1.2	1.1	1.4	0.00	1.1	1.0	1.2	0.02	1.1	1.0	1.2	0.01	1.1	1.0	1.2	0.01	1.1	1.0	1.2	0.02
	SP	1.1	1.0	1.3	0.07	1.2	1.0	1.4	0																				

		Primary analysis (BCG)				Age at BCG vaccine				Age				Mode of Delivery				Maternal BCG				Sex				Immunisation (2 v 3)			
		95% CI				95% CI				95% CI				95% CI				95% CI				95% CI				95% CI			
cytokine	stimulant	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value	GMR	LCI	UCI	p-value
IL-1ra	BCG	1.1	0.7	1.6	0.73	1.2	0.7	2.0	0.59	1.3	0.7	2.3	0.46	1.1	0.7	1.6	0.8	1.1	0.7	1.6	0.8	1.0	0.7	1.6	0.8	1.1	0.7	1.6	0.8
	MTBK	1.4	0.9	2.4	0.14	1.3	0.6	2.7	0.49	2.2	1.0	4.8	0.04	1.5	0.9	2.5	0.1	1.5	0.9	2.5	0.1	1.5	0.9	2.5	0.1	1.5	0.9	2.5	0.1
	SA	1.1	0.7	1.8	0.55	1.0	0.5	1.9	0.97	1.4	0.7	2.9	0.30	1.0	0.7	1.6	0.9	1.0	0.7	1.6	0.8	1.0	0.7	1.6	0.9	1.0	0.7	1.6	0.9
	SP	1.2	0.8	2.0	0.38	1.1	0.5	2.3	0.75	1.5	0.7	3.5	0.29	1.2	0.7	2.0	0.6	1.2	0.7	2.0	0.5	1.2	0.7	2.0	0.5	1.2	0.7	2.0	0.5
	GAS	1.2	0.8	1.9	0.38	1.2	0.6	2.3	0.57	0.9	0.5	1.9	0.83	1.2	0.7	1.9	0.5	1.1	0.7	1.8	0.6	1.2	0.7	1.8	0.6	1.1	0.7	1.8	0.6
	EC	1.1	0.8	1.5	0.58	1.1	0.7	1.8	0.73	1.5	0.9	2.5	0.16	1.1	0.8	1.6	0.6	1.1	0.8	1.6	0.6	1.1	0.8	1.6	0.6	1.1	0.8	1.6	0.6
	HI	1.2	0.9	1.6	0.29	1.1	0.7	1.8	0.63	1.0	0.6	1.6	0.99	1.2	0.8	1.6	0.3	1.2	0.9	1.7	0.3	1.2	0.8	1.6	0.3	1.2	0.9	1.7	0.3
	LM	1.4	0.8	2.2	0.20	1.1	0.6	2.2	0.76	1.6	0.7	3.3	0.24	1.3	0.8	2.2	0.3	1.3	0.8	2.2	0.3	1.3	0.8	2.2	0.2	1.4	0.8	2.2	0.2
	CA	1.2	0.8	1.8	0.48	1.4	0.8	2.7	0.27	1.6	0.8	3.1	0.16	1.2	0.7	1.8	0.5	1.2	0.7	1.8	0.5	1.2	0.7	1.8	0.5	1.2	0.7	1.8	0.5
	PAM3	1.5	1.1	2.2	0.02	1.2	0.7	2.0	0.57	2.3	1.3	4.1	0.00	1.5	1.0	2.2	0.0	1.5	1.0	2.2	0.0	1.5	1.0	2.2	0.0	1.5	1.0	2.2	0.0
	PEPG	0.9	0.6	1.3	0.45	0.9	0.5	1.6	0.85	0.9	0.5	1.6	0.74	0.8	0.6	1.2	0.4	0.8	0.6	1.2	0.4	0.8	0.6	1.2	0.3	0.8	0.6	1.2	0.4
LPS	1.0	0.7	1.4	0.97	1.0	0.6	1.6	0.88	1.3	0.8	2.0	0.34	1.0	0.7	1.4	0.8	1.0	0.7	1.4	0.9	1.0	0.7	1.4	0.8	1.0	0.7	1.4	0.9	
R848	1.0	0.8	1.4	0.82	0.9	0.6	1.4	0.70	1.0	0.6	1.4	0.84	1.0	0.7	1.3	0.9	1.0	0.7	1.3	0.9	1.0	0.7	1.3	0.9	1.0	0.7	1.3	0.9	
IL-6	BCG	1.4	1.0	1.8	0.03	1.6	1.1	2.3	0.02	1.3	0.9	1.9	0.10	1.4	1.0	1.8	0.0	1.4	1.0	1.8	0.0	1.4	1.0	1.8	0.0	1.4	1.0	1.8	0.0
	MTBK	1.1	0.6	2.2	0.78	0.8	0.3	2.3	0.71	1.6	0.6	4.3	0.36	1.2	0.6	2.5	0.6	1.2	0.6	2.4	0.7	1.2	0.6	2.4	0.7	1.2	0.6	2.4	0.6
	SA	1.1	0.9	1.4	0.42	1.3	0.9	1.9	0.13	1.0	0.7	1.4	0.91	1.1	0.9	1.5	0.3	1.1	0.9	1.4	0.3	1.1	0.9	1.5	0.3	1.1	0.9	1.4	0.3
	SP	1.1	0.8	1.3	0.63	1.0	0.7	1.4	0.96	0.9	0.7	1.3	0.62	1.0	0.8	1.3	0.7	1.1	0.8	1.4	0.6	1.1	0.8	1.4	0.6	1.1	0.8	1.4	0.6
	GAS	1.0	0.7	1.4	0.99	1.1	0.7	1.7	0.77	0.8	0.5	1.4	0.46	1.1	0.8	1.5	0.7	1.1	0.8	1.5	0.8	1.0	0.7	1.5	0.8	1.0	0.7	1.5	0.8
	EC	1.1	1.0	1.4	0.15	0.9	0.7	1.2	0.64	1.2	0.9	1.6	0.11	1.1	0.9	1.4	0.2	1.1	0.9	1.4	0.2	1.1	0.9	1.4	0.2	1.1	0.9	1.4	0.2
	HI	1.2	1.0	1.5	0.07	1.0	0.7	1.3	0.98	1.2	0.9	1.5	0.32	1.2	1.0	1.4	0.1	1.2	1.0	1.5	0.1	1.2	1.0	1.5	0.1	1.2	1.0	1.5	0.1
	LM	0.9	0.6	1.3	0.54	0.9	0.5	1.6	0.73	0.8	0.5	1.4	0.44	0.9	0.6	1.4	0.7	0.9	0.6	1.4	0.8	0.9	0.6	1.4	0.8	0.9	0.6	1.4	0.8
	CA	0.9	0.5	1.5	0.64	1.4	0.6	3.1	0.42	0.8	0.3	1.8	0.54	0.9	0.5	1.6	0.8	0.9	0.5	1.6	0.8	0.9	0.5	1.7	0.8	0.9	0.5	1.7	0.8
	PAM3	1.1	0.8	1.6	0.48	0.8	0.5	1.3	0.33	1.0	0.6	1.5	0.84	1.2	0.8	1.7	0.4	1.2	0.8	1.7	0.4	1.2	0.8	1.7	0.3	1.2	0.8	1.7	0.3
	PEPG	1.1	0.9	1.3	0.44	1.0	0.7	1.3	0.80	1.0	0.8	1.4	0.84	1.0	0.8	1.3	0.7	1.1	0.9	1.3	0.6	1.1	0.9	1.3	0.6	1.1	0.9	1.3	0.6
LPS	1.2	1.0	1.4	0.12	1.0	0.8	1.3	0.96	1.2	0.9	1.6	0.17	1.1	0.9	1.4	0.2	1.2	1.0	1.4	0.1	1.2	1.0	1.4	0.1	1.2	1.0	1.4	0.1	
R848	1.1	0.9	1.3	0.19	0.9	0.7	1.2	0.68	1.1	0.9	1.4	0.44	1.1	0.9	1.3	0.4	1.1	0.9	1.3	0.3	1.1	0.9	1.3	0.3	1.1	0.9	1.3	0.3	
IL-8	BCG	1.2	1.0	1.5	0.12	1.3	0.9	1.9	0.13	1.1	0.8	1.5	0.69	1.2	0.9	1.5	0.2	1.2	0.9	1.5	0.2	1.2	0.9	1.5	0.2	1.2	0.9	1.5	0.2
	MTBK	1.1	0.6	2.0	0.82	1.0	0.4	2.4	1.00	1.4	0.6	3.6	0.43	1.1	0.6	2.1	0.7	1.1	0.6	2.1	0.7	1.1	0.6	2.0	0.8	1.1	0.6	2.1	0.7
	SA	1.1	0.9	1.3	0.53	1.3	0.9	1.7	0.12	0.9	0.6	1.2	0.47	1.1	0.8	1.3	0.6	1.1	0.9	1.3	0.6	1.1	0.8	1.3	0.6	1.1	0.9	1.3	0.6
	SP	1.0	0.8	1.3	0.70	1.1	0.8	1.5	0.56	0.8	0.6	1.1	0.27	1.0	0.8	1.2	0.9	1.0	0.8	1.3	0.9	1.0	0.8	1.3	0.9	1.0	0.8	1.3	0.8
	GAS	1.1	0.9	1.4	0.46	1.3	0.9	1.8	0.16	1.0	0.7	1.4	0.82	1.1	0.8	1.4	0.6	1.1	0.9	1.4	0.5	1.1	0.9	1.4	0.5	1.1	0.9	1.4	0.5
	EC	1.2	1.0	1.5	0.05	1.2	0.9	1.6	0.24	1.1	0.8	1.5	0.55	1.1	0.9	1.4	0.3	1.1	0.9	1.4	0.2	1.1	0.9	1.4	0.3	1.1	0.9	1.4	0.2
	HI	1.2	1.0	1.5	0.07	1.1	0.8	1.5	0.51	1.0	0.7	1.4	0.98	1.2	0.9	1.5	0.2	1.2	0.9	1.5	0.2	1.2	0.9	1.5	0.2	1.2	0.9	1.5	0.2
	LM	0.9	0.7	1.3	0.70	1.0	0.7	1.6	0.82	0.9	0.5	1.4	0.53	0.9	0.7	1.3	0.6	0.9	0.7	1.3	0.7	0.9	0.7	1.3	0.7	1.0	0.7	1.3	0.8
	CA	0.9	0.6	1.3	0.65	1.4	0.8	2.5	0.20	0.7	0.4	1.3	0.31	0.9	0.6	1.4	0.7	0.9	0.6	1.4	0.6	0.9	0.6	1.4	0.7	0.9	0.6	1.4	0.7
	PAM3	1.1	0.9	1.5	0.34	1.0	0.7	1.4	0.81	0.9	0.6	1.3	0.66	1.1	0.9	1.5	0.4	1.1	0.9	1.5	0.4	1.1	0.9	1.5	0.4	1.1	0.9	1.5	0.4
	PEPG	1.0	0.8	1.3	0.74	1.0	0.8	1.4	0.79	0.9	0.6	1.2	0.32	1.0	0.8	1.3	1.0	1.0	0.8	1.3	0.9	1.0	0.8	1.3	1.0	1.0	0.8	1.3	1.0
LPS	1.3	1.0	1.6	0.02	1.2	0.9	1.7	0.17	1.1	0.8	1.6	0.52	1.2	1.0	1.5	0.1	1.2	1.0	1.5	0.1	1.2	1.0	1.5	0.1	1.2	1.0	1.5	0.1	
R848	1.1	0.9	1.5	0.31	1.1	0.8	1.6	0.59	1.0	0.7	1.4	0.85	1.1	0.8	1.4	0.6	1.1	0.8	1.4	0.6	1.1	0.8	1.4	0.7	1.1	0.8	1.4	0.7	
IP-10	BCG	1.2	0.9	1.6	0.23	1.2	0.8	1.7	0.38	1.3	0.9	2.0	0.16	1.1	0.9	1.5	0.3	1.1	0.9	1.5	0.3	1.1	0.9	1.5	0.3	1.1	0.9	1.5	0.3
	MTBK	1.0	0.7	1.3	0.77	1.2	0.8	1.8	0.36	1.0	0.6	1.6	0.98	1.0	0.7	1.3	1.0	1.0	0.7	1.3	0.8	1.0	0.7	1.3	0.8	1.0	0.7	1.3	0.9
	SA	0.9	0.7	1.2	0.47	1.1	0.8	1.6	0.54	1.1	0.7	1.6	0.71	1.0	0.7	1.2	0.7	0.9	0.7	1.2	0.7	1.0	0.7	1.2	0.7	1.0	0.7	1.2	0.7
	SP	1.0	0.8	1.3	0.89	1.2	0.8	1.7	0.42	1.3	0.8	1.9	0.25	1.1	0.8	1.4</													

4.3 DISCUSSION

This publication adds to the literature on BCG-induced heterologous immunity in several ways. It showed that alterations in cytokine responses following heterologous stimulation in BCG-vaccinated infants persist beyond the neonatal period and that effect modifiers including sex and maternal vaccination status remain significant. The most notable change in the immune response at this time point was the pattern of IFN- γ responses. The specific, anti-mycobacterial IFN- γ response in BCG-vaccinated infants, which was not seen in neonates is evident at this time point. In contrast, the median IFN- γ response to heterologous pathogens was decreased in BCG-vaccinated infants' due to a higher proportion of IFN- γ 'non-responders' in the BCG-vaccinated group. This finding is of particular relevance in light of a growing body of literature describing individual level variability in IFN- γ responsiveness following BCG vaccination [94, 99, 170].

The production of IFN- γ is central to the immune response following mycobacterial challenge, although it does not correlate directly with BCG efficacy against TB [191, 192]. Differential IFN- γ responses between populations has been suggested as a possible mechanism to explain the geographic variability in the efficacy of BCG [193]. Differential IFN- γ responses following BCG vaccination within populations were not successfully correlated with protection against TB in S. African infants but were associated with a change in the clinical response to *P. falciparum* in a human challenge model of malaria [45, 106]. Whether this variability between and within populations represents a genetically or environmentally determined effect modifier of BCG vaccine remains unclear [194, 195].

Several studies show variation in IFN- γ responses between BCG-vaccinated infants in different geographic locations and concluded that environmental exposures such as *in utero* mycobacteria might be the cause [94, 99, 170]. More recently there is evidence of variability in IFN- γ responses in neonates from a single geographic location, but who are differentiated by mycobacterial exposure in their mothers. Mawa *et al.* found that the response to BCG vaccine differed between infants whose mother had a diagnosis of LTBI compared to controls and in infants whose mothers had a BCG vaccine scar

compared to controls [196]. The immune response of infants of BCG scar positive mothers was characterised by increased IFN- γ , IL-12, IL-10, TNF- α and IL-1 β production when compared to infants of mothers with no BCG scar which is in keeping with the findings of the MIS BAIR study.

However a study in BCG-naïve, TST negative, adult volunteers in Denmark who received BCG vaccination, provides evidence that BCG vaccination can induce 'dichotomous' immune responses within a homogenous population with no prior mycobacterial exposure [197]. Three months following BCG vaccination, there was a clear division between individuals who developed intense skin inflammation, a positive IFN- γ response to PPD and IFN- γ ⁺IL-2⁺ TNF- α ⁺ polyfunctional CD4⁺ and IL-17A⁺CD4⁺ T cells and those individuals who had a low skin inflammation score, no IFN- γ response to PPD and expression of CD8⁺ regulatory T cells. This finding supports an individual level genetic rather than an environmental cause for variability in the IFN- γ response.

In summary, there is a complex relationship between IFN- γ levels prior to BCG vaccination (priming), the magnitude of IFN- γ response post BCG vaccination and subsequent BCG vaccine responses to heterologous stimulation. Furthermore, these effects vary between individuals and between populations and may have important implications for both the efficacy of BCG against TB and the observed beneficial non-specific effects of BCG vaccination. Future studies need to consider (i) the effect of this pattern of response on the power of the study to correlate the BCG vaccine response with clinical correlates of disease (ii) the association of dichotomous responses with protection from or control of infection and (iii) whether the effect is due to underlying genetic polymorphisms or differential environmental exposures.

4.4 LIMITATIONS

The results of this study need to be interpreted acknowledging that all participants had received at least two doses of routine scheduled vaccines subsequent to randomisation to BCG vaccine or no BCG vaccine. The major limitation remains the inability to link these results to clinical correlates of disease or immunological correlates of protection. Although it appeared that the effect of BCG vaccine on IFN- γ

responsiveness was restricted to Gram-negative organisms and LPS. Analysis of the proportion of responders clearly shows that the pattern of individual level responsiveness is the same following heterologous stimulation with Gram-positive organisms but that the lower level of response to these stimulants made the effect more difficult to detect in the given sample size. The use of a cohort study design In chapter 5 addresses this limitation by providing within patient comparisons of heterologous cytokine responses.

Another limitation of the MIS BAIR cytokine analysis reported in chapters 3 and 4 was the failure to run paired biological samples from the same participant on the same assay batch at both time points. This omission precluded any longitudinal analysis of heterologous cytokine responses. Finally, although non-vaccine serotypes of *S. pneumoniae* were deliberately included in the whole blood assay, an effect of individual variation in exposure to this bacterium (with associated presence of antibodies) cannot be excluded.

4.4.1 CONCLUSIONS

Heterologous cytokine responses following neonatal BCG vaccination persist beyond the neonatal period and up to 7 months of age. The significant findings at this time-point are indicative of changes in both innate (MIG, IL-8, IL-1Ra) and cell-mediated immunity (IFN- γ) in BCG-vaccinated infants. The observation that BCG vaccination leads to a higher proportion of infants who do not mount an IFN- γ response following heterologous stimulation is of particular interest. Future research need to consider the potential for genetic or environmentally induced changes in IFN- γ responses on study outcomes and should focus on determining the clinical significance of this effect.

Chapter 5 A SYSTEMS VACCINOLOGY APPROACH TO UNDERSTANDING THE HETEROLOGOUS IMMUNE RESPONSE TO BCG IN INFANTS

5.1 INTRODUCTION

The results in chapters 3 and 4 showed differential cytokine production between BCG-vaccinated and BCG-naïve neonates and infants following *in vitro* whole blood stimulation with heterologous pathogens and TLR ligands [198]. Two key limitations of these studies are (i) the effect of inter-individual variability and (ii) the characterisation of the immune response using a single assay. To overcome these limitations and leverage the insights gained from the cytokine analyses in MIS BAIR participants, the BabyBAIR study was conceptualised. This study compared the immune response to BCG within each infant using a systems vaccinology approach.

The WHO-commissioned review of the immunological basis for the beneficial non-specific effects of BCG vaccine called for studies using a systems vaccinology approach [90]. In systems vaccinology studies, early post vaccination transcriptomic signatures are correlated with the development of protective antibodies (an immunologic correlate) [102, 199] or the development of disease (a clinical correlate)[106].

In the BabyBAIR study, blood was collected prior to (baseline) and post BCG vaccination (7 days and 3 months). The cytokine and flow cytometry analyses in BabyBAIR describe heterologous responses to BCG vaccination i.e. post *in vitro* stimulation with heterologous pathogens, while the RNASeq data describes the unstimulated whole blood transcriptome post BCG vaccination. In the absence of an available clinical or immune correlate of protection, analysis of this data is hypothesis generating.

5.2 PARTICIPANTS AND RECRUITMENT

Participants were recruited from infants attending the RCH for BCG vaccination prior to overseas travel. Exclusion criteria were: (i) fever within the last 48 hours; (ii) any vaccine other than BCG on the day of recruitment; (iii) any household exposure to TB; and (iv) prior BCG vaccination. Infants were vaccinated intradermally with BCG-Denmark (Statens Serum Institute, SSI) 0.05 mL (<12 months of age) or 0.1 mL (≥12 months of age). Infants had blood collected at three time points: (i) prior to and on the same day as BCG vaccination (baseline); (ii) 7 ($\pm$ 2) days post BCG vaccination (7-day);

and (iii) 3 months ($\pm$ 2 weeks) post BCG vaccination (3-month). Only infants with a baseline sample and at least one post-vaccination blood sample were included.

Blood collection was done at RCH or in the participant's home. Blood was collected via a closed loop system into endotoxin-free sodium-heparin tubes (S-monovette, Numbrecht, Germany). Samples were kept at room temperature and protected from direct sunlight during transport to the laboratory. Samples were processed 4 hours ($\pm$ 10%; 24 minutes) after collection.

As a result of the limited blood volume that could be obtained from some infants, blood samples were processed in the following order of priority: (i) whole blood stimulation assay for multiplex cytokine analysis; (ii) whole blood stimulation assay with brefeldin A for flow cytometry; (iii) whole blood storage in TRIzol for RNASeq; (iv) IL-12p40 measurement by ELISA. The final number of blood samples and overlap between assays is described in the results section (Figure 6).

The protocol and participant information and consent form (PICF) for this study are provided as appendix 7a and 7b. This protocol and associated materials were approved by the RCH HREC No. 33128F.

5.3 LABORATORY METHODS

5.3.1 MEASUREMENT OF CYTOKINES FOLLOWING WHOLE BLOOD STIMULATION WITH HETEROLOGOUS PATHOGENS AND TOLL-LIKE RECEPTOR (TLR) LIGANDS

Whole blood *in vitro* stimulation assays were done as described in chapters 3 and 4. The BabyBAIR laboratory standard operating procedure is given as appendix 6. Briefly, whole blood, diluted 1:1 with RPMI-1640 medium (GlutaMAX™ Supplement, HEPES, Gibco, Life Technologies), was stimulated at 37°C (5% CO₂: air) for 20 ($\pm$ 2) hours with BCG-Denmark 75 μ g/mL, killed *Mycobacterium tuberculosis* 1.0 x 10⁶ CFU/mL, *Streptococcus agalactiae* (group B streptococcus / GBS) 1.0 x 10⁷ CFU/mL, *Escherichia coli* 1.0 x 10⁶ CFU/mL, *Haemophilus influenzae* type B 1.0 x 10⁶ CFU/mL, *Staphylococcus aureus* 1.0 x 10⁷ CFU/mL, *Streptococcus pneumoniae* serotype 15C 1.0 x 10⁷ CFU/mL and *Listeria monocytogenes* 1.0 x 10⁷ CFU/mL, *Candida albicans* 1.0 x

10⁶ CFU/mL and the TLR ligands lipopolysaccharide [LPS] 100 ng/mL, resiquimod [R848] 3.5 µg/mL, peptidoglycan [PEPG] 10 µg/mL and (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride [Pam3CSK4] 1 µg/mL (InvivoGen, (USA)).

The following cytokines were measured in supernatants using an xMAP Luminex 200 Analyser as described previously: IFN-γ, TNF-α, IL-6, IL-1β, MIF, IL-8, MIG, MCP-1 (MCAF), MIP-1α, MIP-1β, IL-10, IL-1ra (Bio-Rad, California). IL-12p40 was measured using the Human IL-12/-23 (p40) ELISA development kit (Mabtech, Sweden) as per the manufacturer's instructions. IL-12p40 analysis was done post-hoc on review of the data from the flow cytometry analysis.

5.3.2 FLOW CYTOMETRY FOLLOWING WHOLE BLOOD STIMULATION WITH HETEROLOGOUS PATHOGENS AND TOLL-LIKE RECEPTOR (TLR) LIGANDS.

Whole blood was stimulated as above with the addition of brefeldin A (10 µg/mL, Sigma-Aldrich, USA). Samples were incubated for 4 hours at 37°C before fixation with FACS lysing solution (BD Biosciences, USA) and storage at -80°C. Frozen samples were subsequently thawed at 37°C and permeabilised with Perm2 buffer (BD Biosciences, USA) for 10 min and stained for 30 min in the dark at room temperature using the following fluorochrome-conjugated monoclonal antibodies: CD11c-APC, CD14-V500 (561392), IFN-α-PE (560097) (BD Biosciences, USA); CD66-Biotin (custom made, Biolegend, USA) and subsequently conjugated to BV786-streptavidin (BD Bioscience, USA), CD123-PE-Cy7, HLA-DR-efluor605, IFN-γ-APC-efluor780, IL6-PerCP-efluor710, IL12p40-efluor450, TNF-α-AlexaFluor700 (BD Biosciences, USA). Cells were washed in PBS with 1% BSA after each step, and twice after staining. Cells were counted using a LSRII flow cytometer (BD Biosciences). Compensation was done using CompBead anti-mouse-Ig (BD Biosciences). Cytometer setup, tracking beads (BD Biosciences) and control samples (volunteer adult blood stimulated with R848 in a similar assay) were acquired at each acquisition to determine LSRII baseline and variability.

Flow cytometry data was analysed using FlowJo v.10.2 (TreeStar, Ashland, USA). Granulocytes (CD66+) were removed from the analysis as they have a high auto-

fluorescence that could appear as cytokine producing cells in subsequent gates, producing false positive results. Cells were then separated into antigen presenting cells (HLA-DR+, CD14-) and monocytes (HLADR+/int, CD14+). Antigen presenting cells were further classified as plasmacytoid dendritic cells (CD123) and CD11C/CD14 myeloid dendritic cells (mDCs).

5.3.3 RNA EXTRACTION FROM WHOLE BLOOD

After sampling, whole blood was centrifuged at 5000g for 7 minutes, plasma was removed and the remaining pellet was resuspending in TRIzol (1:1) prior to storage at -80°C. For RNA extraction, frozen TRIzol samples were thawed and incubated for five minutes at room temperature. Chloroform was added at a concentration of 200 µl to 1 ml of TRIzol (80 µl) and incubated at room temperature for three minutes. Samples were then centrifuged at 12,000xg for 15 minutes at 4°C. The resulting aqueous phase was removed and loaded onto a gDNA eliminator column (Qiagen, Netherlands) and centrifuged for 30 seconds at 8000xg. 400 µl of 70% ethanol was added to the gDNA eliminator column (Qiagen). The sample was then transferred 200 µl at a time to RNAeasy spin column (Qiagen), incubated for 3 minutes at room temperature and then centrifuged for 30 seconds at 8000xg. RPE Buffer (Qiagen) in a solution of 1:4 with absolute alcohol was added to the spin column. The RNAeasy spin column was then centrifuged for 15 seconds twice at 10,000xg. Next 500 µl of 70% alcohol is added to the RNAeasy spin column which is now centrifuged at 8000xg for 2 minutes followed by a further 5 minutes centrifugation. Finally, 30 µl of RNase free water was placed on the column which was incubated for 10 minutes at room temperature. RNA was eluted into a collection tube following a further 1 minute centrifugation at 8000xg. All RNA samples were checked for purity using a ND-1000 spectrophotometer (NanoDrop Technologies) and for integrity by electrophoresis on a 2200 BioAnalyzer (Agilent Technologies).

5.3.4 RNA SEQUENCING

Library preparation was done at the Victorian Centre for Clinical Genetics (VCGS), using the TruSeq Stranded total RNA prep kit (Illumina, USA) including globin depletion and

RiboZero depletion of rRNA. RNA was also sequenced at VCGS with NextSeq v2 chemistry (Illumina) using 1x75 bp reads.

5.4 STATISTICAL METHODS

5.4.1 STATISTICAL METHODS FOR CYTOKINE DATA

Statistical analysis was done using Stata version 14.1. Cytokine responses below the lower limit of detection were replaced in a standardised fashion using the same methodology as Chapters 4 and 5. There were no data excluded for being above the upper limit of detection. Data from each cytokine / stimulant pair were visualised to inform the appropriate choice of statistical method (parametric versus non-parametric). Comparisons between baseline and 7-day cytokine values were then done using: (i) paired t-test (means with 95%CI); (ii) paired t-test with sensitivity analysis for the effect of excluding single outliers (means with 95%CI); or (iii) Wilcoxon-rank sum test (median with IQR) based on the observed distribution (Figure 5.1). All comparison between baseline and 3-month samples required non-parametric analysis due to small sample numbers.

Subset analyses to investigate differences in cytokine responses following BCG vaccination between: (i) males and females; and (ii) infants whose last vaccine was live (BCG as first vaccine or any MMR-containing vaccine) and non-live (hepatitis B vaccination or Infanrix Hexa™) were limited to cytokines identified as significantly modified by BCG vaccination in the main analysis. All subset analyses were done using Wilcoxon-rank sum ($p < 0.05$).

Fold-change (7-day value: baseline value) data for each cytokine stimulant pair was grouped by Spearman's ranked correlation and presented as a heatmap. Two participant clusters were identified and Fisher's exact test was done to test for an association between belonging to a given cluster and: (i) sex; (ii) nature of last vaccine administered (live versus non-live); (iii) age; (iv) childcare attendance; (v) prior overseas travel.

	IFN- γ	TNF- α	IL-1 α	IL-1 β	IL-6	IL-8	IL-10	MCP-1	MIF	MIG	MIP-1 α	MIP-1 β	IL-12p40
BCG	WRS	T	T	T	T	T	T	T	T	T(S)	T(S)	T	T
MTBK	WRS	T	T	T	T	T	T(S)	T	T	T	T	T(S)	T
GBS	WRS	T	T	T	T	T	T	T	T	T(S)	T(S)	T	T(S)
EC	WRS	T	T	T	T	T	T	T	T	T(S)	T	T	T(S)
HI	WRS	T	T	T	T	T	T	T	T	T	T	T	T(S)
LM	WRS	T	T	c	T	T	T	T	T	T	T(S)	T	T(S)
SA	WRS	T	T	T(S)	T	T	T(S)	T	T	T(S)	T	T	T
SP	WRS	T	T	T	T	T	T	T	T	T	T	T	N/A
CA	WRS	WRS	T	T(S)	T	T	WRS	T	T	T	WRS	T	WRS
PEPG	WRS	T	T	T	T	T	T	T	T	T	T(S)	T	T
LPS	WRS	T	T	T	T	T(S)	T	T	T	T	T(S)	T	T(S)
PamCys3	WRS	T	T	T	T	T	T	T	T	T	T(S)	T	N/A
R848	WRS	T	T	T	T	T	T	T	T	T	T	T	T(S)
NIL	WRS	WRS	WRS	WRS	WRS	WRS	WRS	WRS	T	T(S)	WRS	T(S)	WRS

Figure 5.1 Overview of statistical methods used for the comparison of 7-day heterologous cytokine responses

WRS = Wilcoxon ranked sum, T = paired t-test, T(S) = paired t-test repeated as a sensitivity analysis.

BCG = Bacille Calmette-Guerin, MTBK = *M. tuberculosis*, EC = *E. coli*, HI = *H. influenzae*, CA = *C. albicans*, GBS = group B streptococcus (*S. agalactiae*), LM = *L. monocytogenes*, SA = *S. aureus*, SP = *S. pneumoniae*, PEPG = Peptidoglycan, PAM = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, LPS = lipopolysaccharide, R848 = resiquimod, NIL = RPMI.

5.4.2 STATISTICAL ANALYSIS OF FLOW CYTOMETRY DATA

The proportion of cytokine-producing cells for each cell type (monocytes, myeloid dendritic cells and plasmacytoid dendritic cells) was compared between baseline and 7-day using Wilcoxon-rank sum test using Prism 5 (GraphPad Software, La Jolla, USA).

5.4.3 ANALYSIS OF RNASEQ DATA

Gene expression fold-change of each subject was obtained by subtracting the 7-day or 3-month log₂ expression values from its corresponding baseline value. Genes were filtered out if no increase or decrease greater than 1.25-fold was observed at either post-vaccination time point. RNASeq raw counts were normalized with DESeq (R statistical software) following a moderated t-test to identify differentially expressed genes between groups. P-values were calculated for each gene over the time course by combining the data for all the participants. The calculations were done for 7-day and 3-month versus baseline, considering a p-value ≤ 0.05 to be significant [200].

Differentially expressed genes were reviewed using Genecards™ gene ontology (GO) platform to identify genes associated with both innate and adaptive immunity.

Differentially upregulated and downregulated gene lists were input into the Geneanalytics™ platform, filtered for human tissue and analysed for associated biological processes, molecular functions and phenotypes. Observations from this preliminary round of enquiry was used to inform the supervised analyses.

The supervised analysis included enrichment analysis with the program Gene Set Enrichment Analysis (GSEA) [201], using the REACTOME genesets [202]. Functional gene set enrichment analysis is a non-parametric method of pathway analysis. The list of differentially expressed genes at each time point was ordered by fold change to determine if an identifiable phenotype, determined by benchmarking against existing annotation databases, was significantly enriched post BCG vaccination compared to baseline. The degree of enrichment, or enrichment score was calculated using the Kolmogorov-Smirnov statistic and significance estimated using a phenotype-based permutation test. The normalised enrichment scores (NES) indicate the strength of the association to a given pathway [201]. Protein-protein interaction networks were built

with up and down regulated genes using the NetworkAnalyst program [203] and an interactome database [204].

5.5 RESULTS

5.5.1 STUDY POPULATION

Demographic features of the study participants included in each analysis are shown in Table 5.1. The majority of patients were of Asian and South East Asian ethnicity. Retention in the study was hampered by multiple sampling time points and a high rate of overseas travel before the 3-month time point.

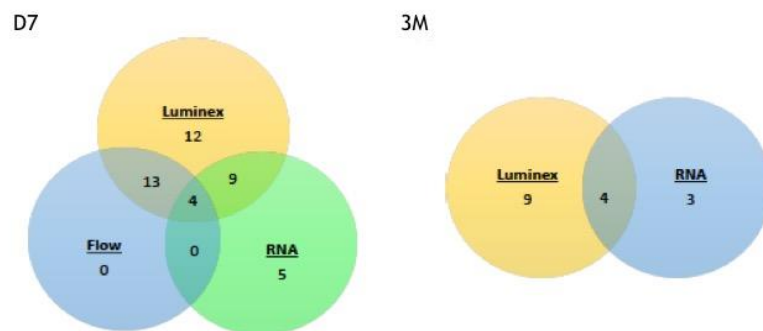


Figure 5.2 Overlap of samples by assay type at each time point in the BabyBAIR study.

Number of samples included in paired analysis at baseline and 7 days (D7) and 3 months (3M.) Luminex = multiplex cytokine analysis on supernatants harvested following whole blood stimulation with heterologous pathogens and TLR. Flow = flow cytometry done on supernatants harvested following whole blood stimulation with heterologous pathogens and TLR ligands. RNA = RNASeq following RNA extraction from whole blood.

		Cytokine Analysis			Flow Cytometry	RNAseq	
		D0 – D7	D0 – 3M	D0 – D7 – 3M	D0 – D7	D0 – D7	D0 – 3M
Paired samples		39	13	10	17	18	7
Sex	Male	16 (41.0)	4 (30.8)	4 (40.0)	5 (29.4)	9 (50)	4 (57.1)
	Female	23 (59.0)	9 (69.2)	6 (60.0)	12 (70.6)	9 (50)	3 (42.9)
Age (years)	Mean (SD)	0.9 (1.3)	0.5 (0.5)	0.5 (0.5)	1.3 (1.8)	0.9 (1.8)	1.9 (2.7)
	≤ 6m	26 (66.6)	9 (69.2)	7 (70)	10 (58.8)	14 (77.8)	4 (57.1)
	> 6m-12m	4 (10.3)	2 (15.4)	1 (10)	3 (17.7)	0 (0)	0 (0)
	> 12m-24m	5 (12.8)	2 (15.4)	2 (20)	4 (23.5)	2 (11.1)	1 (14.3)
	> 24m	4 (10.3)	0 (0)	0 (0)	0 (0)	2 (11.1)	2 (28.6)
Ethnicity	South-East Asian	20 (51.3)	7 (53.8)	5 (50)	9 (52.9)	12 (66.7)	6 (85.7)
	Asian	7 (18.0)	5 (38.5)	4 (40)	2 (11.8)	4 (22.1)	1 (14.3)
	Caucasian	2 (5.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Hispanic	1 (2.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	African	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)
	Middle Eastern	2 (5.1)	0 (0)	0 (0)	1 (5.9)	1 (5.6)	0 (0)
	Mixed	7 (18.0)	1 (7.7)	1 (10.0)	5 (29.4)	1 (5.6)	0 (0)
Infection in last month	Yes	5 (12.8)	0 (0)	0 (0)	3 (17.7)	2 (11.1)	0 (0)
	No	34 (87.2)	13 (100)	10 (100)	14 (82.3)	16 (88.9)	7 (100.0)
Attends childcare	Yes	8 (20.5)	2 (15.4)	1 (10)	5 (29.4)	2 (11.1)	2 (28.6)
	No	31 (79.5)	11 (84.6)	9 (90)	12 (70.6)	16 (88.9)	5 (71.4)
Last vaccine	No prior	5 (12.8)	4 (30.7)	4 (40)	3 (17.7)	1 (5.6)	1 (14.3)
	Hepatitis B	2 (5.1)	2 (15.4)	1 (10)	1 (5.6)	4 (22.2)	1 (14.3)
	Infanrix Hexa + Prevenar 13 + Rotavirus	24 (61.5)	6 (46.2)	4 (40)	8 (47.2)	9 (50.0)	3 (42.9)
	MMR + Prevenar 13	1 (2.6)	0 (0)	0 (0)	1 (5.9)	2 (11.1)	1 (14.3)
	MMRV + Hib	4 (10.3)	1 (7.7)	1 (10)	2 (11.8)	1 (5.6)	1 (14.3)
	Not recorded	3 (7.7)	0 (0)	0 (0)	2 (11.8)	1 (5.6)	0 (0)

Table 5.1 Characteristics of participants in the BabyBAIR study at each time point.

Blood sample at baseline (D0), 7 ±2 days post BCG vaccination (D7) and 3 months ±2 weeks post BCG vaccination (3M).

SD = standard deviation, Infanrix hexa = diphtheria + tetanus + pertussis + *H. influenzae* B (Hib) + inactivated poliovirus + hepatitis B, Prevenar 13= 13 valent conjugated pneumococcal vaccine, MMR (V) = measles + mumps + rubella + (varicella)

5.5.2 CYTOKINE ANALYSIS RESULTS

5.5.2.1 7 days post BCG vaccination

BCG vaccination leads to an 'early' pro-inflammatory response following stimulation with mycobacterial and heterologous pathogens, and Pam3CYSK4

The results of the 7-day cytokine data analysis is shown in Figure 5.3. Compared to baseline, at 7 days post BCG vaccination, participants had decreased concentrations of IL-10 in RPMI-stimulated (nil) samples. Following stimulation with the specific mycobacterial stimulant BCG, TNF- α production was increased 7 days post BCG vaccination. Following stimulation with the heterologous bacteria *E. coli*, *H. influenzae* and group B streptococcus (GBS), IFN- γ and TNF- α production were both increased at 7-days post BCG vaccination compared to baseline. Stimulation with heterologous bacteria also resulted in increased IL-6, IL-1 β and IL-12p40 responses at 7 days compared to baseline. IFN- γ , TNF- α , IL-1 β , IL-6, and IL-10 responses to the TLR ligand Pam3CYSK4 were also increased at 7 days compared to baseline, but this finding was statistically significant only for IFN- γ , TNF- α and IL-10. Changes in chemokine responses at 7 days post BCG vaccination included significant increases in production of MIP-1 β in response to *H. influenzae* and MIG (CXCL9) in response to R848.

5.5.2.2 3 months post BCG vaccination

The development of a 'specific' Th1 response at 3 months post BCG vaccination is accompanied by downregulation of the pro-inflammatory heterologous response

Compared to baseline, at 3 months post BCG vaccination, participants had increased IFN- γ responses in RPMI-stimulated (nil) samples. Following stimulation with BCG and *M. tuberculosis* there was also increased production of IFN- γ . Conversely, compared to baseline, at 3 months post BCG vaccination, participants had decreased production of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) responses following stimulation with multiple heterologous pathogens and TLR-ligands (Figure 5.4). IL-10 responses was also decreased following stimulation with *S. aureus* and *M. tuberculosis*.

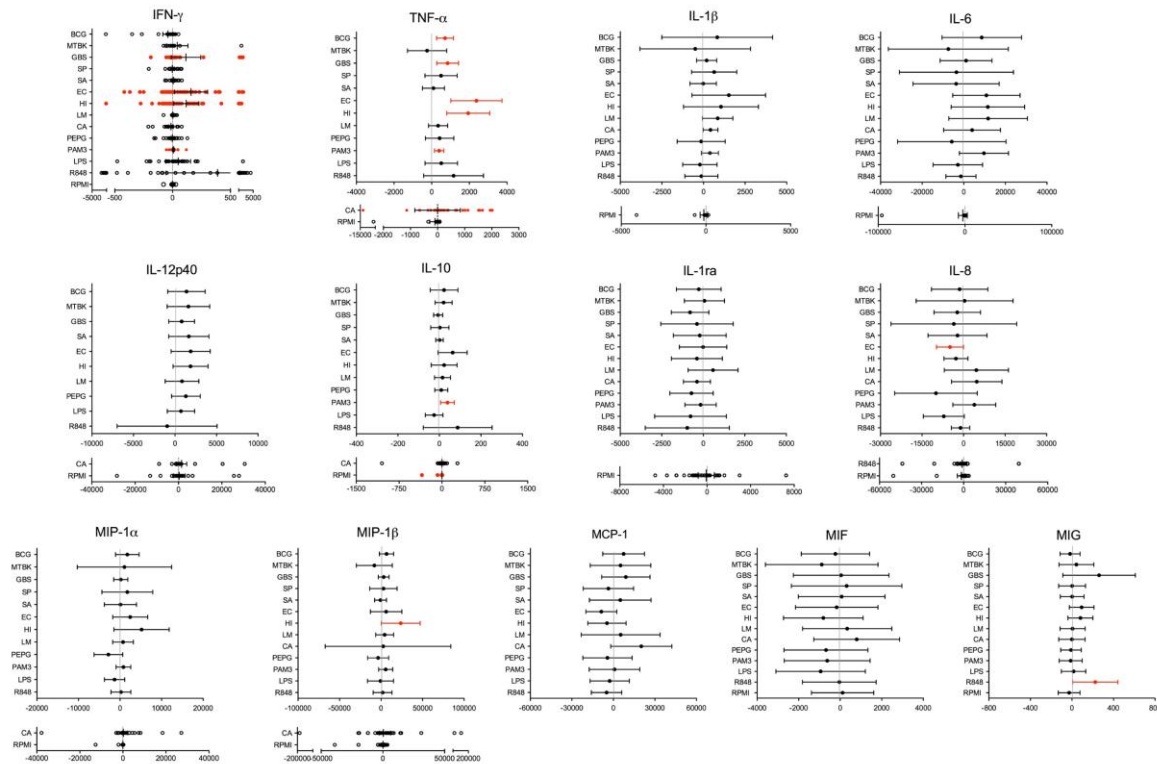


Figure 5.3 Cytokine responses at 7 days post BCG vaccination compared to baseline

All comparisons are Wilcoxon-rank sign tests presented as dotplots with median (IQR). Results with $p < 0.05$ are depicted in red. BCG = Bacille Calmette-Guerin ($n=39$), MTBK = *M. tuberculosis* ($n=33$), EC = *E. coli* ($n=39$), HI = *H. influenzae* ($n=39$), CA = *C. albicans* ($n=35$), GBS = Group B streptococcus ($n=38$), LM = *L. monocytogenes* ($n=34$), SA = *S. aureus* ($n=35$), SP = *S. pneumoniae* ($n=33$), PEPG = Peptidoglycan ($n=38$), PAM = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride ($n=38$), LPS = lipopolysaccharide ($n=38$), R848 = resiquimod ($n=39$), NIL = RPMI ($n=39$)

The production of IFN- γ and MIG (CXCL9) were similarly decreased at 3 months post BCG vaccination compared to baseline in response to stimulation with *E. coli* and *H. influenzae*, however this was statistically significant only for MIG (CXCL9)/*H. influenzae*. There was a general increase in the production of IL-8 at 3 months post BCG vaccination which was statistically significant for responses to stimulation with multiple pathogens (*M. tuberculosis*, *E. coli*, *H. influenzae*, *L. monocytogenes*, *S. aureus*, *S. pneumoniae*) and the TLR ligands (LPS, Pam3CYSK4 and PEPG). There was a similar increase in IL-10 production in response to stimulation with *E. coli* and *H. influenzae* and MIP-1 β in response to *S. aureus* and *S. pneumoniae* (Figure 5.4). The following isolated cytokine/stimulant pairs had significantly increased responses at 3-month: IL-1 α /Pam3CYSK4, MIP-1 α /Pam3CYSK4 and MIF/R848) or decrease (MCP-1/PEPG).

5.5.2.3 Cytokine data from all three time points

Development of the heterologous immune response is mediated by pathogen-specific changes in the production of IL-1 α , IL-10, IL-12p40 and upregulation of IL- 8

The pattern of cytokine response following BCG vaccination varied by pathogen (Figure 5.5). The low sample number (n=10) precluded statistical analysis of the subset of samples with data at all three time points.

Stimulation with mycobacteria led to increased IFN- γ at 7 days (BCG) and 3 months (BCG, *M. tuberculosis*). The Gram negative bacteria, *H. influenzae* and *E. coli* induced a distinctive pattern of cytokine (IFN- γ , TNF- α , IL-12p40) and chemokine (MIG) responses which peaked at 7 days and fell below baseline at 3 months. IL-1 β fell to below baseline at 3 months but it did not peak at 7 days with the other pro-inflammatory cytokines. Stimulation with Gram positive bacteria (*S. aureus*, *S. pneumoniae*, group B streptococcus), and to a lesser extent the intracellular Gram positive bacterium *L. monocytogenes*, led to a similar decrease in pro-inflammatory cytokine (TNF- α , IL-1 β) responses at 3 months compared to baseline, however this was a gradual decline without a peak at 7 days post vaccination. Of note, IL-8 responses were consistently upregulated between 7 days and 3 months for the majority of stimulants.

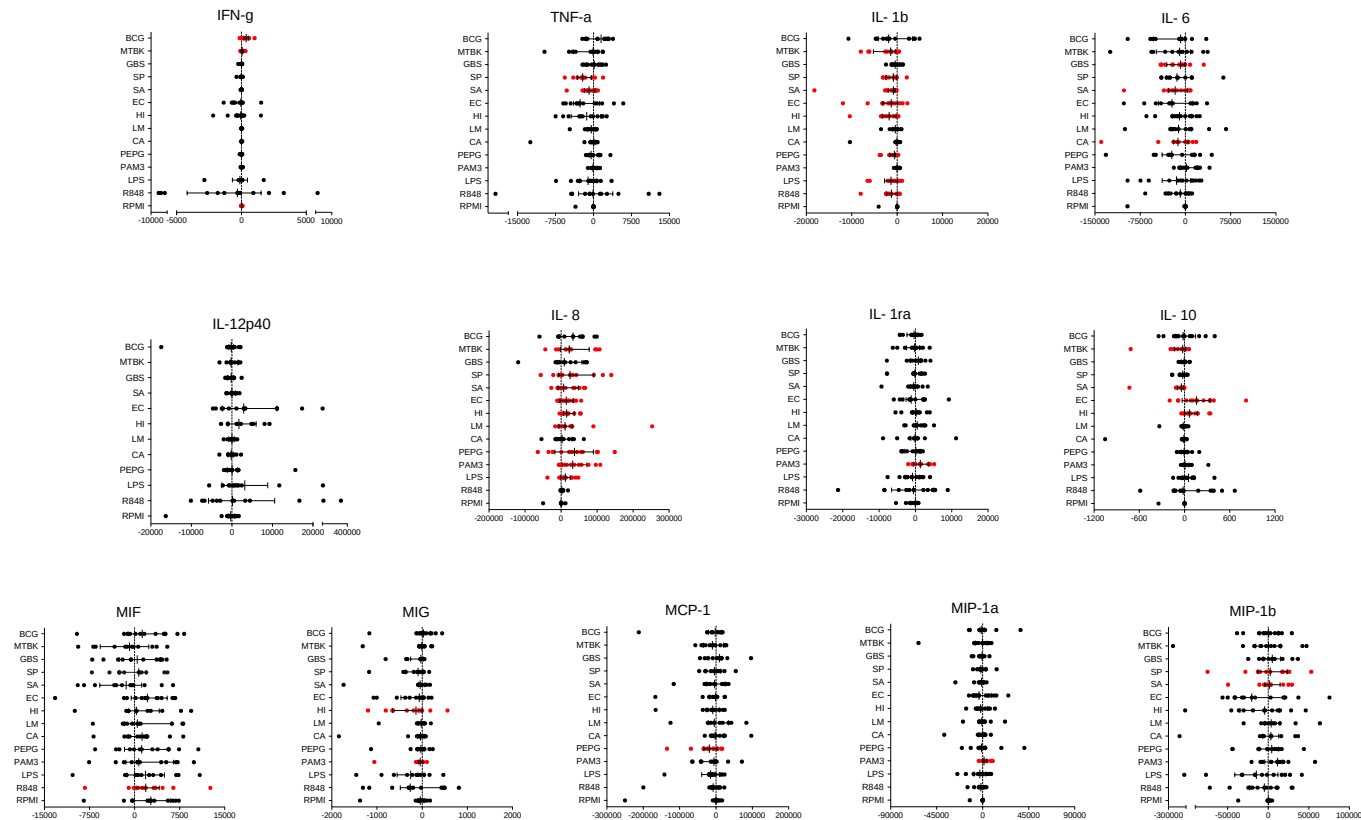


Figure 5.4 Cytokine responses at 3 months post BCG vaccination compared to baseline.

Wilcoxon-rank sign test are presented as dotplots with median (IQR) and t-tests as mean (95% CI). Results with $p < 0.05$ are depicted in red. BCG = Bacille Calmette-Guerin (n=13), MTBK = *M. tuberculosis* (n=12), EC = *E. coli* (n=13), HI = *H. influenzae* (n=12), CA = *C. albicans* (n=12), GBS = Group B streptococcus (n=12), LM = *L. monocytogenes* (n=11), SA = *S. aureus* (n=12), SP = *S. pneumoniae* (n=11), PEPG = Peptidoglycan (n=13), PAM = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride (n=12), LPS = lipopolysaccharide (n=13), R848 = resiquimod (n=13), NIL = RPMI (n=13).

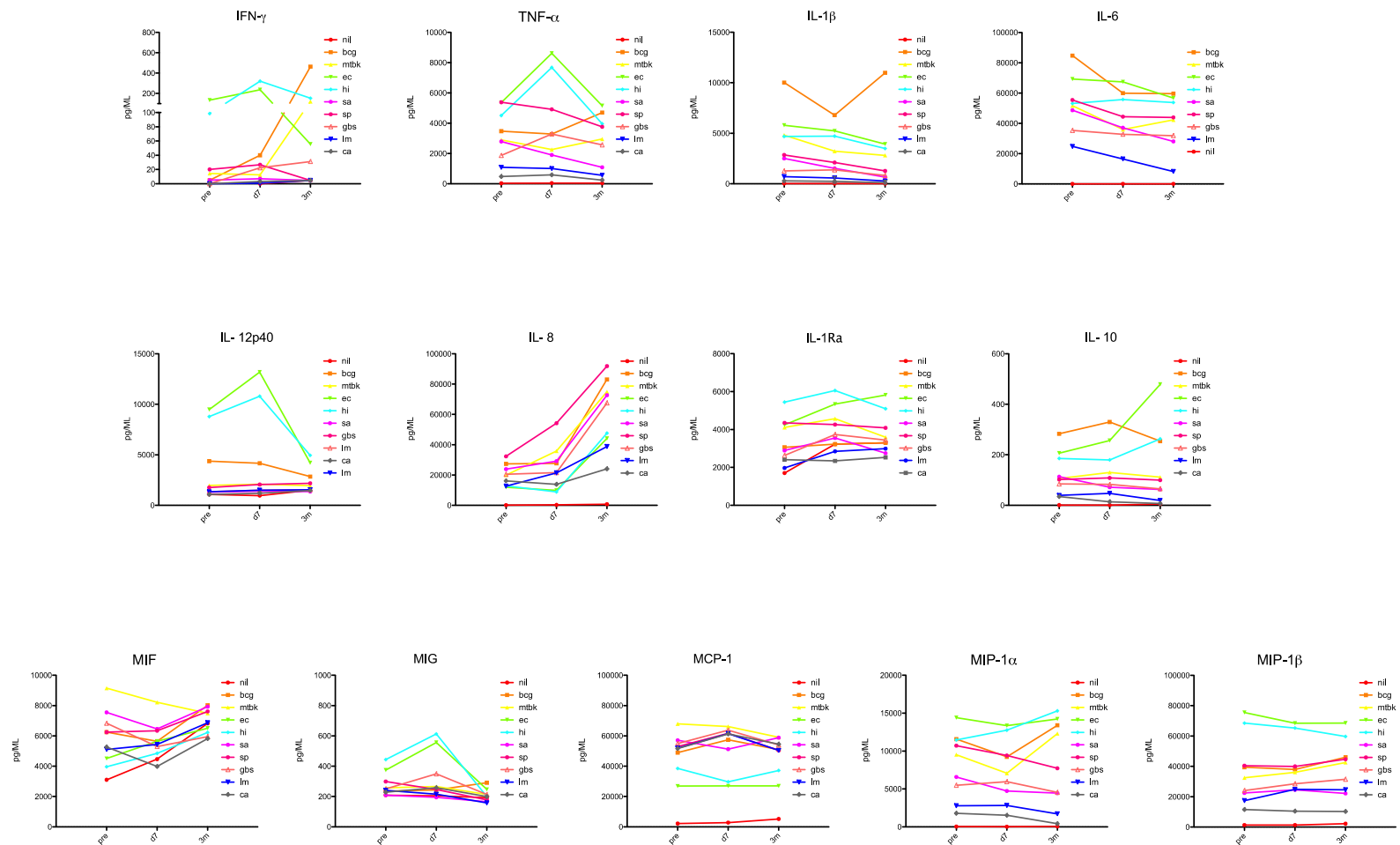


Figure 5.5 Longitudinal expression of heterologous cytokine responses post BCG vaccination

BCG = Bacille Calmette-Guerin, MTBK = *M. tuberculosis*, EC = *E. coli*, HI = *H. influenzae*, CA = *C. albicans*, GBS = Group B streptococcus (*S. agalactiae*), LM = *L. monocytogenes*, SA = *S. aureus*, SP = *S. pneumoniae*, NIL = RPMI

5.5.2.4 Subset analysis for effect modifiers of BCG vaccination

5.5.2.4.1 Sex

The alterations in IFN- γ production at 7 days post BCG vaccination compared to baseline (i.e. increased production in response to *E. coli*, *H. influenzae* and Pam3CYSK4) were similar when the effect of BCG was studied in males and females separately but were no longer statistically significant (Figure 5.6). However, in contrast to male infants, female infants had significantly increased IFN- γ responses to *S. aureus*, *C. albicans* and group B streptococcus (GBS) at 7 days post BCG vaccination. Similarly, at 7 days post BCG vaccination, female infants had increased TNF- α responses to stimulation with *C. albicans* and *L. monocytogenes*, an effect not seen in the main analysis. TNF- α responses to stimulation with group B streptococcus (GBS) was significant across all analyses.

5.5.2.4.2 Last vaccine given

Alterations in IFN- γ production at 7 days post BCG vaccination compared to baseline (*E. coli*, *H. influenzae*) were similar but also no longer statistically significant when analysed by subgroup (i.e. last vaccine live or non-live) (Figure 5.7). However, IFN- γ responses were increased in RPMI-stimulated (nil) samples when the last vaccine given to a participant was non-live, an effect not seen in the primary analysis or when the last vaccine received was live. The effect of BCG vaccination on TNF- α responses following heterologous stimulation was most marked in infants whose last vaccine was a non-live vaccine.

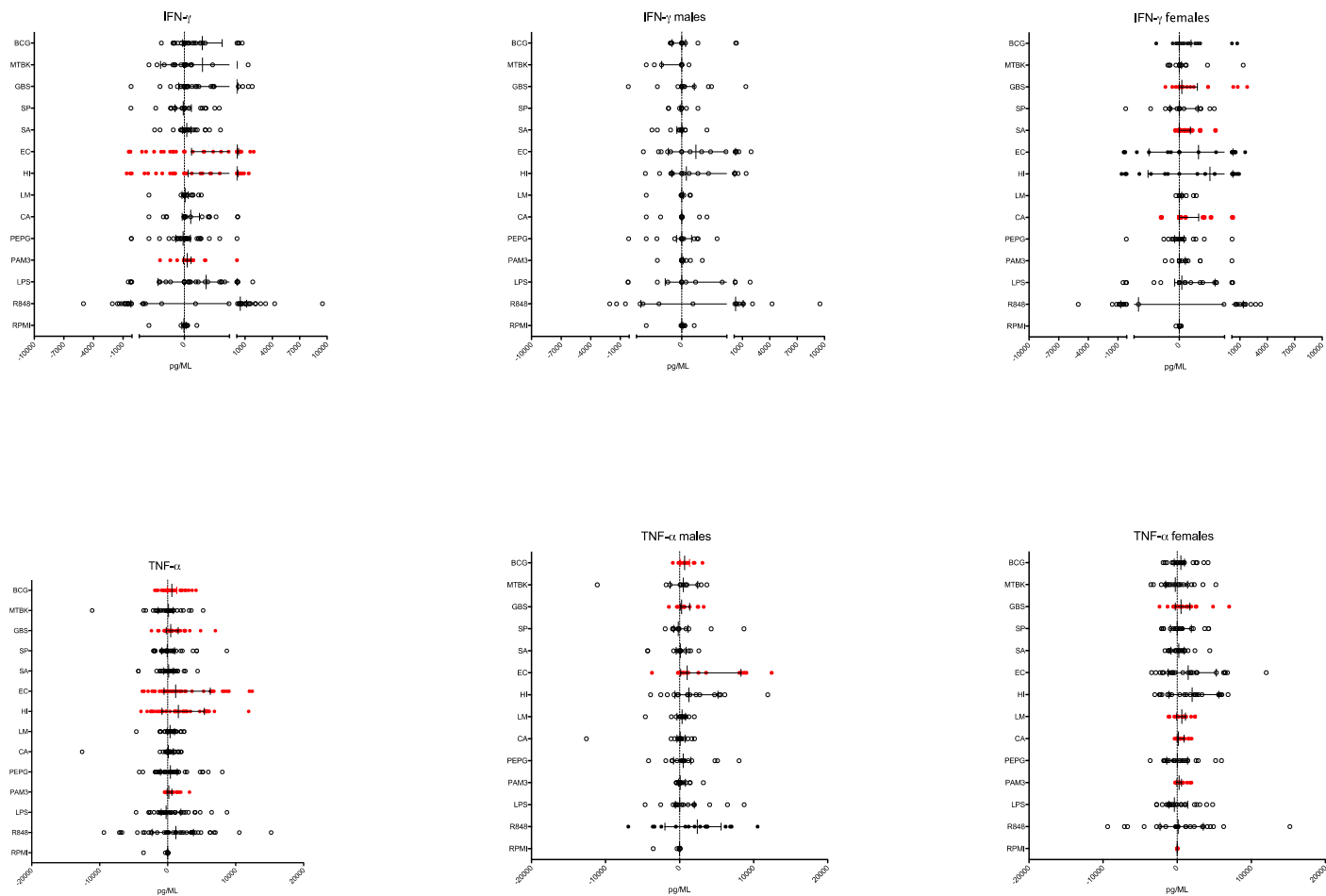


Figure 5.6 The effect of sex at 7 days post BCG vaccination

All comparisons are Wilcoxon-rank sign tests presented as dotplots with median (IQR). Results with $p < 0.05$ are depicted in red.

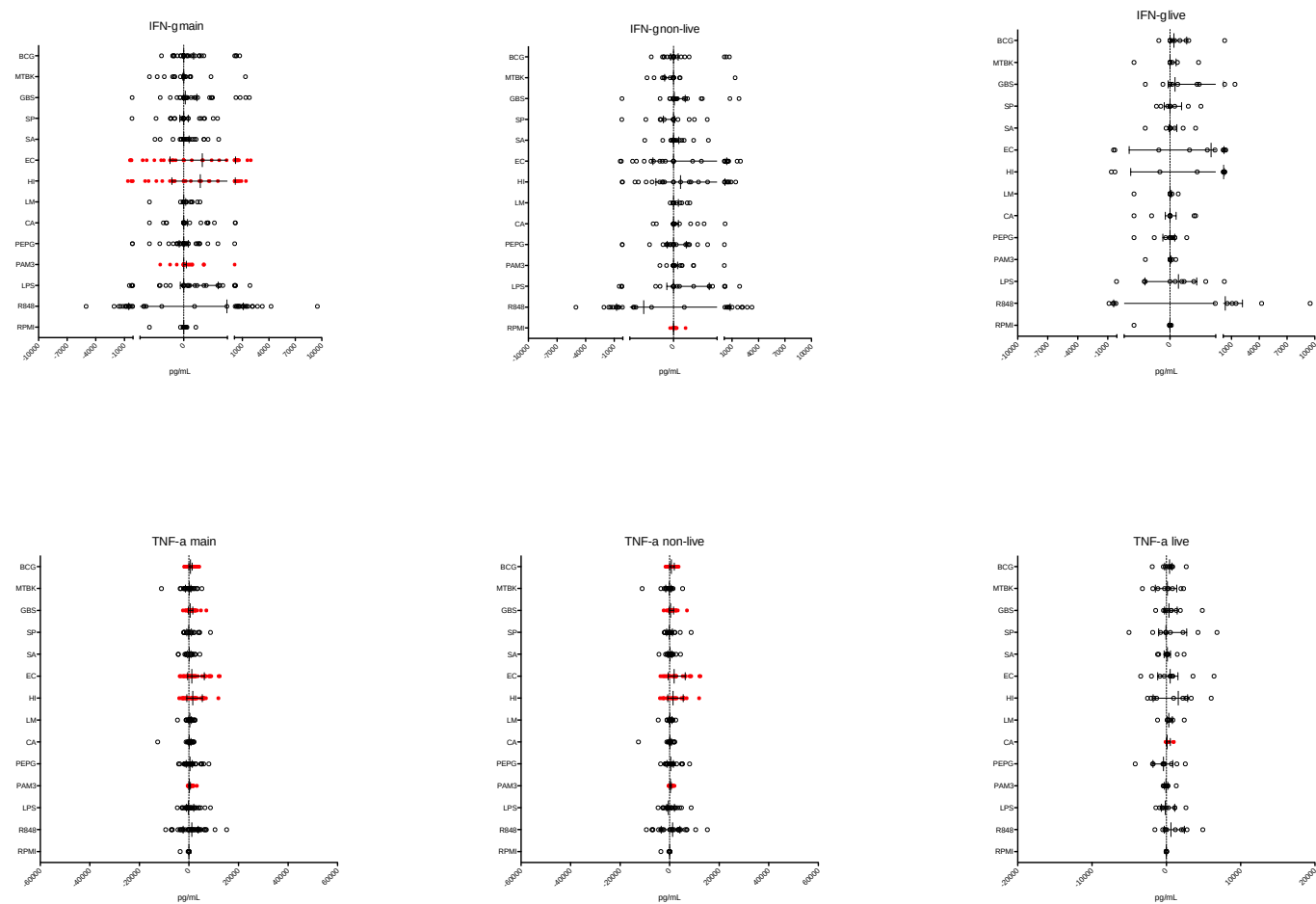


Figure 5.7 The effect of last vaccine received (live versus non-live) at 7 days post BCG vaccination

All comparisons are Wilcoxon-rank sign tests presented as dotplots with median (IQR). Results with $p < 0.05$ are depicted in red.

5.5.3 HIERARCHICAL CLUSTERING OF 7-DAY VERSUS BASELINE CYTOKINE RESPONSES

Fold-change (7-day value: baseline value) cytokine/stimulant data were grouped by Spearman's ranked correlation to investigate uncategorised relationships between cytokine/stimulant pairs (horizontal) and participants (vertical). This analysis was only possible for participants with a complete set of cytokine/stimulant pairs (n=28).

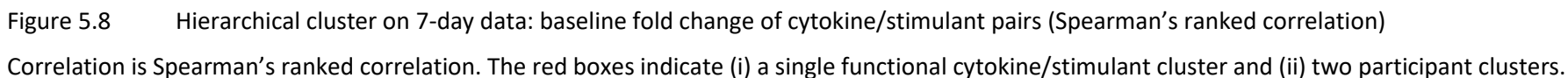
The correlations between cytokine/stimulant pairs were primarily influenced by the effect of each cytokine or each stimulant (Figure 5.8). There is a single functional cluster of correlated cytokine/stimulant responses which overcame this effect and included IFN- γ /*E. coli*, IFN- γ /*H. influenza*, IFN- γ /R848, TNF- α /*E. coli*, TNF- α /*H. influenzae*, TNF- α /BCG, TNF- α /R848, TNF- α /Pam3CYSK4, IL-1 β /Pam3CYSK4, IL-1 β /LPS, IL-1 β /*E. coli*, IL-10/BCG, IL-10/ Pam3CYSK4, IL-1Ra/BCG, MIP-1 α /*E. coli*, MIP-1 β /*E. coli*, IL-6/*E. coli* and IFN- γ /R848 (Figure 5.8).

There were two participant clusters: (i) participants 5029-5047 (cluster 1) and (ii) participants 5004 to 5050 (cluster 2). The division into these clusters was determined primarily by responses in cytokine/stimulant pairs that were differentially upregulated (green) or downregulated (red) (Figure 5.8): there was generalised upregulation of cytokine responses compared to baseline in cluster 2 (Figure 5.8). Attendance at childcare was the only demographic feature which differed significantly between participant clusters (Table 5.2).

Variable		Cluster 1 (n=16)	Cluster 2 (n=12)	p-value*
Sex	Male	5 (50%)	5 (50%)	0.5
	Female	11 (61%)	7 (39%)	
Last vaccine	Non-live	8 (41%)	10 (59%)	0.06
	Live	8 (80%)	2 (20%)	
Childcare	Yes	7 (100%)	0 (0%)	0.01
	No	9 (43%)	12 (57%)	
Travel overseas	Yes	4 (25%)	1 (75%)	0.35
	No	12 (8%)	11 (92%)	
Age	<1y	10 (48%)	11 (52%)	0.2
	>1y	6 (86%)	1 (14%)	

Table 5.2 Comparison of demographic features between participant clusters of cytokine/stimulant data (BabyBAIR)

* Fisher's exact test



5.5.4 FLOW CYTOMETRY RESULTS

Heterologous responses are characterised by an increase in pro-inflammatory and IL-12p40-producing monocytes and myeloid dendritic cells

Seventeen participants had flow cytometry data at both baseline and 7 days post BCG vaccination. Compared to baseline, at 7 days, *in vitro* stimulation with heterologous bacteria *E. coli* and *H. influenzae*, and the TLR ligands LPS and R848, led to a significant increase in the following cell-types; monocytes (IL-12p40⁺), monocytes (IL-12p40⁺/IL-6⁺), monocytes (IL-12p40⁺/TNF- α ⁺), monocytes (IL-6⁺/IL-12p40⁺/TNF- α ⁺), myeloid dendritic cells (mDCs) (IL-6⁺/IL-12p40⁺), mDC (IL-12p40⁺/TNF- α ⁺) and mDC (IL-12p40⁺/IL-6⁺/TNF- α ⁺). The proportion of monocytes (IL-12p40⁺/TNF- α ⁺) was also increased following stimulation with *S. pneumoniae* and PEPG as was the proportion of mDCs (IL-12p40⁺) following stimulation with PEPG and Pam3CYSK4 (Figure 5.9 & 5.10).

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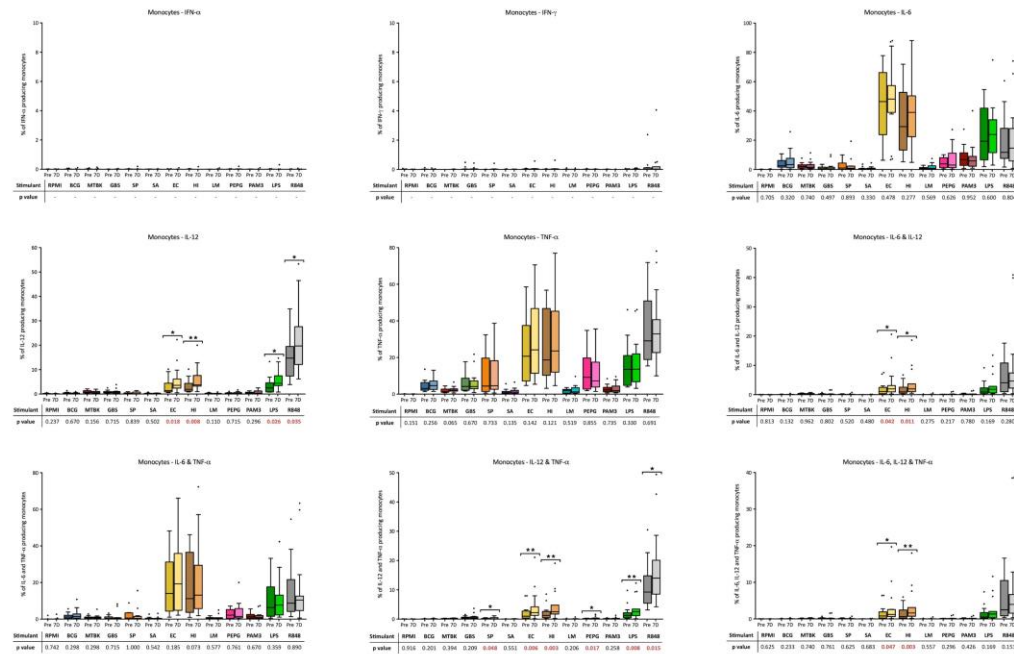


Figure 5.9 Proportion of myeloid dendritic cells at 7 days post BCG vaccination compared to baseline

BCG = Bacille Calmette-Guerin, MTBK = *M. tuberculosis*, EC = *E. coli*, HI = *H. influenzae*, CA = *C. albicans*, GBS = Group B streptococcus (*S. agalactiae*), LM = *L. monocytogenes*, SA = *S. aureus*, SP = *S. pneumoniae*, PEPG = peptidoglycan, PAM = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, LPS = lipopolysaccharide, R848 = resiquimod, NIL = RPMI

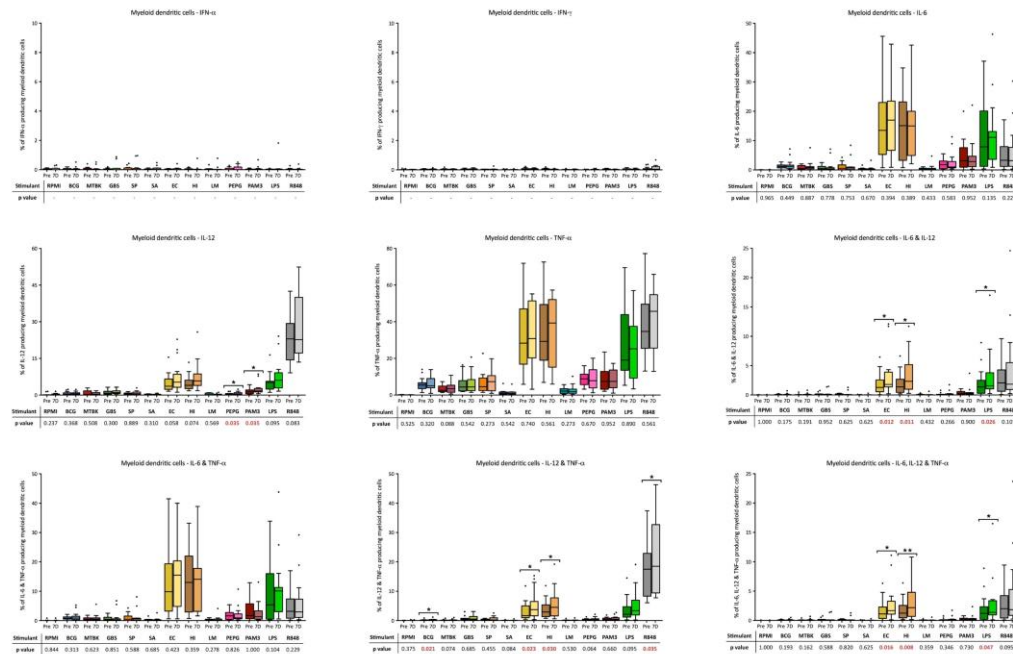


Figure 5.10 Proportion of monocytes at 7 days post BCG vaccination compared to baseline

BCG = Bacille Calmette-Guerin, MTBK = *M. tuberculosis*, EC = *E. coli*, HI = *H. influenzae*, CA = *C. albicans*, GBS = Group B streptococcus (*S. agalactiae*), LM = *L. monocytogenes*, SA = *S. aureus*, SP = *S. pneumoniae*, PEPG = Peptidoglycan, PAM = (S)-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4OH, trihydrochloride, LPS = lipopolysaccharide, R848 = resiquimod, NIL = RPMI

5.5.5 RNASeq RESULTS

There were two stages to the RNASeq analysis of whole blood samples. Firstly, the unsupervised analysis identified differentially expressed genes at each time point compared to baseline. The molecular function of these genes and associated gene sets, defined by time point and direction of change, were investigated to identify those with potentially relevant molecular functions. Secondly, a supervised analysis was done including (i) functional gene set enrichment analysis (fGSEA) and (ii) network analysis.

5.5.5.1 Overview of differentially expressed genes

There were 1733 differentially expressed transcripts in total. The number and direction of differentially expressed genes (DEGs) by comparison is shown in Figure 5.11. There was a predominance of downregulated genes at 7 days and 3 months post BCG vaccination compared to baseline.

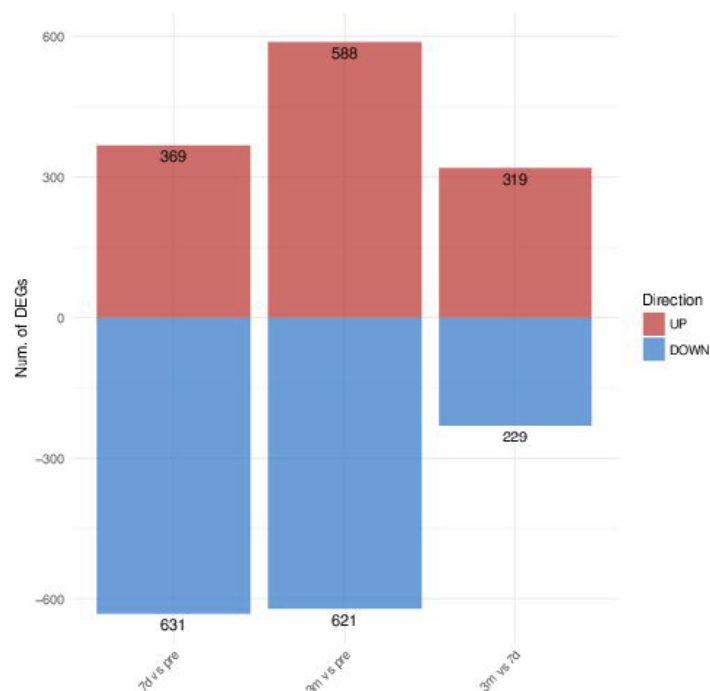


Figure 5.11 Count and direction of DEGS at each time point

5.5.5.2 Differentially expressed genes associated with immune function

GeneCards™ was used to identify transcripts which were protein coding (n=1640) and those with gene ontology (GO) terms and molecular functions associated with innate and adaptive immune function (n=138)[205].

The 138 DEGS GO terms related to immune function are presented in Figure 5.12 in three groups: (i) those which were upregulated at 3 months; (ii) those which had a difference in gene expression only at 7 days; and (iii) those which were downregulated at 3 months.

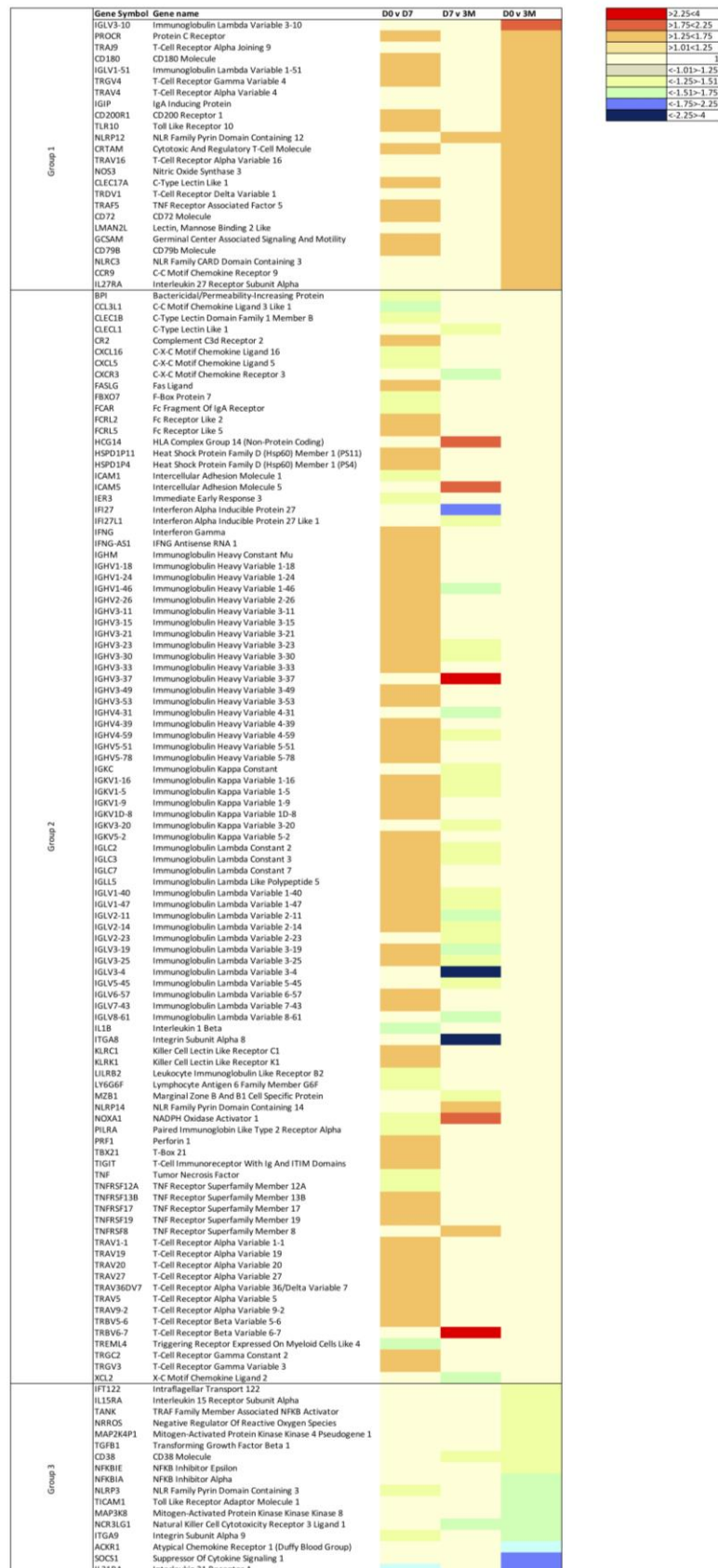


Figure 5.12 DEGs related to immune function (from GO terms) sorted by fold change (3-month: baseline)

Group 1: DEGs upregulated at 3 months, Group 2: DEGs only at 7 days, Group 3: DEGs downregulated at 3 months. Colour depicts magnitude of fold change

5.5.5.3 Functional gene set enrichment analysis

Supervised analysis of RNASeq data was done by functional gene set enrichment analysis (fgSEA). The 30 pathways with the most significantly normalised enrichment scores (NES) are shown in Figure 5.13. All other significantly enriched pathways and associated NES are provided in Tables 5.4-5.6.

5.5.5.3.1 7 days post BCG vaccination

The pathways with the highest NES (i.e. upregulated pathways) at 7 days post BCG vaccination were related to: (i) viral infection (influenza, influenza infection, influenza life cycle, viral mRNA translation, HIV infection); (ii) complement (classical antibody mediated triggering of complement, initial triggering of complement creation of C2/C4 activators and regulation of the complement cascade; and (iii) signalling via the B-cell receptor (signalling through the BCR and CD22 regulated BCR signalling); (iv) cell cycle (cell cycle mitotic, M phase, G2/Mi checkpoints, mitotic G1/S phases, mitotic G2/-G2M phases, separation of sister chromatids and chromosome maintenance) (v) FCεRI mediated activation of NF-κB and (vi) epigenetics (epigenetic regulation of gene expression, HAT acetylate histones) (Figure 5.13). All other immune pathways with positively enriched NES at this time point are shown in Table 21 and all fall within one of the aforementioned categories.

The pathways with the lowest normalised enrichment scores (i.e. downregulated pathways) at 7 days post BCG vaccination were: (i) neutrophil degranulation; (ii) interferon signalling cascades (interferon signalling, IFN-γ signalling, interferon α/β signalling); (iii) the pathway Class 1 MHC mediated antigen presentation and the pathway; (iv) cellular response to external stimuli which includes pathways related to maintenance of homeostasis (macroautophagy) and response to stress (Figure 5.13). All other immune associated pathways with negatively enriched NES scores appear in Table 21 (n=79). This group is predominantly associated with downregulation of TLR signalling (at the cell surface and intracellular), inflammasome assembly and the production of cytokines (including TNF, IL-10, IL-1β, IL-2, IL-4, IL-13 and IL-17).

5.5.5.3.2 3 months post BCG vaccination

The only pathways with persistently positively enriched NES at 3 months post BCG vaccination were those related to viral infection (influenza, influenza infection, influenza life cycle, viral mRNA translation). At 3 months post BCG vaccination, the pathway with the lowest NES were related to: (i) cell division (cell cycle mitotic, Mphase, G2/M checkpoints); (ii) neutrophil degranulation; (iii) the pathway Class 1 MHC mediated antigen presentation; and (iv) IFN signalling. All other immune associated pathways with negatively enriched NES scores which persist at 3 months are shown in Table 21 (n=90). This group is similar to those which are negatively enriched at 7 days and includes pathways associated with TLR signalling (at the cell surface and intracellular), control of IFN signalling (RIG1 regulation of IFN- α / IFN- β , negative regulation of RIG1/MDA5) and the production of cytokines (IFN- γ , TNF- α and IL-2), epigenetics and cell cycle.

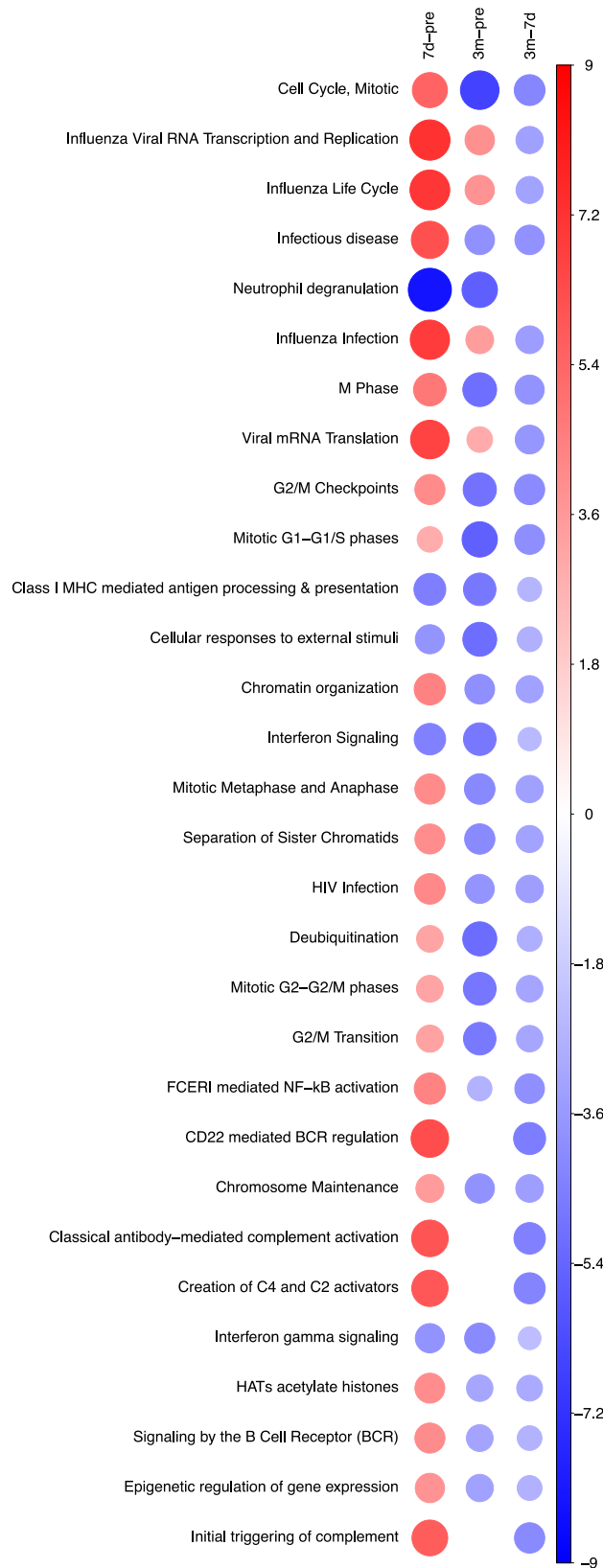


Figure 5.13 Pathways identified by fGSEA as being either positively (red) or negatively (blue) enriched post BCG vaccination.

NES = normalised enrichment score

Pathways	NES 7-day	Pathways	NES 3-month
Influenza Viral RNA Transcription and Replication	7.23236765	Influenza Viral RNA Transcription and Replication	3.88529953
Influenza Life Cycle	7.08818326	Influenza Life Cycle	3.81833342
Influenza Infection	6.88375192	Influenza Infection	3.45390482
Viral mRNA Translation	6.63219111	Viral mRNA Translation	2.93505288
CD22 mediated BCR regulation	6.29195547	Intraflagellar transport	2.29305548
Infectious disease	6.16607712		
Classical antibody-mediated complement activation	5.99487501		
Creation of C4 and C2 activators	5.89188741		
Initial triggering of complement	5.71045362		
Role of phospholipids in phagocytosis	5.61004444		
Cell Cycle, Mitotic	5.48277319		
FCER1 mediated Ca+2 mobilization	5.39878202		
Regulation of Complement cascade	5.20336948		
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	5.14728513		
Complement cascade	5.05865582		
M Phase	4.75072915		
Regulation of actin dynamics for phagocytic cup formation	4.52130465		
Chromatin organization	4.40056452		
FCER1 mediated NF-kB activation	4.34077606		
HIV Infection	4.15968136		
Mitotic Metaphase and Anaphase	4.06399536		
G2/M Checkpoints	4.05492176		
Signaling by the B Cell Receptor (BCR)	4.04824339		
Separation of Sister Chromatids	4.00658893		
HATs acetylate histones	3.99479727		
RNA Polymerase I Transcription	3.96621023		
Epigenetic regulation of gene expression	3.83697429		
HIV Life Cycle	3.75731875		
Positive epigenetic regulation of rRNA expression	3.73995706		
Late Phase of HIV Life Cycle	3.5986373		
Telomere Maintenance	3.53519288		
Chromosome Maintenance	3.49643069		
Fc epsilon receptor (FCER1) signaling	3.48000708		
FCER1 mediated MAPK activation	3.46983278		
G2/M Transition	3.26676348		
Negative epigenetic regulation of rRNA expression	3.22858819		
Host Interactions of HIV factors	3.20614839		
Mitotic G2-G2/M phases	3.19597569		
Deubiquitination	3.18551362		
HIV Transcription Initiation	3.15833231		
HDACs deacetylate histones	3.0490895		
Transcription of the HIV genome	2.9352184		
Mitotic G1-G1/S phases	2.90493977		
DNA Damage/Telomere Stress Induced Senescence	2.77229184		
RMTs methylate histone arginines	2.76439848		
Intraflagellar transport	2.71981362		
E3 ubiquitin ligases ubiquitinate target proteins	2.58627723		
TCR signaling	2.50695871		
Inactivation of APC/C via direct inhibition of the APC/C complex	2.48998913		
Downstream TCR signaling	2.28887267		
DNA methylation	2.26884356		
Formation of HIV-1 elongation complex containing HIV-1 Tat	2.26349199		
HIV Transcription Elongation	2.26349199		
Meiosis	2.23350977		

Table 5.3 Pathways identified by fGSEA with a positively enriched NES post BCG vaccination.

Pathways	NES 7-day	Pathways	NES 3-month
RIP-mediated NFkB activation via ZBP1	-2.0860982	TNF signaling	-2.136474628
TRAF6 mediated IRF7 activation	-2.1804796	TRAF6 mediated NF-kB activation	-2.224379059
ER-Phagosome pathway	-2.1927204	Oncogene Induced Senescence	-2.235601787
Listeria monocytogenes entry into host cells	-2.198435	RIP-mediated NFkB activation via ZBP1	-2.241712814
Regulation of TNFR1 signaling	-2.2451898	PKMTs methylate histone lysines	-2.258059692
Signaling by TGF-beta Receptor Complex	-2.2487211	TRAF6 Mediated Induction of proinflammatory cytokines	-2.283772461
TNF signaling	-2.2489301	Downregulation of TGF-beta receptor signaling	-2.30456248
activated TAK1 mediates p38 MAPK activation	-2.2492076	Late Phase of HIV Life Cycle	-2.321918303
Neurodegenerative Diseases	-2.2494262	E3 ubiquitin ligases ubiquitinate target proteins	-2.420451552
RAF/MAP kinase cascade	-2.2717205	TRAF6 mediated induction of NFkB and MAP kinases upon TLR7/8	-2.435805453
MAP2K and MAPK activation	-2.2743235	HIV Life Cycle	-2.446321116
Signaling by Leptin	-2.2755383	MyD88 dependent cascade initiated on endosome	-2.462653619
Prolonged ERK activation events	-2.3099548	Toll Like Receptor 7/8 (TLR7/8) Cascade	-2.462653619
Signaling by ERBB2	-2.3136498	Toll Like Receptor 9 (TLR9) Cascade	-2.468486901
Signalling to p38 via RIT and RIN	-2.355709	TGF-beta receptor signaling activates SMADs	-2.480327503
Signalling to ERKs	-2.3837601	MyD88 cascade initiated on plasma membrane	-2.529307875
MAP kinase activation in TLR cascade	-2.3925238	Toll Like Receptor 10 (TLR10) Cascade	-2.529307875
Budding and maturation of HIV virion	-2.4380837	Toll Like Receptor 5 (TLR5) Cascade	-2.529307875
Interleukin receptor SHC signaling	-2.4773872	Negative epigenetic regulation of rRNA expression	-2.529965455
Assembly Of The HIV Virion	-2.4845594	Assembly Of The HIV Virion	-2.530715692
IKK complex recruitment mediated by RIP1	-2.485531	MyD88:Mal cascade initiated on plasma membrane	-2.557857753
Interleukin-2 signaling	-2.486541	Toll Like Receptor 2 (TLR2) Cascade	-2.557857753
Diseases associated with the TLR signaling cascade	-2.4919642	Toll Like Receptor TLR1:TLR2 Cascade	-2.557857753
Diseases of Immune System	-2.4919642	Toll Like Receptor TLR6:TLR2 Cascade	-2.557857753
Regulation of TLR by endogenous ligand	-2.5068085	Neurodegenerative Diseases	-2.600184951
Apoptosis	-2.5085126	MyD88-independent TLR3/TLR4 cascade	-2.637643924
Insulin receptor recycling	-2.5088543	Toll Like Receptor 3 (TLR3) Cascade	-2.637643924
The NLRP3 inflammasome	-2.5213442	TRIF-mediated TLR3/TLR4 signaling	-2.637643924
C-type lectin receptors (CLRs)	-2.5242213	Signaling by NOTCH1	-2.658640244
Inflammasomes	-2.5698937	FCER1 mediated NF-kB activation	-2.685497
Interleukin-3, 5 and GM-CSF signaling	-2.5815729	Positive epigenetic regulation of rRNA expression	-2.707113471
Oncogenic MAPK signaling	-2.6336056	Budding and maturation of HIV virion	-2.724659288
Oncogene Induced Senescence	-2.6468535	Telomere Maintenance	-2.75477525
IRAK4 deficiency (TLR2/4)	-2.6488394	DNA Damage/Telomere Stress Induced Senescence	-2.867634538
MyD88 deficiency (TLR2/4)	-2.6488394	Signaling by NOTCH	-2.881116069
IRS-mediated signalling	-2.729046	Toll-Like Receptors Cascades	-2.906040274
G1 Phase	-2.729693	Activated TLR4 signalling	-2.907294018
Insulin receptor signalling cascade	-2.7452126	Toll Like Receptor 4 (TLR4) Cascade	-2.923835723
Interleukin-4 and 13 signaling	-2.7567125	Signaling by ERBB2	-2.933863827
Negative regulators of RIG-I/MDA5 signaling	-2.7663041	Meiosis	-3.054323625
Signaling by Type 1 Insulin-like Growth Factor 1 Receptor	-2.7824328	G0 and Early G1	-3.11691107
Interleukin-17 signaling	-2.8035924	HATs acetylate histones	-3.11946818
IGF1R signaling cascade	-2.8074813	Negative regulators of RIG-I/MDA5 signaling	-3.127528949
Signaling by MET	-2.8295723	Signaling by MET	-3.161401736
ROS, RNS production in phagocytes	-2.8328661	Signaling by TGF-beta Receptor Complex	-3.179554449
Interleukin-1 signaling	-2.8460111	Signaling by the B Cell Receptor (BCR)	-3.189820283
Signaling by BRAF and RAF fusions	-2.8582524	Oncogenic MAPK signaling	-3.241445275
PI3K/AKT activation	-2.8604168	Signaling by BRAF and RAF fusions	-3.243057623
MAPK family signaling cascades	-2.88884	DNA methylation	-3.245478121
Interleukin-10 signaling	-2.8988939	G1 Phase	-3.276277175
Signaling by NOTCH1	-2.913567	FCER1 mediated MAPK activation	-3.278726249
Downstream signaling events of B Cell Receptor (BCR)	-2.9554877	Gastrin-CREB signalling pathway via PKC and MAPK	-3.291207543
Antimicrobial peptides	-2.9816626	Epigenetic regulation of gene expression	-3.307606734
TRAF6 Mediated Induction of proinflammatory cytokines	-2.9899129	Host Interactions of HIV factors	-3.389119881
Gastrin-CREB signalling pathway via PKC and MAPK	-3.0251627	MAPK6/MAPK4 signaling	-3.402485286
MyD88 cascade initiated on plasma membrane	-3.1345597	Apoptosis	-3.407038531

Table 5.4 Pathways identified by fGSEA with a negatively enriched NES post BCG vaccination (P1).

Pathways	NES 7-day	Pathways	NES 3-month
Toll Like Receptor 10 (TLR10) Cascade	-3.1345597	TP53 Regulates Metabolic Genes	-3.436929403
Toll Like Receptor 5 (TLR5) Cascade	-3.1345597	C-type lectin receptors (CLRs)	-3.448012973
Signaling by Insulin receptor	-3.14686675	Disorders of transmembrane transporters	-3.477166172
TP53 Regulates Metabolic Genes	-3.14824528	Regulation of Apoptosis	-3.563930643
Signaling by NOTCH	-3.15885862	Dectin-1 mediated noncanonical NF-kB signaling	-3.62222693
MyD88-independent TLR3/TLR4 cascade	-3.2175911	Interleukin-17 signaling	-3.636006498
Toll Like Receptor 3 (TLR3) Cascade	-3.2175911	HDACs deacetylate histones	-3.638575434
TRIF-mediated TLR3/TLR4 signaling	-3.2175911	Prolonged ERK activation events	-3.668174921
Toll Like Receptor 9 (TLR9) Cascade	-3.47856591	Fc epsilon receptor (FCER1) signaling	-3.675513917
NGF signalling via TRKA from the plasma membrane	-3.48513947	NIK-->noncanonical NF-kB signaling	-3.683012869
TRAF6 mediated induction of NFkB and MAP kinases upon TLR7/8	-3.48732393	MAPK1/MAPK3 signaling	-3.700030379
MyD88 dependent cascade initiated on endosome	-3.4894924	Signalling to ERKs	-3.708204917
Toll Like Receptor 7/8 (TLR7/8) Cascade	-3.4894924	MAPK family signaling cascades	-3.713351866
RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways	-3.54025304	Activation of NF-kappaB in B cells	-3.715505059
MyD88:Mal cascade initiated on plasma membrane	-3.76131084	TCR signaling	-3.738095557
Toll Like Receptor 2 (TLR2) Cascade	-3.76131084	HIV Infection	-3.752702962
Toll Like Receptor TLR1:TLR2 Cascade	-3.76131084	Downstream TCR signaling	-3.760742521
Toll Like Receptor TLR6:TLR2 Cascade	-3.76131084	Signaling by Leptin	-3.778190949
Cellular responses to external stimuli	-3.76906453	Signalling to p38 via RIT and RIN	-3.782748416
Interferon gamma signaling	-3.80106317	Interleukin-2 signaling	-3.788036068
Activated TLR4 signalling	-3.92183114	RAF/MAP kinase cascade	-3.78878595
Signaling by Interleukins	-4.04972109	Interleukin-3, 5 and GM-CSF signaling	-3.790028345
Toll Like Receptor 4 (TLR4) Cascade	-4.10468547	Oxygen-dependent proline hydroxylation of Hypoxia-inducible F	-3.807991739
Interferon alpha/beta signaling	-4.15949858	ER-Phagosome pathway	-3.813130348
Toll-Like Receptors Cascades	-4.34885483	Chromosome Maintenance	-3.837359801
Interferon Signaling	-4.41060661	Downstream signaling events of B Cell Receptor (BCR)	-3.850743334
Class I MHC mediated antigen processing & presentation	-4.52933307	Interleukin receptor SHC signaling	-3.852800535
Neutrophil degranulation	-8.28871151	Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)	-3.874622339
		IRS-mediated signalling	-3.883168494
		Signaling by Interleukins	-3.896400926
		Infectious disease	-3.897506943
		IGF1R signaling cascade	-3.904881528
		Signaling by Insulin receptor	-3.930203481
		Chromatin organization	-3.934231536
		NGF signalling via TRKA from the plasma membrane	-3.949163372
		Insulin receptor signalling cascade	-3.957526558
		Interferon gamma signaling	-4.086389338
		Interleukin-1 signaling	-4.087128601
		Separation of Sister Chromatids	-4.101119989
		RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways	-4.104205007
		Transcriptional regulation by RUNX3	-4.114503986
		Mitotic Metaphase and Anaphase	-4.160672204
		G2/M Transition	-4.703878968
		Class I MHC mediated antigen processing & presentation	-4.717860712
		Interferon Signaling	-4.739814898
		Mitotic G2-G2/M phases	-4.788966997
		Interferon alpha/beta signaling	-4.854976767
		G2/M Checkpoints	-4.935023653
		M Phase	-5.0897989
		Cellular responses to external stimuli	-5.11839287
		Deubiquitination	-5.177671287
		Mitotic G1-G1/S phases	-5.565343664
		Neutrophil degranulation	-5.609145862
		Cell Cycle, Mitotic	-6.62997208

Table 5.5 Pathways identified by fGSEA with a negatively enriched NES post BCG vaccination (P2).

5.5.5.4 Network analysis

Protein-protein interactions were mapped using an interactome database on NetworkAnalyst software. Networks were constructed for upregulated and downregulated differentially expressed genes at both 7 days and 3 months compared to baseline. All gene names and molecular functions were designated using the GeneCards™ program [205].

5.5.5.4.1 Networks upregulated at 7 days post BCG vaccination

There were four upregulated network hubs at 7 days post BCG vaccination (Fig 5.14). The central nodes for each hub along with the number of interactions and their molecular functions are shown in Table 23. Nodes which connected these hubs included *NOTCH2NL* (Notch homolog 2 N-terminal-like), a key constituent of the highly conserved NOTCH-signalling pathway and *PIN* (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1), a membrane protein and a constituent of RIG-like receptor signalling pathway.

5.5.5.4.2 Networks downregulated at 7 days post BCG vaccination

There were six downregulated network hubs at 7 days post BCG vaccination (Fig 5.15). The central nodes for each hub along with the number interactions and their molecular functions are shown in Table 23. Nodes located upstream, included anti-apoptotic members of the TNF-superfamily (*TRAF1*, *TRAF2*), constituents of the TGF- β signalling pathway (*STK3*, *DVL3*), constituents of neutrophil degranulation pathways (*STOM*, *PRAM1*, *OLFM*), IL-4 signalling pathways (*TGF4/NFIL3*), C-lectin pathways (*CLEC7A*) and G-coupled protein receptor pathways (*CXCL5*) and the NF- κ B subunit *REL*.

5.5.5.4.3 Networks upregulated at 3 months post BCG vaccination

The protein-protein interaction network of upregulated genes at 3 months post BCG vaccination are characterised by 12 well discrete network hubs (Fig 5.16). The central nodes for each hub along with the number of interactions and their molecular functions are shown in Table 23.

5.5.5.4.4 Networks downregulated at 3 months post BCG vaccination

The protein-protein interaction network of downregulated genes at 3 months post BCG vaccination are characterised by 6 network hubs (Fig 5.17). The central nodes for each hub along with the number of interactions and their molecular functions are shown in Table 23. Nodes located upstream, were genes involved in innate immune signalling including several which were also seen at 7 days post BCG vaccination including *NFKBIA* (NFKB Inhibitor Alpha) which interacts with REL dimers to inhibit NF- κ B /REL complexes in the control of inflammatory responses, *BCL7B* (BAF Complex Component) which positively regulates apoptosis and *BCL10* (B-cell CLL/lymphoma 7B), an immune adaptor which promotes apoptosis, pro-caspase-9 maturation and activation of NF- κ B via NIK and IKK (non-canonical NF- κ B signalling). Also, notable within these networks are those members of the TNF superfamily which were also present at 7 days post BCG vaccination; *TRAF1* and *TRAF2* as well as two key constituents of the NOD-1 signalling pathway *TRIP6* and *RIPK2*.

Node	Protein	Function	Interactions
7-day upregulated networks			
<i>ZNF446</i>	Zinc finger protein 446	DNA binding and transcription	18
<i>ZNF232</i>	Zinc finger protein 332	DNA binding and transcription	3
<i>FASLG</i>	Fas ligand	Control of apoptosis in cancer and immunity	4
<i>KRT2</i>	Keratin-2	Constituent of cytoskeleton.	5
7-day downregulated networks			
<i>ADAMTSL4</i>	ADAMTSL4 protein	Microtubule formation	12
<i>STX11</i>	Syntaxin 11	Intracellular protein trafficking.	11
<i>TRIM23</i>	Tripartite Motif 23	E3-ubiquitin protein ligase involved	9
<i>CXCL16</i>	C-X-C motif chemokine	Macrophage scavenger receptor	9
<i>GABARAPL2</i>	GABA receptor-associated protein-like 2	Autophagy and TLR signalling	6
<i>NOCTH2NLA</i>	Notch 2 N-terminal Like	Notch signalling	5
<i>KRT4</i>	Keratin 4	Keratinization	5
<i>REL</i>	REL proto-oncogene	Component of the NF- κ B subunit.	5
3-month upregulated			
<i>PDE4DIP</i>	Phosphodiesterase 4D Interacting Protein	Microtubule formation	33
<i>FXR2</i>	Fragile X syndrome-related protein 2	RNA binding during cellular proliferation	31
<i>GOPC</i>	Golgi Associated PDZ And Coiled-Coil Motif Containing protein	Intracellular protein trafficking.	31
<i>RAD51</i>	Recombinase 51	DNA repair and tumorigenesis.	12
<i>TRIM32</i>	E3-ubiquitin protein ligase	Innate immune sensing and control NF- κ B signalling via ubiquitination.	12
<i>TRAF5</i>	TNF receptor associated factor 5	TNF superfamily protein involved in TNF activation	11
<i>TRAF3IP3</i>	TRAF3 interacting protein 3	Modulates of the c-Jun N-terminal kinase signalling	11

<i>DNAJA3</i>	DNAJA3	Activates NF- κ B signalling at the level of I κ B.	10
<i>CEP72</i>	Centrosomal protein 72	Control of cell cycle	10
<i>KLC4</i>	Kinesin light chain 4	Transport of proteins via the Golgi apparatus.	8
<i>MTSU1</i>	Microtubule scaffolding protein	Microtubule formation	5
3-month downregulated			
<i>TSC22D4</i>	TSC22 domain family member 4	DNA-binding transcription factor involved in the HIV life cycle	15
<i>RAD51D</i>	RAD51 paralog D	Homologous recombination repair pathway in cancer	12
<i>VPS37C</i>	VPS37C, ESCRT-I subunit	Constituent of the HIV life cycle and macroautophagy pathways.	
<i>REL</i>	REL Proto-Oncogene	NF- κ B subunit	11
<i>CEP70</i>	Centrosomal protein 70	Cell cycle, mitotic pathways.	10
<i>SPRY2</i>	Sprouty RTK signalling stagonist 2	Tyrosine kinase negative regulation of ERK pathway.	10
<i>NDC80</i>	NDC80 kinetochore component	Cell cycle and chromosome division	8

Table 5.6 DEGS with highest degrees of centrality in the protein-protein interaction analysis

Molecular functions and gene names assigned using GeneCards™

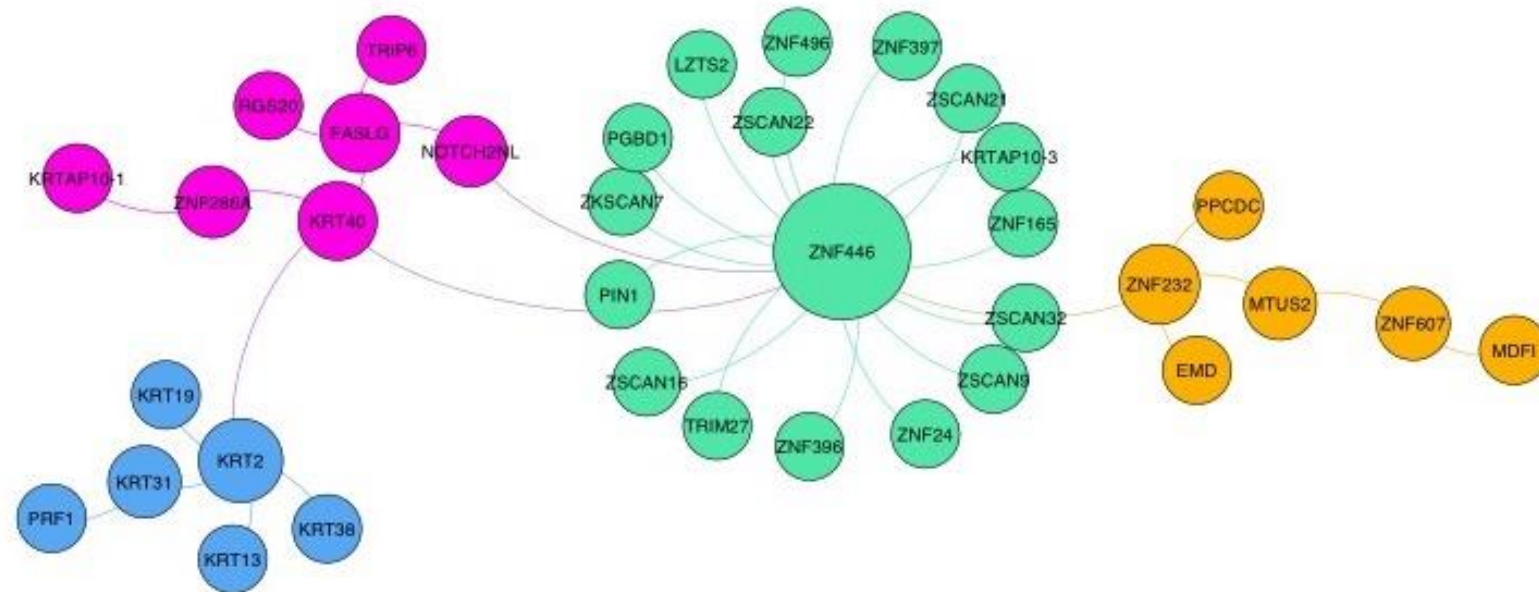


Figure 5.14 Upregulated protein networks at 7 days post BCG vaccination

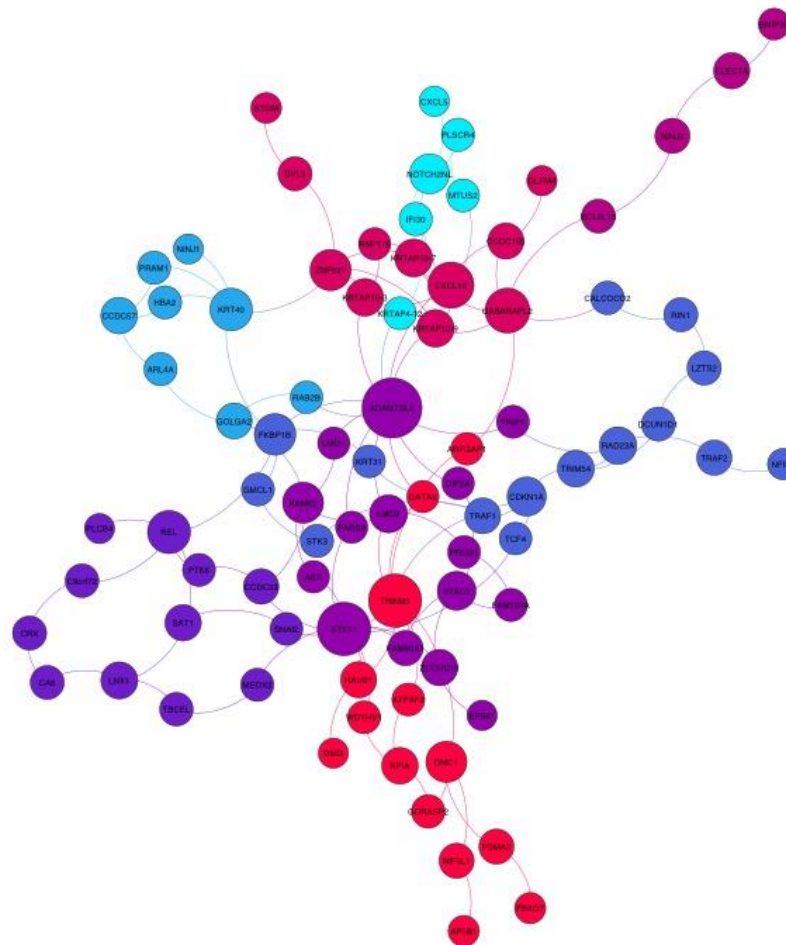


Figure 5.15 Downregulated protein networks at 7 days post BCG vaccination



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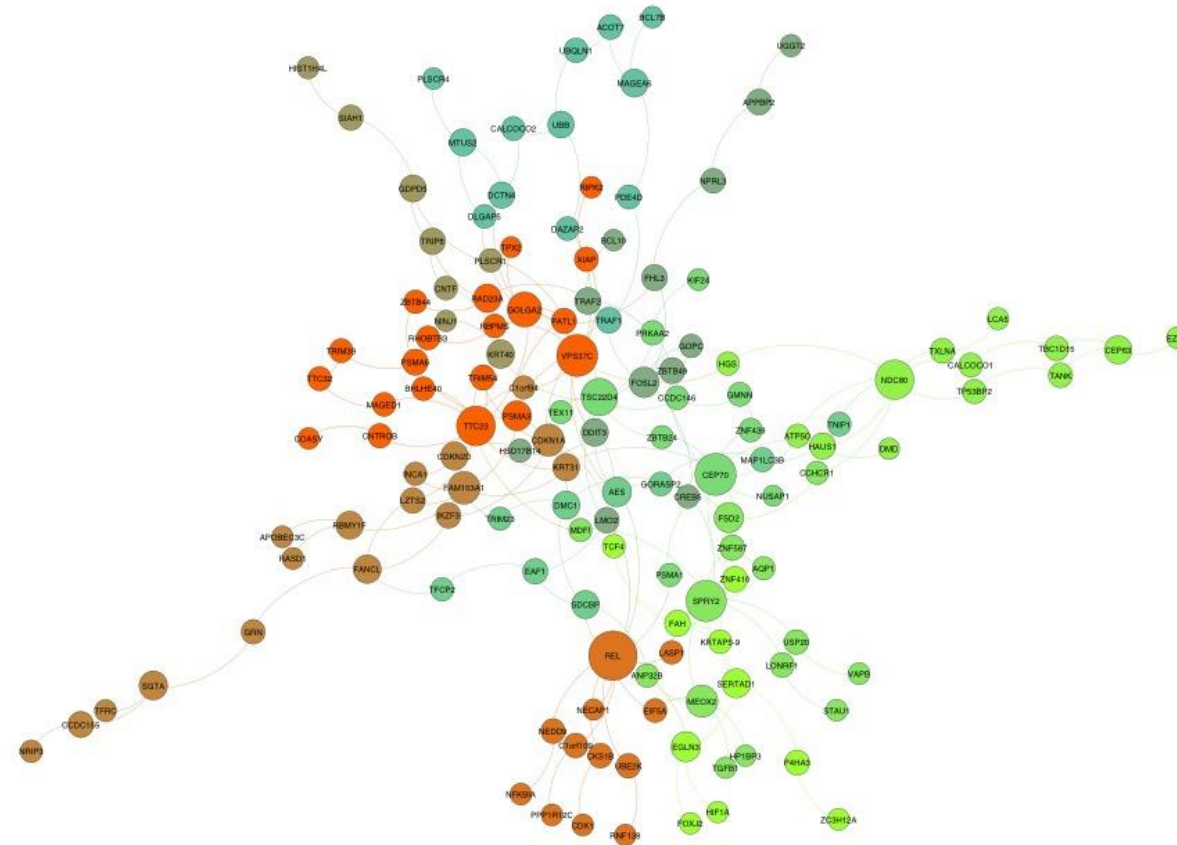


Figure 5.17 Upregulated protein networks at 3 months post BCG vaccination

5.6 DISCUSSION

This is the first study to use a systems vaccinology approach to investigate the heterologous effects of BCG vaccination in infants. The data presented in this study characterises BCG-induced alterations of the infant immune system prior to stimulation (RNASeq) concurrently with BCG-induced heterologous cytokine responses following pathogen exposure *in vitro*. BCG-induced heterologous cytokine response profiles varied over time and were characterised by: (i) transient 'early' pro-inflammatory responses at 7 days post BCG vaccination which were not seen at 3 months; (ii) early pro-inflammatory responses which peaked at 7 days and were downregulated by 3 months post BCG vaccination; and (iii) 'late' cytokines response seen for the first time at 3 months post BCG vaccination, characterised by an increase in immune-regulatory cytokines and a decrease in pro-inflammatory cytokines. In this discussion, alterations in the whole blood transcriptome which follow the same patterns of change over time are examined for hypothetical molecular mechanisms which could underpin the BCG-induced heterologous cytokine responses and ultimately the beneficial clinical effects of BCG vaccine.

The effect of BCG vaccination on infant mortality secondary to sepsis is strongest in the first week following vaccination and decreases in the following 6 months [36]. Thus, characterisation of the early post-BCG vaccination response is central to understanding the mechanism of BCG-induced heterologous immunity. Transient changes in the early BCG-induced immune response (7 days) were characterised by upregulation of gene expression pathways related to the initiation and control of complement and B-cell signalling and the induction of Th1 immunity [206], changes which could be associated with enhanced control of inflammation during bacterial sepsis [207, 208]. There was a concurrent increase in IFN- γ responses following stimulation with Gram-negative bacteria and IL-10 responses following stimulation of TLR1/2. IL-10 is essential to the control of inflammation during intracellular infection [209] and in the case of mycobacterial infection is produced following stimulation of TLR1/2 as seen in our study [210]. It is therefore interesting to hypothesise that baseline changes in complement activation and B-cell signalling could facilitate the

rapid induction of IL-10 and IFN- γ in the context of both bacterial and mycobacterial infection in the early post BCG-vaccination period.

In addition to these transient cytokine responses, there was also BCG-induced upregulation of TNF- α responses at 7 days which fell below baseline by 3 months. These changes were accompanied by alterations of several innate immune gene expression pathways (epigenetics, cell cycle and Fc ϵ RI-mediated control of NF- κ B). In adults, BCG-induced trained immunity leads to epigenetic modifications of the NOD2 pathway and increased heterologous TNF- α responses *in vitro* at 14 days post BCG vaccination [80]. Also in adults, epigenetic modifications of PI3k/AKT pathway was reported with associated decreased cytokine production during experimental yellow fever infection at one month post BCG vaccination [44]. Training of the innate immune system with β -glucan leads to epigenetic modifications which counteract dysregulated inflammation and subsequent immune paralysis seen in sepsis [211]. This effect of trained immunity was offered as an explanation for decreased circulating cytokines in BCG-vaccinated adults during the control of yellow fever viremia [44]. The pattern of BCG-induced heterologous responses seen in the BabyBAIR study and the concurrent alterations in gene expression pathways related to epigenetics provides indirect evidence of a role for trained immunity in the development of BCG-induced heterologous cytokine responses in infants. Studies combining epigenomics, transcriptomics and BCG-induced heterologous cytokine responses will be necessary to confirm the role of trained immunity in this population. Irrespective of the underlying mechanism, it is interesting that beyond the first week post vaccination, BCG-induced heterologous immunity is characterised by downregulation of pro-inflammatory cytokines which may suggest enhanced control of inflammation during infection and improved survival *in vivo*.

Two further gene expression pathways were altered in a similar fashion (upregulated at 7 days post BCG vaccination and downregulated by 3 months post BCG vaccination): “cell cycle” and “Fc ϵ RI mediated control of NF- κ B”. These pathways have not previously been associated with BCG-induced trained immunity in adults and their contribution to the development of BCG-induced heterologous cytokine responses in

the BabyBAIR study is uncertain. Cell cycle was the most significantly altered pathway overall. Although it is tempting to hypothesise that pathways related to cell cycle might be involved in the molecular control of cellular metabolism related to BCG-induced trained immunity [131], no such association was found. It was notable however that the cell cycle pathway was linked to the macro-autophagy pathway on the Interactome™ database [131]. BCG-induced trained immunity has been associated with the induction and control of autophagy during bladder cancer treatment and autophagy provides an attractive mechanism by which BCG vaccine could contribute to enhanced immune regulation during disparate infections [212-215]. In the BabyBAIR study, DEGS associated with the control of autophagy are prominent in the network analyses at both 7 days and 3 months post BCG vaccination, providing further evidence for a potential role for autophagy in the induction of the immune response to BCG vaccine.

FC ϵ RI mediated control of NF- κ B is more easily linked to innate immune regulation and could be important following BCG vaccination. Traditionally, FC ϵ RI cross-linking with IgE has been associated with the induction of Th2 immunity and the development of asthma and allergy [216]. Thus downregulation of this pathway might have implications for the effect of BCG vaccination on atopy. In addition, more recent evidence shows that expression of FC ϵ RI on monocytes and dendritic cells can have immune regulatory effects during infection which vary depending on the context [217]. FC ϵ RI stimulation can lead to increased pro-inflammatory IL-12 responses through antigen presentation [218] or immune suppressive effects through induction of IL-10 from DCs [219], decreased transcription of TLR9/10 [220] and reduction of MCP-1 following LPS stimulation of TLR4 [221]. All of these FC ϵ RI-associated immune signatures were a feature of BCG-induced alterations in the infant immune system in the cytokine, flow-cytometry and RNA-Seq results of this study. Alterations of the FC ϵ RI mediated control of NF- κ B is thus an interesting candidate mechanism by which BCG vaccination could influence outcomes associated with infection, inflammation and atopy.

At 3 months post BCG-vaccination, BCG-induced heterologous cytokine responses which did not peak at 7 days comprised either increases in immune-regulatory cytokines (IL-8, IL-10 and IL-1RA) or decreases in pro-inflammatory cytokines (IL-1 β). This BCG-induced cytokine response profile could hypothetically be supported by the widespread downregulation of innate immune gene expression pathways associated with (i) signalling via TLR, NLR, RLR and NF- κ B and (ii) the production of TNF- α and IL-1 β observed in the whole blood transcriptome at the same time point. Persistent downregulation of the pathway “cellular response to external stimuli” at 3 months post BCG vaccination may again be evidence of a role for autophagy at this stage. In addition, it is interesting to hypothesise that, as with the results of the MIS BAIR study at 7 days, decreased MIG in response to *E. coli* and MCP-1 in response to stimulation of TLR2 might be associated with the concomitant downregulation of the gene expression pathway neutrophil degranulation. Enhanced control of neutrophil-mediated inflammation would be beneficial in the context of neonatal sepsis [198] and the development of disseminated TB [222, 223] making downregulation of this pathway another potential mechanism by which BCG vaccine might lead to enhanced control of inflammation in the context of both heterologous and mycobacterial infection.

Also persistently decreased at 3 months post BCG-vaccination were gene expression pathways associated with interferon signalling. This finding is notable given the deleterious role of excessive type I interferon responses during the immune response to TB [224]. In the BabyBAIR study, type I interferon cytokine responses were not measured meaning it is not possible to confirm this finding by an alternative assay type. However IL-1 β and IL-10 responses to *M. tuberculosis* were significantly decreased at 3 months post BCG vaccination in line with the significant fall in heterologous cytokine responses to IL-1 β at the same time point. The control of IL-1 signalling and resulting IL-1 β responses are central to the concept of BCG-induced trained immunity [225] and negative regulation of IL-1 β by type I interferons is well described in the context of acute bacterial and viral infection [226] and can limit immunopathology [227]. Taken together, these results suggest that BCG-induced

alterations in IL-1 and interferon signalling pathways and their co-regulatory mechanisms is a further potential mechanism of enhanced immune regulation which would be beneficial in the context of both mycobacterial and heterologous infection.

BCG vaccination has been associated with clinical protection against disseminated TB disease [17], mortality secondary to neonatal sepsis [35, 36], mortality secondary to infant malaria and hospitalisation secondary to sepsis and pneumonia [31]. The scope of these clinical effects suggest induction of 'non-specific' heterologous immunity or priming of the immune system rather than classical antibody or T-cell receptor associated cross-reactivity to a given pathogen as the underlying mechanism. In the animal studies of BCG-induced heterologous immunity described in chapter 2, the effector mechanism associated with protection varied depending on the nature of the challenge pathogen [43]. In the BabyBAIR study, there is evidence of divergent IFN- γ responses following mycobacterial and heterologous challenge which was accompanied by a uniform upregulation of IL-8 responses. In summary, the results suggest that BCG vaccination leads to alterations in the infant immune system at baseline which leave it primed to deal with subsequent infection but that the nature of the heterologous response may vary by pathogen. This underlines the limitations of using single assay types or cross-sectional assessments when trying to understand the mechanism of BCG-induced heterologous immunity.

Alterations in the expression of individual genes (DEG) are of limited value without validation in different populations and at different time points. However, several DEGS were identified with molecular functions consistent with the results of accompanying pathway analyses and the overall hypothesis of the study. Table 21 shows a group of DEGS which were persistently up or downregulated at 3 months post BCG vaccination compared to baseline and which have molecular functions with proven associations to clinically beneficial immune modulation during infection in humans. In light of the hypothesis that BCG vaccination protects from infectious morbidity and mortality by altering the inflammatory response during infection, these genes are candidates for future validation studies.

Symbol	Protein	Direction	Function
CD200R1	CD200 Receptor 1	Up	Tolerance to LPS via chromatin modification[228]. Improved survival following meningitis and influenza[228]. Development of chronic intracellular infections through control of inflammation [228].
NLRC3	NLR Family CARD Domain Containing 3	Up	Dampened inflammation and improved outcomes following pseudomonas keratitis [229]. Improved survival following HSV-I infection[230]. NLRC3 knockout mice have increased susceptibility to LPS induced toxic shock[231]. Protective immunity in <i>M. tuberculosis</i> [232].
CD180	CD180 Molecule	Up	B cell recognition of lipopolysaccharide (LPS) and innate response to bacteria[233]. Expression affected by Vitamin D and Vitamin A concentrations of administration[234].
IL27RA	Interleukin 27 Receptor Subunit Alpha	Up	Control of <i>M. tuberculosis</i> and HIV [235, 236].
CCL3L1	C-C Motif Chemokine Ligand 3	Down	Marker of susceptibility to HIV [237]. Marker of susceptibility to asthma via IL-10 expression [238].
ICAM1	Intercellular Adhesion Molecule 1	Down	Susceptibility to severe and cerebral malaria [239, 240].
LILRB2	Leukocyte Ig Like Receptor	Down	Modulation of this mRNA alters response to septic shock in humans [241].
NFKBIE	NFKB Inhibitor Epsilon	Down	Increased Susceptibility to pneumococcal meningitis in humans [242].
ACKR1	Atypical Chemokine Receptor 1	Down	Increased susceptibility to severe malaria [243].

Table 5.7 Differentially expressed genes which are persistently altered at 3 months post BCG vaccination and which have been associated with tolerance or susceptibility to infection in human

5.7 STRENGTHS & LIMITATIONS

The major strength of this study is the longitudinal design allowing within patient comparisons. Furthermore, the use of several classes of clinically relevant heterologous and mycobacterial pathogens in parallel with TLR ligands allowed characterisation of differences in the immune response to different classes of pathogens. The major limitation of this study is the inability to definitively correlate innate immune signatures from the RNASeq analysis with BCG-induced heterologous cytokine responses or protection from clinically relevant infection in the study participants. The concordance between patterns of BCG-induced heterologous cytokine responses and alterations in gene expression pathways over time is interesting but remains hypothesis generating only. A further limitation of this study was the relatively small number of participants with matched data from all three analyses and/or all three time points due to small volumes of blood available and difficulties with retention at multiple time points. Recruitment of participants from travel clinic led to a high number of South East Asian infants and a high likelihood that all mothers were themselves BCG-vaccinated, which may have influenced the results [198].

There are several considerations specific to the interpretation of the RNASeq data. When assessing the whole genome transcriptome response to a vaccine, within-patient comparisons are superior to case-control assessments [244]. Ideally, patients are homogenous with respect to age, ethnicity and concomitant vaccination exposures which, although a limitation of this study, was mitigated by the use of pathway-based methodologies which reference prior gene sets, such as functional gene set enrichment analysis (fGSEA) [201]. Transcriptomic analysis of whole blood may be biased towards transcripts which predominate in red blood cells but this can be mediated by the use of globin depletion techniques such as that applied in this study [245]. In addition, assessment of differential gene expression using RNA-Seq analysis of whole blood post-vaccination can be distorted by transient increases in innate cell

types such as monocytes and neutrophils. On the other hand, transcriptomic analysis of cell subtypes such as PBMCs may be prone to artefact introduced during cell isolation and also require larger volumes of blood than are practical when studying infant immune response using multiple assays [246]. Again, the use of fGSEA methodologies which reference prior datasets helps to mitigate against this effect.

Finally, although consideration is given to the potential effect modification by the last routine vaccine administered on BCG-induced heterologous cytokine responses, it is impossible to differentiate this from any effect modification due to the variation in age of the participants. The numbers were too small for multivariable regression and this should be considered as an exploratory analysis only.

5.8 CONCLUSIONS & FUTURE DIRECTIONS

This study confirms that BCG vaccination in infancy alters cytokine responses following stimulation with heterologous pathogens and TLR ligands and allowed characterisation of the evolution of this response from an early pro-inflammatory to a late anti-inflammatory profile. Consideration of time matched changes in the whole blood transcriptome with cytokine and flow-cytometry responses following both mycobacterial and heterologous stimulation provided a comprehensive assessment of BCG-induced immunity and identified several innate immune pathways which may underpin BCG-induced heterologous cytokine responses. The results of this study support the hypothesis of non-specific heterologous immunity, i.e. protection which is syndrome-specific as opposed to antibody- or receptor-mediated cross-reactivity which is pathogen-specific. Trained immunity, the induction of innate immune memory via epigenetic modification of innate signalling pathways, is an example of non-specific heterologous immunity and the induction of trained immunity following infant BCG vaccination is indirectly supported by the results of this study. In addition, the results of this study indicate that other non-specific alterations in innate immune function may result in induction of effector mechanisms following *in vitro* challenge which differ depending on the nature of the pathogen exposure, underscoring the

importance of systems vaccinology approaches rather than cross-sectional assessments when aiming to describe complex immune mechanisms.

The results of this study support the hypothesis that BCG vaccination leads to beneficial alterations in the inflammatory response during acute bacterial and mycobacterial infection. Future studies should ideally correlate BCG-induced alterations in the immune systems with comprehensive immune-phenotyping during episodes of acute infection *in vivo* compared to 'well' (uninfected) controls.

Replication of these findings in neonates, in different geographic locations and following vaccination with different strains of BCG vaccine would enhance the generalisability of the findings. The results support the paradigm of trained immunity and advocate for combining transcriptomic and epigenetic assessments where possible.

Chapter 6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 OVERVIEW

This chapter provides an overview and critical appraisal of the studies presented in this thesis. The aim is to build on previous discussion by considering the results of the three studies together and to assess the overall contribution of the work to the current understanding of BCG-induced heterologous immunity. Finally, the strengths and limitations of this thesis are highlighted, and recommendations are made for future research.

6.2 SUMMARY OF PRINCIPLE FINDINGS

The cytokine/stimulant responses which were significantly altered by BCG vaccination in the three studies in this thesis are summarised in Table 6.2. The five principle findings in this thesis that will be discussed further below are the following:

1. BCG-induced heterologous cytokine responses differ between neonates and older infants.
2. BCG-induced heterologous immunity in both neonates and infants involves changes which are consistent with an effect on innate immunity.
3. BCG-induced cytokine responses differ depending on the nature of the *in vitro* challenge pathogen (specific vs heterologous).
4. There are several consistent effect modifiers of BCG-induced heterologous immunity in both neonates and older infants.
5. Inter-individual and inter-stimulant variation in cytokine responses impact on the detection of an effect attributable to BCG vaccination.

		MIS BAIR 7-day	BabyBAIR 7-day	MIS BAIR 7-month	BabyBAIR 3-month
Nil	RPMI	↑IL-6	↓IL-10	↓IL-10, IP-10, MIF, IL-8	↑IFN-γ
Intracellular	BCG		↑TNF-α	↑IFN-γ, MIG, IL-6, TNF-α	↑IFN-γ
	MTB			↑IFN-γ, MIG	↑IFN-γ, IL-8, IL-10, ↓IL-1β
	LM	↓MCP-1, IL-1RA, MIP-1β		↓IL-10, MIP-1α, MIP-1β	↑IL-8
	CA	↓MCP-1	↑TNF-α	↑MIG	↓IL-6
Gram+ve	GBS		↑IFN-γ, TNF-α	Not done	↓IL-6
	SP				↑IL-8, MIP-1β, ↓TNF-α, IL-1β
	SA			↑MIG, MIP-1β	↓TNF-α, IL-1β, IL-6 ↑IL-8, IL-10, MIP-1β
	GAS	Not done	Not done	↑MIG, MIF	Not done
Gram -ve	EC	↓MCP-1, MIP-1β	↑IFN-γ, TNF-α, ↓IL-8	↑IL-8, ↓IFN-γ	↑IL-8, IL-10, ↓IL-1β,
	HI		↑IFN-γ, TNF-α, MIP-1β	↓IFN-γ	↑IL-8, IL-10, ↓IL-1β, MIG
TLR-ligands	Pam3		↑IFN-γ, TNF-α IL-10	↑IL-1RA	↑IL-8, IL-1RA, MIP-1α, ↓MIG
	PEPG	↓MCP-1, MIP-1α, MIP-1β, IL-6		↓IFN-γ	↓IL-1β, MCP-1 ↑IL-8,
	LPS			↑IL-8, ↓IFN-γ,	↓IL-1β, ↑IL-8,
	R848	↓MCP-1, MIP-1α, MIP-1β, IL-6, IL-10, IL-1RA	↑MIG		↓IL-1β, ↑MIF

Table 6.1 Summary of statistically significant cytokine results from the MIS BAIR and BabyBAIR studies.

6.2.1 BCG-INDUCED HETEROLOGOUS CYTOKINE RESPONSES DIFFERS BETWEEN NEONATES AND OLDER INFANTS

Immune ontogeny refers to the development of the infant immune system over time [92]. The changing immune system may alter the response to a vaccine depending on the age at which the vaccine is administered. Distinct from this, is the concept that the immune response to a vaccine may change with time since vaccination, i.e. the immediate or early post vaccine response or the late, persistent post vaccine response. Clinical trials have shown that the effect of BCG vaccination on the reduction of all-cause mortality is most prominent in the first weeks of life, i.e. within the first weeks post neonatal BCG vaccination [247]. The cytokine response data from the MIS BAIR study at 7 days is therefore relevant in this context. In addition, comparison of these results with the cytokine response data from the Baby BAIR study at 7 days provides the opportunity to describe the effect of immune ontogeny on this early response. Understanding the role of immune ontogeny on the heterologous response to BCG vaccination has implications for: (i) the administration of BCG vaccine beyond the newborn period and (ii) the appropriateness of extrapolating results from studies in older infants and children, and those in adults, to neonates.

An important caveat to this discussion is the difficulty in interpreting alterations in BCG-induced cytokine responses from studies with different designs even when considering responses from the same time point, i.e. at 7 days post BCG vaccination. As outlined in chapter 3, changes in pro-inflammatory cytokine responses are more easily detected in 'within patient' (BabyBAIR) studies than 'between group' (MIS BAIR) studies. Thus, when cytokine/stimulant responses differ between these two studies, this may be due to either the difference in age of the participants (immune ontogeny) or the difference in study design. For this reason it is helpful to consider changes in the functional profile of cytokine responses (e.g. pro vs anti-inflammatory) rather than individual cytokine/stimulant responses and to hypothesise how the observed differences between infants of different age groups could result from the effect of immune ontogeny on the response to BCG vaccine.

In this thesis, the RPMI-stimulated (nil) cytokine responses represent the effect of BCG vaccination alone, prior to a heterologous *in vitro* challenge. Increased IL-1 β responses in RPMI-stimulated (nil) samples following BCG vaccination have been associated with the subsequent induction of trained immunity and the control of experimental viremia in adults at 1 month post-vaccination [44]. In the MIS BAIR study, at 7 days, BCG-vaccinated neonates had significantly increased IL-6 responses in RPMI-stimulated (nil) samples compared to BCG-naïve infants. While there was an increase in IL-1 β responses in BCG-vaccinated neonates compared to controls this was not statistically significant. Similarly, in a RCT done in Guinea-Bissau, BCG-vaccinated neonates had significantly increased TNF- α and IFN- γ responses and a non-significant increase in IL-1 β responses compared to BCG-naïve controls in RPMI-stimulated (nil) samples at 28 days post-vaccination [96]. While this study presents further evidence of an 'early' pro-inflammatory phenotype in BCG-vaccinated neonates, it is interesting the TNF- α and IFN- γ predominate in this study, an effect which may be due to age (28 days post vaccination) or study location [170]. In the BabyBAIR study, at 7 days in the RPMI-stimulated (nil) samples, there was no increase in a pro-inflammatory cytokine but rather a decrease in the anti-inflammatory cytokine IL-10 compared to pre-vaccination levels, which we concluded resulted in a pro-inflammatory bias [198]. While IL-10, in association with IL-1 α , regulates IL-1 β production, a concomitant increase in IL-1 β was not evident in our results. Therefore, the results of the studies in this thesis suggest that BCG vaccination induces a pro-inflammatory phenotype in the 'early' (7 days) post BCG vaccination period in both neonates (MIS BAIR) and older (BabyBAIR) infants, which manifests differently by age, and is distinct from that seen in adults. IL-6 is central to the initiation and control of inflammation in response to infection in neonates, and in these studies it appears that IL-6, rather than IL-1 β , may be involved in the 'early' immune response to BCG vaccine in this age group [171].

Following heterologous stimulation, there was again evidence of an 'early' pro-inflammatory BCG-induced cytokine response profile which manifested differently depending on age. While neonates (MIS BAIR) exhibited a pro-inflammatory bias characterised by lower levels of anti-inflammatory cytokines (IL-10, IL-1 α), older infants (BabyBAIR) mounted a more classical pro-inflammatory (TNF- α , IFN- γ) response

akin to that previously associated with BCG-induced trained immunity in adults [93]. One potential explanation for the absence of a classical pro-inflammatory response in the MIS BAIR study, is that the neonatal immune system is a relatively TNF- α deficient state [156, 248]. The mechanism by which a reduction in the anti-inflammatory cytokines IL-10 and IL-1Ra mediate a pro-inflammatory bias is uncertain from the results of the MIS BAIR study and warrants further investigation. However, the results of the studies in this thesis suggest that the measurement of cytokines associated with BCG-induced heterologous immunity in studies done in older infants or adults may not be relevant as markers of the heterologous effects of BCG vaccine in the neonatal age group.

In the MIS BAIR study at 7 days, IL-6 was again significantly altered during the heterologous response to BCG vaccination. The decreased levels of IL-6 in BCG-vaccinated neonates compared to controls might support the induction of a pro-inflammatory bias in BCG-vaccinated neonates as discussed above, as IL-6 is pleiotropic cytokine which has both anti- and pro-inflammatory potential, particularly in the neonatal period [92]. On the other hand, the lower levels of IL-6 in response to heterologous stimulation in BCG-vaccinated infants may represent enhanced regulation of inflammation in the context of neonatal infection, analogous to that seen at 1 month post BCG vaccination in adults during the control of experimental viraemia [44]. In neonates, during episodes of infection, declining IL-6 levels on day two are associated with enhanced response to antibiotics and improved outcomes [171], suggesting that any mechanism which controls IL-6 responses might be beneficial in the context of neonatal infection. It is therefore plausible that the reduction of heterologous IL-6 responses in BCG-vaccinated neonates is a consequence of the rapid induction of enhanced immune regulation in the context of pathogen exposure. If true, the measurement of this effect at 7 days post BCG-vaccination suggests that modulation of the innate immune system by BCG occurs more rapidly in neonates than in adults or older infants when mediated by IL-1 β , where it was not demonstrated until a month post vaccination [44, 80]. Kleinnijenhuis *et al.* demonstrated evidence of trained immunity and increased TNF- α production in response to heterologous stimulation (*S. aureus*, *E. coli*, LPS and *C. albicans*) after incubation of PBMCs with BCG

in vitro. However, *S. aureus* and *C. albicans* induced levels of IL-1 β were not significantly increased in PBMCS isolated from adult volunteers until 1 month post BCG vaccination [80].

The production of chemokines, as distinct from cytokines, and their role in the control of neonatal infection is less well defined. In the MIS BAIR study, at 7 days, BCG-induced heterologous immunity was associated with decreased production of monocyte-derived chemokines and specifically MCP-1. MCP-1 is involved in neutrophil chemotaxis during infection, the development of disseminated rather than latent TB disease and in the development of multiple sclerosis [249]. As such it is an interesting candidate to regulate both BCG-induced heterologous and anti-mycobacterial immunity in the newborn period. Interestingly, in the BabyBAIR study, at 7 days post BCG vaccination there was no effect on MCP-1 production and where chemokines were altered, their production was increased. While it is not possible to draw conclusions on the potential clinical relevance of this variation in BCG-induced heterologous chemokine responses between neonates and older infants at 7 days, it is plausible that given the prominent role for neutrophils during aberrant inflammatory processes in the neonatal period [250], the downregulation of chemokine responses in BCG-vaccinated neonates in the MIS BAIR study compared to controls would be particularly beneficial in this age group. This makes it an interesting candidate immune signature to correlate with clinical outcomes during neonatal infection following BCG vaccination.

Mortality in perinatal and neonatal sepsis is associated with a combination of hyper-inflammation (unchecked TLR signalling, marked leucocytosis) and concomitant immune suppression [250]. Thus, any intervention which counteracts or regulates the development of these two syndromes would be beneficial. As discussed above, the studies in this thesis support an early BCG-induced pro-inflammatory cytokine response in both neonates and older infants, although the manifestations of this vary by age at the time of vaccination. Furthermore, neonates appear to develop a concomitant downregulation of IL-6 and chemokine responses, which may be indicative of enhanced immune regulation during pathogen exposure in this age group.

In the absence of a clinical correlate of disease it is not possible to conclusively determine the clinical significance of these results. However, it is plausible that a combination of; (i) pro-inflammatory bias which leads to enhanced pathogen clearance (ii) modification of the inflammatory response to infection and (iii) decreased neutrophil-mediated inflammation would be particularly beneficial in counteracting the pathophysiological changes in neonatal sepsis. In addition, these results highlight the importance of considering the effect of immune ontogeny in the design and interpretation of future studies of BCG-induced heterologous immunity in this population.

6.2.2 BCG-INDUCED HETEROLOGOUS IMMUNITY IN BOTH NEONATES AND INFANTS INVOLVES CHANGES WHICH ARE CONSISTENT WITH AN EFFECT ON INNATE IMMUNITY

BCG vaccine alters TLR signalling in animal and *in vitro* models (Table 3.3). There are several results from the studies in this thesis which suggest that TLR-signalling contributes to BCG-induced heterologous cytokine responses in neonates and infants. Firstly, significant BCG-induced alterations in the early pro-inflammatory response in neonates (MIS BAIR) was only seen following stimulation with TLR ligands, which may reflect the relative importance of innate immunity in the newborn period. Secondly, significant BCG-induced alterations in the late (3-7 month) response were characterised by the same pattern of change following stimulation with both TLR ligands and heterologous pathogens. For example, in the MIS BAIR study at 7 months, alterations in IFN- γ responses were similar following stimulation with Gram-negative bacteria (*E. coli*, *H. influenzae*) and the TLR-ligands 2 and 4 (PEPG, LPS). Toll-like receptors 2 and 4 are central to the control of Gram-negative sepsis, thus concordance in cytokine responses following these stimulations is not surprising but does suggest a role for signalling via TLR2 and TLR4 in mediating the observed BCG-induced heterologous cytokine responses. Of note, blockade of TLR4 (LPS) and TLR2 (PAM3) had no adverse effect on the ability of BCG to 'train' monocytes and induce heterologous pro-inflammatory cytokine responses in adults, whereas NOD2 blockade did. These results suggest a minimal role for TLRs in BCG-induced trained immunity in adults. Our observation that stimulation of TLRs lead to differential cytokine responses

in BCG-vaccinated infants indicates that either (i) trained immunity is not the underlying mechanism for this observation in infants or (ii) that trained innate immunity is mediated via epigenetic changes in TLR signalling rather than NOD2 signalling in this age group. This provides further evidence for the influence of age and immune maturation on the innate immune response to BCG vaccine.

Trained immunity is a potential mechanism for BCG-induced heterologous immunity based on studies done in adult participants [44, 45]. Trained immunity refers to epigenetic modification of immune pathways leading to the development of innate immune memory and enhanced protection against subsequent infection [251-253]. In the absence of epigenetic analyses, the studies presented in this thesis cannot conclusively associate alterations in BCG-induced heterologous cytokine responses with the induction of trained immunity. However, the pattern of altered BCG-induced heterologous cytokine responses seen in the BabyBAIR study is consistent with those previously described in the context of trained immunity in adults [44, 93] and is accompanied by concomitant alterations in gene pathways associated with epigenetic modification. Taken together, these results provide indirect evidence for the role of trained immunity in the development of BCG-induced heterologous cytokine responses in neonates and infants.

The results of the BabyBAIR study show that, in infants, BCG vaccination leads to widespread changes in the transcriptional profile of whole blood which persist up to 3 months post vaccination. Several hypothetical molecular mechanisms underpinning BCG-induced heterologous cytokine responses related to the innate immune system were identified in the BabyBAIR study, including control of signalling through NLRs, RLRs, TLRs and complement, the induction of autophagy and the control of neutrophil degranulation. In contrast, systems vaccinology studies of BCG-induced heterologous immunity in adults failed to identify significant transcriptomic changes in PBMCs at 1 month post BCG vaccination and concluded that, in the context of trained immunity, BCG vaccine did not lead to transcriptional changes in monocytes prior to heterologous challenge [44]. The presence of widespread alterations in the transcriptional profile of infants, not seen in adults, may be due to the methodological

difference between the studies, including the use of whole blood (in contrast to PBMCs) or may indicate that BCG-induced transcriptional changes are different in infants compared to adults.

In summary, the results of this thesis are consistent with a role for innate immunity in mediating the beneficial non-specific effects of BCG vaccination, particularly in neonates and in the early post vaccination period. Immune regulatory cytokines of the innate immune system including IL-1Ra may be important in the evolution of BCG-induced heterologous cytokine responses over time. While the results of the BabyBAIR study support the paradigm of trained immunity in infants, the unsupervised assessment of the whole blood transcriptome indicated widespread alterations in pathways associated with the control of innate immunity and inflammation not previously described in adults

6.2.3 BCG-INDUCED CYTOKINE RESPONSES DIFFER DEPENDING ON THE NATURE OF THE *IN VITRO* CHALLENGE PATHOGEN (SPECIFIC VS HETEROLOGOUS).

Trained immunity has been suggested as a potential mechanism to explain both the heterologous and the specific, anti-mycobacterial effects of BCG vaccine [254-256]. In adults, BCG-induced trained immunity has been associated with the control of heterologous infection *in vivo* and the control of mycobacterial growth *in vitro* [44, 105]. While epigenetic changes indicative of trained immunity were identified in both contexts, the effector mechanisms with which they correlated varied by pathogen [44, 105]. Similarly, in the BabyBAIR study at 3 months and the MIS BAIR study at 7 months, there was evidence that BCG vaccination had significant but opposing effects on mycobacterial and heterologous IFN- γ responses, despite a consistent pattern of IL-8 responses to both classes of pathogen. As discussed previously, we cannot conclude that these alterations in heterologous cytokine responses are due to the induction of trained immunity, but irrespective of the underlying mechanism, it is clear that immunological correlates of BCG vaccine identified as significant in the response to a single pathogen may not be transferrable to studies in other contexts.

In addition, our review of BCG-induced heterologous immunity in animals (chapter 2), highlighted that decreased mortality following intracellular bacterial (mycobacteria, salmonella, listeria) infection were generally associated with increased pathogen clearance and sequestration in the spleen (i.e. prevention of dissemination). In contrast, during challenge models of extracellular bacteria and viruses, mortality was decreased irrespective of the circulating pathogen load in the blood, suggesting a role for control of inflammation rather than pathogen clearance in this context [43, 75]. Thus it is plausible that BCG vaccination leads to changes in the immune system at baseline which result in the induction of disparate effector mechanisms which are tailored to the nature of the subsequent pathogen challenge.

This observation can also be explained by the induction of resistance and tolerance to heterologous infection following BCG vaccination. These pathways may be inter-dependent or independent and the mechanism may vary between pathogen types [138, 139]. The widespread BCG-induced alterations in the whole blood transcriptome of infants from the BabyBAIR study suggest that changes in the infant immune system go beyond those thus far identified in association with trained immunity in adults. Furthermore, in this model of BCG-induced immunity, correlation of post BCG vaccination changes at baseline with subsequent evidence of enhanced immune regulation in the context of any infection (rather than with a particular effector mechanism or survival from a particular disease) may have more power to detect a beneficial effect of BCG vaccine and may represent a more holistic approach to understanding the range of host-pathogen interactions which take place following BCG vaccination of infants [138].

6.2.4 THERE ARE SEVERAL CONSISTENT EFFECT MODIFIERS OF BCG-INDUCED HETEROLOGOUS IMMUNITY IN BOTH NEONATES AND OLDER INFANTS (SEX, MATERNAL BCG).

The beneficial effect of BCG vaccination on all-cause mortality is greatest in boys in the early post-natal period and in girls later in infancy [247]. The finding in the MIS BAIR study at 7 days, that BCG-vaccinated boys had decreased production of MIF in response to heterologous stimulation is interesting in light of the role of MIF in

neonatal immunity and response to infection [257, 258]. Blockade of MIF responses during established infection is associated with improved mortality in a model of neonatal mouse sepsis [182]. Consistent with a later beneficial clinical effect of BCG in girls, in MIS BAIR study at 7 months post BCG vaccination, girls had increased heterologous pro-inflammatory cytokine responses and concomitant upregulation of IL-1Ra responses, which was not observed in boys. As discussed above, IL-1 signalling is central to the hypothesis of BCG-induced trained immunity and subsequent protection from infection in adults thus significant changes in the key regulatory cytokine IL-1Ra which are more marked in female rather than male infants is notable. These results suggest that, similar to the results of the primary analysis, immune ontogeny may lead to the differences in the effect of sex on BCG-induced heterologous immunity and the variability of the clinical effect by sex at different ages.

Maternal BCG vaccination status was an effect modifier on the risk of hospitalisation due to infection in the first year of life in a Danish RCT of BCG vaccination at birth [38]. As BCG vaccination is not routinely administered in Australia, and consequently only a proportion of participants' mothers had been BCG vaccinated, the MIS BAIR study provided an opportunity to investigate the influence of maternal BCG vaccination on infant cytokine responses following stimulation with pathogens and TLR-ligands in both BCG-vaccinated infants and controls in both the early (7 days) and late (7 months) post BCG vaccination period. Prior discourse in the literature has suggested that maternal BCG vaccination might influence the infant immune response to BCG vaccination through epigenetic modifications of the fetal immune system or trans-placental transfer of antibodies, neither of which have been studied in great detail to date. In the MIS BAIR study at 7 months, maternal BCG vaccination led to a generalised increase in TNF- α responses to bacterial stimulation *in vitro* irrespective of infant BCG vaccination status which suggests that an independent effect on fetal immunity (i.e. epigenetics) is more likely than a prime-boost type mechanism (antibody mediated). Irrespective of the underlying mechanism, studies of BCG-induced heterologous immunity need to consider the potential for maternal BCG status to influence outcomes.

A major finding of this thesis was the observation of an effect of BCG vaccination on individual level IFN- γ responsiveness following heterologous stimulation *in vitro*. Whether this is due to a genetic or environmental predisposition is unclear. Potential factors that might contribute to this observation include SNPs, maternal BCG vaccination, season of birth and mode of delivery, and, in the relevant setting, maternal LTBI, maternal helminth infection and placental malaria. It is possible that either responders or non-responders represent a sub-group of patients in whom the clinical benefits of BCG vaccination are more prominent. Identifying such groups and the associated effect modifier is important for (i) determining populations in which BCG vaccination is likely to have the most pronounced clinical effect and (ii) the design of studies with adequate power to identify the mechanisms for the heterologous effects of BCG vaccination.

6.2.5 INTER-INDIVIDUAL AND INTER-STIMULANT VARIATION IN CYTOKINE RESPONSES IMPACT THE DETECTION OF AN EFFECT ATTRIBUTABLE TO BCG VACCINATION.

As previously discussed, interpretation of cytokine response data from unpaired (independent) groups is affected by inter-individual variability to a greater extent than that from paired (within individual) studies. Cytokine/stimulant pairs characterised by highly dynamic response profiles, such as pro-inflammatory cytokines, were more susceptible to this effect. The statistical methodology developed as part of this thesis addressed these considerations by: (i) assessing the distribution of individual cytokine/stimulant pairs (rather than the data as a whole) prior to selecting statistical test in both the MIS BAIR and the BabyBAIR studies and (ii) incorporating the RPMI (nil) stimulated value as a covariate in the analysis of the independent group data in MIS BAIR. This methodology controlled for variability introduced by an individual's baseline immune phenotype and their responsiveness to a given antigen, thus maximising the chance of detecting an effect attributable to BCG vaccine in the MIS BAIR study. Despite this approach, the results of cytokine response data from the two studies are not directly comparable. The concordance of individual cytokine/stimulant pairs is less important than the concordance of cytokine response profiles with similar functional capacity. This understanding has underpinned the interpretation of the results in this

thesis and has facilitated observations of changes in the nature of cytokine response profiles which are associated with age and/or time since vaccination.

Although studies comparing independent groups suffer from the disadvantage of variability between individual, they have some advantages. The larger numbers of participants in the MIS BAIR study allowed assessment of the influence of effect modifiers (such as sex and maternal BCG vaccination) through interaction and subset analyses which are limited in smaller cohort studies. The relatively large sample size also facilitated detection of the significant changes in individual level IFN- γ responsiveness following heterologous stimulation. This was complementary to the BabyBAIR study which had smaller numbers but allowed a more in-depth analysis of the mechanism underlying the non-specific effect of BCG.

6.2.6 STRENGTHS AND LIMITATIONS

The individual strengths and limitations of each study are discussed in detail in the relevant chapters. Overall, the major strength of this thesis is the interpretation of data from two study types done using consistent laboratory methodologies in the same geographical location. As detailed above, the MIS BAIR study provided proof of principle that BCG vaccination altered heterologous cytokine responses in both neonates and older infants and allowed evaluation of effect modifiers while the BabyBAIR study facilitated investigation of potential underlying mechanisms.

A further strength of this thesis was the development and use of a whole blood assay which included several classes of clinically relevant pathogens alongside TLR ligands and mycobacterial antigens. This combination led to the conclusion that innate immunity and specifically TLR signalling had a role in mediating BCG-induced heterologous immunity and that the manifestations of this response may vary between pathogens.

The main limitation of this work is the inability to definitively assign clinical relevance to the immune signatures which were altered by BCG vaccination. The gene expression pathways and patterns of cytokine response identified in the course of this thesis suggest a role for trained immunity but also suggest widespread transcriptional

changes which could hypothetically be associated with immune regulation of the innate immune system and survival benefit in the context of infection-related inflammation.

A further limitation of this thesis was the inability to fully characterise the longitudinal evolution of BCG-induced heterologous cytokine responses. In the MIS BAIR study there was a population of infants with cytokine response data at two time points (7 days and 7 months) but differences in the multiplex cytokine kits batches made direct comparison of results over time impossible. In the BabyBAIR study, visualisation of dynamic changes in heterologous cytokine responses over time was informative but due to difficulties with retention of infants at multiple sampling points, the overall number of paired samples across three time points was limited and precluded any statistical analysis of observed trends. This highlights the difficulty of doing systems vaccinology studies in infants and children and the need for careful planning of the choice of time point and blood sampling to maximise retention.

6.2.7 FUTURE RESEARCH

Nested-immunological studies in trials of BCG vaccine should incorporate capacity to identify the cause of death in both BCG-vaccinated and BCG-naïve infants with respect to pathogen and mechanism (i.e. hyper-inflammation versus immune-suppression). The development and validation of an immunological correlate of protection from heterologous infection following BCG-vaccination which is a composite signature of enhanced immune regulation in the context of infection may be an innovative approach which avoids the limitations of measuring the effector responses relevant to a single pathogen challenge.

Future studies should be designed giving adequate consideration to (i) the role of immune ontogeny and (ii) environmental and genetic determinants of the immune response to BCG vaccination in a given population. Evaluation of the immunological and clinical response to BCG vaccination with these effects in mind may identify the subgroups of participants most likely to benefit from the non-specific effects of BCG vaccination. This has the potential to extend the scope of BCG vaccination beyond

routine immunisation to intervention or adjuvant intervention for specific diseases or in certain groups of patient. The results of the studies in this thesis suggest several gene expression pathways and immune mechanisms which could be prioritised or validated in future studies of BCG-induced heterologous immunity.

6.2.8 CONCLUSIONS

BCG vaccine is a powerful immune-modulator which leads to a reduction in all-cause infant mortality in high mortality settings. The studies in this thesis (i) confirm that BCG vaccination alters the immune response to heterologous pathogens *in vitro* in neonates and infants and (ii) provides hypothetical molecular mechanisms underpinning these changes through the comparison of alterations in the whole blood transcriptome following BCG-vaccination with concurrent changes in BCG-induced heterologous cytokine responses. Furthermore, the results of this thesis show that BCG-induced heterologous immunity varies between individuals and is modified by immune ontogeny, sex and maternal BCG vaccine status. Quantifying the nature and contribution of effect modifiers on the immune response may allow identification of those patients most likely to benefit from the heterologous effects of BCG vaccination.

Overall, this thesis supports the hypothesis that BCG vaccination leads to non-specific, heterologous immunity which improves survival during infection through more effective initiation and control of inflammation. This effect may be 'syndrome specific' rather than pathogen specific and this needs to be considered in the development of appropriate correlates of heterologous protection. Further understanding of the heterologous effects of BCG vaccination might also contribute to the understanding of the protective efficacy of BCG against TB.

BCG vaccination provides a cheap, safe and effective mechanism to achieve gains in the reduction of infant mortality in high-mortality settings. Maximising the beneficial effect of BCG through a greater understanding of the mechanism of action should be the goal of future combined clinical and immunological studies. The ability to modulate the infant immune system in a pathogen-independent manner is particularly attractive in the era of anti-microbial resistance.

Bibliography

1. Strachan, D.P., *Hay fever, hygiene, and household size*. BMJ, 1989. **299**(6710): p. 1259-60.
2. Rook, G.A., *Review series on helminths, immune modulation and the hygiene hypothesis: the broader implications of the hygiene hypothesis*. Immunology, 2009. **126**(1): p. 3-11.
3. Marchant, A., et al., *Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination*. J Immunol, 1999. **163**(4): p. 2249-55.
4. Freyne, B. and N. Curtis, *Does neonatal BCG vaccination prevent allergic disease in later life?* Arch Dis Child, 2014. **99**(2): p. 182-4.
5. Steenhuis, T.J., et al., *Bacille-Calmette-Guerin vaccination and the development of allergic disease in children: a randomized, prospective, single-blind study*. Clin Exp Allergy, 2008. **38**(1): p. 79-85.
6. Koppen, S., et al., *No epidemiological evidence for infant vaccinations to cause allergic disease*. Vaccine, 2004. **22**(25-26): p. 3375-85.
7. Balicer, R.D., et al., *Is childhood vaccination associated with asthma? A meta-analysis of observational studies*. Pediatrics, 2007. **120**(5): p. e1269-77.
8. Arnoldussen, D.L., M. Linehan, and A. Sheikh, *BCG vaccination and allergy: a systematic review and meta-analysis*. J Allergy Clin Immunol, 2011. **127**(1): p. 246-53, 253 e1-21.
9. El-Zein, M., et al., *Does BCG vaccination protect against the development of childhood asthma? A systematic review and meta-analysis of epidemiological studies*. International Journal of Epidemiology, 2010. **39**(2): p. 469-486.
10. Thostesen, L.M., et al., *Neonatal BCG has no effect on allergic sensitization and suspected food allergy until 13 months*. Pediatr Allergy Immunol, 2017. **28**(6): p. 588-596.
11. Thostesen, L.M., et al., *Neonatal BCG vaccination and atopic dermatitis before 13 months of age: A randomized clinical trial*. Allergy, 2018. **73**(2): p. 498-504.
12. Thostesen, L.M., et al., *Neonatal BCG vaccination has no effect on recurrent wheeze in the first year of life: A randomized clinical trial*. J Allergy Clin Immunol, 2017. **140**(6): p. 1616-1621.e3.
13. Naeslund, C., *Resultats des experiences de vaccination par le BCG poursuivie dane le Norbotten (Suede)*. Institut Pasteur. 1932, Paris: Masson et Cie.
14. Dye, C., *Making wider use of the world's most widely used vaccine: Bacille Calmette-Guerin revaccination reconsidered*. J R Soc Interface, 2013. **10**(87): p. 20130365.
15. Colditz, G.A., et al., *Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature*. JAMA, 1994. **271**(9): p. 698-702.
16. Colditz, G.A., et al., *The efficacy of bacillus Calmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature*. Pediatrics, 1995. **96**(1 Pt 1): p. 29-35.
17. Trunz, B.B., P. Fine, and C. Dye, *Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness*. Lancet, 2006. **367**(9517): p. 1173-80.

18. Andersen, P. and T.M. Doherty, *The success and failure of BCG - implications for a novel tuberculosis vaccine*. Nat Rev Microbiol, 2005. **3**(8): p. 656-62.
19. McShane, H., *Tuberculosis vaccines: beyond bacille Calmette-Guerin*. Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1579): p. 2782-9.
20. Roth, A., et al., *Tuberculin reaction, BCG scar, and lower female mortality*. Epidemiology, 2006. **17**(5): p. 562-8.
21. Calmette, A., *Preventive Vaccination Against Tuberculosis with BCG*. Proc R Soc Med, 1931. **24**(11): p. 1481-90.
22. Shann, F., *The non-specific effects of vaccines*. Arch Dis Child, 2010. **95**(9): p. 662-7.
23. Aaby, P., et al., *Sex-differential non-specific effects of BCG and DTP in Cebu, The Philippines*. International Journal of Epidemiology, 2009. **38**(1): p. 320-3.
24. Aaby, P., et al., *Vaccinia scars associated with better survival for adults. An observational study from Guinea-Bissau*. Vaccine, 2006. **24**(29-30): p. 5718-25.
25. Aaby, P., et al., *Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period?* J Infect Dis, 2011. **204**(2): p. 245-52.
26. Aaby, P., et al., *Sex differential effects of routine immunizations and childhood survival in rural Malawi*. Pediatr Infect Dis J, 2006. **25**(8): p. 721-7.
27. Biering-Sorensen, S., et al., *Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact*. Pediatr Infect Dis J, 2012. **31**(3): p. 306-8.
28. Kristensen, I., P. Aaby, and H. Jensen, *Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa*. BMJ, 2000. **321**(7274): p. 1435-8.
29. Roth, A., et al., *BCG vaccination scar associated with better childhood survival in Guinea-Bissau*. Int J Epidemiol, 2005. **34**(3): p. 540-7.
30. Roth, A., et al., *Low birth weight infants and Calmette-Guerin bacillus vaccination at birth: community study from Guinea-Bissau*. Pediatr Infect Dis J, 2004. **23**(6): p. 544-50.
31. Storgaard, L., et al., *Development of BCG Scar and Subsequent Morbidity and Mortality in Rural Guinea-Bissau*. Clin Infect Dis, 2015. **61**(6): p. 950-9.
32. Vaugelade, J., et al., *Non-specific effects of vaccination on child survival: prospective cohort study in Burkina Faso*. Bmj, 7478. **329**(7478): p. 4.
33. Higgins, J.P., et al., *Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review*. Bmj, 2016. **355**: p. i5170.
34. Jayaraman, K., et al., *Two Randomized Trials of the Effect of the Russian Strain of Bacillus Calmette-Guerin Alone or With Oral Polio Vaccine on Neonatal Mortality in Infants Weighing <2000 g in India*. Pediatr Infect Dis J, 2019. **38**(2): p. 198-202.
35. Biering-Sorensen, S., et al., *Early BCG-Denmark and Neonatal Mortality Among Infants Weighing <2500 g: A Randomized Controlled Trial*. Clin Infect Dis, 2017. **65**(7): p. 1183-1190.
36. Schaltz-Buchholzer, F., et al., *Early BCG Vaccination, Hospitalizations, and Hospital Deaths: Analysis of a Secondary Outcome in 3 Randomized Trials from Guinea-Bissau*. J Infect Dis, 2019. **219**(4): p. 624-632.

37. Stensballe, L.G., et al., *BCG vaccination at birth and early childhood hospitalisation: a randomised clinical multicentre trial*. Arch Dis Child, 2017. **102**(3): p. 224-231.
38. Stensballe, L.G., et al., *BCG Vaccination at Birth and Rate of Hospitalization for Infection Until 15 Months of Age in Danish Children: A Randomized Clinical Multicenter Trial*. J Pediatric Infect Dis Soc, 2018.
39. de Castro, M.J., J. Pardo-Seco, and F. Martinon-Torres, *Nonspecific (Heterologous) Protection of Neonatal BCG Vaccination Against Hospitalization Due to Respiratory Infection and Sepsis*. Clin Infect Dis, 2015. **60**(11): p. 1611-9.
40. Hollm-Delgado, M.G., E.A. Stuart, and R.E. Black, *Acute lower respiratory infection among Bacille Calmette-Guerin (BCG)-vaccinated children*. Pediatrics, 2014. **133**(1): p. e73-81.
41. Stensballe, L.G., et al., *Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study*. Vaccine, 1251. **23**(10): p. 1251-7.
42. Rodrigues, A., et al., *Community cohort study of rotavirus and other enteropathogens: are routine vaccinations associated with sex-differential incidence rates?* Vaccine, 2006. **24**(22): p. 4737-46.
43. Freyne, B., A. Marchant, and N. Curtis, *BCG-associated heterologous immunity, a historical perspective: intervention studies in animal models of infectious diseases*. Trans R Soc Trop Med Hyg, 2015. **109**(1): p. 52-61.
44. Arts, R.J.W., et al., *BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity*. Cell Host Microbe, 2018. **23**(1): p. 89-100.e5.
45. Walk, J., et al., *Outcomes of controlled human malaria infection after BCG vaccination*. Nat Commun, 2019. **10**(1): p. 874.
46. Haahr, S., et al., *Non-specific effects of BCG vaccination on morbidity among children in Greenland: a population-based cohort study*. Int J Epidemiol, 2016. **45**(6): p. 2122-2130.
47. Jason, J., et al., *Clinical and immune impact of Mycobacterium bovis BCG vaccination scarring*. Infect Immun, 2002. **70**(11): p. 6188-95.
48. Aaby, P. and C.S. Benn, *Non-specific and sex-differential effects of routine vaccines: what evidence is needed to take these effects into consideration in low-income countries?* Hum Vaccin, 2011. **7**(1): p. 120-4.
49. Thostesen, L.M., et al., *Bacillus Calmette-Guerin immunisation at birth and morbidity among Danish children: A prospective, randomised, clinical trial*. Contemp Clin Trials, 2015. **42**: p. 213-8.
50. Flanagan, K.L., et al., *Sex differences in the vaccine-specific and non-targeted effects of vaccines*. Vaccine, 2011. **29**(13): p. 2349-54.
51. Darboe, F., et al., *Minimal Sex-Differential Modulation of Reactivity to Pathogens and Toll-Like Receptor Ligands following Infant Bacillus Calmette-Guerin Russia Vaccination*. Front Immunol, 2017. **8**: p. 1092.
52. Breiman, R.F., et al., *Effect of infant immunisation on childhood mortality in rural Bangladesh: analysis of health and demographic surveillance data*. Lancet, 2004. **364**(9452): p. 2204-11.

53. Nissen, T.N., et al., *Bacillus Calmette-Guerin vaccination at birth and in vitro cytokine responses to non-specific stimulation. A randomized clinical trial*. Eur J Clin Microbiol Infect Dis, 2018. **37**(1): p. 29-41.
54. Jensen, K.J., et al., *Heterologous and sex differential effects of administering vitamin A supplementation with vaccines*. Trans R Soc Trop Med Hyg, 2015. **109**(1): p. 36-45.
55. Arts, R.J., et al., *Vitamin A induces inhibitory histone methylation modifications and down-regulates trained immunity in human monocytes*. J Leukoc Biol, 2015. **98**(1): p. 129-36.
56. Diness, B.R., et al., *Effect of high-dose vitamin A supplementation on the immune response to Bacille Calmette-Guerin vaccine*. Am J Clin Nutr, 2007. **86**(4): p. 1152-9.
57. Hsu, J.W., et al., *1 alpha, 25-dihydroxylvitamin D3 promotes Bacillus Calmette-Guerin immunotherapy of bladder cancer*. Oncotarget, 2013. **4**(12): p. 2397-406.
58. Jeon, K., et al., *Severe vitamin D deficiency is associated with non-tuberculous mycobacterial lung disease: a case-control study*. Respirology, 2013. **18**(6): p. 983-8.
59. Nelson, C.D., et al., *Regulation of Mycobacterium-specific mononuclear cell responses by 25-hydroxyvitamin D3*. PLoS ONE [Electronic Resource], 2011. **6**(6): p. e21674.
60. Nonnecke, B.J., et al., *In vitro effects of 1,25-dihydroxyvitamin D3 on interferon- and tumor necrosis factor-alpha secretion by blood leukocytes from young and adult cattle vaccinated with Mycobacterium bovis BCG*. International Journal for Vitamin and Nutrition Research, 2003. **73**(4): p. 235-244.
61. Salahuddin, N., et al., *Vitamin D accelerates clinical recovery from tuberculosis: results of the SUCCINCT Study [Supplementary Cholecalciferol in recovery from tuberculosis]. A randomized, placebo-controlled, clinical trial of vitamin D supplementation in patients with pulmonary tuberculosis*. BMC Infect Dis, 2013. **13**: p. 22.
62. Behr, M.A., *Correlation between BCG genomics and protective efficacy*. Scand J Infect Dis, 2001. **33**(4): p. 249-52.
63. Ritz, N., et al., *The influence of bacille Calmette-Guerin vaccine strain on the immune response against tuberculosis: a randomized trial*. American Journal of Respiratory & Critical Care Medicine, 2012. **185**(2): p. 213-22.
64. Ritz, N., et al., *Influence of BCG vaccine strain on the immune response and protection against tuberculosis*. FEMS Microbiol Rev, 2008. **32**(5): p. 821-41.
65. Brewer, T.F. and G.A. Colditz, *Relationship between bacille Calmette-Guerin (BCG) strains and the efficacy of BCG vaccine in the prevention of tuberculosis*. Clin Infect Dis, 1995. **20**(1): p. 126-35.
66. Garly, M.L., et al., *BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa. A non-specific beneficial effect of BCG? Vaccine*, 2003. **21**(21-22): p. 2782-90.
67. Anderson, E.J., et al., *The influence of BCG vaccine strain on mycobacteria-specific and non-specific immune responses in a prospective cohort of infants in Uganda*. Vaccine, 2012. **30**(12): p. 2083-9.

68. Shann, F., *Substantial benefits from finding the most effective BCG strain*. Lancet Respir Med, 2016. **4**(7): p. e35.
69. Biering-Sorensen, S., et al., *Variation of growth in the production of the BCG vaccine and the association with the immune response. An observational study within a randomised trial*. Vaccine, 2015. **33**(17): p. 2056-65.
70. Mawa, P.A., et al., *Maternal BCG scar is associated with increased infant proinflammatory immune responses*. Vaccine, 2017. **35**(2): p. 273-282.
71. Berendsen, M.L.T., et al., *Maternal Priming: Bacillus Calmette-Guerin (BCG) Vaccine Scarring in Mothers Enhances the Survival of Their Child With a BCG Vaccine Scar*. J Pediatric Infect Dis Soc, 2019.
72. Rehmann, B. and E.C. Shin, *Private aspects of heterologous immunity*. J Exp Med, 2005. **201**(5): p. 667-70.
73. Selin, L.K., et al., *Memory of mice and men: CD8+ T-cell cross-reactivity and heterologous immunity*. Immunol Rev, 2006. **211**: p. 164-81.
74. Fanning, M.M., *Immunology of BCG vaccine*. Asian Pac J Allergy Immunol, 1984. **2**(2): p. 262-71.
75. Freyne, B., A. Marchant, and N. Curtis, *BCG-associated heterologous immunity, a historical perspective: experimental models and immunological mechanisms*. Trans R Soc Trop Med Hyg, 2015. **109**(1): p. 46-51.
76. Mathurin, K.S., et al., *CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses*. Journal of virology, 2009. **83**(8): p. 3528-3539.
77. Mackaness, G.B., *THE IMMUNOLOGICAL BASIS OF ACQUIRED CELLULAR RESISTANCE*. The Journal of Experimental Medicine, 1964. **120**(1): p. 105-120.
78. Mackaness, G.B., *THE INFLUENCE OF IMMUNOLOGICALLY COMMITTED LYMPHOID CELLS ON MACROPHAGE ACTIVITY IN VIVO*. The Journal of Experimental Medicine, 1969. **129**(5): p. 973-992.
79. van Dissel JT, et al., *Divergent changes in antimicrobial activity after immunologic activation of mouse peritoneal macrophages*. Journal of Immunology, 1987. **139**(5): p. 1665-1672.
80. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(43): p. 17537-17542.
81. Howard, J.G., et al., *The effect of mycobacterium tuberculosis (BCG) infection on the resistance of mice to bacterial endotoxin and salmonella enteritidis infection*. Clinique Medicale Propedeutique d l'Hopital Broussais, 1959.
82. Nakamura, M. and W.R. Cross, *Susceptibility of rabbits immunized with Mycobacterium bovis (BCG) or Mycobacterium phlei to Shigella keratoconjunctivitis*. Infect Immun, 1972. **6**(6): p. 1025-30.
83. Suenaga, T., et al., *Effect of Mycobacterium tuberculosis BCG infection on the resistance of mice to ectromelia virus infection: participation of interferon in enhanced resistance*. Infection & Immunity, 1978. **20**(1): p. 312-4.
84. Larson, C.L., et al., *Herpesvirus hominis type 2 infections in rabbits: effect of prior immunization with attenuated Mycobacterium bovis (BCG) cells*. Infect Immun, 1972. **6**(4): p. 465-8.

85. Clark, I.A., A.C. Allison, and F.E. Cox, *Protection of mice against Babesia and Plasmodium with BCG*. Nature, 1976. **259**(5541): p. 309-11.
86. Kleinert, R.D.V., et al., *Yellow Fever: Integrating Current Knowledge with Technological Innovations to Identify Strategies for Controlling a Re-Emerging Virus*. Viruses, 2019. **11**(10).
87. Sattler, R.A., et al., *Animal Models of Lassa Fever*. Pathogens, 2020. **9**(3).
88. Dubos, R.J. and R.W. Schaedler, *Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections I. Protective effects*. J Exp Med, 1957. **106**(5): p. 703-17.
89. Parra, M., et al., *Molecular analysis of non-specific protection against murine malaria induced by BCG vaccination*. PLoS One, 2013. **8**(7): p. e66115.
90. Kandasamy, R., et al., *Non-specific immunological effects of selected routine childhood immunisations: systematic review*. Bmj, 2016. **355**: p. i5225.
91. Corbett, N.P., et al., *Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells*. PLoS One, 2010. **5**(11): p. e15041.
92. Dowling, D.J. and O. Levy, *Ontogeny of early life immunity*. Trends Immunol, 2014. **35**(7): p. 299-310.
93. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes*. Proc Natl Acad Sci U S A, 2012. **109**(43): p. 17537-42.
94. Lalor, M.K., et al., *BCG vaccination induces different cytokine profiles following infant BCG vaccination in the UK and Malawi*. Journal of Infectious Diseases, 2011. **204**(7): p. 1075-1085.
95. Lalor, M.K., et al., *Complex cytokine profiles induced by BCG vaccination in UK infants*. Vaccine, 2010. **28**(6): p. 1635-41.
96. Jensen, K.J., et al., *Heterologous immunological effects of early BCG vaccination in low-birth-weight infants in Guinea-Bissau: a randomized-controlled trial*. J Infect Dis, 2015. **211**(6): p. 956-67.
97. Smith, S.G., et al., *Whole Blood Profiling of Bacillus Calmette-Guerin-Induced Trained Innate Immunity in Infants Identifies Epidermal Growth Factor, IL-6, Platelet-Derived Growth Factor-AB/BB, and Natural Killer Cell Activation*. Front Immunol, 2017. **8**: p. 644.
98. Hanekom, W.A., *The immune response to BCG vaccination of newborns*, in *Human Immunology: Patient-Based Research*, R.M. Steinman, Editor. 2005. p. 69-78.
99. Djuardi, Y., et al., *A longitudinal study of BCG vaccination in early childhood: the development of innate and adaptive immune responses*. PLoS One, 2010. **5**(11): p. e14066.
100. Kleinnijenhuis, J., et al., *Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity*. J Innate Immun, 2014. **6**(2): p. 152-8.
101. Kleinnijenhuis, J., et al., *BCG-induced trained immunity in NK cells: Role for non-specific protection to infection*. Clin Immunol, 2014. **155**(2): p. 213-9.
102. Nakaya, H.I. and B. Pulendran, *Vaccinology in the era of high-throughput biology*. Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1671).

103. Pulendran, B., *Systems vaccinology: probing humanity's diverse immune systems with vaccines*. Proc Natl Acad Sci U S A, 2014. **111**(34): p. 12300-6.
104. Matsumiya, M., et al., *Gene expression and cytokine profile correlate with mycobacterial growth in a human BCG challenge model*. J Infect Dis, 2015. **211**(9): p. 1499-509.
105. Joosten, S.A., et al., *Mycobacterial growth inhibition is associated with trained innate immunity*. J Clin Invest, 2018.
106. Fletcher, H.A., et al., *Human newborn bacille Calmette-Guerin vaccination and risk of tuberculosis disease: a case-control study*. BMC Med, 2016. **14**: p. 76.
107. Wilson, M.E., H.V. Fineberg, and G.A. Colditz, *Geographic latitude and the efficacy of bacillus Calmette-Guerin vaccine*. Clin Infect Dis, 1995. **20**(4): p. 982-91.
108. Oosterlinck, W. and K. Decaestecker, *Current strategies in the treatment of non-muscle-invasive bladder cancer*. Expert Rev Anticancer Ther, 2012. **12**(8): p. 1097-106.
109. Paolillo, A., et al., *The effect of Bacille Calmette-Guerin on the evolution of new enhancing lesions to hypointense T1 lesions in relapsing remitting MS*. J Neurol, 2003. **250**(2): p. 247-8.
110. Faustman, D.L., et al., *BCG treatment of long-term type 1 diabetics*. Diabetes, 2011. **60**: p. A596.
111. Morales, A., D. Eidinger, and A.W. Bruce, *Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors*. J Urol, 1976. **116**(2): p. 180-3.
112. Babjuk, M., et al., *EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2016*. Eur Urol, 2017. **71**(3): p. 447-461.
113. Kelley, D.R., et al., *Intravesical bacillus Calmette-Guerin therapy for superficial bladder cancer: effect of bacillus Calmette-Guerin viability on treatment results*. J Urol, 1985. **134**(1): p. 48-53.
114. Redelman-Sidi, G., M.S. Glickman, and B.H. Bochner, *The mechanism of action of BCG therapy for bladder cancer--a current perspective*. Nat Rev Urol, 2014. **11**(3): p. 153-62.
115. De Boer, E.C., et al., *Leukocytes and cytokines in the urine of superficial bladder cancer patients after intravesical immunotherapy with bacillus Calmette-Guerin*. In Vivo, 1991. **5**(6): p. 671-7.
116. De Boer, E.C., et al., *Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guerin*. Cancer Immunol Immunother, 1991. **33**(6): p. 411-6.
117. Suttman, H., et al., *Mechanisms of bacillus Calmette-Guerin mediated natural killer cell activation*. J Urol, 2004. **172**(4 Pt 1): p. 1490-5.
118. Luo, Y., X. Chen, and M.A. O'Donnell, *Role of Th1 and Th2 cytokines in BCG-induced IFN-gamma production: cytokine promotion and simulation of BCG effect*. Cytokine, 2003. **21**(1): p. 17-26.
119. O'Donnell, M.A., et al., *Role of IL-12 in the induction and potentiation of IFN-gamma in response to bacillus Calmette-Guerin*. J Immunol, 1999. **163**(8): p. 4246-52.

120. Naoe, M., et al., *Bacillus Calmette-Guerin-pulsed dendritic cells stimulate natural killer T cells and gammadeltaT cells*. Int J Urol, 2007. **14**(6): p. 532-8; discussion 538.
121. Ludwig, A.T., et al., *Tumor necrosis factor-related apoptosis-inducing ligand: a novel mechanism for Bacillus Calmette-Guerin-induced antitumor activity*. Cancer Res, 2004. **64**(10): p. 3386-90.
122. Suttman, H., et al., *Neutrophil granulocytes are required for effective Bacillus Calmette-Guerin immunotherapy of bladder cancer and orchestrate local immune responses*. Cancer Res, 2006. **66**(16): p. 8250-7.
123. Schamhart, D.H., et al., *Urinary cytokines reflecting the immunological response in the urinary bladder to biological response modifiers: their practical use*. Eur Urol, 2000. **37 Suppl 3**: p. 16-23.
124. Ristori, G., et al., *Effects of Bacille Calmette-Guerin after the first demyelinating event in the CNS*. Neurology, 2014. **82**(1): p. 41-8.
125. *TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group*. Neurology, 1999. **53**(3): p. 457-65.
126. Gregory, A.P., et al., *TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis*. Nature, 2012. **488**(7412): p. 508-511.
127. Yong, J., et al., *BCG vaccine-induced neuroprotection in a mouse model of Parkinson's disease*. PLoS One, 2011. **6**(1): p. e16610.
128. Faustman, D.L., et al. *Proof-of-concept, randomized, controlled clinical trial of Bacillus-Calmette-Guerin for treatment of long-term type 1 diabetes*. PloS one, 2012. **7**, e41756 DOI: 10.1371/journal.pone.0041756.
129. Kuhreiber, W.M., et al., *Long-term reduction in hyperglycemia in advanced type 1 diabetes: the value of induced aerobic glycolysis with BCG vaccinations*. NPJ Vaccines, 2018. **3**: p. 23.
130. Qin, H.Y., P. Chaturvedi, and B. Singh, *In vivo apoptosis of diabetogenic T cells in NOD mice by IFN-gamma/TNF-alpha*. International Immunology, 2004. **16**(12): p. 1723-32.
131. Arts, R.J.W., et al., *Immunometabolic Pathways in BCG-Induced Trained Immunity*. Cell Rep, 2016. **17**(10): p. 2562-2571.
132. De Rosa, V., et al., *Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants*. Nat Immunol, 2015. **16**(11): p. 1174-84.
133. Kishore, M., et al., *Regulatory T Cell Migration Is Dependent on Glucokinase-Mediated Glycolysis*. Immunity, 2018. **48**(4): p. 831-832.
134. Petrovsky, N., D. Silva, and D.A. Schatz, *Prospects for the prevention and reversal of type 1 diabetes mellitus*. Drugs, 2002. **62**(18): p. 2617-2635.
135. Schneider, D.S. and J.S. Ayres, *Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases*. Nat Rev Immunol, 2008. **8**(11): p. 889-95.
136. Leulier, F. and B. Lemaitre, *Toll-like receptors--taking an evolutionary approach*. Nat Rev Genet, 2008. **9**(3): p. 165-78.

137. Clark, I.A., *How TNF was recognized as a key mechanism of disease*. Cytokine Growth Factor Rev, 2007. **18**(3-4): p. 335-43.
138. Ayres, J.S. and D.S. Schneider, *Tolerance of infections*. Annu Rev Immunol, 2012. **30**: p. 271-94.
139. Lazzaro, B.P. and D.S. Schneider, *The genetics of immunity*. Genetics, 2014. **197**(2): p. 467-70.
140. Smolen, K.K., et al., *Pattern recognition receptor-mediated cytokine response in infants across 4 continents*. J Allergy Clin Immunol, 2014. **133**(3): p. 818-26 e4.
141. Newport, M.J., *The genetic regulation of infant immune responses to vaccination*. Front Immunol, 2015. **6**: p. 18.
142. Li, Y., et al., *Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi*. Nat Med, 2016. **22**(8): p. 952-60.
143. Reikie, B.A., et al., *Ontogeny of Toll-like receptor mediated cytokine responses of South African infants throughout the first year of life*. PLoS One, 2012. **7**(9): p. e44763.
144. Kampmann, B. and C.E. Jones, *Factors influencing innate immunity and vaccine responses in infancy*. Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1671).
145. Currie, A.J., et al., *Preterm infants have deficient monocyte and lymphocyte cytokine responses to group B streptococcus*. Infect Immun, 2011. **79**(4): p. 1588-96.
146. Schultz, C., et al., *Immature anti-inflammatory response in neonates*. Clin Exp Immunol, 2004. **135**(1): p. 130-6.
147. Afrazi, A., et al., *Toll-like receptor 4-mediated endoplasmic reticulum stress in intestinal crypts induces necrotizing enterocolitis*. J Biol Chem, 2014. **289**(14): p. 9584-99.
148. Hackam, D.J., et al., *Innate immune signaling in the pathogenesis of necrotizing enterocolitis*. Clin Dev Immunol, 2013. **2013**: p. 475415.
149. Li, J., et al., *Tumor necrosis factor alpha mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present*. J Neurosci, 2008. **28**(20): p. 5321-30.
150. Volpe, J.J., *Postnatal sepsis, necrotizing enterocolitis, and the critical role of systemic inflammation in white matter injury in premature infants*. J Pediatr, 2008. **153**(2): p. 160-3.
151. Nguyen, M., et al., *Acquisition of adult-like TLR4 and TLR9 responses during the first year of life*. PLoS One, 2010. **5**(4): p. e10407.
152. Kollmann, T.R., et al., *Neonatal innate TLR-mediated responses are distinct from those of adults*. J Immunol, 2009. **183**(11): p. 7150-60.
153. Kollmann, T.R., et al., *Innate immune function by Toll-like receptors: distinct responses in newborns and the elderly*. Immunity, 2012. **37**(5): p. 771-83.
154. Martino, D.J., et al., *Evidence for age-related and individual-specific changes in DNA methylation profile of mononuclear cells during early immune development in humans*. Epigenetics, 2011. **6**(9): p. 1085-94.
155. Goriely, S., et al., *Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes*. J Immunol, 2001. **166**(3): p. 2141-6.
156. Levy, O., et al., *Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha*

- induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol, 2004. 173(7): p. 4627-34.*
157. Shey, M.S., et al., *Optimization of a whole blood intracellular cytokine assay for measuring innate cell responses to mycobacteria. J Immunol Methods, 2012. 376(1-2): p. 79-88.*
158. Arko-Mensah, J., et al., *TLR2 but not TLR4 signalling is critically involved in the inhibition of IFN-gamma-induced killing of mycobacteria by murine macrophages. Scandinavian Journal of Immunology, 2007. 65(2): p. 148-57.*
159. D'Avila, H., et al., *Toll-like receptor-2-mediated C-C chemokine receptor 3 and eotaxin-driven eosinophil influx induced by Mycobacterium bovis BCG pleurisy. Infection and Immunity, 2007. 75(3): p. 1507-1511.*
160. Doz, E., et al., *Acylation determines the toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGIRR-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. Journal of Biological Chemistry, 2007. 282(36): p. 26014-25.*
161. Fremont, C.M., et al., *Control of Mycobacterium bovis BCG infection with increased inflammation in TLR4-deficient mice. Microbes & Infection, 2003. 5(12): p. 1070-81.*
162. Heldwein, K.A., et al., *TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. Journal of leukocyte biology, 2003. 74(2): p. 277-286.*
163. Lasunskaja, E.B., et al., *Mycobacteria directly induce cytoskeletal rearrangements for macrophage spreading and polarization through TLR2-dependent PI3K signaling. Journal of Leukocyte Biology, 2006. 80(6): p. 1480-90.*
164. von Meyenn, F., et al., *Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. Immunobiology, 2006. 211(6-8): p. 557-565.*
165. Andersson, M., et al., *Mycobacterium bovis bacilli Calmette-Guerin regulates leukocyte recruitment by modulating alveolar inflammatory responses. Innate immunity, 2012. 18(3): p. 531-40.*
166. Kiemer, A.K., et al., *Attenuated activation of macrophage TLR9 by DNA from virulent mycobacteria. Journal of Innate Immunity, 2009. 1(1): p. 29-45.*
167. Mendez-Samperio, P., L. Belmont, and E. Miranda, *Mycobacterium bovis BCG Toll-Like Receptors 2 and 4 Cooperation Increases the Innate Epithelial Immune Response. Archives of Medical Research, 2008. 39(1): p. 33-39.*
168. Miyazaki, J., et al., *Uroepithelial cells can directly respond to Mycobacterium bovis bacillus Calmette-Guerin through Toll-like receptor signalling. BJU International, 2006. 97(4): p. 860-864.*
169. Alter-Koltunoff, M., et al., *Innate immunity to intraphagosomal pathogens is mediated by Interferon Regulatory Factor 8 (IRF-8) that stimulates the expression of macrophage-specific Nramp1 through antagonizing repression by c-Myc. Journal of Biological Chemistry, 2008. 283(5): p. 2724-2733.*
170. van den Biggelaar, A.H., et al., *Neonatal innate cytokine responses to BCG controlling T-cell development vary between populations. Journal of Allergy & Clinical Immunology, 2009. 124(3): p. 544-50, 550.e1-2.*

171. Jekarl, D.W., et al., *Procalcitonin as a diagnostic marker and IL-6 as a prognostic marker for sepsis*. Diagn Microbiol Infect Dis, 2013. **75**(4): p. 342-7.
172. Watkins, M.L., et al., *Exposure of cord blood to Mycobacterium bovis BCG induces an innate response but not a T-cell cytokine response*. Clinical & Vaccine Immunology: CVI, 2008. **15**(11): p. 1666-73.
173. O'Donnell, M.A., et al., *Role of IL-12 in the induction and potentiation of IFN-gamma in response to bacillus Calmette-Guerin*. Journal of Immunology, 1999. **163**(8): p. 4246-52.
174. Gopal, R., et al., *IL-23-dependent IL-17 drives Th1-cell responses following Mycobacterium bovis BCG vaccination*. European Journal of Immunology, 2012. **42**(2): p. 364-373.
175. Sugisaki, K., et al., *Nonspecific and immune-specific up-regulation of cytokines in rabbit dermal tuberculous (BCG) lesions*. Journal of Leukocyte Biology, 1998. **63**(4): p. 440-450.
176. Blanden, R.V., M.J. Lefford, and G.B. Mackaness, *The host response to Calmette-Guerin bacillus infection in mice*. Journal of Experimental Medicine, 1979. **129**(5): p. 1079-107.
177. Jarisch, R. and I. Sandor, *[Therapy of recurrent herpes simplex and its surveillance by MIF determination: with levamisole, BCG, urushiol and herpes antigen vaccine (author's transl)]*. Arch Dermatol Res, 1977. **258**(2): p. 151-9.
178. Salvin, S.B. and J. Nishio, *Cellular origin of migration inhibitory factor released into the circulation of intact tuberculous mice*. Federation Proceedings, 1975. **34**(3): p. No. 4355.
179. Cruz, A., et al., *BCG vaccination inducing long-term protection against Mycobacterium tuberculosis correlates with the differentiation of IL-17/IL-2/TNF producing T cells*. Immunology, 2012. **137**: p. 596.
180. Marchant, A., et al. *Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination*. Journal of Immunology, 1999. **163**, 2249-2255.
181. Chen, K.Y.H., et al., *Innate immune responses following Kawasaki disease and toxic shock syndrome*. PLoS One, 2018. **13**(2): p. e0191830.
182. Roger, T., et al., *High expression levels of macrophage migration inhibitory factor sustain the innate immune responses of neonates*. Proc Natl Acad Sci U S A, 2016. **113**(8): p. E997-1005.
183. Decker, M.L., et al., *Cytokine profiling in healthy children shows association of age with cytokine concentrations*. Sci Rep, 2017. **7**(1): p. 17842.
184. Decker, M.L., M.P. Grobusch, and N. Ritz, *Influence of Age and Other Factors on Cytokine Expression Profiles in Healthy Children-A Systematic Review*. Front Pediatr, 2017. **5**: p. 255.
185. Dorea, J.G., *Breast-feeding and responses to infant vaccines: constitutional and environmental factors*. Am J Perinatol, 2012. **29**(10): p. 759-75.
186. Lee, Y.C., et al., *Influence of genetic and environmental factors on the immunogenicity of Hib vaccine in Gambian twins*. Vaccine, 2006. **24**(25): p. 5335-40.

187. Moore, S.E., et al., *Effect of month of vaccine administration on antibody responses in The Gambia and Pakistan*. Trop Med Int Health, 2006. **11**(10): p. 1529-41.
188. Ter Horst, R., et al., *Host and Environmental Factors Influencing Individual Human Cytokine Responses*. Cell, 2016. **167**(4): p. 1111-1124.e13.
189. Bennett, S. and E.M. Riley, *The statistical analysis of data from immunoepidemiological studies*. J Immunol Methods, 1992. **146**(2): p. 229-39.
190. Joosten, S.A., et al., *Mycobacterial growth inhibition is associated with trained innate immunity*. J Clin Invest, 2018. **128**(5): p. 1837-1851.
191. Mittrucker, H.W., et al., *Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis*. Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(30): p. 12434-12439.
192. Kagina, B.M., et al., *Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns*. Am J Respir Crit Care Med, 2010. **182**(8): p. 1073-9.
193. Dockrell, H.M., S.G. Smith, and M.K. Lalor, *Variability between countries in cytokine responses to BCG vaccination: what impact might this have on protection?* Expert Rev Vaccines, 2012. **11**(2): p. 121-4.
194. Newport, M.J., et al., *Genetic regulation of immune responses to vaccines in early life*. Genes Immun, 2004. **5**(2): p. 122-9.
195. Finan, C., et al., *Natural variation in immune responses to neonatal Mycobacterium bovis Bacillus Calmette-Guerin (BCG) Vaccination in a Cohort of Gambian infants*. PLoS One, 2008. **3**(10): p. e3485.
196. Mawa, P.A., et al., *The impact of maternal infection with Mycobacterium tuberculosis on the infant response to bacille Calmette-Guerin immunization*. Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1671).
197. Boer, M.C., et al., *Mycobacterium bovis BCG Vaccination Induces Divergent Proinflammatory or Regulatory T Cell Responses in Adults*. Clin Vaccine Immunol, 2015. **22**(7): p. 778-88.
198. Freyne, B., et al., *Neonatal BCG vaccination influences cytokine responses to Toll-like receptor ligands and heterologous antigens*. J Infect Dis, 2018.
199. Querec, T.D., et al., *Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans*. Nat Immunol, 2009. **10**(1): p. 116-125.
200. Anders, S. and W. Huber, *Differential expression analysis for sequence count data*. Genome Biol, 2010. **11**(10): p. R106.
201. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles*. Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15545-50.
202. Fabregat, A., et al., *The Reactome Pathway Knowledgebase*. Nucleic Acids Res, 2018. **46**(D1): p. D649-d655.
203. Xia, J., E.E. Gill, and R.E. Hancock, *NetworkAnalyst for statistical, visual and network-based meta-analysis of gene expression data*. Nat Protoc, 2015. **10**(6): p. 823-44.
204. Rolland, T., et al., *A proteome-scale map of the human interactome network*. Cell, 2014. **159**(5): p. 1212-1226.

205. Ben-Ari Fuchs, S., et al., *GeneAnalytics: An Integrative Gene Set Analysis Tool for Next Generation Sequencing, RNAseq and Microarray Data*. Omics, 2016. **20**(3): p. 139-51.
206. Arbore, G., et al., *T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4(+) T cells*. Science, 2016. **352**(6292): p. aad1210.
207. Merle, N.S., et al., *Complement System Part II: Role in Immunity*. Front Immunol, 2015. **6**: p. 257.
208. Kelly-Scumpia, K.M., et al., *B cells enhance early innate immune responses during bacterial sepsis*. J Exp Med, 2011. **208**(8): p. 1673-82.
209. Couper, K.N., D.G. Blount, and E.M. Riley, *IL-10: the master regulator of immunity to infection*. J Immunol, 2008. **180**(9): p. 5771-7.
210. Shey, M.S., et al., *Maturation of innate responses to mycobacteria over the first nine months of life*. J Immunol, 2014. **192**(10): p. 4833-43.
211. Novakovic, B., et al., *beta-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance*. Cell, 2016. **167**(5): p. 1354-1368.e14.
212. Castillo, E.F., et al., *Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation*. Proc Natl Acad Sci U S A, 2012. **109**(46): p. E3168-76.
213. Maurer, K., V.J. Torres, and K. Cadwell, *Autophagy is a key tolerance mechanism during Staphylococcus aureus infection*. Autophagy, 2015. **11**(7): p. 1184-6.
214. Orvedahl, A., et al., *Autophagy protects against Sindbis virus infection of the central nervous system*. Cell Host Microbe, 2010. **7**(2): p. 115-27.
215. Wang, L., et al., *Autophagy and Ubiquitination in Salmonella Infection and the Related Inflammatory Responses*. Front Cell Infect Microbiol, 2018. **8**: p. 78.
216. Galli, S.J. and M. Tsai, *IgE and mast cells in allergic disease*. Nat Med, 2012. **18**(5): p. 693-704.
217. Shin, J.S. and A.M. Greer, *The role of Fc epsilon RI expressed in dendritic cells and monocytes*. Cell Mol Life Sci, 2015. **72**(12): p. 2349-60.
218. Novak, N., et al., *Fc epsilon RI engagement of Langerhans cell-like dendritic cells and inflammatory dendritic epidermal cell-like dendritic cells induces chemotactic signals and different T-cell phenotypes in vitro*. J Allergy Clin Immunol, 2004. **113**(5): p. 949-57.
219. Novak, N., T. Bieber, and N. Katoh, *Engagement of Fc epsilon RI on human monocytes induces the production of IL-10 and prevents their differentiation in dendritic cells*. J Immunol, 2001. **167**(2): p. 797-804.
220. Schroeder, J.T., K.L. Chichester, and A.P. Bieneman, *Toll-like receptor 9 suppression in plasmacytoid dendritic cells after IgE-dependent activation is mediated by autocrine TNF-alpha*. J Allergy Clin Immunol, 2008. **121**(2): p. 486-91.
221. Platzer, B., et al., *Dendritic cell-bound IgE functions to restrain allergic inflammation at mucosal sites*. Mucosal Immunol, 2015. **8**(3): p. 516-32.
222. Leisching, G.R., *Susceptibility to Tuberculosis Is Associated With PI3K-Dependent Increased Mobilization of Neutrophils*. Front Immunol, 2018. **9**: p. 1669.
223. Liu, C.H., H. Liu, and B. Ge, *Innate immunity in tuberculosis: host defense vs pathogen evasion*. Cell Mol Immunol, 2017. **14**(12): p. 963-975.

224. Wassermann, R., et al., *Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1*. Cell Host Microbe, 2015. **17**(6): p. 799-810.
225. Moorlag, S., et al., *The role of the interleukin-1 family in trained immunity*. Immunol Rev, 2018. **281**(1): p. 28-39.
226. Mayer-Barber, K.D. and B. Yan, *Clash of the Cytokine Titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses*. Cell Mol Immunol, 2017. **14**(1): p. 22-35.
227. Castiglia, V., et al., *Type I Interferon Signaling Prevents IL-1beta-Driven Lethal Systemic Hyperinflammation during Invasive Bacterial Infection of Soft Tissue*. Cell Host Microbe, 2016. **19**(3): p. 375-87.
228. Vaine, C.A. and R.J. Soberman, *The CD200-CD200R1 inhibitory signaling pathway: immune regulation and host-pathogen interactions*. Adv Immunol, 2014. **121**: p. 191-211.
229. Guo, L., et al., *NLRC3 promotes host resistance against Pseudomonas aeruginosa-induced keratitis by promoting the degradation of IRAK1*. Int J Mol Med, 2017. **40**(3): p. 898-906.
230. Zhang, L., et al., *NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING*. Immunity, 2014. **40**(3): p. 329-41.
231. Schneider, M., et al., *The innate immune sensor NLRC3 attenuates Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-kappaB*. Nat Immunol, 2012. **13**(9): p. 823-31.
232. Hu, S., et al., *NLRC3 negatively regulates CD4+ T cells and impacts protective immunity during Mycobacterium tuberculosis infection*. PLoS Pathog, 2018. **14**(8): p. e1007266.
233. Dong, G., et al., *Ligation of CD180 contributes to endotoxic shock by regulating the accumulation and immunosuppressive activity of myeloid-derived suppressor cells through STAT3*. Biochim Biophys Acta Mol Basis Dis, 2019. **1865**(3): p. 535-546.
234. Longtine, M.S., et al., *Calcitriol regulates immune genes CD14 and CD180 to modulate LPS responses in human trophoblasts*. Reproduction, 2017. **154**(6): p. 735-744.
235. Ruiz-Riol, M., et al., *Identification of Interleukin-27 (IL-27)/IL-27 Receptor Subunit Alpha as a Critical Immune Axis for In Vivo HIV Control*. J Virol, 2017. **91**(16).
236. Torrado, E., et al., *Interleukin 27R regulates CD4+ T cell phenotype and impacts protective immunity during Mycobacterium tuberculosis infection*. J Exp Med, 2015. **212**(9): p. 1449-63.
237. Huik, K., et al., *CCL3L1 copy number is a strong genetic determinant of HIV seropositivity in Caucasian intravenous drug users*. J Infect Dis, 2010. **201**(5): p. 730-9.
238. Lee, H., et al., *Copy number variation of CCL3L1 influences asthma risk by modulating IL-10 expression*. Clin Chim Acta, 2011. **412**(23-24): p. 2100-4.

239. Cunningham, D.A., et al., *ICAM-1 is a key receptor mediating cytoadherence and pathology in the Plasmodium chabaudi malaria model*. Malar J, 2017. **16**(1): p. 185.
240. Mustafa, K.M.F., et al., *In vitro inhibition and reversal of Plasmodium falciparum cytoadherence to endothelium by monoclonal antibodies to ICAM-1 and CD36*. Malar J, 2017. **16**(1): p. 279.
241. Venet, F., et al., *Modulation of LILRB2 protein and mRNA expressions in septic shock patients and after ex vivo lipopolysaccharide stimulation*. Hum Immunol, 2017. **78**(5-6): p. 441-450.
242. Lundbo, L.F., et al., *Genetic Variation in NFKBIE Is Associated With Increased Risk of Pneumococcal Meningitis in Children*. EBioMedicine, 2016. **3**: p. 93-99.
243. Rappoport, N., et al., *The Duffy antigen receptor for chemokines, ACKR1, -'Jeanne DARC' of benign neutropenia*. Br J Haematol, 2019. **184**(4): p. 497-507.
244. O'Connor, D. and A.J. Pollard, *Characterizing vaccine responses using host genomic and transcriptomic analysis*. Clin Infect Dis, 2013. **57**(6): p. 860-9.
245. Debey, S., et al., *A highly standardized, robust, and cost-effective method for genome-wide transcriptome analysis of peripheral blood applicable to large-scale clinical trials*. Genomics, 2006. **87**(5): p. 653-64.
246. Debey, S., et al., *Comparison of different isolation techniques prior gene expression profiling of blood derived cells: impact on physiological responses, on overall expression and the role of different cell types*. Pharmacogenomics J, 2004. **4**(3): p. 193-207.
247. Biering-Sorensen, S., et al., *Rapid Protective Effects of Early BCG on Neonatal Mortality Among Low Birth Weight Boys: Observations From Randomized Trials*. J Infect Dis, 2018. **217**(5): p. 759-766.
248. Levy, O., et al., *The adenosine system selectively inhibits TLR-mediated TNF- α production in the human newborn*. J Immunol, 2006. **177**(3): p. 1956-66.
249. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview*. J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.
250. Hibbert, J.E., A. Currie, and T. Strunk, *Sepsis-Induced Immunosuppression in Neonates*. Front Pediatr, 2018. **6**: p. 357.
251. Levy, O. and J.L. Wynn, *A prime time for trained immunity: innate immune memory in newborns and infants*. Neonatology, 2014. **105**(2): p. 136-41.
252. Mitroulis, I., et al., *Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity*. Cell, 2018. **172**(1-2): p. 147-161.e12.
253. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity*. Science, 2014. **345**(6204): p. 1251086.
254. Blok, B.A., et al., *Trained innate immunity as underlying mechanism for the long-term, nonspecific effects of vaccines*. J Leukoc Biol, 2015. **98**(3): p. 347-56.
255. Kaufmann, E., et al., *BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis*. Cell, 2018. **172**(1-2): p. 176-190.e19.
256. Lerm, M. and M.G. Netea, *Trained immunity: a new avenue for tuberculosis vaccine development*. J Intern Med, 2016. **279**(4): p. 337-46.

257. Calandra, T., *Macrophage migration inhibitory factor and host innate immune responses to microbes*. Scand J Infect Dis, 2003. **35**(9): p. 573-6.
258. Clark, I. and M. Awburn, *Migration inhibitory factor in the cerebral and systemic endothelium in sepsis and malaria*. Crit Care Med, 2002. **30**(5 Suppl): p. S263-7.

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RESEARCH ARTICLE

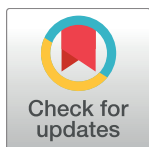
Innate immune responses following Kawasaki disease and toxic shock syndrome

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Abstract

The pathogenesis of Kawasaki disease (KD) remains unknown and there is accumulating evidence for the importance of the innate immune system in initiating and mediating the host inflammatory response. We compared innate immune responses in KD and toxic shock syndrome (TSS) participants more than two years after their acute illness with control participants to investigate differences in their immune phenotype. Toxic shock syndrome shares many clinical features with KD; by including both disease groups we endeavoured to explore changes in innate immune responses following acute inflammatory illnesses more broadly. We measured the *in vitro* production of interferon (IFN)- γ , tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-1 receptor antagonist (IL-1ra), and IL-10 following whole blood stimulation with toll-like receptor and inflammasome ligands in 52 KD, 20 TSS, and 53 control participants in a case-control study. Analyses were adjusted for age, sex, and unstimulated cytokine concentrations. Compared to controls, KD participants have reduced IL-1ra production in response to stimulation with double stranded RNA (geometric mean ratio (GMR) 0.37, 95% CI 0.15, 0.89, $p = 0.03$) and increased IL-6 production in response to incubation with LyovectTM (GMR 5.48, 95% CI 1.77, 16.98, $p = 0.004$). Compared to controls, TSS participants have increased IFN- γ production in response to peptidoglycan (GMR 4.07, 95% CI 1.82, 9.11, $p = 0.001$), increased IL-1 β production to lipopolysaccharide (GMR 1.64, 95% CI 1.13, 2.38, $p = 0.01$) and peptidoglycan (GMR 1.61, 95% CI 1.11, 2.33, $p = 0.01$), and increased IL-6 production to peptidoglycan (GMR 1.45, 95% CI 1.10, 1.92, $p = 0.01$). Years following the acute illness, individuals with previous KD or TSS exhibit a pro-inflammatory innate immune phenotype suggesting a possible underlying immunological susceptibility or innate immune memory.

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Abbreviations: KD, Kawasaki disease; TSS, toxic shock syndrome; GMR, geometric mean ratio; IFN- γ , interferon- γ ; TNF- α , tumour necrosis factor- α ; IL-6, interleukin-6; IL-1ra, IL-1 receptor antagonist; ITPKC, inositol-trisphosphate-kinase C; NLRP3, nucleotide-binding domain and leucine-rich repeat pyrin domain contain 3; TLR, toll-like receptor; LPS, lipopolysaccharide; EDTA, ethylenediamine tetraacetic acid; hsCRP, high sensitivity C-reactive protein; PGN, peptidoglycan; NOD, nucleotide-binding oligomerization domain; RIG, retinoic acid-inducible gene; STING, Stimulator of the interferon gamma gene; Poly I:C, polyinosinic-polycytidylic acid; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; dsRNA, double stranded RNA; TSST-1, toxic shock syndrome toxin-1.

Introduction

Kawasaki disease (KD) is a systemic vasculitis most commonly affecting pre-school children characterised by fever, rash, conjunctivitis, lymphadenopathy, mucosal and peripheral changes in the hands and feet.[1] Its pathogenesis remains unknown, leading to many uncertainties in the diagnosis and treatment of the disease. The main complication of KD is the development of coronary artery aneurysms.[2] It is the leading cause of acquired heart disease in children living in industrialised countries.

Although it is widely believed that KD is caused by an infectious trigger, there is growing evidence that genetic factors play a role in determining susceptibility and severity of the disease.[2] Evidence for host susceptibility comes from epidemiological data. The incidence of KD remains highest in children of Asian ethnicity with Japan reporting over 10 times the incidence rate compared to United States for children under five years of age.[3, 4] This increased incidence is evident in children of Asian migrants living in low incidence countries, and in first degree relatives of KD patients. [5–7] Furthermore, genome-wide association and candidate gene studies have identified a number of associations with genes involved in the regulation of the inflammatory process, including *ITPKC* and *ORAI1*, which regulate intracellular calcium-signalling pathways.[8–11] These genetic associations support the widely accepted hypothesis that KD occurs in genetically susceptible individuals in whom microbial pathogens or environmental agents trigger an aberrant inflammatory response.[2]

There is increasing interest in the role of the innate immune system in the generation of this aberrant inflammatory response in KD.[12] During the acute illness, peripheral neutrophils and monocytes increase and infiltrate affected arterial walls, leading to acute self-limited necrotising arteritis.[13–16] Acutely, KD patients have increased toll-like receptor (TLR) mRNA levels and upregulation of genes in the IL-1 pathway.[17, 18] Furthermore, human and murine data implicate a critical role for nucleotide-binding domain and leucine-rich repeat pyrin domain contain 3 (NLRP3) inflammasome activation in acute KD, and in the development of coronary artery aneurysms. Modulation of IL-1 is therefore a potential therapeutic target.[19–22]

Little is known about the immune responses to microbial pathogens years after an acute episode of KD. We hypothesised that an abnormal inflammatory phenotype in KD patients, measured years after the acute illness, might provide clues to the nature of the aberrant immune response in acute KD and reveal targets for anti-inflammatory treatment.

Toxic shock syndrome (TSS), a prototypical illness caused by bacterial superantigens, shares many clinical and immunological features with KD.[23] In particular, KD shock syndrome, characterised by myocardial dysfunction, coronary artery involvement and resistance to standard therapy, is the most difficult to clinically differentiate from TSS.[24] There is also emerging interest in the role of the innate immune system in initiating and mediating the host inflammatory response in the development of sepsis.[25] Similar to KD, there is likely an underlying host immunological susceptibility as not all individuals exposed to the causative exotoxin develop TSS.[26, 27] By including both diseases, we were able to study changes in innate immune responses following acute inflammatory illnesses more broadly.

We compared the innate immune responses in participants with previous KD or TSS with control participants at least two years after their acute illness. Our aim was to define an innate immune phenotype that might suggest either an underlying immunological susceptibility and/or evidence of innate immune memory.

Material and methods

Participants and enrolment

Participants between six and 30 years of age who had KD or TSS at least two years previously were recruited from The Royal Children's Hospital (RCH) and Monash Medical Centre (MMC), Melbourne, Australia. A research letter of invitation was sent out to families meeting the inclusion criteria following a medical record search.

The KD group included participants with a history of acute KD that fulfilled either the American Heart Association diagnostic criteria for KD,[28] and/or had abnormal coronary artery dimensions that met the Japanese Ministry of Health Criteria for abnormal dilatation [29] on an echocardiogram performed within two months of disease onset. Height is not routinely measured on children acutely hospitalised in Australia and so it was not possible to generate coronary artery z-scores, which are based on calculated body surface area.[30] The KD group were therefore defined by widely accepted and robust criteria.

The TSS group included patients who fulfilled the Centers for Disease Control and Prevention case definition for either probable or definite TSS.[31] Control participants were unrelated healthy individuals of similar age and sex recruited from The Royal Children's Hospital Melbourne clinics, or unrelated friends of KD and TSS participants. Exclusion criteria for all groups were treatment for hypertension and/or dyslipidaemia, or chronic auto-inflammatory conditions. Assessments were deferred for at least four weeks following an acute febrile illness. The study was approved by the human research ethics committee at RCH (Approval No 31090) and Monash (11293X) and written informed consent was obtained from the parents or adult participants at enrolment. Participants attended the Murdoch Children's Research Institute (Melbourne, Australia) on a single occasion between September 2013 and December 2015 during which anthropometric measurements (BC 418, Tanita, Tokyo, Japan), as well as demographic and clinical details were obtained. Venous blood was collected into lipopolysaccharide (LPS)-free sodium heparin, serum gel, and ethylenediamine tetraacetic acid (EDTA) tubes (Sarstedt, Germany). High sensitivity C-reactive protein (hsCRP) (Abbott Architect, IL, USA) was measured from the serum gel tube according to manufacturer's protocol.

In vitro stimulation assays

To standardise assays, all TLR and inflammasome ligands were pre-prepared in robotically-filled 8-well strips within 96-well plates (Corning, USA) at the University of British Columbia, Vancouver, as previously described.[32] The wells in the TLR strips contained the following ligands: 3.5 µg/mL R848 (Resiquimod) (TLR 7/8 ligand, InvivoGen, USA); 100 ng/ml LPS (TLR 4, InvivoGen); 10 µg/ml peptidoglycan (PGN) (nucleotide-binding oligomerization domain (NOD) 1/2, InvivoGen); 100 µg/ml polyinosinic-polycytidylic acid (Poly I:C) (TLR 3, Amersham Biosciences, UK); 20 µg/ml cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) (Stimulator of the interferon gamma gene (STING) ligand, InvivoGen) with Lyovect[™] (InvivoGen); 2 ng/mL double stranded RNA (dsRNA) (Retinoic acid-inducible gene (RIG) 1, InvivoGen) with Lyovect[™]; and RPMI 1640 (Gibco, USA) and Lyovect[™] (unstimulated). The NLRP3 inflammasome strip-wells contained 100 µg/ml Zymosan (Sigma-Aldrich), 100 ng/ml LPS (InvivoGen), 5 mM/mL adenosine triphosphate (ATP) (InvivoGen), and RPMI (unstimulated).

One hour after collection, heparinised fresh blood was diluted with RPMI to a final 1:1 dilution by adding 180 µl of the diluted blood to each well of the pre-prepared thawed and centrifuged strips containing 20 µl of the specific ligand per well. Samples stimulated with NLRP3 inflammasome ligands were incubated for two hours, while samples containing TLR ligands

were incubated for 24 hours at 37°C in 5% CO₂. Pre-prepared ATP was thawed and added to the Zymosan and LPS wells on the inflammasome strips 30 minutes before harvesting.[33] Following incubation, the strip-wells were centrifuged at 600 g for 5 minutes and the supernatants stored in microtubes (VWR, USA) at -80°C for future batched analysis.

Cytokine analysis

The choice of cytokines measured was based on innate immune pathways for each ligand. Cytokine concentrations from TLR ligand-stimulated supernatants were measured using an x-MAP immunobead-based multiplexed assay (Bio-Plex Pro Human Cytokine Group 1 Assay, Bio-Rad, USA) according to manufacturer's protocol. Samples from KD, TSS, and control participants were scrambled across plates. To ensure that cytokine concentrations of stimulated samples were within the detectable range, samples were diluted 1:4 for the measurement of interferon (IFN)- γ , tumour necrosis factor (TNF)- α , and interleukin (IL)-10 and 1:100 for the measurement of IL-1 β , IL-1 receptor antagonist (IL-1ra), and IL-6. In preliminary experiments, IFN- α and IFN- β concentrations from samples diluted 1:4 were below the detection limit for the majority of samples in 8 out of 10 participants (3 KD, 3 TSS and 4 control participants) despite measurable concentrations for other cytokines. These two cytokines were therefore not measured for the remaining participants.

Interleukin-1 β levels were measured in the NLRP3 inflammasome ligand-stimulated supernatant using an enzyme-linked immunosorbent assay (Mabtech, Australia) according to the manufacturer's protocol using samples diluted 1:50.

Statistical analysis

Statistical analysis was done using Stata 13.0 (Stata Corporation, Texas). Cytokine concentrations below the detection limit were designated a value half the lowest detection limit for that specific cytokine. Outlier participants were defined as those having two or more unstimulated cytokine concentrations greater than two standard deviations above the mean value of the other participants and were excluded.

Cytokine concentrations were analysed as continuous variables and were log transformed to achieve a normal distribution. Geometric mean (95% CI) is the mean of the logarithmic values retransformed back to picogram/ml (pg/ml) using the exponential function. Univariate comparisons of cytokine concentrations were done using 2-tailed *t*-test.

Multi-variable linear regression was used to quantify the differences between groups adjusting for unstimulated cytokine concentrations (either with RPMI or Lyovect[™]), age and sex with results expressed as geometric mean ratio (GMR).

Cytokine concentrations from the inflammasome strip-wells could not be transformed into a normal distribution and, therefore, comparisons between participant groups were done using the Kruskal-Wallis test after nil correction.

Results

In total, 60 KD, 22 TSS, and 60 control participants were recruited. Ninety two eligible KD participants were approached and 60 were recruited. The number of participants with coronary artery abnormalities in the recruited (35/60 (58.3%)) and the declined group (18/32 (56.3%)) were similar.

Two participants (one TSS and one control) were considered outliers and another two KD participants were excluded on the basis that they had a febrile illness on the day of the assessment. A further 13 participants (6 KD, 1 TSS, 6 controls) were excluded due to insufficient blood to complete stimulations assays. There were, therefore, 52 KD, 20 TSS, and 53 control

participants included in the final analysis. There were no differences in age, sex, anthropometry, pubertal status and hsCRP levels between the groups (Table 1).

Thirty nine (75%) participants met the American Heart Association complete diagnostic criteria for KD. Thirteen participants (25%) met the incomplete diagnostic criteria and fulfilled the Japanese Ministry of Health echocardiogram criteria for coronary artery abnormality. There were 20 (38.5%) participants with no history of coronary artery abnormalities, 18 (34.6%) with resolved dilatation or coronary artery aneurysms, and 14 (26.9%) with persistent coronary artery aneurysms. Ten (19.2%) were resistant to the first dose of intravenous immunoglobulin. All received a second dose of intravenous immunoglobulin and two (3.8%) received steroids in addition.

Of the 20 TSS participants in the final analysis nine (45%) met the definite TSS diagnostic criteria and 11 (55%) met the probable TSS criteria.

Toll-like receptor ligand stimulation

Compared to controls, KD participants had reduced IL-1 α production in response to dsRNA (GMR 0.37, 95% CI 0.15, 0.89, $p = 0.03$). There were no differences in pro-inflammatory cytokine production in response to TLR ligands between KD and control participants (Fig 1).

Compared to controls, TSS participants had increased IFN- γ production in response to PGN stimulation (GMR 4.07, 95% CI 1.82, 9.11, $p = 0.001$), increased IL-1 β production to LPS (GMR 1.64, 95% CI 1.13, 2.38, $p = 0.01$) and PGN (GMR 1.61, 95% CI 1.11, 2.33, $p = 0.01$), and increased IL-6 production to PGN (GMR 1.45, 95% CI 1.10, 1.92, $p = 0.01$) after adjusting for unstimulated cytokine concentrations, age and sex. There were no differences in anti-inflammatory cytokine production between TSS and control participants (Fig 1).

Unadjusted geometric means of cytokines following TLR ligand stimulations are shown in S1 Table. The direction and magnitude of change were similar to the adjusted analysis with one exception. In the unadjusted analysis, there was an increased production of IL-6 in response to dsRNA stimulation in KD participants which was not statistically significant after adjusting for unstimulated cytokine concentrations, age and sex.

Table 1. Participant characteristics.

	Kawasaki disease (n = 52)	Toxic shock syndrome (n = 20)	Control (n = 53)
Age (years)	15.5 $\pm$ 5.8	15.6 $\pm$ 5.6	14.9 $\pm$ 6.1
Sex (male (%))	30 (57.7%)	12 (57.1%)	26 (48.2%)
Height (cm)	158.7 $\pm$ 19.2	162.2 $\pm$ 19.5	155.3 $\pm$ 17.1
Weight (kg)	54.0 $\pm$ 18.1	59.7 $\pm$ 23.3	51.1 $\pm$ 18.3
Post-pubertal ^a (n %)	24 (46.2%)	10 (47.6%)	20 (37.0%)
Age at acute illness (years)	3.3 $\pm$ 3.0	8.7 $\pm$ 4.5	N/A
Time since acute illness (years)	12.2 $\pm$ 5.8	5.9 $\pm$ 3.1	N/A
High sensitive CRP (mg/L)	0.6 (0.3–1.4)	0.5 (0.3–0.7)	0.4 (0.2–0.8)
Ethnicity (n%)			
Caucasians	40 (69.8%)		37 (73.3%)
Asians	9 (17.0%)		9 (17.1%)
Others	7 (13.2%)		3 (9.6%)

Mean $\pm$ SD for normally distributed data, median (interquartile range) for skewed data

^aself-reported Tanner stage 5

CRP = C-reactive protein

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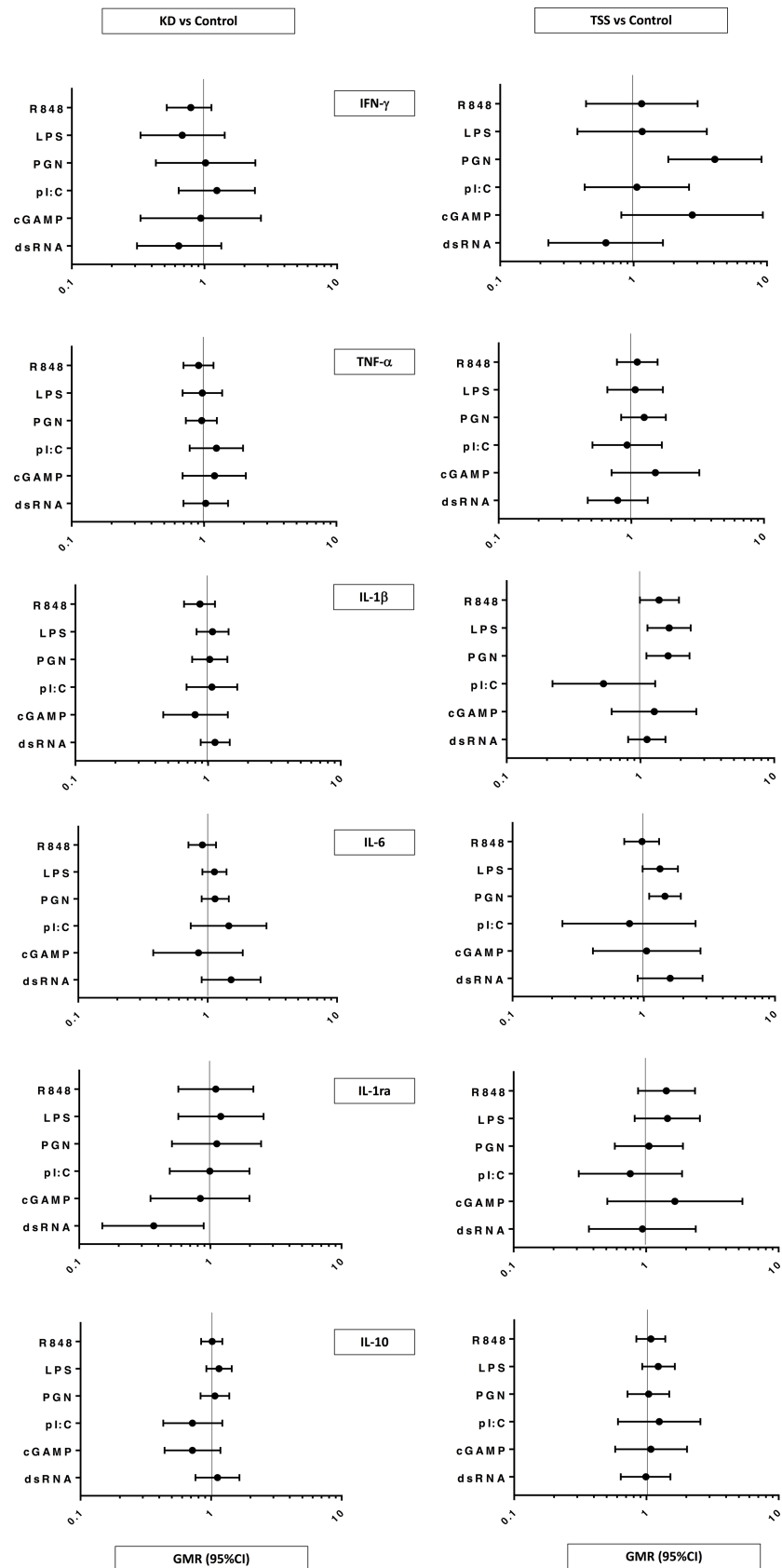


Fig 1. Geometric mean ratio (GMR) and 95% CI of *in vitro* toll-like receptor induced cytokine concentrations in 52 Kawasaki disease (KD), 20 toxic shock syndrome (TSS) and 53 control participants adjusted for age, sex and unstimulated cytokine concentrations. Abbreviations: IFN, interferon; TNF, tumour necrosis factor; IL, interleukin; IL-1ra, receptor antagonist; R848, Resiquimod; LPS, lipopolysaccharide; PGN, peptidoglycan; pI:C, polyinosinic-polycytidylic acid; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; dsRNA, double stranded RNA.

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Cytokine responses in the ‘unstimulated’ wells incubated with Lyovect™ were consistently higher than those observed in the wells incubated with RPMI with the notable exception of IFN- γ (S1 Table). Compared with the controls, KD participants had a higher geometric mean IL-6 concentration in samples incubated with Lyovect™, and the TSS participant group had a higher geometric mean IL-1ra concentration in samples incubated with RPMI (S1 Table and Fig 2).

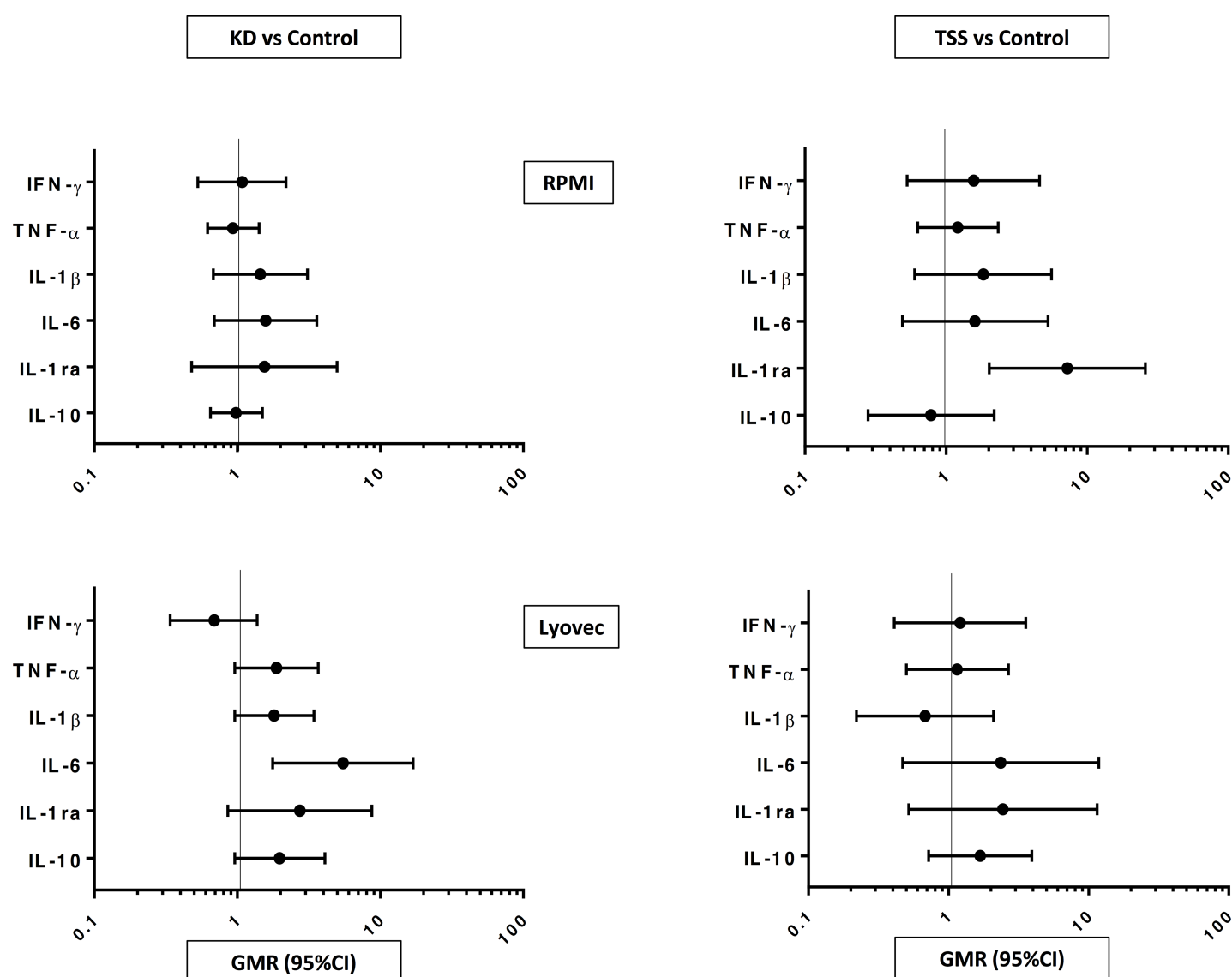


Fig 2. Geometric mean ratio (GMR) and 95% CI of *in vitro* cytokine concentrations in the unstimulated RPMI and Lyovect™ wells of 52 Kawasaki disease (KD), 20 toxic shock syndrome (TSS) and 53 control participants. Abbreviations: IFN, interferon; TNF, tumour necrosis factor; IL, interleukin; IL-1ra, receptor antagonist.

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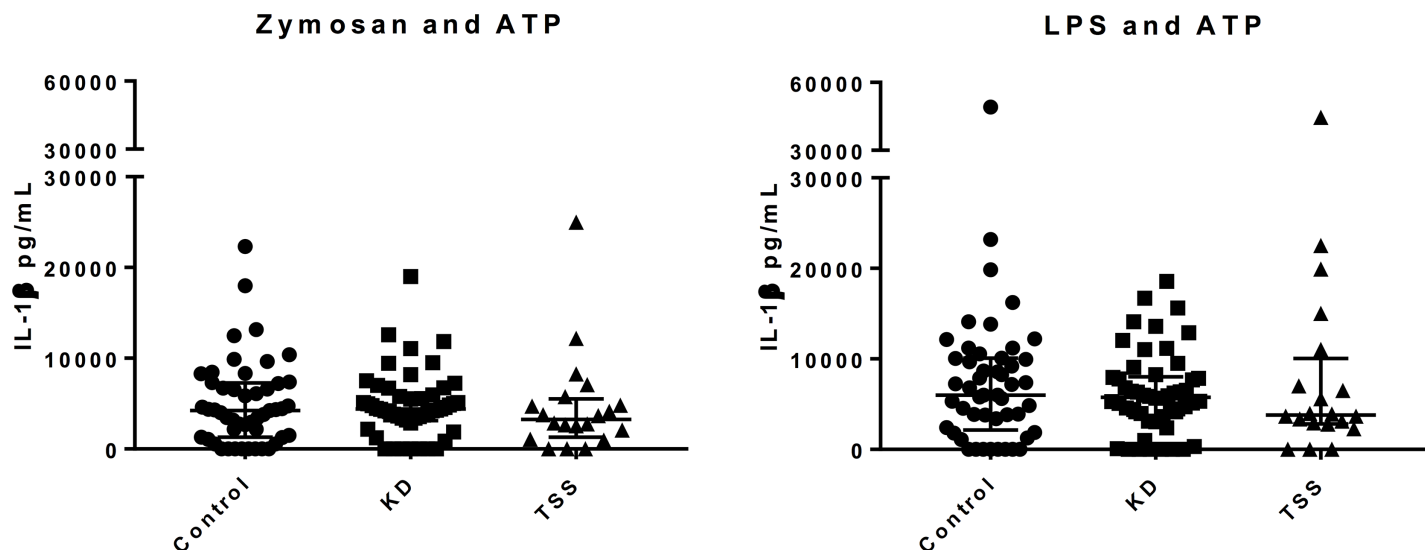


Fig 3. Scatter plot of interleukin (IL)-1 β concentrations in 52 Kawasaki disease (KD), 20 toxic shock syndrome (TSS) and 53 control participants following *in vitro* stimulations of the nucleotide-binding domain and leucine-rich repeat pyrin domain containing 3 (NLRP3) inflammasome. The median and interquartile ranges are marked by the bars. Abbreviations: pg, picogram; ATP, adenosine triphosphate.

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Inflammasome ligand stimulation

No differences were found in IL-1 β production between KD, TSS, and control participants in response to Zymosan plus ATP (median (IQR): 4388.25 (3189.00, 5785.25) vs 3249.85 (1559.53, 5276.95) vs 4229.20 (1304.70, 7185.30) pg/ml, $p = 0.6$) and LPS plus ATP (5760.10 (3632.55, 7941.00) vs 3777.48 (2843.43, 9021.60) vs 5995.10 (2396.80, 10032.40) pg/ml, $p = 0.7$) stimulations of the NLRP3 inflammasome (Fig 3).

Discussion

This is the first study to report the innate immune responses in individuals with previous KD and TSS years after the acute illness. The observed decreased IL-1 α production in KD participants with a prototypical viral ligand stimulation is consistent with data implicating the role of IL-1 β in the pathogenesis of KD. Studies have shown elevated IL-1 β in acute KD especially in treatment resistant individuals, increased transcript abundance for IL-1 β related genes, and protection from coronary arteritis in IL-1 β receptor-deficient mice.[19, 20, 34] Randomised controlled trials using an IL-1 β receptor antagonist and monoclonal antibody inhibition of IL-1 β in KD are underway.[19–22] We found no differences in IL-1 β production in KD participants following both TLR and inflammasome stimulation, which is consistent with studies in convalescent KD.[19] Inflammasome activation and resulting elevated levels of IL-1 β are most likely transient acute inflammatory responses.

The observation that cytokine concentrations in samples incubated with Lyovec™ were higher than those incubated with RPMI suggests that this lyophilized cationic lipid-based transfection reagent stimulates cytokine responses. Cationic lipids such as Lyovec™ are able to activate pro-inflammatory pathways, in particular, intracellular calcium-signalling pathways. [35] These pathways have been implicated in the pathogenesis of KD.[9, 19] The finding that Lyovec™ was associated with a greater IL-6 response in the KD group compared to the control group may point to a genetically determined difference in the response to intracellular pattern recognition receptor stimulation by lipids from the cell wall of bacteria or fungi. This might

suggest that KD patients have an underlying different inflammatory response involving these pathways.

This study does not provide evidence for either a conventional or superantigen as the initial trigger for KD as superantigens were not used as a ligand in our study. Superantigens will likely be neutralised with the use of whole blood required to activate the innate immune system in our study.

Toxic shock syndrome participants had an increased pro-inflammatory cytokine response to bacterial ligands, which is consistent with reported cytokine responses to TSS toxin (TSST)-1 *in vitro*. [36] Most cases of TSS are caused by exotoxins produced by *Staphylococcus aureus* and *Streptococcus pyogenes*, which act as ‘superantigens’ binding directly to the major histocompatibility complex class II molecule and T-cell receptor, bypassing the conventional antigen-binding sites. [23] Conceivably, similar to LPS and PGN ligand stimulation in our study, these bacteria would also stimulate the host innate immune system through activation of TLR 2 and NOD receptors [37]

The underlying immunological and/or genetic susceptibility determinants of TSS are poorly understood. The prevalence of antibody to TSST-1 in the population far outweighs the number of TSS cases. [26, 27] The observed increased pro-inflammatory cytokine response to TLR 2 and NOD receptor stimulation in TSS may be due to either an underlying genetic immunological susceptibility or an altered immune response secondary to the past TSS illness. The possibility that our results reflect an underlying genetic immunological susceptibility is supported by the finding of a strong genetic influence on *in vitro* LPS-induced cytokine responses in meningococcal disease in a study of first degree relatives. [38]

Of note, compared to control participants, IL-1 β production following inflammasome stimulation was not increased in TSS participants despite increased production following TLR stimulation, which theoretically share the same pathway for IL-1 activation. It is possible that LPS and PGN may have stimulated the activation of caspase (which in turn activates IL-1) through other less well characterised pathways, such as RIG-I or IFN- γ inducible protein 16. [39] Furthermore, differences in methodologies between the TLR and NLRP3 inflammasome strip stimulation assays, especially in the incubation time, make direct comparison of results difficult.

The strengths of our study are the use of well-characterised KD and TSS participants, and rigorously standardised assays. Limitations of our study include its retrospective design and therefore inability to differentiate whether the innate immune response differences preceded KD or TSS (reflecting potential underlying immunosusceptibility) versus those attributable to the effect of diseases (innate immune memory). We are also unable to ascertain whether these innate immune responses persist into later life and were related to time following the acute illness. Furthermore, the purpose of this study was to explore whether there are changes in the innate immune response in two acute illnesses with overlapping clinical features, rather than to directly compare KD and TSS, moreover, the sample size is insufficient.

As this is an exploratory study, analyses have not been adjusted for multiple comparisons. The exploratory nature of this study necessarily required the inclusion of numerous cytokines measures to investigate multiple innate immune pathways. We did not adjust each measure by severity indicators (such as coronary artery status, peak inflammatory markers, duration of fever, and treatment resistance) as the small number of participants in this subset analysis would risk Type 1 and Type 2 errors. The impact of disease severity on innate immune responses would best be explored in future studies with sufficient sample size focusing on a small number of implicated cytokines.

There is increasing interest in the concept that innate immune cells also have the capacity to enhance their response following repeated stimulation by microbial pathogens through

epigenetic programming.[40] Innate immune memory has important implications for understanding host response to pathogens and auto-inflammatory conditions. Hyper-inflammatory innate immune responses, particularly noted in TSS participants, may indicate innate immune memory and warrant further investigation.

In summary, years following the acute illness, individuals with previous KD and TSS have a decreased anti-inflammatory and increased pro-inflammatory response respectively to innate immune stimulation, suggesting a possible underlying immunological susceptibility or innate immune memory. Further genetic and immunological studies may unravel the mechanisms underlying these responses, which have implications for improved diagnostics and treatment.

Supporting information

S1 Table. Geometric mean (95% CI) of stimulated *in vitro* cytokine concentrations in pg/ml.
(DOCX)

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References

1. Kawasaki T, Kosaki F, Okawa S, Shigematsu I, Yanagawa H. A new infantile acute febrile mucocutaneous lymph node syndrome (MLNS) prevailing in Japan. *Pediatrics*. 1974; 54(3):271–6. Epub 1974/09/01. PMID: 4153258.
2. Newburger JW, Takahashi M, Burns JC. Kawasaki Disease. *J Am Coll Cardiol*. 2016; 67(14):1738–49. Epub 2016/04/09. <https://doi.org/10.1016/j.jacc.2015.12.073> PMID: 27056781.
3. Nakamura Y, Yashiro M, Uehara R, Sadakane A, Tsuboi S, Aoyama Y, et al. Epidemiologic features of Kawasaki disease in Japan: Results of the 2009–2010 nationwide survey. *J Epidemiol*. 2012; 22

- (3):216–21. Epub 2012/03/27. <https://doi.org/10.2188/jea.JE20110126> PMID: 22447211; PubMed Central PMCID: PMC3798622.
4. Uehara R, Belay ED. Epidemiology of Kawasaki disease in Asia, Europe, and the United States. *J Epidemiol.* 2012; 22(2):79–85. Epub 2012/02/07. <https://doi.org/10.2188/jea.JE20110131> PMID: 22307434; PubMed Central PMCID: PMC3798585.
5. Fujita Y, Nakamura Y, Sakata K, Hara N, Kobayashi M, Nagai M, et al. Kawasaki Disease in Families. *Pediatrics.* 1989; 84(4):666–9. PubMed Central PMCID: PMC2780128. PMID: 2780128
6. Uehara R, Yashiro M, Nakamura Y, Yanagawa H. Clinical features of patients with kawasaki disease whose parents had the same disease. *Arch Pediatr Adolesc Med.* 2004; 158(12):1166–9. <https://doi.org/10.1001/archpedi.158.12.1166> PubMed Central PMCID: PMC15583102. PMID: 15583102
7. Uehara R, Yashiro M, Nakamura Y, Yanagawa H. Kawasaki disease in parents and children. *Acta Paediatrica.* 2003; 92(6):694–7. <https://doi.org/10.1111/j.1651-2227.2003.tb00602.x> PubMed Central PMCID: PMC12856980. PMID: 12856980
8. Lin MT, Wang JK, Yeh JI, Sun LC, Chen PL, Wu JF, et al. Clinical Implication of the C Allele of the ITPKC Gene SNP rs28493229 in Kawasaki Disease: Association With Disease Susceptibility and BCG Scar Reactivation. *Pediatr Infect Dis J.* 2011; 30(2):148–52. <https://doi.org/10.1097/INF.0b013e3181f43a4e> PMID: 20805785.
9. Onouchi Y, Gunji T, Burns JC, Shimizu C, Newburger JW, Yashiro M, et al. ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms. *Nat Genet.* 2008; 40(1):35–42. <https://doi.org/10.1038/ng.2007.59> PubMed Central PMCID: PMC2876982. PMID: 18084290
10. Onouchi Y, Suzuki Y, Suzuki H, Terai M, Yasukawa K, Hamada H, et al. ITPKC and CASP3 polymorphisms and risks for IVIG unresponsiveness and coronary artery lesion formation in Kawasaki disease. *Pharmacogenomics J.* 2013; 13(1):52–9. Epub 2011/10/12. <https://doi.org/10.1038/tpj.2011.45> PMID: 21987091.
11. Onouchi Y, Fukazawa R, Yamamura K, Suzuki H, Kakimoto N, Suenaga T, et al. Variations in ORAI1 Gene Associated with Kawasaki Disease. *PLoS One.* 2016; 11(1):e0145486. Epub 2016/01/21. <https://doi.org/10.1371/journal.pone.0145486> PMID: 26789410; PubMed Central PMCID: PMC4720480.
12. Hara T, Nakashima Y, Sakai Y, Nishio H, Motomura Y, Yamasaki S. Kawasaki disease: A matter of innate immunity. *Clin Exp Immunol.* 2016; 186(2):134–43. Epub 2016/06/28. <https://doi.org/10.1111/cei.12832> PMID: 27342882.
13. Biezeveld MH, Geissler J, Weverling GJ, Kuipers IM, Lam J, Ottenkamp J, et al. Polymorphisms in the mannose-binding lectin gene as determinants of age-defined risk of coronary artery lesions in Kawasaki disease. *Arthritis Rheum.* 2006; 54(1):369–76. Epub 2005/12/31. <https://doi.org/10.1002/art.21529> PMID: 16385529.
14. Furukawa S, Matsubara T, Yabuta K. Mononuclear cell subsets and coronary artery lesions in Kawasaki disease. *Arch Dis Child.* 1992; 67(6):706–8. Epub 1992/06/01. PMID: 1378258; PubMed Central PMCID: PMC3798622.
15. Ye F, Foell D, Hirono KI, Vogl T, Rui C, Yu X, et al. Neutrophil-derived S100A12 is profoundly upregulated in the early stage of acute Kawasaki disease. *Am J Cardiol.* 2004; 94(6):840–4. Epub 2004/09/18. <https://doi.org/10.1016/j.amjcard.2004.05.076> PMID: 15374807.
16. Orenstein JM, Shulman ST, Fox LM, Baker SC, Takahashi M, Bhatti TR, et al. Three linked vasculopathic processes characterize Kawasaki disease: a light and transmission electron microscopic study. *PLoS One.* 2012; 7(6):e38998. Epub 2012/06/23. <https://doi.org/10.1371/journal.pone.0038998> PMID: 22723916; PubMed Central PMCID: PMC3377625.
17. Huang Y, Li S, Huang L, Chen P, Lin Y, Lin C, et al. Identifying genetic hypomethylation and upregulation of toll-like receptors in Kawasaki disease. *Oncotarget.* 2017; 8(7):11249–58. <https://doi.org/10.18632/oncotarget.14497> PMID: 28061462; PubMed Central PMCID: PMC5355262.
18. Hoang LT, Shimizu C, Ling L, Naim AN, Khor CC, Tremoulet AH, et al. Global gene expression profiling identifies new therapeutic targets in acute Kawasaki disease. *Genome Med.* 2014; 6(11):541. Epub 2015/01/24. <https://doi.org/10.1186/s13073-014-0102-6> PMID: 25614765; PubMed Central PMCID: PMC4279699.
19. Alphonse MP, Duong TT, Shumitsu C, Hoang TL, McCrindle BW, Franco A, et al. Inositol-Triphosphate 3-Kinase C Mediates Inflammasome Activation and Treatment Response in Kawasaki Disease. *J Immunol.* 2016; 197(9):3481–89. Epub 2016/10/04. <https://doi.org/10.4049/jimmunol.1600388> PMID: 27694492 PubMed Central PMCID: PMC3337219.
20. Lee Y, Schulte DJ, Shimada K, Chen S, Crother TR, Chiba N, et al. Interleukin-1 β Is Crucial for the Induction of Coronary Artery Inflammation in a Mouse Model of Kawasaki Disease. *Circulation.* 2012; 125(12):1542–50. <https://doi.org/10.1161/CIRCULATIONAHA.111.072769> PMID: 22361326; PubMed Central PMCID: PMC3337219.

21. Tremoulet AH, Jain S, Kim S, Newburger J, Arditi M, Franco A, et al. Rationale and study design for a phase I/IIa trial of anakinra in children with Kawasaki disease and early coronary artery abnormalities (the ANAKID trial). *Contemp Clin Trials*. 2016; 48:70–5. Epub 2016/04/16. <https://doi.org/10.1016/j.cct.2016.04.002> PMID: 27080929.
22. Burns JC, Kone-Paut I, Kuijpers T, Shimizu C, Tremoulet A, Arditi M. Review: Found in Translation: International Initiatives Pursuing Interleukin-1 Blockade for Treatment of Acute Kawasaki Disease. *Arthritis & rheumatology (Hoboken, NJ)*. 2017; 69(2):268–76. Epub 2016/10/30. <https://doi.org/10.1002/art.39975> PMID: 27792871; PubMed Central PMCID: PMC45274552.
23. Llewellyn M, Cohen J. Superantigens: microbial agents that corrupt immunity. *Lancet Infect Dis*. 2002; 2(3):156–62. Epub 2002/04/12. PMID: 11944185.
24. Yim D, Ramsay J, Kothari D, Burgner D. Coronary Artery Dilatation in Toxic Shock-Like Syndrome: The Kawasaki Disease Shock Syndrome. *Pediatr Cardiol*. 2010; 31(8):1232–5. <https://doi.org/10.1007/s00246-010-9771-0> PMID: 20706709.
25. Luan YY, Dong N, Xie M, Xiao XZ, Yao YM. The significance and regulatory mechanisms of innate immune cells in the development of sepsis. *J Interferon Cytokine Res*. 2014; 34(1):2–15. Epub 2013/09/07. <https://doi.org/10.1089/jir.2013.0042> PMID: 24006870; PubMed Central PMCID: PMC3887438.
26. Park JY, Kim JS, Woo H. Prevalence of antibody to toxic shock syndrome toxin-1 in burn patients. *Ann Lab Med*. 2015; 35(1):89–93. Epub 2015/01/02. <https://doi.org/10.3343/alm.2015.35.1.89> PMID: 25553286; PubMed Central PMCID: PMC4272971.
27. Parsonnet J, Hansmann MA, Delaney ML, Modern PA, DuBois AM, Wieland-Alter W, et al. Prevalence of Toxic Shock Syndrome Toxin 1-Producing *Staphylococcus aureus* and the Presence of Antibodies to This Superantigen in Menstruating Women. *J Clin Microbiol*. 2005; 43(9):4628–34. <https://doi.org/10.1128/JCM.43.9.4628-4634.2005> PMID: 16145118; PubMed Central PMCID: PMC1234102.
28. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: A statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation*. 2004; 110(17):2747–71. <https://doi.org/10.1161/01.CIR.0000145143.19711.78> PMID: 15505111.
29. JCS Joint Working Group. Guidelines for Diagnosis and Management of Cardiovascular Sequelae in Kawasaki Disease (JCS 2013). *Circ J*. 2014; 78(10):2521–62. Epub 2014/09/23. PMID: 25241888.
30. Zhang YQ, Chen SB, Huang GY, Zhang HY, Huang MR, Wang SS, et al. Coronary artery indexed diameter and z score regression equations in healthy Chinese Han children. *J Clin Ultrasound*. 2014; 43(1):39–46. Epub 2014/07/01. <https://doi.org/10.1002/jcu.22176> PMID: 24975134.
31. Case definitions for infectious conditions under public health surveillance. Centers for Disease Control and Prevention. *MMWR Recomm Rep*. 1997; 46(RR-10):1–55. Epub 1997/05/02. PMID: 9148133.
32. Jansen K, Blimkie D, Furlong J, Hajjar A, Rein-Weston A, Crabtree J, et al. Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation. *J Immunol Methods*. 2008; 336(2):183–92. Epub 2008/06/21. <https://doi.org/10.1016/j.jim.2008.04.013> PMID: 18565537; PubMed Central PMCID: PMC2570701.
33. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol*. 2016; 13(2):148–59. Epub 2015/11/10. <https://doi.org/10.1038/cmi.2015.95> PMID: 26549800; PubMed Central PMCID: PMC4786634.
34. Weng KP, Hsieh KS, Ho TY, Huang SH, Lai CR, Chiu YT, et al. IL-1B polymorphism in association with initial intravenous immunoglobulin treatment failure in Taiwanese children with Kawasaki disease. *Circ J*. 2010; 74(3):544–51. Epub 2010/01/19. PMID: 20081319.
35. Loney C, Vandenbranden M, Ruyschaert JM. Cationic lipids activate intracellular signaling pathways. *Advanced drug delivery reviews*. 2012; 64(15):1749–58. Epub 2012/05/29. <https://doi.org/10.1016/j.addr.2012.05.009> PMID: 22634161.
36. Kum WW, Cameron SB, Hung RW, Kalyan S, Chow AW. Temporal sequence and kinetics of proinflammatory and anti-inflammatory cytokine secretion induced by toxic shock syndrome toxin 1 in human peripheral blood mononuclear cells. *Infect Immun*. 2001; 69(12):7544–9. Epub 2001/11/14. <https://doi.org/10.1128/IAI.69.12.7544-7549.2001> PMID: 11705931; PubMed Central PMCID: PMC98845.
37. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature*. 2007; 449(7164):819–26. <https://doi.org/10.1038/nature06246> PMID: 17943118.
38. Westendorp RGJ, Langermans JAM, Huizinga TWJ, Elouali AH, Verweij CL, Boomsma DI, et al. Genetic influence on cytokine production and fatal meningococcal disease. *Lancet*. 1997; 349(9046):170–3. [http://dx.doi.org/10.1016/S0140-6736\(96\)06413-6](http://dx.doi.org/10.1016/S0140-6736(96)06413-6) PMID: 9111542.
39. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol*. 2016; 16(7):407–20. Epub 2016/06/14. <https://doi.org/10.1038/nri.2016.58> PMID: 27291964.

40. Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. *Science*. 2016; 352(6284):427. <https://doi.org/10.1126/science.aaf1098> PMID: [27102489](https://pubmed.ncbi.nlm.nih.gov/27102489/); PubMed Central PMCID: PMC5087274.

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Manuscripts

Note from the Editors: Instructions for reviewers of study protocols

Since launching in 2011, BMJ Open has published study protocols for planned or ongoing research studies. If data collection is complete, we will not consider the manuscript.

Publishing study protocols enables researchers and funding bodies to stay up to date in their fields by providing exposure to research activity that may not otherwise be widely publicised. This can help prevent unnecessary duplication of work and will hopefully enable collaboration. Publishing protocols in full also makes available more information than is currently required by trial registries and increases transparency, making it easier for others (editors, reviewers and readers) to see and understand any deviations from the protocol that occur during the conduct of the study.

The scientific integrity and the credibility of the study data depend substantially on the study design and methodology, which is why the study protocol requires a thorough peer-review.

BMJ Open will consider for publication protocols for any study design, including observational studies and systematic reviews.

Some things to keep in mind when reviewing the study protocol:

- Protocol papers should report planned or ongoing studies. The dates of the study should be included in the manuscript.
- Unfortunately we are unable to customize the reviewer report form for study protocols. As such, some of the items (i.e., those pertaining to results) on the form should be scored as Not Applicable (N/A).
- While some baseline data can be presented, there should be no results or conclusions present in the study protocol.
- For studies that are ongoing, it is generally the case that very few changes can be made to the methodology. As such, requests for revisions are generally clarifications for the rationale or details relating to the methods. If there is a major flaw in the study that would prevent a sound interpretation of the data, we would expect the study protocol to be rejected.

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Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR) A randomised, controlled trial to determine the non-specific effects of neonatal BCG vaccination in a low-mortality setting

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ABSTRACT

Introduction Bacillus Calmette-Guérin (BCG) vaccination reduces all-cause infant mortality in high-mortality settings by more than can be attributed to protection against tuberculosis. This is proposed to result from non-specific protection against non-vaccine targeted ('off-target') infections. There is also evidence that BCG protects against allergic diseases.

Methods The Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR) is a phase III multicentre, single-blinded, randomised controlled trial. A total of 1438 healthy neonates will be randomised to receive either BCG vaccination or no BCG vaccination in the first 10 days of life. Measures of allergy, eczema, infection, and asthma will be obtained from parent-completed questionnaires 3 monthly in the first year and 6-monthly from one to five years of age, and clinical assessments at one and five years of age. Biological samples will also be collected for future immunological studies.

Analysis Primary outcome: The proportion of participants with measures of allergy and infection (atopic sensitisation, eczema, lower respiratory tract infection) at one and five years of age, and asthma at five years of age. Secondary outcomes: (1) the proportion of participants with additional measures of allergy, eczema, asthma and infections; (2) medication use for eczema and asthma; (3) the severity and age of onset of eczema and asthma; (4) the number of episodes of infection; (5) hospitalisations for infections; and (6) laboratory measures of immune responses.

Ethics and dissemination This trial has ethical and governance approval from Mercy Health Human Research Ethics Committee (HREC, No. R12-28) and Royal Children's Hospital HREC (No. 33025) with additional governance approval from Barwon Health and St John of God, Geelong, Victoria. Results of this trial will be published in peer-reviewed journals and presented at scientific conferences.

Trial Registration number: Clinical trials.gov NCT01906853

ARTICLE SUMMARY

Strengths and limitations of this study

- Infections remain a leading cause of mortality in infants and allergic diseases and asthma are increasing causes of infant/child morbidity
- Large-scale randomised trials for non-specific beneficial effects of neonatal BCG vaccination in high-income countries are lacking
- Neonatal BCG vaccination could be a safe and low-cost means to improve infant health
- No placebo was given to the control group because the development of a scar in BCG vaccinated infants prevents the blinding of parents to infants' randomisation group.
- BCG was followed by multiple administrations of non-live vaccines, and this might attenuate the beneficial non-specific effects of BCG

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INTRODUCTION

The Bacillus Calmette-Guérin (BCG) vaccine is given to more than 85 percent of infants worldwide to protect against tuberculosis (TB).¹ In addition to protecting against TB, vaccination with BCG-Denmark reduces all-cause neonatal mortality in a high-mortality setting,²⁻⁴ likely by protecting against non-mycobacterial infections.^{4,5}

Observational studies suggest that the beneficial ‘non-specific’ (heterologous) effects of BCG on the developing immune system may also reduce the prevalence of allergic disease and asthma in children.⁶ However, meta-analyses of observational studies have had inconsistent findings.⁷⁻⁹ The two randomised controlled trials (RCTs) that have investigated the effect of BCG on infant allergic disease both found that BCG vaccination reduced infant eczema (medication for eczema at 18 months of age;¹⁰ clinically diagnosed eczema at 13 months of age¹¹) but did not have a statistically significant effect on the prevalence of allergic sensitisation or food allergy at 13 or 18 months of age. However, in these studies, allergic outcomes were determined by parent questionnaire and serum IgE, rather than clinical assessment. Studies assessing the impact of BCG vaccination on asthma have had more consistent findings with two meta-analyses concluding the BCG reduces the risk of asthma by 14-27%.^{7,9}

As a result of a reduction in the prevalence of TB in several high- and middle-income countries, BCG has been removed from routine vaccination schedules. This might have contributed to the increased prevalence of allergic diseases over the past three decades.¹²⁻¹⁶ It is proposed that, by acting as an early life microbial stimulus,¹⁷ BCG prevents allergy by skewing the developing immune system in predisposed individuals away from the T helper (Th) 2 type immune response typically associated with allergy.^{6,8}

BCG vaccination induces potent Th1 responses in neonates. In adults, it induces trained immunity in innate immune cells^{18,19} and promotes Th1 and Th17 responses to non-mycobacterial pathogens.²⁰⁻²²

Two large observational studies provide further evidence that BCG vaccination protects against non-mycobacterial infections, particularly sepsis and respiratory infections.^{23,24} However, BCG-mediated protection against these non-vaccine targeted or ‘off-target’ infections might be limited by subsequent vaccination with non-live vaccines which are proposed to counteract the beneficial non-specific effects of BCG.^{4,25,26} Studies in a low-mortality setting, including a recent RCT of neonatal BCG vaccination,²⁷ and an observational study of over 19,000 infants,²⁸ support this hypothesis with protective effects of BCG against off-target infections most evident in early infancy, before administration of non-live vaccines.²⁹ Moreover, in the RCT, the protective effect of BCG was only evident in infants of BCG-vaccinated mothers.²⁷

Subsequent to a 2004 WHO recommendation, an increasing number of countries have implemented routine neonatal hepatitis B vaccination.³⁰ Therefore, the potential impact of this vaccine on the beneficial non-specific effects of neonatal BCG vaccination also requires consideration.

Australia, where routine BCG vaccination was halted in the 1980s - has one of the highest rates of infant allergic disease globally.³¹⁻³³ It therefore represents an ideal setting in which to assess the impact of BCG on allergic disease. The Melbourne Infant Study: BCG for Allergy

and Infection Reduction (MIS BAIR) is an RCT to investigate whether neonatal BCG vaccination reduces the prevalence of allergic and infectious disease.

STUDY AIMS

Primary aims

To determine whether neonatal BCG vaccination compared with no BCG vaccination, reduces allergic disease, infection and asthma in infants and children in Australia.

Secondary aims

To evaluate the immunological mechanisms underlying the non-specific effects of BCG by comparing the immune responses in BCG-vaccinated to those in BCG-naïve infants.

METHODS AND ANALYSIS

Study design and setting

This is a phase III multicentre, single-blinded, RCT of neonatal BCG vaccination compared with no BCG vaccination. Clinical trials.gov NCT01906853. (Recruitment status: completed).

The study population will be healthy neonates 0 to 10 days of age born at one of the study site hospitals in Victoria, Australia. Recent studies of the burden of allergic diseases in this population have reported high a prevalence of eczema (28%³⁴), allergic sensitisation (18%) and clinically significant food allergy (10.4%³⁵) at 12 months of age, as well as high rates (22%) of asthma by 4-5 years.³⁶

Study sites comprise: the Murdoch Children's Research Institute (MCRI), Melbourne; Royal Children's Hospital (RCH) Melbourne; Mercy Hospital for Women, Heidelberg; Werribee Mercy Hospital, Werribee; University Hospital Geelong, Geelong; and St John of God Geelong Hospital, Geelong.

Patient and Public Involvement

Patients and public were not involved in the design of this study. The results of this study will be disseminated to study participants via participant newsletter.

Eligibility criteria

The inclusion criteria comprise: healthy neonates up to 10 days of age; birth weight greater than 1500 grams; gestational age equal to or greater than 32 weeks, mothers testing HIV negative during pregnancy, English-speaking mother and parent/legal guardian able to complete questionnaires in English and attend study visit. The exclusion criteria comprise: any indication for BCG vaccination in the first year of life as per the Australian national guidelines³⁷; known or suspected HIV infection; infant at risk of immunodeficiency; serious underlying illness (including fever) or medical instability; skin infection or other skin condition; need for treatment with hepatitis B immunoglobulin; multiple-birth of more than twins; older sibling in the study.

Intervention

Infants will be randomised to receive BCG vaccination or no BCG vaccination in the first 10 days of life. Infants randomised to the BCG vaccination group will be administered a single

intradermal injection of 0.05 mL *Mycobacterium bovis* BCG vaccine, Danish Strain 1331(1-4 x 10⁵ colony forming units) over the left deltoid within 24 hours of randomisation.

Reasons for withdrawal

Reasons for withdrawal from the study will be recorded along with demographic data. These may include: protocol violations; indication for participant to receive BCG vaccine within the first year of life;³⁷ serious adverse event (SAE) or other adverse event (AE); or guardian/parent request.

Data and sample collection

Timing of data and sample collection in MIS BAIR is summarised in Table 1. Web-based questionnaires will be administered to parents at the time of recruitment, randomisation and when the participant is 3, 6, 9 and 12 months of age as well as 6-monthly up to 5 years of age using the Research Electronic Data Capture (REDCap) platform.³⁸ Infant perinatal data, including infections, medications and hospital admission will be obtained from the birth site records. Participants will be invited to optional study visits for biological sample collection 7 days (± 4 days) after randomisation and at 6 months (5-8 months) of age. Infants will be invited for clinical assessment and biological sample collection at 1 year (between 11 and 24 months) and 5 years (between 5 and 6 years) of age.

Table 1: Data and sample collection schedule

Infant age	Antenatal	0-10d	3m	6m	9m	1y	1.5y	2y	2.5y	3y	3.5y	4y	4.5y	5y
Recruitment & randomisation														
Eligibility check and consent	✓	✓												
Baseline questionnaire	✓	✓												
Birth questionnaire		✓												
Randomisation ± vaccination		✓												
Full blood examination		✓ ^a												
Post randomisation														
Perinatal hospital data		✓												
Parent questionnaire			✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Clinical eczema assessment						✓								✓
Skin prick test						✓								✓
Oral food challenge						✓ ^b								✓ ^b
Biological sample collection		✓ ^c		✓ ^d		✓								✓

^a soon after birth; ^b if indicated by skin prick test result; ^c 7±4 days post randomisation; ^d 5-8 months of age; d, days; m, months; y, year

Parent questionnaires

To collect data on potential confounding factors and for stratification prior to randomisation, baseline parent questionnaires will be used to collect data on demographic, environmental, prenatal, and birth factors (Table 2). When the participants are 3, 6, 9 and 12 months of age, questionnaires will be provided to parents to collect data on diet, medications and potential environmental confounders, and to collect data for primary and secondary outcomes (Table 3).

Table 2: Baseline questionnaire data

Family and demographic	Family history of allergic disease (allergy, eczema, hay fever, asthma), maternal BCG vaccination, parent education, parent country of birth, ethnicity.

Household environment	Size, composition, smoking, pets.
Maternal prenatal	Age, weight, height, prior pregnancies, vaccinations, smoking, antibiotics, vitamin D, probiotics other medications or supplements.
Birth	Mode of delivery*, birth site*, plurality*, birth complications, gestational age, weight, sex, antibiotics or other medications during labour.

* Required for stratification prior to randomisation

Table 3: Three- and six-monthly questionnaire outcome data

Illnesses	Any episode of illness, infant age, duration of illness, symptoms, medial consultations and hospital admissions, diagnosis and any treatments/medications.
Eczema	Symptoms of eczema, infant age at onset of eczema, distribution of eczema, use of eczema medications, medical consultations and hospital admissions. Includes modified U.K. working party's diagnostic criteria for atopic dermatitis and modified patient-oriented eczema measure (POEM) tool questions. ^{52 53}
Allergies	Any episodes of food or other allergies, infant age, allergen, symptoms, severity, diagnosis and any treatment.
Asthma	International study of asthma and allergies in childhood (ISAAC) questions, ^{54 55} asthma medication usage, symptom severity, acute exacerbations and health care utilisation, including hospitalisations
Diet	Breast milk feeding, formula milk feeding, food introduction, dietary supplements.
Other	Household composition, childcare, pets and other animals, household smoking, drinking water source, vaccinations, other medications or supplements, other diseases/disabilities, BCG complications, overseas travel, non-illness associated hospital admissions.

Clinical assessments

Allergic sensitisation: Skin prick testing (SPT) to the following panel of allergens will be assessed: food allergens - cow's milk, raw egg, peanut, sesame, cashew, hazelnut, shellfish, walnut (at 5-year visit only); other allergens - *Dermatophagoides pteronyssinus* 1 (house dust mite), cat, dog, *Alternaria tenuis* (mould) and rye grass pollen. SPT will be done according to standard guidelines.³⁹ For each food allergen tested, data will also be collected on prior ingestion, exposure, reactions and tolerance (after the SPT wheal size is assessed).

Eczema: Severity of eczema will be assessed using Scoring of Atopic Dermatitis (SCORAD).⁴⁰

Oral food challenge (OFC): Participants with a wheal diameter ≥ 1 mm greater than the negative control to selected food allergens during SPT will be invited to have an OFC as detailed in supplementary Figure 1. OFCs will be done at MCRI according to Australasian Society of Clinical Immunology and Allergy (ASCI) guidelines as used by the RCH Allergy Clinic and the HealthNuts study.⁴¹ The different pathways that will occur during an OFC detailed in supplementary Figure 2.

Other: Weight, height, skin, eye and hair colour, BCG vaccination site including scar measurement and photograph. BCG vaccination site assessment will occur after all other assessments are complete.

Biological sample collection

Full blood examination: A capillary blood sample will be collected from participants soon after birth as a preliminary screen for primary T-cell immunodeficiencies. This may be done before or after randomisation.

Peripheral blood: Peripheral blood samples will be collected at 7 days, 7 months, 1 year and 5 years of age. These samples will be used for immediate immune stimulation experiments as well as separation and storage of peripheral blood mononuclear cells and granulocytes, plasma and plasma-depleted cells at -80°C or in liquid nitrogen, as appropriate. These samples will be retained for future immunological analysis.

Stool: From the day of birth participant’s parents will be requested to collect a stool sample on each day (up to seven samples) and store it in a domestic freezer until collection at the 7-day post randomisation study visit. Additional stool samples will be collected during the 7-month and 1-year study visits or by parents prior to the one-year study visit using an in-home stool collection kit provided by the study team.

Outcomes

Primary outcomes

The primary outcomes are the proportions of infants with the following measures of allergic disease and infection at 1 year and 5 years of age:

- atopic sensitisation (positive SPT) to one or more of a panel of food and aeroallergens
- eczema
- lower respiratory tract infection (LRTI)
- asthma (5 years of age only)

Secondary outcomes

The secondary outcomes are the following additional measures of allergy and infection:

Allergy

The proportion of infants with clinical food allergy (Supplementary figures 1-2); atopic sensitisation with ≥ 3 mm wheal diameter; atopic sensitisation to multiple allergens; parent report of food allergy to any food; atopic sensitisation to egg allergen; atopic wheeze.

Eczema

Severity of eczema; age of onset of eczema; and proportions of clinically diagnosed eczema and steroid use for eczema.

Infections

Proportion of infants with or frequency of the following: any infection; upper respiratory tract infection; LRTI; diarrhoea with vomiting; rash with fever; fever; hospitalisation for infection; hospitalisation for respiratory tract infection.

Asthma,

The proportion of infants with current asthma, asthma severity and medication use for asthma

Sample size and power calculation

This study aims to randomise a total of 1,438 participants. This sample size is calculated based on an assumed minimum 80% retention rate, resulting in an expected minimum 1,150 infants with 1-year data available. With a final sample size of 575 in each arm, this study is powered to detect a minimum 35% reduction in atopic sensitisation, 25% reduction in eczema and 25% reduction in LRTI with a power of 80% in the first year of life and a minimum 37% reduction in atopic sensitisation, 26% reduction in eczema, 26% reduction in LRTI and 27% reduction in asthma with a power of 80% at five years of age. These differences are based on the previously reported prevalence of atopic sensitisation, eczema, LRTI and asthma in Australian infants at 1 and 5 years of age.^{35 36 42}

Recruitment

Recruitment will occur in two stages: (1) early consent will be sought from pregnant women attending antenatal clinics or in the postnatal ward at a study site; (2) additional pregnant women or mothers interested in participating but not being cared for at a study site may also be recruited if they contact the research team antenatally or within 10 days of delivery. Consent to participate in the study will be verbally confirmed after the birth of antenatally recruited infants and prior to randomisation.

Randomisation

Recruited neonates will only be randomised after confirmation that they still fulfil inclusion criteria and do not meet any exclusion criteria. Randomisation to BCG-vaccination or no BCG vaccination will be done in a 1:1 ratio using the REDCap randomisation function.³⁸ The randomisation schedule will be established by a statistician external to the study using random permuted blocks with a minimum of three different block sizes. Randomisation will be stratified by: (i) site (hospital); (ii) method of delivery (Caesarean vs non-Caesarean); and (iii) plurality of birth (twins vs singletons). Twins will be assigned to the same intervention arm.

Blinding

As BCG vaccination results in the formation of a scar at the vaccination site in 93-99% of infants,⁴³⁻⁴⁶ blinding with the use of a placebo is not possible. For blinding of clinical assessments, prior to commencement of the 1- and 5-year study visits, each participant's left upper arm will be covered with a bandage by the parent or a member of the study team not involved with the assessment to hide the potential scar site. Clinical assessments will be done by a member of the study team or clinical staff member who was not involved in the randomisation of the participant.

Statistical analysis

Statistical analysis of primary and secondary outcomes will be overseen by the trial statistician. Primary analysis will be by intention to treat, including all randomised participants where outcome data are available. Data will be collected according to Consolidated Standards of Reporting Trials (CONSORT) guidelines for reporting randomised trials.⁴⁷

Outcomes

Primary outcomes

Comparison between BCG-vaccinated and BCG-naïve infants will be estimated using binary regression adjusted for the stratification factors used during randomisation. Results will be presented as risk difference with 95% confidence intervals (CI).

Secondary outcomes

Comparison between the BCG-vaccinated and BCG-naïve infants will be estimated using binary linear or Poisson regression adjusted for the stratification factors used during randomisation. Results will be presented as risk difference with 95% CI.

Missing data

If the proportion of missing data is less than 5%, the primary analysis will be a complete case analysis. Otherwise, the frequency and patterns of missing data will be examined and, if appropriate, multiple imputation models will be conducted for the outcome variables. Fifty completed data sets will be imputed by chained equations including all the children initially randomised. The primary outcome, strata variable (mode of birth: vaginal/caesarean), and the variables predictive of missingness and allergy, eczema, infection or asthma will be included in the imputation model.

Subgroup analysis

Prior to any subgroup analysis, adjusted models including the stratification factors used in randomisation, the randomisation assignment and the subgroup variable as covariates will be used to estimate the interaction between the intervention and the subgroup variable. Where these models provide evidence that the intervention varies between subgroups, specific subgroup estimates and confidence intervals will be presented obtained from the adjusted model. The subgroups are presence or absence of BCG scar; timing of BCG administration; maternal BCG vaccination; sex; mode of delivery; season of birth; timing of hepatitis B vaccination. For allergy, eczema and asthma outcomes, an additional subgroup variable, family history of allergic disease or asthma, will be assessed.

Data monitoring and auditing

The independent Data Safety and Monitoring Committee (DSMC), consisting of an independent statistician, neonatologist and paediatric infectious diseases consultant will meet to review data and participant safety 6-monthly.

Risks

The potential risks of participation in this study may be related to: (1) adverse reactions to BCG vaccination: subcutaneous abscess, exaggerated local reaction, lymphadenitis, keloid scarring, osteitis and disseminated infection.⁴⁸ In Australia, adverse reactions to BCG vaccine occur in 15.3 out of 10,000 doses;⁴⁹ (2) blood collection: discomfort, bruising and rarely minor infection or blood clots (3) clinical assessments of allergy: anaphylactic reactions may occur during OFC, during SPT (rare) and as a late reaction to OFC (from further allergen exposures during the subsequent week). The protocols and workflows for SPT and OFC mitigate the risk of anaphylactic reactions and their safety has been demonstrated in previous clinical trials (supplementary Figures 1 and 2).⁴¹ All AE will be recorded, and SAEs reported to the study site HREC and the DSMC.

Outlook and significance

The findings of MIS BAIR will provide evidence as to whether neonatal BCG vaccination, a low-cost, readily available intervention, reduces the prevalence of allergies and infections in the first one and five years of life, and asthma in the first five years of life. An immune

priming benefit of neonatal BCG against these diseases would have considerable public health implications and thus inform guidelines for BCG vaccine policies worldwide.

Limitations

The potential limitations of MIS BAIR include the inability to blind the parent(s)/guardian(s) to the infant's randomisation assignment. This may lead to bias or lower compliance if parent(s)/guardian(s) are disappointed with the randomisation assignment. However, blinding will be done for the 13-month study clinical assessments from which several of the primary and secondary outcomes measures of allergy and eczema are obtained. We expect that there will be greater recruitment of participants with a family history of allergic disease, which may limit the generalisability of our findings. We will therefore collect data in relation to family history to enable us to consider for this in later analyses. BCG was followed by multiple administrations of non-live vaccines, and this might attenuate the beneficial non-specific effects of BCG.^{50 51}

ETHICS AND DISSEMINATION

This trial has been approved by the Mercy Health Human Research Ethics Committee (HREC, No. R12-28) and RCH HREC (No. 33025) with governance approval from Barwon Health and St John of God, Geelong. A record of consents and refusals will be maintained on the study REDCap database. Parent(s)/guardian(s) of participants will be informed of their option to withdraw from the study at any time. An electronic or hard copy parent /guardian information and consent form (PGICF) will be completed a parent/guardian as part of the consent process. Results of this trial will be published in peer-reviewed journals and presented at scientific conferences.

Contributors NC is the lead investigator and responsible for study conception, design and funding acquisition. KG developed the trial database. KG, NC, BF, SD, LC, CZ developed the ethics application and all other authors provided critical evaluation and revision. DC, KG, PV, CM, VA, NC developed the recruitment methods. KG, BF, VA, ALP, KA, PV developed and SD, MS, NC contributed to the design of the questionnaires. KG, KA developed and ALP, NC, PV contributed to the clinical assessment methods. CZ, NC, BF, SG, KF developed and KG, NM, RRB, PV contributed to biological sample collection and processing methods. SD developed the statistical analysis plan. NC, KG, KA, FS, VA, NM, MS contributed to and ALP, KF, RRB, PV, DC provided critical evaluation and revision. NM drafted the manuscript, co-ordinated manuscript preparation and revision. All authors provided critical evaluation and revision of the manuscript, and have approved the final version of this manuscript.

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Patient consent Parent/guardian consent obtained

Ethics approval Mercy Health HREC and RCH HREC

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES

1. World Health Organization. Immunization coverage estimates by WHO region: BCG: World Health Organization; 2018 [updated 19th July 2018. Available from: <http://apps.who.int/gho/data/view.main.81500?lang=en> accessed 1st June 2019 2019.

2. Higgins JP, Soares-Weiser K, Lopez-Lopez JA, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. *BMJ* 2016;355:i5170. doi: 10.1136/bmj.i5170 [published Online First: 2016/10/16]

3. Pollard AJ, Finn A, Curtis N. Non-specific effects of vaccines: plausible and potentially important, but implications uncertain. *Arch Dis Child* 2017;102(11):1077-81. doi: 10.1136/archdischild-2015-310282 [published Online First: 2017/05/16]

4. Biering-Sorensen S, Aaby P, Lund N, et al. Early BCG-Denmark and Neonatal Mortality Among Infants Weighing <2500 g: A Randomized Controlled Trial. *Clin Infect Dis* 2017;65(7):1183-90. doi: 10.1093/cid/cix525 [published Online First: 2018/03/27]

5. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis* 2011;204(2):245-52. doi: 10.1093/infdis/jir240 [published Online First: 2011/06/16]

6. Freyne B, Curtis N. Does neonatal BCG vaccination prevent allergic disease in later life? *Arch Dis Child* 2014;99(2):182-4. doi: 10.1136/archdischild-2013-305655 [published Online First: 2014/01/15]

7. Arnoldussen DL, Linehan M, Sheikh A. BCG vaccination and allergy: a systematic review and meta-analysis. *J Allergy Clin Immunol* 2011;127(1):246-53, 53 e1-21. doi: 10.1016/j.jaci.2010.07.039 [published Online First: 2010/10/12]

8. Rousseau MC, Parent ME, St-Pierre Y. Potential health effects from non-specific stimulation of the immune function in early age: the example of BCG vaccination. *Pediatr Allergy Immunol* 2008;19(5):438-48. doi: 10.1111/j.1399-3038.2007.00669.x [published Online First: 2008/01/03]

9. El-Zein M, Parent ME, Benedetti A, et al. Does BCG vaccination protect against the development of childhood asthma? A systematic review and meta-analysis of epidemiological studies. *Int J Epidemiol* 2010;39(2):469-86. doi: 10.1093/ije/dyp307 [published Online First: 2009/10/14]

10. Steenhuis TJ, van Aalderen WM, Bloksma N, et al. Bacille-Calmette-Guerin vaccination and the development of allergic disease in children: a randomized, prospective, single-blind study. *Clin Exp Allergy* 2008;38(1):79-85. doi: 10.1111/j.1365-2222.2007.02859.x [published Online First: 2007/10/25]

11. Thostesen LM, Kjaergaard J, Pihl GT, et al. Neonatal BCG vaccination and atopic dermatitis before 13 months of age: A randomized clinical trial. *Allergy* 2018;73(2):498-504. doi: 10.1111/all.13314 [published Online First: 2017/09/21]

12. Asher MI, Montefort S, Bjorksten B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006;368(9537):733-43. doi: 10.1016/S0140-6736(06)69283-0 [published Online First: 2006/08/29]

13. Mullins RJ. Paediatric food allergy trends in a community-based specialist allergy practice, 1995-2006. *Med J Aust* 2007;186(12):618-21. [published Online First: 2007/06/20]

14. Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol* 2003;112(6):1203-7. doi: 10.1016/S0091 [published Online First: 2003/12/06]

15. Gupta R, Sheikh A, Strachan D, et al. Increasing hospital admissions for systemic allergic disorders in England: analysis of national admissions data. *BMJ* 2003;327(7424):1142-3. doi: 10.1136/bmj.327.7424.1142 [published Online First: 2003/11/15]

16. Downs SH, Marks GB, Sporik R, et al. Continued increase in the prevalence of asthma and atopy. *Arch Dis Child* 2001;84(1):20-23. [published Online First: 2000/12/22]

17. Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;299(6710):1259-60. [published Online First: 1989/11/18]

18. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe* 2011;9(5):355-61. doi: 10.1016/j.chom.2011.04.006 [published Online First: 2011/05/18]

19. Novakovic B, Messina N, Curtis N. Chapter 6 - The Heterologous Effects of Bacillus Calmette-Guérin (BCG) Vaccine and Trained Innate Immunity. In: Faustman DL, ed. *The Value of BCG and TNF in Autoimmunity (Second Edition)*. Second edition. ed: Academic Press 2018:71-90.

20. Marchant A, Goetghebuer T, Ota MO, et al. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. *J Immunol* 1999;163(4):2249-55. [published Online First: 1999/08/10]
21. Kleinnijenhuis J, Quintin J, Preijers F, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J Innate Immun* 2014;6(2):152-8. doi: 10.1159/000355628 [published Online First: 2013/11/07]
22. Messina NL, Zimmermann P, Curtis N. The impact of vaccines on heterologous adaptive immunity. *Clin Microbiol Infect* 2019 doi: 10.1016/j.cmi.2019.02.016 [published Online First: 2019/02/24]
23. de Castro MJ, Pardo-Seco J, Martinon-Torres F. Nonspecific (Heterologous) Protection of Neonatal BCG Vaccination Against Hospitalization Due to Respiratory Infection and Sepsis. *Clin Infect Dis* 2015;60(11):1611-9. doi: 10.1093/cid/civ144 [published Online First: 2015/03/01]
24. Hollm-Delgado MG, Stuart EA, Black RE. Acute lower respiratory infection among Bacille Calmette-Guerin (BCG)-vaccinated children. *Pediatrics* 2014;133(1):e73-81. doi: 10.1542/peds.2013-2218 [published Online First: 2014/01/01]
25. Aaby P, Mogensen SW, Rodrigues A, et al. Evidence of Increase in Mortality After the Introduction of Diphtheria-Tetanus-Pertussis Vaccine to Children Aged 6-35 Months in Guinea-Bissau: A Time for Reflection? *Front Public Health* 2018;6:79. doi: 10.3389/fpubh.2018.00079 [published Online First: 2018/04/05]
26. Aaby P, Ravn H, Benn CS. The WHO Review of the Possible Nonspecific Effects of Diphtheria-Tetanus-Pertussis Vaccine. *Pediatr Infect Dis J* 2016;35(11):1247-57. doi: 10.1097/INF.0000000000001269 [published Online First: 2016/10/19]
27. Kjaergaard J, Birk NM, Nissen TN, et al. Nonspecific effect of BCG vaccination at birth on early childhood infections: a randomized, clinical multicenter trial. *Pediatr Res* 2016;80(5):681-85. doi: 10.1038/pr.2016.142 [published Online First: 2016/11/02]
28. Haahr S, Michelsen SW, Andersson M, et al. Non-specific effects of BCG vaccination on morbidity among children in Greenland: a population-based cohort study. *Int J Epidemiol* 2016;45(6):2122-30. doi: 10.1093/ije/dyw244 [published Online First: 2016/01/01]
29. Benn CS, Sorup S. Commentary: BCG has no beneficial non-specific effects on Greenland. An answer to the wrong question? *Int J Epidemiol* 2016;45(6):2131-33. doi: 10.1093/ije/dyw299 [published Online First: 2016/11/20]
30. World Health Organization. Global Hepatitis Report 2017. Geneva: World Health Organization, 2017:83.
31. Loh W, Tang MLK. The Epidemiology of Food Allergy in the Global Context. *Int J Environ Res Public Health* 2018;15(9) doi: 10.3390/ijerph15092043 [published Online First: 2018/09/21]
32. Peters RL, Koplin JJ, Gurrin LC, et al. The prevalence of food allergy and other allergic diseases in early childhood in a population-based study: HealthNuts age 4-year follow-up. *J Allergy Clin Immunol* 2017;140(1):145-53 e8. doi: 10.1016/j.jaci.2017.02.019 [published Online First: 2017/05/19]
33. Prescott SL, Pawankar R, Allen KJ, et al. A global survey of changing patterns of food allergy burden in children. *World Allergy Organ J* 2013;6(1):21. doi: 10.1186/1939-4551-6-21 [published Online First: 2013/12/07]
34. Martin PE, Koplin JJ, Eckert JK, et al. The prevalence and socio-demographic risk factors of clinical eczema in infancy: a population-based observational study. *Clin Exp Allergy* 2013;43(6):642-51. doi: 10.1111/cea.12092 [published Online First: 2013/05/29]
35. Osborne NJ, Koplin JJ, Martin PE, et al. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol* 2011;127(3):668-76 e1-2. doi: 10.1016/j.jaci.2011.01.039 [published Online First: 2011/03/08]
36. Australian Centre for Asthma Monitoring. Asthma in Australia 2011. AIHW Asthma Series: no 4 Cat no ACM 22. Canberra: Australian Institute of Health and Welfare, 2011.
37. The BCG vaccine: information and recommendations for use in Australia. National Tuberculosis Advisory Committee update October 2012. *Commun Dis Intell Q Rep* 2013;37(1):E65-72. [published Online First: 2013/07/12]
38. Harris PA, Taylor R, Thielke R, et al. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;42(2):377-81. doi: 10.1016/j.jbi.2008.08.010 [published Online First: 2008/10/22]

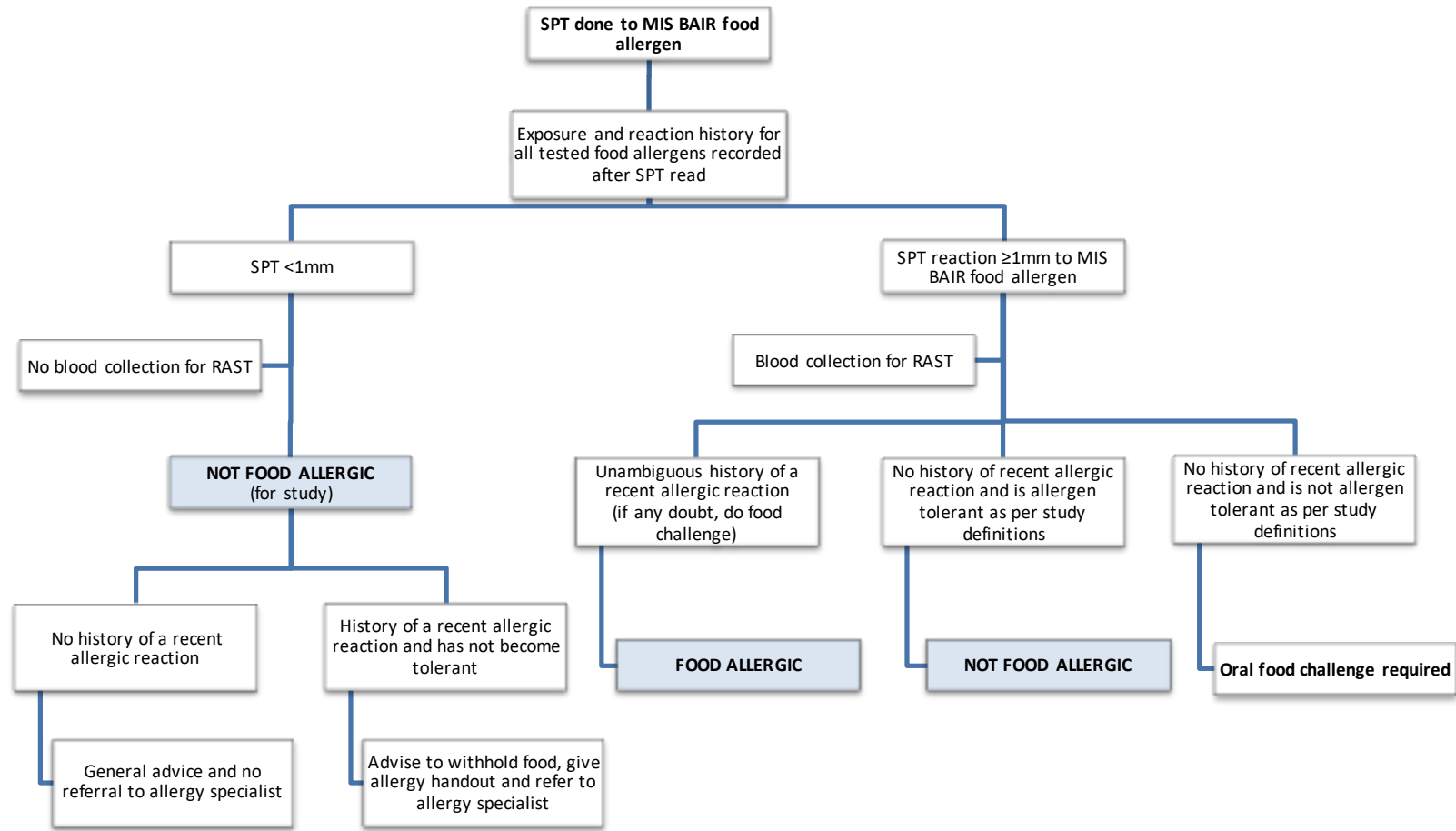
39. Australasian Society of Clinical Immunology and Allergy (ASCIA) SPTWP. Skin prick testing for the diagnosis of allergic disease: A manual for practitioners Sydney: ASCIA; 2016 [Available from: <https://allergy.org.au/hp/papers/skin-prick-testing/>].
40. Pucci N, Novembre E, Cammarata MG, et al. Scoring atopic dermatitis in infants and young children: distinctive features of the SCORAD index. *Allergy* 2005;60(1):113-6. doi: 10.1111/j.1398-9995.2004.00622.x [published Online First: 2004/12/04]
41. Osborne NJ, Koplin JJ, Martin PE, et al. The HealthNuts population-based study of paediatric food allergy: validity, safety and acceptability. *Clin Exp Allergy* 2010;40(10):1516-22. doi: 10.1111/j.1365-2222.2010.03562.x [published Online First: 2010/07/09]
42. Kusel MM, de Klerk NH, Holt PG, et al. Role of respiratory viruses in acute upper and lower respiratory tract illness in the first year of life: a birth cohort study. *Pediatr Infect Dis J* 2006;25(8):680-6. doi: 10.1097/01.inf.0000226912.88900.a3 [published Online First: 2006/07/29]
43. Anderson EJ, Webb EL, Mawa PA, et al. The influence of BCG vaccine strain on mycobacteria-specific and non-specific immune responses in a prospective cohort of infants in Uganda. *Vaccine* 2012;30(12):2083-9. doi: 10.1016/j.vaccine.2012.01.053 [published Online First: 2012/02/04]
44. Birk NM, Nissen TN, Ladekarl M, et al. The association between Bacillus Calmette-Guerin vaccination (1331 SSI) skin reaction and subsequent scar development in infants. *BMC Infect Dis* 2017;17(1):540. doi: 10.1186/s12879-017-2641-0 [published Online First: 2017/08/05]
45. Frankel H, Byberg S, Bjerregaard-Andersen M, et al. Different effects of BCG strains - A natural experiment evaluating the impact of the Danish and the Russian BCG strains on morbidity and scar formation in Guinea-Bissau. *Vaccine* 2016;34(38):4586-93. doi: 10.1016/j.vaccine.2016.07.022 [published Online First: 2016/08/06]
46. Funch KM, Thyssen SM, Rodrigues A, et al. Determinants of BCG scarification among children in rural Guinea-Bissau: A prospective cohort study. *Hum Vaccin Immunother* 2018;14(10):2434-42. doi: 10.1080/21645515.2017.1421879 [published Online First: 2018/01/03]
47. Turner L, Shamseer L, Altman DG, et al. Consolidated standards of reporting trials (CONSORT) and the completeness of reporting of randomised controlled trials (RCTs) published in medical journals. *Cochrane Database Syst Rev* 2012;11:MR000030. doi: 10.1002/14651858.MR000030.pub2 [published Online First: 2012/11/16]
48. World Health Organization. BCG vaccine: WHO position paper - February 2018 Weekly epidemiological record. Switzerland: World Health, Organization, 2018:73-96.
49. Hendry AJ, Dey A, Beard FH, et al. Adverse events following immunisation with bacille Calmette-Guerin vaccination: baseline data to inform monitoring in Australia following introduction of new unregistered BCG vaccine. *Commun Dis Intell Q Rep* 2016;40(4):E470-E74. [published Online First: 2017/01/04]
50. Aaby P, Kollmann TR, Benn CS. Nonspecific effects of neonatal and infant vaccination: public-health, immunological and conceptual challenges. *Nat Immunol* 2014;15(10):895-9. doi: 10.1038/ni.2961 [published Online First: 2014/09/19]
51. Shann F. BCG vaccination in developing countries. *BMJ* 2010;340:c809. doi: 10.1136/bmj.c809 [published Online First: 2010/03/17]
52. Williams HC, Burney PG, Hay RJ, et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br J Dermatol* 1994;131(3):383-96. [published Online First: 1994/09/01]
53. Charman CR, Venn AJ, Williams HC. The patient-oriented eczema measure: development and initial validation of a new tool for measuring atopic eczema severity from the patients' perspective. *Arch Dermatol* 2004;140(12):1513-9. doi: 10.1001/archderm.140.12.1513 [published Online First: 2004/12/22]
54. Mallol J, Crane J, von Mutius E, et al. The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three: a global synthesis. *Allergol Immunopathol (Madr)* 2013;41(2):73-85. doi: 10.1016/j.aller.2012.03.001 [published Online First: 2012/07/10]
55. Beasley R, The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 1998;351(9111):1225-32. [published Online First: 1998/06/27]

Melbourne Infant Study: BCG for Allergy and Infection Reduction (MIS BAIR) A randomised, controlled trial to determine the non-specific effects of neonatal BCG vaccination in a low-mortality setting

*NL Messina, *K Gardiner, S Donath, K Flanagan, AL Ponsonby, F Shann, R Robins-Browne, B Freyne, V Abruzzo, C Morison, L Cox, S Germano, C Zufferey, KJ Allen, P Vuillermin, M South, D Casalez, N Curtis

Supplementary Figures

For peer review only



Total dose of allergen as per food challenge protocol

- Cows milk: 160 ml
- Raw egg white: 1x 60 g egg
- Sesame: 2 teaspoons
- Peanut: 2 teaspoons
- Cashew: 2 teaspoons
- Hazelnut: 2 teaspoons
- Shellfish: 1 peeled prawn (~15 g)

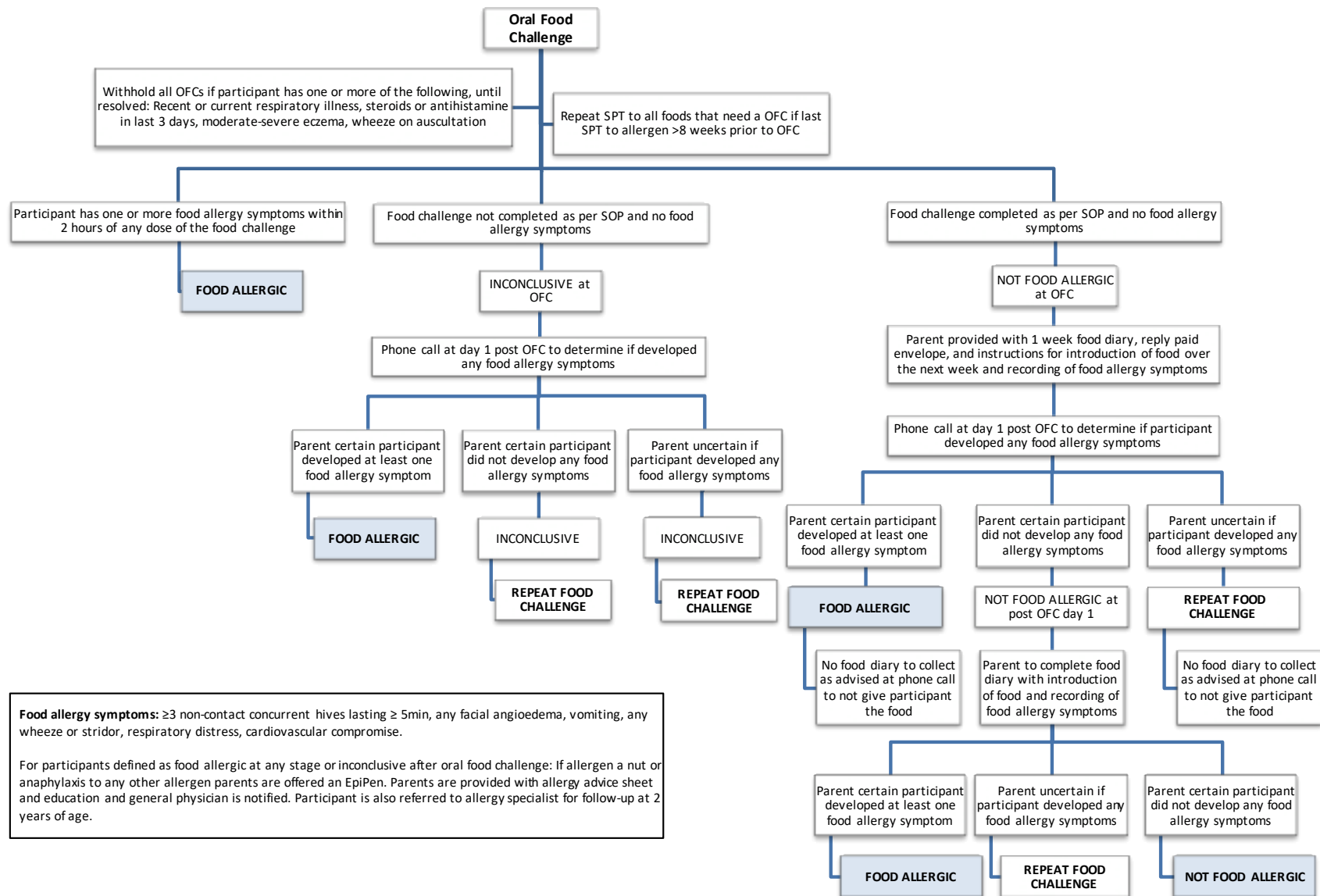
Definitions

History of a recent allergic reaction: In the past 1-2 months (1 month for egg or milk, 2 months for any other allergen), at least one of the following reactions within 30 minutes of exposure to the MIS BAIR food allergen: ≥3 non-contact concurrent hives lasting ≥5 mins, vomiting (not gagging), facial angioedema, wheeze or stridor, respiratory distress, cardiovascular compromise (pale and floppy infant, cardiac arrest).

Allergen tolerance: In the past 1-2 months (1 month for egg or milk, 2 months for any other allergen), infant has eaten equivalent to ≥ 'total dose of food challenge protocol' of allergen on 2 separate occasions without reaction.

Adequate exposure: Food eaten not just touched and food in form that is recognizable (e.g. not mixed nuts) unless infant has previously tolerated all the other food in the dish.

Supplementary Figure 1: MIS BAIR skin prick test flow chart
SPT skin prick test, RAST radioallergosorbent test



Supplementary Figure 2: MIS BAIR oral food challenge flow chart
 OFC oral food challenge, SPT skin prick test, SOP standard operating procedure



SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____2 & 4_____
	2b	All items from the World Health Organization Trial Registration Data Set	Available at Clinical trials.gov
Protocol version	3	Date and version identifier	Approved HREC application
Funding	4	Sources and types of financial, material, and other support	_____11_____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	_____1 & 11_____
	5b	Name and contact information for the trial sponsor	_____1_____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	_____11_____

5d Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) _____11_____

Introduction

Background and rationale 6a Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention _____3_____

6b Explanation for choice of comparators _____3_____

Objectives 7 Specific objectives or hypotheses _____4_____

Trial design 8 Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) _____4_____

Methods: Participants, interventions, and outcomes

Study setting 9 Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained _____4_____

Eligibility criteria 10 Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) _____4_____

Interventions 11a Interventions for each group with sufficient detail to allow replication, including how and when they will be administered _____5_____

11b Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) _____NA_____

11c Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) _____5_____

11d Relevant concomitant care and interventions that are permitted or prohibited during the trial _____NA_____

1	Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	7-8
6	Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5
10	Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	8
13	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	8

15 **Methods: Assignment of interventions (for controlled trials)**

17 Allocation:

19	Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	8
25	Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	8
30	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8
33	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	9
36		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	9

40 **Methods: Data collection, management, and analysis**

1	Data collection	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related	___5-7___
2	methods		processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of	
3			study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known.	
4			Reference to where data collection forms can be found, if not in the protocol	
5				
6		18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be	___9___
7			collected for participants who discontinue or deviate from intervention protocols	
8				
9	Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality	Approved HREC
10			(eg, double data entry; range checks for data values). Reference to where details of data management	application
11			procedures can be found, if not in the protocol	
12				
13	Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the	9 & clinical
14			statistical analysis plan can be found, if not in the protocol	trials.gov
15				
16		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	___9___
17				
18		20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any	
19			statistical methods to handle missing data (eg, multiple imputation)	___9___
20				
21				
22				
23	Methods: Monitoring			
24				
25	Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of	___10___
26			whether it is independent from the sponsor and competing interests; and reference to where further details	
27			about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not	
28			needed	
29				
30		21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim	___NA___
31			results and make the final decision to terminate the trial	
32				
33				
34	Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse	___10___
35			events and other unintended effects of trial interventions or trial conduct	
36				
37	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent	___10___
38			from investigators and the sponsor	
39				
40				

Ethics and dissemination

1	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___10___
2				
3				
4	Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	___NA___
5				
6				
7				
8	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Approved HREC application
9				
10		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Approved HREC application
11				
12				
13				
14	Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	5 & Approved HREC application
15				
16				
17				
18	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	___11___
19				
20				
21	Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Approved HREC application
22				
23				
24	Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Approved HREC application
25				
26				
27	Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___11___
28				
29		31b	Authorship eligibility guidelines and any intended use of professional writers	___NA___
30				
31		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	___NA___
32				
33				
34				
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36	Appendices			
37				
38	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Approved HREC application
39				
40				
41				
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1	Biological	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular	7 & Approved
2	specimens		analysis in the current trial and for future use in ancillary studies, if applicable	HREC application

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4 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.

5 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons

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Biological sex influences antibody responses to routine vaccinations in the first year of life

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Abstract

Aim: We investigated the effect of early-life factors, namely sex, delivery mode, feeding method and antibiotic exposure, on antibody responses to routine vaccinations administered during the first year of life.

Methods: One and seven months after the primary course of routine vaccines and 1 month after routine vaccines at 12 months of age, antibodies against 26 vaccine antigens were measured in 398 healthy infants. The geometric mean concentration (GMC) of antibodies (adjusted for effect modifiers with multiple linear regression) and the seroprotection rate for each vaccine were compared for each early-life factor.

Results: Sex had an influence on GMCs. Antibody concentrations were significantly lower at 7 months of age in females for tetanus and filamentous haemagglutinin and at 13 months of age for pertactin. In contrast, at 13 months of age, antibody concentrations were significantly higher in females for polio type 3, pneumococcal serotype 6A and measles. Sex did not have an influence on seroprotection rates. Delivery mode, feeding method and antibiotic exposure did not exert a substantial influence on vaccine antibody concentrations.

Conclusion: There is a difference between males and females in the humoral response to routine vaccinations in the first year of life.

KEYWORDS

antibodies, humoral, immunisation, immunoglobulin, infant, titre

Abbreviations: BCG, Bacille Calmette–Guérin; CI, confidence interval; DTPa, diphtheria–tetanus–acellular pertussis vaccine; FHA, filamentous haemagglutinin; GMC, geometric mean concentration; GMR, geometric mean ratio; HepA, hepatitis A; HepB, hepatitis B; Hib, *Haemophilus influenzae* type b; Ig, immunoglobulin; IPV, inactivated polio vaccine; IQR, interquartile range; MenC, meningococcus C; MIS BAIR, The Melbourne Infant Study: BCG for Allergy and Infection Reduction; ORV, oral rotavirus vaccine; PCV13, 13-valent conjugate pneumococcal vaccine; Pn, pneumococcal serotype; PRN, pertactin; PT, pertussis toxin.

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1 | INTRODUCTION

Vaccination is one of the most cost-effective, life-saving medical interventions. However, there is substantial variation between individuals in the magnitude of the immune response to vaccination, which might have implications for both protective efficacy and duration of protection. At the age of 6 months, for example, antibody responses to pneumococcal and *Haemophilus influenzae* type b (Hib) vaccines may vary up to 40-fold.¹

A number of intrinsic host, perinatal and extrinsic factors are likely to contribute to the variation in vaccine responses.² One of the intrinsic factors that has been most investigated is biological sex. But studies to date have almost exclusively been in adults. Other intrinsic factors include polymorphisms in genes encoding major histocompatibility complexes or pattern recognition receptors, such as Toll-like or RIG-like receptors.² Perinatal factors that might influence vaccine responses include gestational age, birth-weight^{3,4} and feeding method (formula vs breastfed). Potential extrinsic factors include exposure to probiotics and antibiotics. While there is evidence for a beneficial effect of probiotics on vaccine responses,² to date, only one study has investigated the effect of antibiotics on vaccine responses in humans. This study reported that administration of antibiotics before vaccination did not alter serum immunoglobulin (Ig) A concentrations to oral rotavirus vaccine (ORV) in adults.⁵

Determining the influence of intrinsic and extrinsic factors on vaccine antibody responses has implications for optimising vaccine recommendations and individualising vaccine schedules.

In this study, we investigated the effect of biological sex, mode of delivery, feeding method and antibiotic exposure on antibody responses to routine vaccinations given in the first year of life.

2 | METHODS

2.1 | Study design and population

Participants were a subset of 471 healthy infants from The Melbourne Infant Study: Bacille Calmette-Guérin (BCG) for Allergy and Infection Reduction (MIS BAIR) (<http://misbair.org>). In this trial, infants were recruited at birth from 2013 to 2016 and randomised to receive neonatal BCG vaccination or no intervention to investigate whether BCG protects from childhood allergies. Inclusion criteria included the following: >32 weeks' gestation and birthweight >1500 g. Details about feeding and antibiotic exposure were prospectively collected by parent questionnaire.

From the subset of participants whose parent/guardian provided consent, blood samples were collected at study visits at 7 and/or 13 months of age (designed to be 4 weeks after the administration of routine scheduled vaccinations). Only participants who had a blood taken 28 ± 14 days after their 6-month and/or 12-month routine vaccinations were included in the analysis of immediate post-vaccination responses. For those in whom persistence of antibodies to their 6-month vaccines was

Key notes

- There are substantial differences between individuals in the immune response to vaccination.
- In this study, we found that there are differences between males and females in antibody responses to routine vaccinations in the first year of life.
- In contrast, delivery mode, feeding method and antibiotic exposure did not affect antibody responses.

measured, blood was taken 7 months $\pm$ 23 days after their 6-month vaccinations.

2.2 | Infant vaccination

All infants received routine vaccinations according to the Australian National Immunisation Program: at birth: intramuscular hepatitis B (HepB) vaccine (H-B-Vax II Paediatric[®]; bioCSL); at 6 weeks, 4 months and 6 months of age: intramuscular combined diphtheria-tetanus-acellular pertussis (DTPa)-HepB-inactivated polio (IPV)-Hib vaccine (Infanrix[®] Hexa; GlaxoSmithKline), intramuscular conjugate pneumococcal vaccine (PCV13) (Prevenar13[®]; Wyeth) and ORV (RotaTeq[®]; Merck); and at 12 months of age: subcutaneous measles-mumps-rubella (MMR) vaccine (Priorix[®]; GlaxoSmithKline) and intramuscular combined Hib and conjugated meningococcal C vaccine (Menitorix[®]; GlaxoSmithKline). Approximately half of the infants received intradermal BCG-Denmark (Statens Serum Institut, Copenhagen) shortly after birth as part of the MIS BAIR. Vaccine records were obtained from individual vaccination records and/or the Australian Immunisation Register.

2.3 | Blood collection and antibody assay

Following collection (S-monovette[®]; Sarstedt), plasma was stored at -80°C until analysis at the National Institute for Health and Environment, in Bilthoven, the Netherlands. IgG antibodies against 26 vaccine antigens (diphtheria, tetanus, pertussis [pertussis toxin [PT], filamentous haemagglutinin [FHA], pertactin [PRN]], polio (types 1, 2, 3), Hib, pneumococcus (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F), meningococcus type C (MenC), measles, mumps and rubella were measured using fluorescent bead-based multiplex immune assays (Luminex xMAP technology). In all assays, international or in-house reference controls and blanks were included on each plate. All analyses were performed with a BioPlex 200 in combination with Bio-Plex manager software (Bio-Rad Laboratories).

2.4 | Categorisation of participants

Infants were categorised by biological sex (male or female), delivery mode (vaginal vs caesarean section), feeding method and antibiotic

exposure using information collected from hospital records and parent questionnaires. Feeding method was categorised as follows: (a) infants who did or did not receive formula before leaving their birth hospital; (b) infants who were or were not still receiving breastmilk at 1 month of age; and (c) infants who were or were not still receiving breastmilk at 6 months of age. Antibiotic exposure was categorised as follows: (a) infants whose mother did or did not receive antibiotics during delivery and (b) infants who did or did not receive systemic antibiotics before their first routine vaccinations scheduled at the age of 6 weeks (assessed individually).

2.5 | Statistical analysis

In each group, the proportion of infants with an antibody concentration above the standard protective correlate value (seroprotection rate) was calculated for each vaccine.⁶⁻⁸ The Clopper-Pearson method was used to estimate the 95% confidence intervals (CIs) of the seroprotection rates. The rates were compared between groups using Fisher's exact test and the 95% confidence interval (CI) for differences in proportions estimated. For FHA and PRN, no standard protective correlate value exists. In each group, the geometric mean concentration (GMC) for each vaccine antibody was calculated. The geometric mean ratio (GMR) with 95% confidence interval (CI) was obtained as the anti-logged coefficient from a linear regression with log-concentrations as outcome and early-life factors (biological sex, delivery mode, feeding choice and antibiotic exposure) as covariates (univariate regression). Effect modification was assessed using multiple linear regression with the following pre-specified factors: maternal age, maternal diphtheria-tetanus-pertussis (dTpa, Boostrix®; GlaxoSmithKline) and trivalent influenza vaccination in pregnancy, birthweight, gestational age, infant BCG and MMR vaccination status, age at first DTPa-HepB-IPV-Hib/ PCV13 vaccination, age at sampling and time interval between 6-month or 12-month vaccination and blood sampling. The GMR for each vaccine antigen was adjusted for those factors that had an effect on vaccine antigen response. As the Hib-MenC vaccine includes a tetanus toxoid (as carrier protein) and a Hib component, infants who had received this vaccine before blood sampling at 13 months of age were excluded from the analysis of persistence of antibodies against these two vaccine antigens. A 5% significance level was used. All statistical analyses were done using R version 3.4.3.

2.6 | Ethics

Informed consent was obtained from participants' parents or guardians. The study was approved by the Royal Children's Hospital Human Research Ethics Committee (HREC, authorisation, 38124A).

3 | RESULTS

Of the 471 participants, seven were excluded because they were a twin of another participant, five because extended parental consent

to use the blood samples was not available, four because they were not vaccinated according to the routine schedule and one because vaccine records were not available (Figure 1). Of the remaining 454 participants, 365 had blood taken in the predefined time frame. At 7 months of age, 91 were included in the final analysis. At 13 months of age, 307 were included for measurement of persistence of antibodies against the primary course of vaccines ending at 6 months and 141 for antibody responses to the 12-month vaccines. The background characteristics of the 365 included participants are summarised in Table 1. The median gestational age was 39.3 weeks (interquartile range [IQR] 38.4-40.4) and the median birthweight 3.45 kg (IQR 3.13-3.76).

Of the 365 infants, 179 (49%) were male, 137 (37%) were born by caesarean section, and 100 (28%) had received formula milk before discharge from their birth hospital. At 1 month of age, 302 (83%) infants were still receiving breastmilk, and at 6 months of age, this number was 263 (72%). In total, 70 (19%) mothers received antibiotics during delivery: 36 (51%) because they were colonised with group B streptococcus, 27 (39%) because of prolonged rupture of membranes, 5 (7%) because of caesarean section delivery and for 2 (3%) the reason was not specified. The administered antibiotics were benzylpenicillin (*n* = 30), amoxycillin (*n* = 14), ampicillin (*n* = 11), cefazolin (*n* = 5), cefotaxime (*n* = 1), metronidazole (*n* = 1) and not specified (*n* = 11).

Antibiotics were administered to 30 (8%) infants before their first routine vaccination schedule at 6 weeks of life. These infants received only one course of antibiotics each. The majority (*n* = 22, 73%) received antibiotics within the first week of life (intravenous benzylpenicillin and gentamicin [*n* = 18], intravenous benzylpenicillin, gentamicin and vancomycin [*n* = 1], intravenous antibiotics not documented [*n* = 1], oral cephalixin [*n* = 1] and oral flucloxacillin [*n* = 1]). The duration of administration varied between 2 and 7 days (mean 3, median 3). Of the remaining eight infants, four were admitted to hospital for intravenous antibiotics (azithromycin [*n* = 1] and antibiotics not documented [*n* = 3]) between 2 and 5 weeks of age (mean 3.5, median 3.5). The duration of administration was less than 5 days (*n* = 2 infants) and between 5 and 10 days (*n* = 2 infants). The infant who received intravenous azithromycin was continued on oral azithromycin for 3 days. Additionally, two infants were continued on oral antibiotics (not documented), one for 5 days and one for more than 10 days. There were four infants who received oral antibiotics after the first week of life (age range 2-4, mean 3.8, median 4 weeks) (amoxicillin [*n* = 2], cephalixin [*n* = 2]). The duration of administration was 5-10 days for all.

3.1 | Effect of sex on vaccine responses

At 7 months of age, females had a lower seroprotection rate against Hib compared with male infants (66% [95% CI 51.6%-79.6%] vs 86% [95% CI 72.1%-94.7%]; *P* = .05). However, overall there were no statistically significant differences between males and females in seroprotection rates at 7 or 13 months of age (Table 2a and Figure 2).

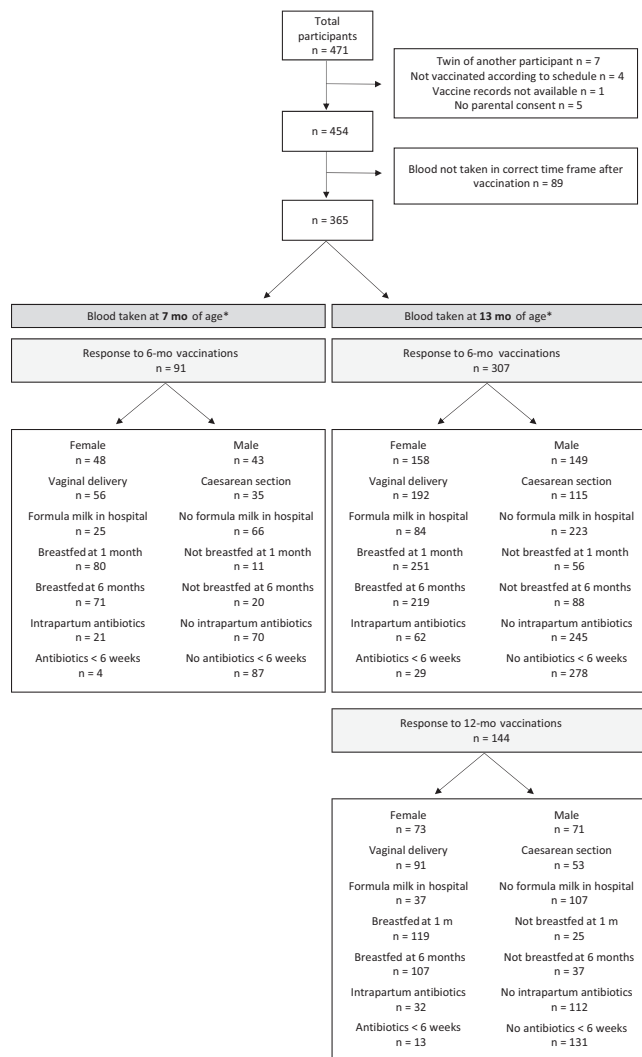


FIGURE 1 Selection of participants. *33 participants had their blood analysed at both 7 and 13 mo of age

After adjustment for pre-specified factors, the female:male GMR was below one (indicating lower average antibody concentrations) in females for tetanus and FHA (Table 2b and Figure 2) at 7 months of age and for PRN at 13 months of age. These findings were statistically significant. At 13 months of age, adjusted average antibody concentrations were statistically significantly higher in females for polio type 3, pneumococcus serotype 6A and measles (Table 2b and Figure 2).

3.2 | No effect of delivery mode on vaccine responses

At 7 months of age, seroprotection rates were lower in infants born vaginally than in those born by caesarean section for the majority of vaccine antigens. However, none of these differences reached statistical significance. At 13 months of age, there were no differences in seroprotection rates between infants born vaginally or by caesarean section (Table S1a and Figure S1). At 13 months of age, infants

born vaginally had a higher average antibody concentration against Hib (Table S1b and Figure S1).

3.3 | No effect of feeding method on vaccine responses

At 7 and 13 months of age, there were no statistically significant differences in seroprotection rates or adjusted average antibody concentrations between infants who received or did not receive formula milk in first few days of life or between infants who were still receiving breastmilk at 1 or 6 months of age and those who were not (Tables S2a-S4b and Figures S2-S4). The only exception was a statistically significant higher adjusted average antibody concentration (ie, a GMR greater than one) against pneumococcus serotype 7F at 7 months of age in infants who did not receive formula milk during the first few days of life compared to those who did (Table S2b and Figure S2), and higher antibody concentration for pneumococcus serotype 19F and MenC at 13 months of age in infants who were still receiving breast at 6 months of age (Tables S4b and Figure S4).

3.4 | Minimal effect of intrapartum or infant antibiotic exposure on vaccine responses

At 7 and 13 months of age, there were no differences in seroprotection rates between infants whose mothers did or did not receive antibiotics during delivery (Table S5a and Figure S5). Infants whose mother received intrapartum antibiotics had a significantly higher adjusted average antibody concentration against PRN at 7 and 13 months of age (Tables S5b and Figure S5). Antibiotic exposure before their first routine vaccination scheduled at 6 weeks of age did not influence seroprotection rates or average antibody concentrations (Table S6a,b and Figure S6).

4 | DISCUSSION

This study is the first to investigate the effect of multiple different early-life factors on antibody responses to routine vaccinations in infancy. We found that infant biological sex had an impact on responses but delivery mode, feeding method, intrapartum antibiotics and antibiotic exposure before the first routine immunisation did not.

Our finding that females had lower antibody responses against tetanus and higher responses against polio (type 3) is consistent with previous studies in adults.⁹⁻¹¹ Previous studies have also reported female adults to have higher antibody responses to dengue, HepA, HepB, rabies, smallpox and trivalent influenza vaccines, while male adults have been reported to have higher responses to diphtheria and conjugated meningococcus A vaccines.² The only studies which have previously been done in infants reported higher antibody responses in females to HepB, Hib and PCV13.² Aside from a higher average antibody concentration in females against one pneumococcal serotype (6A), we did not find sex differences in the response to pneumococcal vaccination. This contrasts with studies in adults, in

TABLE 1 Characteristics of study participants

	Total cohort	Samples at 7 mo of age for antibodies		Samples at 13 mo of age for persistence of antibodies		Samples at 13 mo of age for antibodies	
		to primary course of vaccines ending at 6 mo of age				to 12-mo vaccines	
	(n = 365) n (%) or median (IQR)	(n = 91) n (%) or median (IQR)	(n = 307) n (%) or IQR			(n = 144) n (%) or IQR	
Sex (male)	179 (49)	43 (47)	149 (49)			71 (49)	
Gestational age (wk)	39.3 (38.4-40.4)	39.5 (38.6-40.5)	39.3 (38.4-40.3)			39.2 (38.4-40.4)	
Birthweight (kg)	3.45 (3.13-3.76)	3.48 (3.10-3.78)	3.45 (3.13-3.76)			3.41 (3.13-3.67)	
Caesarean section	136 (37)	35 (38)	115 (37)			53 (37)	
Received formula milk in hospital in first week of life	100 (27)	25 (27)	84 (27)			37 (26)	
Still receiving breast at 1 mo of age	302 (83)	80 (88)	251 (82)			119 (83)	
Still receiving breast at 6 mo of age	263 (72)	71 (78)	219 (71)			107 (74)	
Antibiotics during admission for delivery							
Intrapartum antibiotics	70 (19)	21 (23)	62 (20)			32 (22)	
Antibiotics before first vaccination	30 (8)	4 (4)	29 (9)			13 (9)	
BCG-vaccinated	198 (54)	45 (49)	166 (54)			76 (53)	
Maternal dTpa vaccination in pregnancy	174 (48)	46 (51)	146 (48)			67 (47)	
Maternal influenza vaccination in pregnancy	208 (57)	55 (60)	171 (56)			84 (58)	
Age at routine vaccination (d)							
6-wk vaccines	45 (43-494) ^a	45 (43-48) ^a	45 (43-49)			45 (43-48)	
4-mo vaccines	125 (120-131)	123 (117-127)	125 (121-132)			124 (120-131)	
6-mo vaccines	190 (184-202)	187 (181-193)	191 (185-204)			190 (185-201)	
12-mo vaccines	376 (369-383)	-	-			376 (369-383)	
Interval between (d)							
6-mo vaccines and 7-mo blood sample	-	28 (21-37)	-			-	
6-mo vaccines and 13-mo blood sample	-	-	209 (190-224)			-	
12-mo vaccines and 13-mo blood sample	-	-	-			28 (22-36)	
Age at blood sampling (d)	-	218 (208-227)	400 (389-418)			406 (397-414)	

Abbreviations: BCG, Bacille Calmette-Guérin; dTpa, diphtheria-tetanus-acellular pertussis vaccine.

^aAge for one participant not available.

TABLE 2 (a) Seroprotection rates at 7 and 13 mo of age in female and male infants. (b) Geometric mean antibody concentrations (GMCs) and geometric mean antibody ratios (GMRs) at 7 and 13 mo of age in female and male infants

(a)	Antibodies to primary course of vaccines ending at 6 mo of age measured at 7 mo of age							Antibodies to primary course of vaccines ending at 6 mo of age measured at 13 mo of age						
	Vaccine antigen	Protective correlate	Female (n = 48)		Male (n = 43)		Two-sided P-value	Female (n = 158)		Male (n = 149)		Difference % (95% CI)	Two-sided P-value	
			% (n); (95% CI)	% (n); (95% CI)	% (n); (95% CI)	% (n); (95% CI)		% (n); (95% CI)	% (n); (95% CI)					
	Diphtheria	0.1 IU/mL ^a	97.9 (47); (88.9, 99.9)	95.3 (41); (84.1, 99.4)	2.6 (-6.9, 13.7)	.60	98.7 (156); (95.5, 99.8)	98.0 (146); (94.2, 99.6)	0.7 (-2.7, 4.6)	.68				
	Tetanus	0.1 IU/mL ^a	100 (48); (92.6, 100)	100 (43); (91.8, 100)	0 (-7.5, 8.3)	-	100 (85); (96.8, 100) ^b	100 (78); (95.4, 100) ^b	0 (-4.3, 4.7) ^b	-				
	PT	25 IU/mL	89.6 (43); (77.3, 96.5)	90.7 (39); (77.9, 97.4)	-1.1 (-14.4, 12.7)	1	28.5 (45); (21.6, 36.2)	31.5 (47); (24.2, 39.7)	-3.1 (-13.3, 7.2)	.62				
	Hib	0.15 µg/mL	66.7 (32); (51.6, 79.6)	86.0 (37); (72.1, 94.7)	-19.4 (-36.0, -1.7)	.05	80.0 (68) (69.9, 87.9) ^b	73.1 (57); (61.8, 82.5) ^b	6.9 (-6.1, 20.1) ^b	.36 ^b				
	Polio type 1	0.23 IU/mL	100 (48); (92.6, 100)	100 (43); (91.8, 100)	0 (-7.5, 8.3)	-	100 (158); (97.7, 100)	100 (149); (97.6, 100)	0 (-2.4, 2.5)	-				
	Polio type 2	0.29 IU/mL	100 (48); (92.6, 100)	100 (43); (91.8, 100)	0 (-7.5, 8.3)	1	100 (158); (97.7, 100)	99.3 (148); (96.3, 100)	0.7 (-1.7, 3.7)	.49				
	Polio type 3	0.12 IU/mL	100 (48); (92.6, 100)	100 (43); (91.8, 100)	0 (-7.5; 8.3)	-	100 (158); (97.7, 100)	100 (149); (97.6, 100)	0 (-2.4, 2.5)	-				
	Pn 1	0.35 µg/mL	95.8 (46); (85.7, 99.5)	97.7 (42); (87.7, 99.9)	-1.8 (-12.1, 8.4)	1	92.4 (146); (87.1, 96.0)	95.3 (142); (90.6, 98.1)	-2.9 (-8.7, 2.7)	.35				
	Pn 3	0.35 µg/mL	97.9 (47); (88.9, 99.9)	100 (43); (91.8, 100)	-2.1 (-11.0, 6.3)	1	70.3 (111); (62.5, 77.3)	69.1 (103); (61.0, 76.4)	1.1 (-9.1, 11.4)	.90				
	Pn 4	0.35 µg/mL	66.7 (32); (51.6, 79.6)	72.1 (31); (56.3, 84.7)	-5.4 (-24.0, 13.8)	.65	24.1 (38); (17.6, 31.5)	21.5 (32); (15.2, 28.9)	2.6 (-6.9, 12.0)	.68				
	Pn 5	0.35 µg/mL	95.8 (46); (86.7, 99.5)	100 (43); (85.6, 99.5)	-4.2 (-14.1, 4.3)	.50	89.2 (141); (83.3, 93.6)	84.6 (126); (77.7, 90.0)	4.7 (-2.9, 12.5)	.24				
	Pn 6A	0.35 µg/mL	97.9 (47); (88.9, 99.9)	97.7 (42); (87.7, 99.9)	0.2 (-8.9, 10.3)	1	88.0 (139); (81.9, 92.6)	85.2 (127); (78.5, 90.5)	2.7 (-5.0, 10.6)	.50				
	Pn 6B	0.35 µg/mL	83.3 (40); (69.8, 92.5)	95.3 (41); (84.2, 99.4)	-12.0 (-25.8, 1.1)	.10	68.4 (108); (60.5, 75.5)	59.7 (89); (51.4, 67.7)	8.6 (-2.1, 19.2)	.12				
	Pn 7F	0.35 µg/mL	100 (48); (92.6, 100)	100 (43); (91.8, 100)	0 (-7.5, 8.3)	-	99.4 (157); (96.5, 100)	99.3 (148); (96.3, 100)	0 (-2.9, 3.1)	1				
	Pn 9V	0.35 µg/mL	97.9 (47); (88.9, 99.9)	97.7 (42); (87.7, 99.9)	0.2 (-8.9, 10.2)	1	79.1 (125); (71.9, 85.2)	72.5 (108); (64.6, 79.5)	6.6 (-3.0, 16.2)	.18				
	Pn 14	0.35 µg/mL	91.7 (44); (80.0, 97.7)	90.7 (39); (77.8, 97.4)	1.0 (-11.8, 14.6)	1	84.8 (134); (78.2, 90.0)	87.9 (131); (81.2, 92.3)	-3.1 (-10.9, 4.7)	.51				
	Pn 18C	0.35 µg/mL	95.8 (46); (85.7, 99.5)	95.3 (41); (84.2, 99.4)	0.5 (-10.0, 11.9)	1	78.5 (124); (71.2, 84.6)	75.2 (112); (67.4, 81.9)	3.3 (-6.2, 12.8)	.50				
	Pn 19A	0.35 µg/mL	93.8 (45); (82.8, 98.7)	93.0 (40); (80.9, 98.5)	0.7 (-11, 13.3)	1	47.5 (75); (39.5, 55.6)	52.3 (78); (44.0, 60.6)	-4.9 (-15.9, 6.3)	.42				
	Pn 19F	0.35 µg/mL	100 (46); (92.6, 100)	100 (43); (91.8, 100)	0 (-7.5; 8.3)	-	98.7 (156); (95.5, 99.8)	100 (149); (97.6, 100)	-1.3 (-4.5, 1.3)	.50				
	Pn 23F	0.35 µg/mL	91.7 (44); (80.0, 97.7)	95 (41); (84.2, 99.4)	-3.7 (-15.7, 8.3)	.68	62.7 (99); (54.6, 70.2)	59.7 (89); (51.4, 67.7)	2.9 (-8.0, 13.8)	.64				
	Antibodies to 12-mo vaccines measured 13 mo of age													
	Vaccine antigen	Protective correlate	Female (n = 73)		Male (n = 71)		Difference % (95% CI)		Two-sided P-value					
			% (n); (95% CI)		% (n); (95% CI)									
	Measles	0.12 IU/mL	100 (73); (95.1, 100)		95.8 (68); (88.1, 99.1)		4.2 (-0.9, 11.7)		.12					
	Mumps	45 IU/mL	58.9 (43); (46.8, 70.3)		62.0 (44); (49.7, 73.2)		-3.1 (-18.8, 12.9)		.74					
	Rubella	10 IU/mL	91.8 (67); (83.0, 96.9)		81.7 (58); (70.7, 89.9)		10.1 (-1.1, 21.8)		.09					
	MenC	2 µg/mL	100 (73); (95.1, 100)		98.6 (70); (92.4, 100)		1.4 (-3.7, 7.6)		.49					
	Hib	0.15 µg/mL	98.6 (72); (92.6, 100)		98.6 (70); (92.5, 100)		0 (-6.1, 6.4)		1					
	Tetanus	0.01 IU/mL	100 (73); (95.1, 100)		100 (71); (94.3, 100)		0 (-5.0, 5.1)		-					

(Continues)

TABLE 2 (Continued)

Vaccine antigen	Antibodies to primary course of vaccines ending at 6 mo of age measured at 7 mo of age						Antibodies to primary course of vaccines ending at 6 mo of age measured at 13 mo of age					
	Female (n = 48)			Male (n = 43)			Female (n = 158)			Male (n = 149)		
	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value
Diphtheria ^c	0.38 (0.30, 0.48)	0.92 (0.67, 1.27)	.61	0.41 (0.33, 0.52)	0.77 (0.58, 1.03)	.08	0.08 (0.07, 0.09)	1.05 (0.83, 1.32)	.69	0.08 (0.06, 0.09)	1.05 (0.83, 1.32)	.29
Tetanus ^c	0.98 (0.82, 1.18)	0.77 (0.58, 1.03)	.08	1.27 (1.01, 1.60)	0.77 (0.58, 1.03)	.03	0.41 (0.32, 0.52) ^b	0.85 (0.60, 1.22) ^b	.38 ^b	0.48 (0.37, 0.61) ^b	0.78 (0.56, 1.10) ^b	.16
PT ^c	63.68 (51.21, 79.18)	0.82 (0.58, 1.13)	.23	77.99 (60.10, 101.22)	0.82 (0.58, 1.13)	.31	16.67 (14.26, 19.47)	0.92 (0.73, 1.15)	.45	18.19 (15.37, 21.54)	0.92 (0.73, 1.15)	.87
FHA ^c	52.41 (42.42, 64.76)	0.66 (0.47, 0.92)	.02	79.28 (60.59, 103.73)	0.66 (0.47, 0.92)	.04	16.96 (14.50, 19.83)	0.80 (0.65, 0.99)	.04	21.17 (18.28, 24.52)	0.80 (0.65, 0.99)	.11
PRN ^c	54.00 (42.26, 69.00)	0.79 (0.53, 1.17)	.24	68.66 (49.36, 95.50)	0.79 (0.53, 1.17)	.29	10.13 (8.39, 12.24)	0.71 (0.54, 0.93)	.02	14.24 (11.73, 17.28)	0.71 (0.54, 0.93)	.02
Hib ^d	0.40 (0.25, 0.64)	0.58 (0.30, 1.11)	.10	0.68 (0.44, 1.06)	0.58 (0.30, 1.11)	.21	1.5 (0.80, 2.51) ^b	1.82 (0.90, 3.70) ^b	.09 ^b	0.82 (0.51, 1.33) ^b	1.51 (0.88, 2.61) ^b	.14 ^b
Polio type 1 ^c	33.75 (24.34, 46.81)	0.95 (0.61, 1.47)	.81	35.59 (26.50, 47.80)	0.95 (0.61, 1.47)	.86	11.07 (9.28, 13.19)	0.98 (0.76, 1.26)	.86	11.32 (9.41, 13.61)	0.98 (0.76, 1.26)	.82
Polio type 2 ^c	65.36 (49.45, 86.40)	1.08 (0.69, 1.69)	.73	60.43 (42.14, 86.66)	1.08 (0.69, 1.69)	.57	22.73 (18.74, 27.57)	1.11 (0.83, 1.48)	.47	20.46 (16.54, 25.31)	1.11 (0.83, 1.48)	.25
Polio type 3 ^c	23.43 (17.03, 32.24)	1.21 (0.76, 1.92)	.42	19.41 (13.69, 27.52)	1.21 (0.76, 1.92)	.38	10.17 (8.29, 12.48)	1.32 (0.98, 1.77)	.06	7.71 (6.25, 9.53)	1.32 (0.98, 1.77)	.03
Pn 1 ^d	3.97 (2.86, 5.50)	0.82 (0.53, 1.25)	.34	4.87 (3.70, 6.40)	0.82 (0.53, 1.25)	.55	1.18 (1.03, 1.35)	1.03 (0.85, 1.25)	.78	1.15 (1.00, 1.33)	1.03 (0.85, 1.25)	.58
Pn 3 ^d	1.37 (1.15, 1.64)	0.90 (0.68, 1.20)	.48	1.52 (1.21, 1.90)	0.90 (0.68, 1.20)	.51	0.57 (0.50, 0.65)	1.14 (0.95, 1.37)	.15	0.50 (0.44, 0.57)	1.14 (0.95, 1.37)	.09
Pn 4 ^d	0.53 (0.41, 0.69)	1.06 (0.75, 1.51)	.73	0.50 (0.40, 0.63)	1.06 (0.75, 1.51)	.61	0.22 (0.20, 0.25)	1.05 (0.90, 1.22)	.52	0.21 (0.19, 0.23)	1.05 (0.90, 1.22)	.39
Pn 5 ^d	2.91 (2.17, 3.89)	0.84 (0.57, 1.23)	.36	3.46 (2.71, 4.43)	0.84 (0.57, 1.23)	.65	0.96 (0.83, 1.12)	1.09 (0.89, 1.34)	.41	0.88 (0.76, 1.02)	1.09 (0.89, 1.34)	.22
Pn 6A ^d	4.82 (3.66, 6.34)	1.00 (0.70, 1.44)	1.00	4.81 (3.81, 6.08)	1.00 (0.70, 1.44)	.97	1.08 (0.93, 1.25)	1.16 (0.93, 1.45)	.19	0.93 (0.78, 1.10)	1.16 (0.93, 1.45)	.04
Pn 6B ^d	1.64 (1.04, 2.59)	0.72 (0.38, 1.36)	.31	2.27 (1.46, 3.54)	0.72 (0.38, 1.36)	.29	0.56 (0.47, 0.68)	1.20 (0.93, 1.56)	.17	0.47 (0.39, 0.57)	1.20 (0.93, 1.56)	.08
Pn 7F ^d	5.72 (4.51, 7.27)	0.86 (0.63, 1.20)	.38	6.62 (5.31, 8.26)	0.86 (0.63, 1.20)	.29	1.93 (1.70, 2.18)	1.06 (0.89, 1.26)	.55	1.83 (1.61, 2.07)	1.06 (0.89, 1.26)	.29
Pn 9V ^d	2.40 (1.83, 3.16)	0.95 (0.67, 1.36)	.79	2.52 (2.01, 3.16)	0.95 (0.67, 1.36)	.94	0.63 (0.55, 0.72)	1.12 (0.92, 1.36)	.26	0.57 (0.49, 0.65)	1.12 (0.92, 1.36)	.12
Pn 14 ^d	2.54 (1.82, 3.56)	0.90 (0.54, 1.53)	.70	2.81 (1.86, 4.26)	0.90 (0.54, 1.53)	.65	1.09 (0.93, 1.29)	1.09 (0.87, 1.37)	.46	1.00 (0.85, 1.18)	1.09 (0.87, 1.37)	.28

(Continues)

TABLE 2 (Continued)

Vaccine antigen	Antibodies to primary course of vaccines ending at 6 mo of age measured at 7 mo of age						Antibodies to primary course of vaccines ending at 6 mo of age measured at 13 mo of age					
	Female (n = 48)			Male (n = 43)			Female (n = 158)			Male (n = 149)		
	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Adjusted ^e GMR (95% CI)	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Adjusted ^f GMR (95% CI)
Pn 18C ^d	2.68 (1.96, 3.65)	0.82 (0.55, 1.21)	.31	3.27 (2.59, 4.13)	0.82 (0.55, 1.21)	0.83 (0.56, 1.22)	0.69 (0.60, 0.78)	1.11 (0.92, 1.34)	.28	0.62 (0.54, 0.71)	1.16 (0.97, 1.40)	1.16 (0.97, 1.40)
Pn 19A ^d	1.77 (1.34, 2.34)	1.06 (0.71, 1.58)	.77	1.67 (1.25, 2.22)	1.06 (0.71, 1.58)	1.14 (0.76, 1.69)	0.48 (0.38, 0.60)	1.06 (0.79, 1.42)	.70	0.45 (0.37, 0.54)	1.10 (0.83, 1.47)	1.10 (0.83, 1.47)
Pn 19F ^d	10.20 (7.71, 13.50)	0.77 (0.53, 1.11)	.17	13.22 (10.40, 16.79)	0.77 (0.53, 1.11)	0.80 (0.56, 1.15)	3.21 (2.72, 3.79)	1.07 (0.84, 1.36)	.58	3.00 (2.52, 3.58)	1.11 (0.87, 1.40)	1.11 (0.87, 1.40)
Pn 23F ^d	2.27 (1.64, 3.15)	0.94 (0.58, 1.52)	.81	2.41 (1.69, 3.44)	0.94 (0.58, 1.52)	0.96 (0.60, 1.52)	0.63 (0.52, 0.77)	1.26 (0.96, 1.66)	.10	0.50 (0.41, 0.61)	1.27 (0.97, 1.68)	1.27 (0.97, 1.68)
Antibodies to 12-mo vaccines measured 13 mo of age												
Vaccine antigen	Female (n = 73)			Male (n = 71)			Female (n = 73)			Male (n = 71)		
	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Adjusted ^e GMR (95% CI)	GMC (95% CI)	Unadjusted GMR (95% CI)	Two-sided P-value	GMC (95% CI)	Unadjusted GMR (95% CI)	Adjusted ^e GMR (95% CI)
Measles ^c	3.66 (3.05-4.40)	1.47 (1.03, 2.10)	.03	2.49 (1.83-3.41)	1.47 (1.03, 2.10)	1.45 (1.01, 2.09)	3.66 (3.05-4.40)	1.47 (1.03, 2.10)	.03	2.49 (1.83-3.41)	1.45 (1.01, 2.09)	1.45 (1.01, 2.09)
Mumps ^c	58.93 (43.31, 80.20)	1.03 (0.67, 1.58)	.90	57.40 (42.07, 78.32)	1.03 (0.67, 1.58)	0.94 (0.65, 1.35)	58.93 (43.31, 80.20)	1.03 (0.67, 1.58)	.90	57.40 (42.07, 78.32)	0.94 (0.65, 1.35)	0.94 (0.65, 1.35)
Rubella ^c	60.78 (44.83, 82.40)	1.35 (0.85, 2.14)	.20	45.04 (31.74, 63.92)	1.35 (0.85, 2.14)	1.25 (0.86, 1.81)	60.78 (44.83, 82.40)	1.35 (0.85, 2.14)	.20	45.04 (31.74, 63.92)	1.25 (0.86, 1.81)	1.25 (0.86, 1.81)
MenC ^d	19.47 (16.47, 23.00)	1.23 (0.94, 1.60)	.14	15.89 (12.84, 19.66)	1.23 (0.94, 1.60)	1.27 (0.97, 1.65)	19.47 (16.47, 23.00)	1.23 (0.94, 1.60)	.14	15.89 (12.84, 19.66)	1.27 (0.97, 1.65)	1.27 (0.97, 1.65)
Hib ^d	18.21 (13.08, 25.35)	1.40 (0.84, 2.33)	.20	13.01 (8.74, 19.38)	1.40 (0.84, 2.33)	1.53 (0.93, 2.53)	18.21 (13.08, 25.35)	1.40 (0.84, 2.33)	.20	13.01 (8.74, 19.38)	1.53 (0.93, 2.53)	1.53 (0.93, 2.53)
Tetanus ^c	0.83 (0.64, 1.08)	0.70 (0.47, 1.04)	.07	1.19 (0.88, 1.60)	0.70 (0.47, 1.04)	0.73 (0.49, 1.08)	0.83 (0.64, 1.08)	0.70 (0.47, 1.04)	.07	1.19 (0.88, 1.60)	0.73 (0.49, 1.08)	0.73 (0.49, 1.08)

Bold denotes results with P -value $< .05$.Abbreviations: BCG, Bacille Calmette-Guérin; CI, confidence interval; FHA, filamentous haemagglutinin; GMC, geometric mean antibody concentration; GMR, geometric mean antibody ratio; Hib, *Haemophilus influenzae* type b; MenC, meningococcus C; MMR, measles-mumps-rubella; Pn, pneumococcus serotype; PRN, pertactin; PT, pertussis toxin.^a0.01 IU/mL at 13 mo of age.^bIncludes only participants who have not had Hib-MenC.^cIU/mL.^dμg/mL.^eAdjusted for maternal dTpa (diphtheria-tetanus-aceullar pertussis) and trivalent influenza vaccine during pregnancy, gestational age, BCG vaccination status, age at first DTPa-HepB-IPV-Hib/ PCV13 vaccination, age at sampling and time between vaccination and sampling using multiple linear regression.^fAdjusted for maternal dTpa (diphtheria-tetanus-aceullar pertussis) and trivalent influenza vaccine during pregnancy, gestational age, BCG and MMR vaccination status, age at first DTPa-HepB-IPV-Hib/ PCV13 vaccination, age at sampling and time between vaccination and sampling using multiple linear regression.

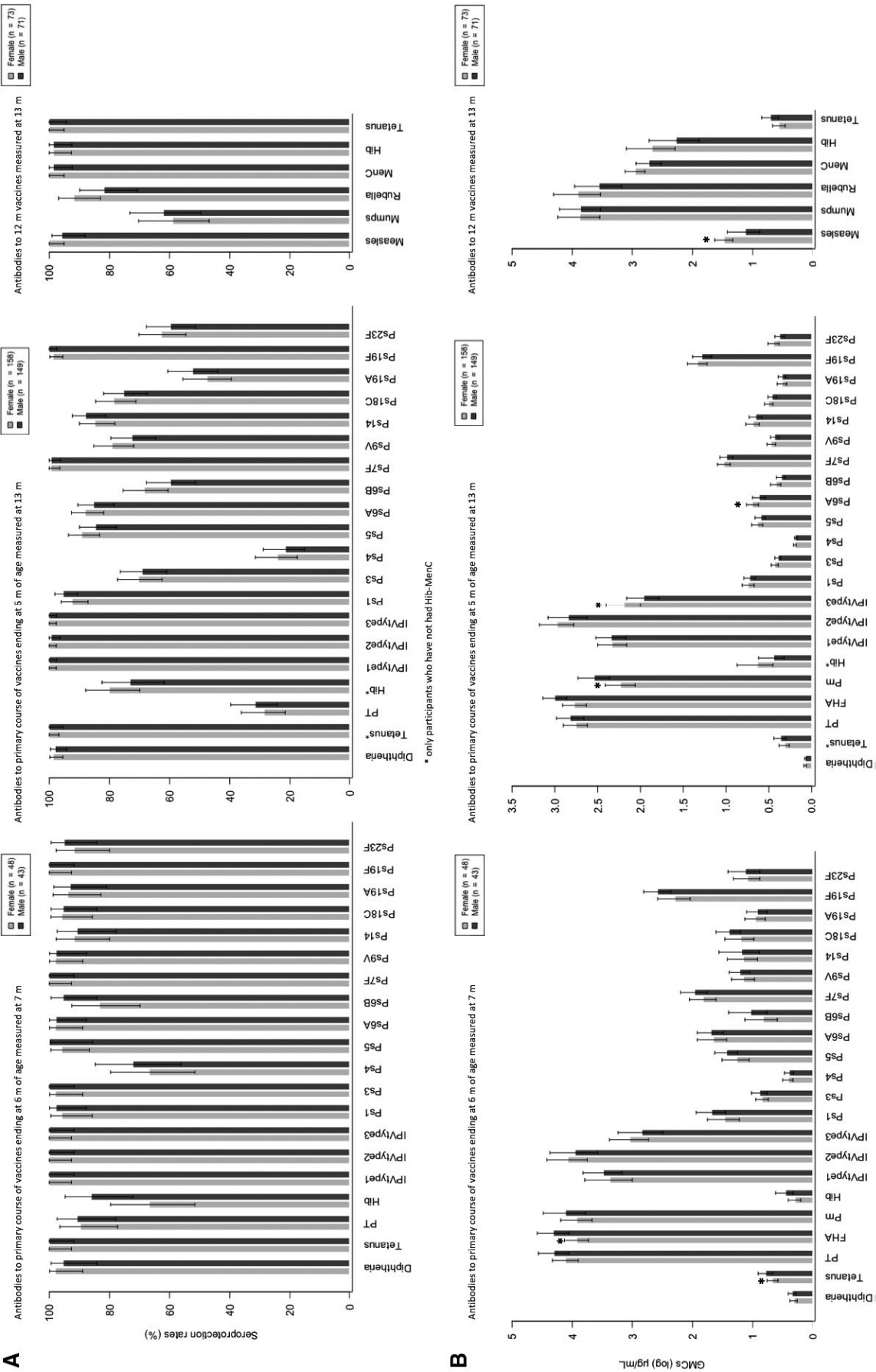


FIGURE 2 Seroprotection rates and geometric mean antibody concentrations (GMCs) in female and male infants

which males have generally been reported to have higher antibody responses to pneumococcal vaccines.²

In our study, we found that female infants had higher antibody levels against measles at 13 months of age than corresponding males. Previous studies investigating sex differences in responses to MMR vaccination report inconsistent findings. Some studies found higher antibody responses to measles, mumps and rubella vaccination in females, while others found transiently lower GMCs to rubella and lower seroconversion rates to measles vaccination in females.² Other studies did not find any sex differences in antibody responses to mumps and rubella, rubella vaccination. Notably, even though females are often reported to have higher antibody responses, faster waning of antibodies has been observed for antibodies against HepA and pneumococcus in females.²

One mechanism through which sex can affect vaccine responses is through hormones: oestradiol, progesterone and testosterone all influence the function of immune cells.¹² However, as sex differences in vaccine responses also occur before puberty and after menopause, other factors might also be involved. Many genes for proteins involved in the immune responses are encoded on the X chromosome, and mutations and polymorphisms of X-linked genes therefore have a greater impact in males.¹³ A further mechanism through which sex differences in vaccine responses might occur is through epigenetic programming, which is also influenced by sex hormones.¹²

The microbiota, especially the intestinal microbiota, plays a crucial role in the development and regulation of the immune system, and its composition affects how individuals respond to vaccinations.¹⁴ Sex hormones play a key role in bacterial-host interactions,¹⁵ and sex differences in the microbiota might therefore influence immune responses.^{16,17} As delivery mode and feeding method strongly affect the composition of the infant microbiota,¹⁸ these factors might be expected to influence vaccine responses. However, in our study, we did not observe any significant impact of delivery mode or feeding method on vaccine responses.

Maternal antibodies may inhibit infant antibody responses to some vaccines.¹⁹ Although the majority of maternal antibody transfer is through the placenta, breastmilk also contains antibodies that might interfere with immune responses. Small studies have reported that after routine vaccination, breastfed infants have higher serum IgG levels to diphtheria,²⁰ Hib^{21,22} and oral polio virus (OPV)^{20,23} vaccination, and higher salivary IgA levels to tetanus, diphtheria and OPV vaccination.²⁰ For ORV, reduced seroconversion rates were observed in breastfed infants compared with formula-fed infants^{24,25}; however, this was not confirmed in subsequent studies.²⁶ Infants with mixed feeding are sometimes classified as breastfed and sometimes as non-breastfed²⁶ and IgA levels, as well as the neutralising activity of IgA in breastmilk, may depend on geographic location.²⁷ These factors could explain some of the inconsistency in results from different studies.

The strengths of our study include the ability to adjust for a large number of possible confounding factors, the standardisation in the vaccines given and the wide range of measured antibody responses. The limitations of the study are the sample size and risk of type-2 error. Furthermore, we did not analyse antibody responses to the HepB vaccine, as this antigen was not included in our multiplex assay and we had insufficient sample volume for alternative assays. We

also did not evaluate the effect of antibiotics that might have been transferred through breastmilk on infant vaccine responses.

As most vaccines induce antibody concentrations above the protective threshold in the majority of infants, small differences in antibody concentrations between groups of individuals are unlikely to be clinically significant. However, differences in antibody concentrations might be relevant for the duration of protection. Nonetheless, when developing new vaccines and designing vaccine schedules, recognising factors which might influence vaccine antibody responses offers ways to optimise vaccine immunogenicity and efficacy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Ritz N, Mui M, Balloch A, Curtis N. Non-specific effect of Bacille Calmette-Guerin vaccine on the immune response to routine immunisations. *Vaccine*. 2013;31:3098-3103.
2. Zimmermann P, Curtis N. Factors that influence the immune response to vaccination. *Clin Microbiol Rev*. 2019;32(2):pii: e00084-18.
3. Han K, Shao X, Zheng H, et al. Revaccination of non- and low- responders after a standard three dose hepatitis B vaccine schedule. *Hum Vaccin Immunother*. 2012;8:1845-1849.
4. Omenaca F, Garcia-Sicilia J, Garcia-Corbeira P, Boceta R, Torres V. Antipolyribosyl ribitol phosphate response of premature infants to primary and booster vaccination with a combined diphtheria-tetanus-acellular pertussis-hepatitis B-inactivated polio virus/*Haemophilus influenzae* type b vaccine. *Pediatrics*. 2007;119:e179-e185.
5. Harris VC, Haak BW, Handley SA, et al. Effect of antibiotic-mediated microbiome modulation on rotavirus vaccine immunogenicity: a human, randomized-control proof-of-concept trial. *Cell Host Microbe*. 2018;24(2):197-207.e4.
6. Plotkin SA. Correlates of protection induced by vaccination. *Clin Vaccine Immunol*. 2010;17:1055-1065.
7. Freidl GS, Tostmann A, Curvers M, et al. Immunity against measles, mumps, rubella, varicella, diphtheria, tetanus, polio, hepatitis A and hepatitis B among adult asylum seekers in the Netherlands, 2016. *Vaccine*. 2018;36:1664-1672.
8. Schepp RM, Berbers GA, Ferreira JA, Reimerink JH, van der Klis FR. A novel multiplex poliovirus binding inhibition assay applicable for large serosurveillance and vaccine studies, without the use of live poliovirus. *J Virol Methods*. 2017;241:15-23.
9. Stark K, Schonfeld C, Barg J, Molz B, Vornwald A, Bienzle U. Seroprevalence and determinants of diphtheria, tetanus and

- poliomyelitis antibodies among adults in Berlin, Germany. *Vaccine*. 1999;17:844-850.
10. Hainz U, Jenewein B, Asch E, Pfeiffer KP, Berger P, Grubeck-Loebenstien B. Insufficient protection for healthy elderly adults by tetanus and TBE vaccines. *Vaccine*. 2005;23:3232-3235.
 11. Gergen PJ, McQuillan GM, Kiely M, Ezzati-Rice TM, Sutter RW, Virella G. A population-based serologic survey of immunity to tetanus in the United States. *N Engl J Med*. 1995;332:761-766.
 12. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol*. 2016;16:626-638.
 13. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol*. 2008;8:737-744.
 14. Zimmermann P, Curtis N. The influence of the intestinal microbiome on vaccine responses. *Vaccine*. 2018;36:4433-4439.
 15. Garcia-Gomez E, Gonzalez-Pedrajo B, Camacho-Arroyo I. Role of sex steroid hormones in bacterial-host interactions. *Biomed Res Int*. 2013;2013:928290.
 16. Fransen F, van Beek AA, Borghuis T, et al. The impact of gut microbiota on gender-specific differences in immunity. *Front Immunol*. 2017;8:754.
 17. Vemuri R, Sylvia KE, Klein SL, et al. The microgenderome revealed: sex differences in bidirectional interactions between the microbiota, hormones, immunity and disease susceptibility. *Semin Immunopathol*. 2019;41(2):265-275.
 18. Zimmermann P, Curtis N. Factors influencing the intestinal microbiome during the first year of life. *Pediatr Infect Dis J*. 2018;37(12):e315-e335.
 19. Voysey M, Kelly DF, Fanshawe TR, et al. The influence of maternally derived antibody and infant age at vaccination on infant vaccine responses: an individual participant meta-analysis. *JAMA Pediatr*. 2017;171:637-646.
 20. Pickering LK, Granoff DM, Erickson JR, et al. Modulation of the immune system by human milk and infant formula containing nucleotides. *Pediatrics*. 1998;101:242-249.
 21. Pabst HF, Spady DW. Effect of breast-feeding on antibody response to conjugate vaccine. *Lancet*. 1990;336:269-270.
 22. Silfverdal SA, Bodin L, Ulanova M, Hahn-Zoric M, Hanson LA, Olcen P. Long term enhancement of the IgG2 antibody response to *Haemophilus influenzae* type b by breast-feeding. *Pediatr Infect Dis J*. 2002;21:816-821.
 23. Hahn-Zoric M, Fulconis F, Minoli I, et al. Antibody responses to parenteral and oral vaccines are impaired by conventional and low protein formulas as compared to breast-feeding. *Acta Paediatr Scand*. 1990;79:1137-1142.
 24. Pichichero ME. Effect of breast-feeding on oral rhesus rotavirus vaccine seroconversion: a metaanalysis. *J Infect Dis*. 1990;162:753-755.
 25. Chilengi R, Simuyandi M, Beach L, et al. Association of maternal immunity with rotavirus vaccine immunogenicity in zambian infants. *PLoS One*. 2016;11:e0150100.
 26. Glass RI, Ing DJ, Stoll BJ, Ing RT. Immune response to rotavirus vaccines among breast-fed and nonbreast-fed children. *Adv Exp Med Biol*. 1991;310:249-254.
 27. Moon SS, Wang Y, Shane AL, et al. Inhibitory effect of breast milk on infectivity of live oral rotavirus vaccines. *Pediatr Infect Dis J*. 2010;29:919-923.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Appendix 3

The published reviews in Chapter 2 were based on a systematic review of the literature as outlined by the search strategy below. The aim was to review the existing literature relating to non-specific stimulation of the innate immune system by BCG vaccine of *M. bovis* as a stimulant. Due to the complexity of the review output, the results were split into two papers presented in Chapter 2.

P: Human, Animal, In vitro

I: BCG vaccination or BCG vaccine or *M. bovis* administered as an immune stimulant, modulator or vaccination.

C: Any experimental control present where same outcomes measured in the absence of BCG vaccine or *M. bovis*.

O: Mortality or Morbidity in *in vitro* models. Evidence of “non-specific” innate cellular activity i.e. against heterologous pathogens (non-TB) or cancer cells.

1 PubMed

1)Search (((((BCG) OR Bacillus Calmette Guerin) OR Bacille Calmette Guerin) OR Bacille Calmette-Guerin) OR Bacillus Calmette-Guerin) OR Mycobacterium Bovis

+ Search (immune) AND #1 3757

+Search (non-specific) AND #1 330

+Search (immune modulation) AND #1 100

+ Search (immune response) AND #1 2007

+ Search (immune response) AND (non-specific) 51

+ Search (immune) +(non-specific) 95

+ Search (immune stimulation) + (non-specific) 2

+ Search (immune modulation) + (non-specific) (2)

2. Medline

1. exp Leukocytes, Mononuclear/

2. exp Cytokines/

3. Mycobacterium bovis/im

4. exp Macrophages/im

5. (non specific or heterologous or non targeted).tw.
6. BCG Vaccine/ge, im [Genetics, Immunology]
7. (6 or 3) and (1 or 2 or 4) and 5
8. (*BCG Vaccine/ge, im or *Mycobacterium bovis/im) and (exp *Leukocytes, Mononuclear/ or exp *Cytokines/ or exp *Macrophages/im)
9. (BCG Vaccine/ or Mycobacterium bovis/) and 5
10. *vaccines/
11. 10 and 5
12. 7 or 8 or 9 or 11

Medline (MeSH)

1. exp Leukocytes, Mononuclear/
2. exp Cytokines/
3. Mycobacterium bovis/im
4. exp Macrophages/im
5. (non specific or heterologous or non targeted or trained).tw.
6. BCG Vaccine/ge, im [Genetics, Immunology]
7. (6 or 3) and (1 or 2 or 4) and 5
8. (*BCG Vaccine/ge, im or *Mycobacterium bovis/im) and (exp *Leukocytes, Mononuclear/ or exp *Cytokines/ or exp *Macrophages/im)
9. (BCG Vaccine/ or Mycobacterium bovis/) and 5
10. *vaccines/
11. 10 and 5
12. 7 or 8 or 9 or 11

3. EmBase

+ trained = 70 new

1. exp lymphocyte/
2. exp cytokine/ec [Endogenous Compound]
3. epigenetics/
4. exp innate immunity/
5. histone/ec [Endogenous Compound]
6. toll like receptor/ec [Endogenous Compound]
7. bcg vaccination/

8. *Mycobacterium bovis*/
9. (nonspecific or heterologous or non targeted).tw.
10. (7 or 8) and (1 or 2 or 3 or 4 or 5 or 6) and 9
11. (*bcg vaccination/ or **Mycobacterium bovis*/) and (exp *lymphocyte/ or exp *cytokine/ec or *epigenetics/ or exp *innate immunity/ or *histone/ec or *toll like receptor/ec)
12. (7 or 8) and 9
13. *vaccine/
14. 9 and 13
15. 10 or 11 or 12 or 14

4. Web of Science

Topic = (BCG vaccine) (269)

Refined by: Web of Science Categories=(IMMUNOLOGY OR INFECTIOUS DISEASES OR MEDICINE RESEARCH EXPERIMENTAL) AND Document Types=(EDITORIAL MATERIAL OR LETTER OR CORRECTION OR BOOK CHAPTER OR PROCEEDINGS PAPER OR MEETING ABSTRACT OR NEWS ITEM)

Databases=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED, IC Timespan=All Years

#1 MeSH descriptor: [immunity, innate] explode all trees

#2 Mesh descriptor:[BCG Vaccine] explode all tress

#3 Mesh descriptor:[Lymphocytes]explode all tress

#4 Mesh descriptor:[*Mycobacterium bovis*] explode all tress

#5 #2 or #4 and (#1 or #3)

#6 "lymphocytes" or "immune response" or "cytokine" or "epigenetics" or "gamma interferon" or "histone" or "interleukin" or "toll like receptor" or "tumour necrosis factor" or "nitrous oxide dependent"

#7 "BCG vaccine or "*Mycobacterium Bovis*" or "Bacille Calmette Guerin" or *Bacillus Calmette Guerin*"

#8 "non specific" or "heterologous"

#9 (#2 or #4 or #7) and (#1 or #3 or #6)

MIS BAIR whole blood assay optimization experiment 1

Conditions to be tested:

Round versus flat bottom 96-well plates

Blood dilutions: 1:2, 1:5, 1:10

Heat killed *M. tuberculosis* (MTBk) concentrations: 3.2×10^6 CFU/mL
($\sim 6.4 \times 10^5$ /200 μ L), 3.2×10^5 CFU/mL ($\sim 6.4 \times 10^4$ /200 μ L)

4 hours versus 18 hours

Luminex assay supernatant volume: 25/30/40/50 μ L

Methods:

1. Donated blood at room temperature pre-stimulation
2. Set up stimulation in 4 x 96-well plates as follows:
 - a. Plate A: Round / 4 hours
 - b. Plate B: Round / 18 hours
 - c. Plate C: Flat / 4 hours
 - d. Plate D: Flat / 18 hours
3. Dilutions:
 - 250 μ L blood/well, dilution in Roswell Park Memorial Institute Media (RPMI), 5mL needed for each dilution (2.5mL+1mL+0.5mL => $\sim$ 5mL total blood);
 - Stock MTBk is 10^8 CFU/mL $\sim$ 10^5 CFU/ μ L:
 - a. 6.4 μ L in 3.2×10^6 /mL wells
 - b. 6.4 μ L in 3.2×10^5 /mL wells of 1:10 diluted MTBk (50 μ L stock in 450 μ L RPMI)
4. After incubation, centrifuge plates at 500g for 5 minutes.
5. Transfer supernatant in corresponding pre-labelled Eppendorf tubes.
6. Transfer to -80°C freezer.
7. Analyse samples by using Millipore r TNF/IL-6 kits.
8. Supernatants tested at 25/30/40/50 μ L to determine aliquot size.

[illegible]

MIS BAIR whole blood assay optimization experiment 2

Conditions to be tested:

Round vs flat bottom 96-well plates

Blood dilutions: 1:2, 1:3, 1:4

Heat-killed *M. tuberculosis* (MTBk) concentrations: 3.2×10^6 CFU/mL ($\sim 6.4 \times 10^5$ /200 μ L), 3.2×10^7 CFU/mL ($\sim 6.4 \times 10^6$ /200 μ L),

4 hours vs 18 hours

Donor 1 v Donor 2

Methods:

1. Blood at room temperature before stimulating it.
2. Set up stimulation in 4 x 96-well plates as follows:

		Blood dilution 1:2			Blood dilution 1:3			Blood dilution 1:4				
	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
	PBS			PBS			PBS			PBS		PBS
MTBk 3.2×10^7/mL	PBS	1:2 64 μ L	1:2 64 μ L	PBS	1:3 64 μ L	1:3 64 μ L	PBS	1:4 64 μ L	1:4 64 μ L	PBS		PBS
	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
MTBk 3.2×10^6/mL	PBS	1:2 6.4 μ L	1:2 6.4 μ L	PBS	1:3 6.4 μ L	1:3 6.4 μ L	PBS	1:4 6.4 μ L	1:4 6.4 μ L	PBS		PBS
	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
	PBS	RPMI	RPMI		RPMI	RPMI		RPMI	RPMI			PBS
	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

PBS=Phosphate buffered solution, RPMI=Roswell Park Memorial Institute Medium

- a. Plate A: Round / 4 hours / Donor 1
- b. Plate B: Round / 18 hours/ Donor 1
- c. Plate C: Flat / 4 hours / Donor 1
- d. Plate D: Flat / 18 hours / Donor 1

- [illegible]

MIS BAIR whole blood assay optimization experiment 3

Conditions to be tested

Round 96 well trays

Blood dilutions: 1:2 and 1:5

Heat killed organisms at 1×10^6 CFU/mL

4 hours vs 18 hours

Heat-Killed organisms

1. RPMI
2. Heat-killed *M. tuberculosis* (MTBk)
3. *E. coli* 12087149
4. *E. coli* 12033126
5. *S. agalactiae* 12107998
6. *S. agalactiae* 11204704
7. *S. pneumoniae* 12095322
8. *S. pneumoniae* 12091368
9. *S. aureus* 13023035
10. *S. aureus* 11191447
11. *L. monocytogenes* 8178712
12. *C. albicans* 11145487
13. *C. albicans* 10005321
14. *S. pyogenes* PMP 1015
15. *S. pyogenes* PMP 1003

To make a final concentration of 1×10^6 CFU/mL in final volume of 200 μ l

Organism	Stock CFU/mL	Dilution	CFU/mL post dilution	Blood (μ L)
2	1×10^8	1:2	5×10^7	4: 196
3	4.4×10^{10}	1:10 → 1:10 → 1:10	4.4×10^7	4.5: 195.5
4	4.8×10^{10}	1:10 → 1:10 → 1:10	4.8×10^7	4.2: 195.8
5	7.6×10^9	1:10 → 1:10	7.6×10^7	2.6: 197.4
6	1×10^{10}	1:10 → 1:10 → 1:2	5×10^7	4: 196

7	3.7×10^9	1:10 → 1:10	3.7×10^7	5.4: 194.6
8	1×10^9	1:10 → 1:2	5×10^7	4: 196
9	8.8×10^{10}	1:10 → 1:10 → 1:10	8.8×10^7	2.2: 197.8
10	4.5×10^{10}	1:10 → 1:10 → 1:10	4.5×10^7	4.4: 195.6
11	2.4×10^{10}	1:10 → 1:10 → 1:5	4.8×10^7	4.2: 195.8
12	7×10^9	1:10 → 1:10	7×10^7	2.8: 197.2
13	7×10^9	1:10 → 1:10	7×10^7	2.8: 197.2
14	5.4×10^9	1:10 → 1:10	5.4×10^7	3.7: 196.3
15	6.02×10^9	1:10 → 1:10	6.02×10^7	3.3: 196.7

Method

1. Blood at room temperature (RT °C) before stimulation
2. Set up stimulation in two 96 well plates as follows (numbers correspond to heat-killed organism list)

1/2 blood dilution	1	1	1	2	2	2	3	3	3	4	4	4
	5	5	5	6	6	6	7	7	7	8	8	8
	9	9	9	10	10	10	11	11	11	12	12	12
	13	13	13	14	14	14	15	15	15	16	16	16
1/5 blood dilution	1	1	1	2	2	2	3	3	3	4	4	4
	5	5	5	6	6	6	7	7	7	8	8	8
	9	9	9	10	10	10	11	11	11	12	12	12
	13	13	13	14	14	14	15	15	15	PBS	PBS	PBS

- Plate A: 4 hours
 - Plate B: 18 hours
3. Dilutions: 200µL blood/well 20 mL needed for each dilution (14 LI whole blood total)
 - a. 1:2: 10 mL blood: 10 mL RPMI
 - b. 1:5: 4 mL blood: 16 mL RPMI
 4. Heat killed organisms: make dilutions as directed in the table above and add the appropriate amount to each well.

5. Incubate plate A for 4 hours, and plate B for 18 hours. Tape plates with parafilm before incubation.
6. After incubation, centrifuge plates at 500g for 5 minutes.
7. Transfer supernatant to pre-labeled Eppendorf tubes, pool triplicates in 1 tube.
8. Transfer tubes to -80 °C freezer.

MIS BAIR whole blood assay optimization experiment 4

Conditions to be tested

Round 96 well trays x 2

Blood dilutions: 1:2

Donor 1 versus Donor 2.

Heat killed organisms at multiple CFU/mL (Table 1).

18 hours incubation

Method

1. Blood at RT °C before stimulation
2. Set up stimulation in a 96-well plate as follows as outlined below
200µl blood/well (78 wells) =15.6ml total. 8mL whole blood: 8mL RPMI= 16mL 1:2 dilution. To achieve 1:2 dilution: 100µL blood +20 µl bug + 80 µl RPMI = 10mL blood + 8mL RPMI.
3. Thaw heat-killed organisms at room temperature for 2 hours. Dilutions as per table and add 20µl to each well. Bacteria vortexed for 30 seconds at each dilution. If 10^6 required 10^7 added as the bug : blood ratio is a further 1:10 dilution.
4. Blood added to plates using multichannel pipette and bacteria:blood re-suspended x 4 at this point.
5. Tape plates with parafilm and Incubate at 37°C, 5%CO₂ for 18 hours.
6. Centrifuge plates 500xg for 5minutes
7. Transfer supernatant to pre-labeled Eppendorf tubes, pool duplicates in one tube.
8. Transfer tubes to -80°C freezer

Heat killed organisms

	Stock concentration	Amount required	Dilution factor	Dilution 1	Dilution factor	Dilution 2	Dilution factor	Dilution 3	Dilution factor	Dilution 4	CFU/ml post dilution	Final volume bugs (μl)	Final volume blood (μl)
<i>E. coli</i> A (12087149)	4.40E+10	10 ⁵	20 10μl bacteria +190μl	2.20E+09	20	1.10E+08	10	1.10E+07	10	1.10E+06	10 ⁶	20	180
		10 ⁶									10 ⁷	20	180
<i>E. coli</i> B (12033126)	4.80E+10	10 ⁵	20	2.40E+09	20	1.20E+08	10	1.20E+07	10	1.20E+06	10 ⁶	20	180
		10 ⁶									10 ⁷	20	180
<i>S. agalactiae</i> A (12107998)	7.60E+09	10 ⁶	30 10μl bacteria +290μl	2.53E+08	20	1.27E+07					10 ⁷	20	180
		10 ⁷			2	1.27E+08					10 ⁸	20	180
<i>S. agalactiae</i> B (11204704)	1.00E+10	10 ⁶	10	1.00E+09	10	1.00E+08	10	1.00E+07			10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
<i>S. pneumoniae</i> A (12095233)	3.70E+09	10 ⁶	20	1.85E+08	10	1.85E+07					10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
<i>S. pneumoniae</i> B (12091368)	1.00E+09	10 ⁶	10	1.00E+08	10	1.00E+07					10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
<i>S. aureus</i> A (13023035)	8.80E+10	10 ⁶	40 10μl bacteria +390μl	2.20E+09	20	1.10E+08	10	1.10E+07			10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
		10 ⁸			2	1.10E+09					10 ⁹	20	180

<i>S. aureus</i> B (11191224)	4.50E+10	10 ⁶	40	1.13E+09	10	1.13E+08	10	1.13E+07			10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
		10 ⁸									10 ⁹	20	180
<i>L. monocytogenes</i> (8178712)	2.40E+10	10 ⁷	20	1.20E+09	10	1.20E+08					10 ⁸	20	180
		10 ⁸									10 ⁹	20	180
<i>C. albicans</i> A (11145487)	7.00E+09	10 ⁶	35	2.00E+08	20	1.00E+07					10 ⁷	20	180
<i>C. albicans</i> B (10005321)	7.00E+09	10 ⁶	35	2.00E+08	20	1.00E+07					10 ⁷	20	180
<i>S. pyogenes</i> A (PMP1015)	5.40E+09	10 ⁶	5	1.08E+09	10	1.08E+08	10	1.08E+07			10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
		10 ⁸									10 ⁹	20	180
<i>S. pyogenes</i> B (PMP1003)	6.00E+09	10 ⁶	6	1.00E+09	10	1.00E+08	10	1.00E+07			10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
		10 ⁸									10 ⁹	20	180
<i>H. influenzae</i> B (12261579)	6.65E+09	10 ⁶	30	2.22E+08	20	1.11E+07					10 ⁷	20	180
		10 ⁷			2	1.11E+08					10 ⁸	20	180
<i>H. influenzae</i> non-B (10327055)	1.58E+10	10 ⁶	10	1.58E+09	10	1.58E+08	10	1.58E+07			10 ⁷	20	180
		10 ⁷									10 ⁸	20	180
Influenza 1 A(H3N2)	1.00E+07	10 ⁴	10	1.00E+06	10	1.00E+05					10 ⁵	20	180
		10 ⁵									10 ⁶	20	180
Influenza 2 A(H1N1)	1.00E+07	10 ⁴	10	1.00E+06	10	1.00E+05					10 ⁵	20	180
		10 ⁵									10 ⁶	20	180

Table 1: Heat killed organisms; stock concentrations, dilution factors and final concentrations. NB Decision on diluent amount to be added to reach desired concentration based on how much final volume required to add to all wells in experiment.

Final Plate Design

	1	2	3	4	5	6 (RNA)	7	8	9	10	11	12
A	PBS	RPMI	RPMI	GBS B 10 ⁷	GBS B 10 ⁷	RPMI	SA B 10 ⁶	SA B 10 ⁶	GAS A 10 ⁷	GAS A 10 ⁷	HIB 10 ⁶	HIB 10 ⁶
B	PBS	EC A 10 ⁵	EC A 10 ⁵	SP A 10 ⁶	SP A 10 ⁶	RPMI	SA B 10 ⁷	SA B 10 ⁷	GAS A 10 ⁸	GAS A 10 ⁸	HIB 10 ⁷	HIB 10 ⁷
C	PBS	EC A 10 ⁶	EC A 10 ⁶	SP A 10 ⁷	SP A 10 ⁷	RPMI	SA B 10 ⁸	SA B 10 ⁸	GAS B 10 ⁶	GAS B 10 ⁶	HINB 10 ⁶	HINB 10 ⁶
D	PBS	EC B 10 ⁵	EC B 10 ⁵	SP B 10 ⁶	SP B 10 ⁶	EC A 10 ⁶	LM 10 ⁷	LM 10 ⁷	GAS B 10 ⁷	GAS B 10 ⁷	HINB 10 ⁷	HINB 10 ⁷
E	PBS	EC B 10 ⁶	EC B 10 ⁶	SP B 10 ⁷	SP B 10 ⁷	EC A 10 ⁶	LM 10 ⁸	LM 10 ⁸	GAS B 10 ⁸	GAS B 10 ⁸	Flu 1 10 ⁴	Flu 1 10 ⁴
F	PBS	GBS A 10 ⁶	GBS A 10 ⁶	SA A 10 ⁶	SA A 10 ⁶	EC A 10 ⁶	CA 10 ⁶	CA 10 ⁶	GAS C 10 ⁶	GAS C 10 ⁶	Flu 1 10 ⁵	Flu 1 10 ⁵
G	PBS	GBS A 10 ⁷	GBS A 10 ⁷	SA A 10 ⁷	SA A 10 ⁷	PBS	CA B 10 ⁶	CA B 10 ⁶	Flu 2 10 ⁴	Flu 2 10 ⁴	PBS	PBS
H	PBS	GBS B 10 ⁶	GBS B 10 ⁶	SA A 10 ⁸	SA A 10 ⁸	PBS	GAS A 10 ⁶	GAS A 10 ⁶	Flu 2 10 ⁵	Flu 2 10 ⁵	PBS	PBS

PBS=Phosphate Buffered Solution, RPMI=Roswell Park Memorial Institute Medium-1640, GBS=Group B streptococcus (*S. agalactiae*), EC= *E. coli*, SP= *S. pneumoniae*, SA=*S. aureus*, LM=*L. monocytogenes*, CA=*C. albicans*, GAS=Group A streptococcus (*S. pyogenes*), HIB= *H. influenzae* type-B, HINB= *H. influenzae* non-type B

MIS BAIR whole blood assay optimization experiment 5

Conditions to be tested

Round 96 well trays x 2

Blood dilutions: 1:2.

Three participants:

P1 (BCG vaccinated): 5mL blood

P2 (BCG vaccinated): 1.5mL blood

P3 (BCG naïve): 2mL blood

Heat killed organisms at multiple CFU/mL as per table.

18 hours incubation.

Method

1. Blood at RT °C before stimulation
2. Set up stimulation in a 96-well plate as outlined below 200 µL blood/well (max 60 wells) = 4.8mL total.
3. To achieve 1:2: 100 µL blood + 20 µL bug + 80 µL RPMI = 5 mL blood + 4mLRPMI (5:4). (or 2.5mL blood + 2mL RPMI) i.e. volume available / 5 x 4
4. Heat killed organisms: Thawed at room temperature for 2 hours. Dilutions as per table and add 20µL to each well
5. Bacteria vortexed for 30 seconds at each dilution. If 10⁶ required 10⁷ added as the bug: blood ratio is a further 1:10 dilution.
6. Blood added to plates using multichannel pipette and bacteria / blood re-suspended x 4 at this point.
7. Tape plates with parafilm and Incubate at 37°C, 5%CO₂ for 18 hours.
8. Centrifuge plates 500g for 5minutes
9. Transfer supernatant to pre-labeled Eppendorf tubes, pool duplicates in one tube.
10. Transfer tubes to -80°C freez

Preparation of stock aliquots of various antigen concentrations (10x50μL aliquots)

	Stock	Amount required	Dilution factor	Dilution 1	Dilution factor	Dilution 2	Dilution factor	Dilution 3	Dilution factor	Dilution 4	Final Volume	CFU/ml post dilution	Final volume bugs (µl)	Final volume blood (µl)	Final CFU/ml
E. coli A (12087149)	4.40E+10	10 ⁶	20 10µl bacteria +190µl	2.20E+09	20 10µl bacteria + 190µl RMPI	1.10E+08	50µl bacteria +450µl	1.10E+07			500µl	10 ⁷	20	180	10 ⁸
S. agalactiae A (12107998)	7.60E+09	10 ⁶	30 10µl bacteria +290µl	2.53E+08	20 25µl bacteria +475µl 2	1.27E+07					500µl	10 ⁷	20	180	10 ⁸
		10 ⁷			250µl bacteria +250µl	1.27E+08					500µl	10 ⁸	20	180	10 ⁷
S. pneumoniae A (12095233)	3.70E+09	10 ⁶	20 20µl bacteria +480µl RPMI	1.85E+08	10 50µl bacteria +450µl	1.85E+07					500µl	10 ⁷	20	180	10 ⁸
		10 ⁷									500µl	10 ⁸	20	180	10 ⁷
		10 ⁶			20 20µl bacteria +480µl RPMI	1.10E+08		1.10E+07			500µl	10 ⁷	20	180	10 ⁸
S. aureus A (13023035)	8.80E+10	10 ⁷	40 10µl bacteria +390µl	2.20E+09			10 50µl bacteria +450µl				500µl	10 ⁸	20	180	10 ⁷
		10 ⁸			2 250µl bacteria +250µl RPMI	1.10E+09					500µl	10 ⁹	20	180	10 ⁸
L. monocytogenes (8178712)	2.40E+10	10 ⁷	20 20µl bacteria +480µl RPMI	1.20E+09	10 50µl bacteria +450µl	1.20E+08					500µl	10 ⁸	20	180	10 ⁷

C. albicans A (11145487)	7.00E+09	10 ⁶	35 10µl bacteria +340µl	2.00E+08	20 20µl bacteria +480µl RPMI	1.00E+07			500µl	10 ⁷	20	180	10 ⁸
S. pyogenes A (PMP1015)	5.40E+09 Only thawed 50µl	10 ⁷ 10 ⁸	5 50µl bacteria +200µl RPMI	1.08E+09	10 50µl bacteria +450µl	1.08E+08			500µl	10 ⁸	20	180	10 ⁷
		10 ⁸							200µl	10 ⁹	20	180	10 ⁸
S. pyogenes B (PMP1003)	6.00E+09	10 ⁶ 10 ⁷ 10 ⁸	6 100µl bacteria +500µl RPMI	1.00E+09	10 50µl bacteria +450µl	1.00E+08			500µl	10 ⁸	20	180	10 ⁷
		10 ⁸							600µl	10 ⁹	20	180	10 ⁸
H. influenzae (12261579)	6.65E+09	10 ⁶	30 20µl bacteria +580µl	2.22E+08	20 20µl bacteria +480µl RPMI	1.11E+07			500µl	10 ⁷	20	180	10 ⁸
H. influenza non-B (10327055)	1.58E+10	10 ⁶	10 20µl bacteria +180µl	1.58E+09	10 50µl bacteria +450µl	1.58E+08	10 50µl bacteria +450µl	1.58E+07	500µl	10 ⁷	20	180	10 ⁸
PPD	1mg/ml	20µl/ml									4µl	200µl	20µl/ml
LPS	500mg/ml	10ng/ml									4ul	200ul	10ng/ml

Heat killed organisms; stock concentrations, dilution factors and final concentrations. Decision on diluent amount to be added to reach desired concentration based on how much final volume required to add to all wells in experiment.

	1	2	3	4	5	6 (RNA)	7	8	9	10	11	12
A	LPS	PPD	RPMI	EC A 10 ⁶	HIB 10 ⁶	CA A 10 ⁶	GBS A 10 ⁶	SA A 10 ⁷	GAS A 10 ⁷	SP A 10 ⁶	HINB 10 ⁶	LM 10 ⁷
B	SA A 10 ⁶	GBS A 10 ⁶	SP A 10 ⁷	SA A 10 ⁸	Flu 1 10 ⁵	Flu 1 10 ⁶	Flu 2 10 ⁵	Flu 2 10 ⁶	GAS A 10 ⁸	GAS B 10 ⁷	GAS B 10 ⁸	RPMI
C	LPS	PPD	RPMI	EC A 10 ⁶	HIB 10 ⁶	CA A 10 ⁶	GBS A 10 ⁶	SA A 10 ⁷	GAS A 10 ⁷	SP A 10 ⁶	HINB 10 ⁶	LM 10 ⁷
D	SA A 10 ⁶	GBS A 10 ⁷	SP A 10 ⁷	SA A 10 ⁸	Flu 1 10 ⁵	Flu 1 10 ⁶	Flu 2 10 ⁵	Flu 2 10 ⁶	GAS A 10 ⁸	GAS B 10 ⁷	GAS B 10 ⁸	RPMI
E	LPS	PPD	RPMI	EC A 10 ⁶	HIB 10 ⁶	CA A 10 ⁶	GBS A 10 ⁶	SA A 10 ⁷	GAS A 10 ⁷	SP A 10 ⁶	HINB 10 ⁶	LM 10 ⁷
F	SA A 10 ⁶	GBS A 10 ⁷	SP A 10 ⁷	SA A 10 ⁸	Flu 1 10 ⁵	Flu 1 10 ⁶	Flu 2 10 ⁵	Flu 2 10 ⁶	GAS A 10 ⁸	GAS B 10 ⁷	GAS B 10 ⁸	RPMI
G	LPS	PPD	RPMI	EC A 10 ⁶	HIB 10 ⁶	CA A 10 ⁶	GBS A 10 ⁶	SA A 10 ⁷	GAS A 10 ⁷	SP A 10 ⁶	HINB 10 ⁶	LM 10 ⁷
H	SA A 10 ⁶	GBS A 10 ⁷	SP A 10 ⁷	SA A 10 ⁸	Flu 1 10 ⁵	Flu 1 10 ⁶	Flu 2 10 ⁵	Flu 2 10 ⁶	GAS A 10 ⁸	GAS B 10 ⁷	GAS B 10 ⁸	RPMI

LPS=Lipopolysaccharide, PPD=Purified protein derivative, RPMI=Roswell Park Memorial Institute Medium-1640, EC= *E. coli*, HIB= *H. influenzae* type-B, CA=*C. albicans*, GBS=Group B streptococcus (*S. agalactiae*), SA=*S. aureus*, SP= *S. pneumoniae*, HINB= *H. influenzae* non-type B, LM=*L. monocytogenes*, , GAS=Group A streptococcus (*S. pyogenes*),



SOP TITLE:	BabyBAIRS blood strip and priority SOP		
SOP Number:		Version / Status:	8.0
Author Name	Bridget Freyne	Review Date	09/05/2016

Document History

Revision	Modified by	Date	Description of Change
8	Bridget	07/05/16	Priority list updated

1. TITLE

Infant blood: Strip stimulation procedure and blood volume priority testing.

2. PURPOSE/AIMS OF PROCEDURE

The purpose of this SOP is to document the procedures for setting up the infant blood-stimulation procedure using strips, and outlining the blood volume priority list for testing.

3. ASSOCIATED POLICY/ PROCEDURES

- I. Waste Management Policy (MCRI1123)
- II. Waste Procedures (MCRI1120)
- III. Incident Investigation & Reporting Procedure (MCRI1103)
- IV. First Aid Procedure (MCRI1111)
- V. Infant blood: Preparation of cell free plasma for Vitamin D testing (MISBAIR####)

4. SAFETY/RISK ASSESSMENT

It is essential that precaution be taken while working with and collecting biological specimens. Appropriate personal protective equipment (PPE) should be worn at all times to ensure safety of staff. Please follow MCRI guidelines (MCRI1103 & MCRI1111) if any incidents occur such as torn gloves, eye splash, and scalpel blade injury.

5. PERSONAL PROTECTIVE EQUIPMENT AND MATERIALS REQUIRED

PPE

On-site provision

- Safety glasses
- Gown
- Gloves

MATERIALS

- Infant blood in Sodium-Heparin monovette
- Strips and plate frame
- Sterile 96 well plate covers (Corning CLS3099-50EA)
- Scalpel blade
- 37 °C incubator (5 % CO₂:air)
- RPMI + Glutamax1 (Gibco #72400-047)
- 20 mM EDTA (Sigma solution 0.5 M, used for VITA)
- FACS lysing solution (BD 349202)
- 15 ml Falcon tubes
- Pipettes
- Filter tips (Axygen)
- Permanent markers
- 1.5 ml microfuge tubes
- Falcon® 5mL Round Bottom Polystyrene Test Tube, with Snap Cap, Sterile (Corning/BD)
- Falcon #352058 2ml RNase free eppendorf tubes
- TriZol Reagent



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6. Check the time point of the sample.

Pre-BCG (Label=Pre)	7 days post BCG (Label=7d)	3 mo post BCG (Label=3M)
2 X 4mL in Na Heparin: • Strips • Transcriptomics • Vitamin D • 1,25-OH Vitamin D • Epimers	2x4 mL in Na Heparin: • Strips • Transcriptomics • Vitamin D • 1,25-OH • Epimer	2X4mL in Na Heparin: • Strips • Transcriptomics • Vitamin D • 1,25-OH • Epimer

7. PROCEDURE

- Upon arrival of the infant blood, note down the following information into Redcap:
 - Study number
 - Time and date of blood collection
 - Time and date of blood arrival
 - Volume of blood collected (weight of tube with blood-weight of empty tube with sticker/1.06)
 - Lab staff who will be processing sample
 - Lab staff who will be storing sample

The infant blood must be processed at 4 hours post collection. Assay and strips should be prepared at 3.5 hours.

- Blood volume priority list

#	Priority: highest to lowest	Incubation	Dilution	Blood Volume Required (µl)	RPMI Volume Required (µl)	Total Blood Volume Required (µl)
1	Vitamin D+RNA (Trizol)	na	na	500	0	500
2	Strip 1 Well A	Short term	1/2	106.25	85	606.25
3	Strip X Well A		1/2	106.25	85	712.5
4	Strip 1 (Wells B-H)		1/2	743.75	595	1456.25
5	PBMC	na	n/a	1mL		2456.25
6	Strip 2 (C-D)	Short term	1/2	212.5	170	2668.75
7	Strip X (B)			106.25	85	2775
8	Strip 2 (E-H)			425	340	3200
9	1,25, OH VitD + Transcriptomics	na	na	500	-	3700
10	VitD Epimer + Transcriptomics	na	na	500	-	4200
11	Extra aliquot for PBMCs (remaining)	na	na	remaining	-	Remaining



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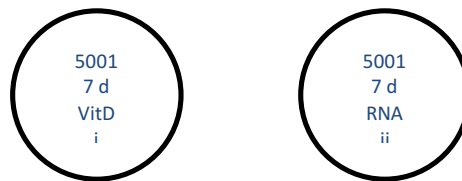
* Determine, based on total blood volume available, which strips are required. Plates of strips will be pre-cut and the underside of well 'A' colour-coded. Stored in the minus 30 freezer, Tissue Culture Lab EL5.1.21, bottom drawer.

5. Prepare strips

- Place required strips into a plate frame and allow contents to thaw in 37 °C incubator for 10 minutes. Record, in your laboratory notebook, the plate and strip number written on the aluminum plate sealer.
- Pre-warm RPMI in 15 ml Falcon tube in 37 °C incubator for 10 minutes. The volume required of RPMI will be based on how many strips will be used (Table 1)
- Spin strips in plate frame and centrifuge at 250 × g for 5 minutes. Use a spare frame with blank strips as a balance

6. If determined sufficient blood for whole assay prepare Vitamin D / Transcriptomics aliquots. Spray hood, gloves, pipette etc with RNA away spray. Use RNase-free 1.5mL eppendorfs.

- Aliquot 500ul into pre-labeled eppendorf (label as RNA as pellet will be left in this tube). Spin at 5000g for 7 min. (1,25 and Epimer same process)



- Spray down fume hood, pipette and gloves with RNA away spray. After removal of plasma from 3 Vitamin D samples add 500ul of TRIzol to each pellet and invert several times immediately to mix well. Store at -80

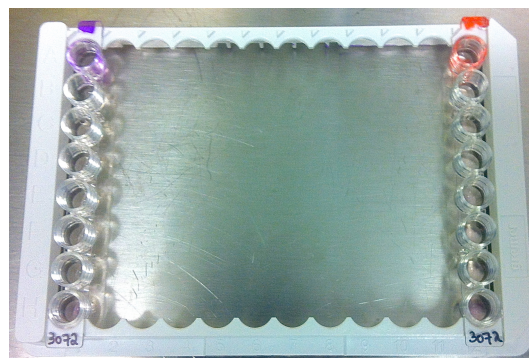
7. Process strip 1A & 2A

Remove aluminum plate sealer in biohazard class II hood.

- Label the top of the strip with the correct colour (Table 2) and write the study number on the bottom of the strip (Fig 1)
- Invert blood tube 4-5 times to mix blood before diluting in RPMI. Add correct amount of blood to pre-warmed RPMI according to the Table 1. Mix by gentle pipetting up and down 5 × using a filter tip on a P1000 pipette:
 - For example, if Strip 1A is being used add 850 µl of blood to 680 µl of pre-warmed RPMI and mix by gentle pipetting, up and down 5 × using a filter tip on a P1000 pipette. If both Strip 1A and Strip 2A are being used add 1700 µl of blood to 1360 µl of pre-warmed RPMI and mix by gentle pipetting, up and down 5 × using a filter tip on a P1000 pipette.

Strip 1A	Purple
Strip 2A	Orange
Strip 1B	Blue
Strip 2B	Red
Strip 3	Green
Strip 4	Black

Colour-code





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For Strip 1A & 2A:

- Add 180 µl of diluted blood to each well and mix by gently pipetting, up and down 5x using a filter tip on a P200 pipette
- Fill up wells always from A to H
- Record which wells have been used for incubation (redcap)
- Place empty strips on either side of experiment strips and fill with PBS 200 µl
- Cover the plate with the lid and seal underside with plate holder and damp towel and incubate for **20 hrs +/- 2 hrs** in 37 °C incubator (5% CO₂:air)

	Strip 1A	Strip 2A	Strip X									
A	Nil 1	GBS A	BCG									
B	BCG	S aur B	MTBk									
C	E coli	C alb A										
D	R848	ppp-ly										
E	H. inf B	MTbk										
F	LPS	S pne										
G	PEPG	L mono										
H	PAM3	Nil 2										

- After **20 hour +/- 2 hours** incubation, remove plates from incubator and centrifuge at 600 × g for 5 minutes
- Collect 100 µl of supernatant from each well and aliquot 60 µl of supernatant into 2 separate pre-labelled 1.5 ml microcentrifuge tubes, store in minus 80 freezer
- Each tube needs to be labeled on the top with study ID number, blood sample type (Pre, 7D,

WASTE DISPOSAL CONSIDERATIONS

All clinical waste including clinical specimens, laboratory cultures and equipment must be disposed of into double-bagged yellow infectious waste bags, clearly marked "Infectious Waste" and marked with the biohazard symbol as per MCRI waste management policy and procedures (MCRI1123, MCRI1120).



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Author Name	Bridget Freyne	Review Date	09/05/2016

BCGNSE2013

Does BCG vaccination strengthen the immune system against childhood infections?

Version 8.0, 2nd August 2016

CONFIDENTIAL

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STATEMENT OF COMPLIANCE

This document is a protocol for a clinical research study. The study will be conducted in compliance with all stipulations of this protocol, the conditions of ethics committee approval, the NHMRC National Statement on Ethical Conduct in Human Research (2007) and the Note for Guidance on Good Clinical Practice (CPMP/ICH-135/95).

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PROTOCOL SYNOPSIS

Title	Investigating the immunological basis for the non-specific effects of BCG vaccination
Hypothesis	BCG vaccination modifies the immune response to subsequent (<i>in vitro</i>) challenge with viral and bacterial pathogens unrelated to TB in healthy children and young adults.
Aims	1. To compare the (<i>in vitro</i>) immune response to infectious pathogens prevalent in childhood before and after BCG vaccination in children. 2. To compare this response in children with that measured in young adults. To compare the response in adults immunised with BCG with adults immunised with influenza vaccine as controls. 3. To determine the optimal experimental conditions to assess the (<i>in vitro</i>) immune response in this setting.
Study Design and Methodology	Prospective case-control study of the <i>in vitro</i> immune response to common infectious pathogens of childhood in 100 BCG-vaccinated children and young adults. Confirmation of eligibility / Recruitment and Consent Participants A) Children referred to RCH infectious diseases / immunisation clinics for BCG vaccination. Children attending clinics at RCH for BCG vaccination will be identified and asked to participate in the study. A brief information sheet (Appendix C) will be sent to parents prior to the clinic. At a scheduled clinical phone call the study team paediatrician who works at the BCG clinic will also ask them if they have any queries regarding the study. Formal consent will be sought at the clinic appointment. If they agree to participation, they (or the infant's legally acceptable representative) will sign and date an informed consent form prior to any study assessments / procedures being conducted. B) Medical students and employees requiring BCG vaccination (usually prior to medical elective in TB endemic areas) or who have volunteered for the study. Medical students from The University of Melbourne and employees of the Royal Children's Hospital and the Murdoch Children's Research Institute who are travelling to TB endemic areas on medical elective will be asked to participate. Medical students who are prepared to receive BCG without a specific

	indication may also be included. They will be asked to sign an informed consent form prior BCG immunisation at RCH. Medical students or employees who are having routine influenza vaccine will be used as control participants. Once informed consent is obtained, a brief questionnaire will be done. A convenient appointment for the next relevant time point will be established. <i>Time point 1: Pre-Vaccination Blood Sample (OPTIONAL)</i> <i>(1 week (+/-4 days) prior to vaccination)</i> A blood sample (maximum volume 5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood collection 1 week prior to administration of the BCG vaccine. A convenient appointment for <i>time point 3</i> will be established. <i>Time point 2: Pre-Vaccination Blood Sample (CORE)</i> <i>(less than 24hr prior to vaccination – usually immediately after consent obtained)</i> A blood sample (maximum volume 5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood collection 1 week prior to administration of the BCG vaccine. A convenient appointment for <i>time point 4</i> will be established. <i>Time point 3: Post vaccination blood sample (CORE)</i> <i>7+/- 4 days post vaccination</i> A blood sample (maximum volume 1-5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood at RCH. A convenient appointment for <i>time point 5</i> may be established. A member of the study team will meet study participants at this visit to receive the results of the Vitamin D quantification and arrange any follow up required (details outlined below section 7). <i>Time point 4: Post vaccination blood sample (OPTIONAL)</i> <i>14+/- 4 days post vaccination</i> A blood sample (maximum volume 1-5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood at RCH. A convenient appointment for <i>time point 6</i> may be established. A member of the study team will meet study participants at this visit to receive the results of the Vitamin D quantification and arrange any follow up required (details outlined below section 7).

	Time point 5: post vaccination blood sample (OPTIONAL)3 months +/- 4 weeks post vaccination A blood sample (maximum volume 1-5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood at RCH. A brief questionnaire pertaining to the healing and development of the BCG scar, travel or inter-current illnesses or vaccinations will be done at the time of attendance or by telephone / email. All blood samples collected will be processed at the MCRI Infectious Diseases & Immunity laboratories and incubated with different concentrations of infective antigens (killed <i>S. aureus</i>, <i>E. coli</i>, <i>S. pneumoniae</i>, RSV, Rotavirus, group B streptococcus, <i>C. albicans</i>). Immunological analyses, including cytokine analysis of supernatants and flow cytometry, and other measures of the immune response will be used to quantify and compare responses before and at the time points after BCG vaccination.
Planned Sample Size	The sample size of is feasible and in line with adult studies which successfully demonstrated an enhanced immunological response to non-specific (ie non-TB related) infective antigens post BCG vaccination. The proposed sample size is 100 participants, 60 children and 40 adults.
Statistical Methods	Whole blood assays and measurement of immunological responses will be done at the first two the time points in all patients. Patients who have not yet travelled to TB endemic areas will have a blood sample checked at 3 months. This will enable us to establish the kinetics of the immune response to non-specific infectious antigens in healthy individuals and whether these responses vary with time and between age groups. Non-parametric tests will be used to compare median responses at each time point, between each stimulant and between children and adults (Kruskal-Wallis for multiple groups; Mann Whitney U test for two-group comparisons).

GLOSSARY OF ABBREVIATIONS

ABBREVIATION	TERM
AE	Adverse Event
BCG	Bacille Calmette-Guérin
CRF	Case Report Form

GCP	Good Clinical Practice
LAR	Legally Acceptable Representative
MCRI	Murdoch Children's Research Institute
RCH	Royal Children's Hospital (Melbourne)
SAE	Serious Adverse Event

1. INVESTIGATORS AND FACILITIES

1.1 Study Location/s

Murdoch Childrens Research Institute
50 Flemington Road
Parkville VIC 3052

The Royal Children's Hospital, Melbourne
50 Flemington Road
Parkville VIC 3052

1.2 Study Management

The Principal Investigator will coordinate the study. Informed consent discussions, eligibility assessment and collection of demographic information will be delegated to doctors and nurses working at the immunisation clinic. Participants will attend the phlebotomy department at RCH for blood sampling. Handling and processing of blood samples will be delegated to the Postdoctoral Scientist on the research team.

1.2.1 Principal Investigator

Professor Nigel Curtis, Professor of Paediatric Infectious Diseases, Department of Paediatrics, The University of Melbourne; Head of Infectious Diseases Unit, The Royal Children's Hospital Melbourne; Leader, Infectious Diseases & Microbiology Group, Murdoch Childrens Research Institute

1.2.2 Associate Investigators

Dr. Bridget Freyne, PhD student, Department of Paediatrics, The University of Melbourne, Infectious Diseases & Microbiology Group, Murdoch Children's Research Institute

Dr Christel Zufferey. Postdoctoral Fellow, Infectious Diseases & Microbiology Group, Murdoch Children's Research Institute

1.2.3 Statistician

Prof Susan Donath,

Clinical and Epidemiological Biostatistics Unit, University of Melbourne and Murdoch Childrens Research Institute.

1.2.4 Internal Trial Committees

Not applicable

1.2.5 Independent Safety and Data Monitoring Committee

Not applicable

1.3 Sponsor

Murdoch Children's Research Institute

1.4 Funding and resources

This study is funded through Infection and Immunity theme funding from the Murdoch Children's Research Institute.

2. INTRODUCTION AND BACKGROUND

2.1 Background Information

There is a rapidly expanding body of evidence showing that BCG exerts beneficial "non-specific effects" distinct from protection against TB. It is postulated that BCG vaccine modulates the immune system and this explains the observed reduction in all cause mortality in BCG-vaccinated infants (1-3). In animal studies, BCG vaccine prevents illness from disparate infections including mycobacteria (*M. leprae*) (4), bacteria (eg *Shigella flexneri*) (5), viruses (eg *Vaccinia*) (6), and protozoa (eg *Plasmodium chaduari*) (7).

Observational studies show a protective effect of BCG vaccination on infant mortality. Furthermore, the increased deaths in the non-BCG-vaccinated group were attributable to diseases other than TB, including pneumonia, diarrhoea and septicaemia. This suggests that BCG vaccination in childhood confers protection against multiple infections (1).

A recent study published in PNAS by our collaborator, Dr Mihai Netea (Nijmegen Institute for Infection, inflammation and Immunity, The Netherlands), shows that BCG vaccination of adult volunteers lead to enhanced release of cytokines including IFN- γ , TNF- α and IL-1 β in response to unrelated bacterial (*S. aureus*) and fungal (*Candida albicans*) pathogens (8). This study provides evidence that BCG vaccination modulates the human immune response to antigens unrelated to TB. Moreover, the authors described the persistence of this effect up to a year post vaccination. This provides a potential *in vitro* correlate for the non-specific effects of BCG vaccine. Such an effect has not yet been tested in children.

2.2 Research Question

Does BCG vaccination modify the immune response to subsequent (*in vitro*) challenge with viral and bacterial pathogens unrelated to TB in healthy children and adults.

2.3 Rationale for Current Study

Observational evidence points to a reduction in childhood mortality from sepsis and pneumonia in children vaccinated with BCG. *In vitro* work in adults has demonstrated that BCG vaccine induces enhanced response to multiple infective antigens. This study would provide the first data in children. Furthermore we will use

pathogens that have not been previously studied in this context, in particular viruses and bacteria that typically affect infants and children.

3. STUDY OBJECTIVES

3.1 Primary Objective

- To determine if there is an enhanced immune response to *in vitro* stimulation with infectious pathogens in blood samples taken from children and adults before and after BCG vaccination.

3.2 Secondary Objectives

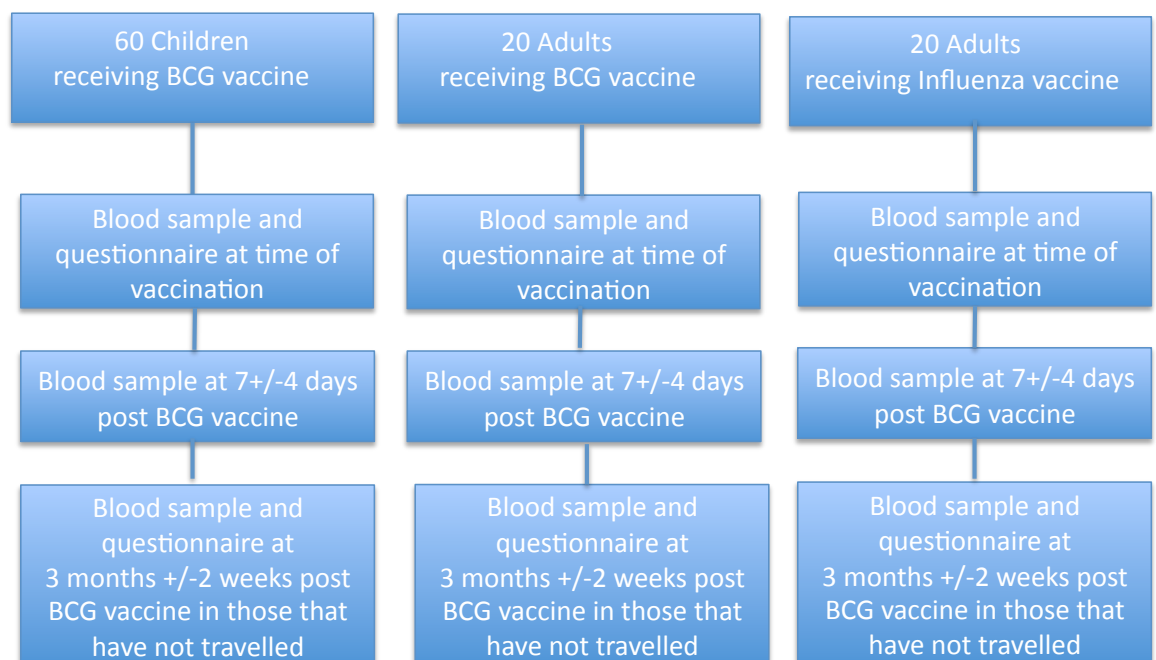
- To compare the response in children with that in young adults.
- To determine the optimal experimental conditions required in assessing the immune response to these pathogens.

4. STUDY DESIGN

4.1 Type of Study

This is a prospective case-control study assessing the immune response to non-specific antigens before and after BCG vaccination *in vitro*. We will compare the response in adults and children. The influenza-vaccinated control group is essential to show that any observed effect is specific to BCG vaccination.

4.2 Study design diagram



4.3 Number of subjects

The proposed study number of is in line with previous research carried out in adults. This number will allow confirmation and reproducibility of any observed effect. This number is feasible in terms of the proposed recruitment strategy and cost of the laboratory analysis. The numbers required in each group (60, 20, 20) reflect the number of paired adequate samples required for analysis. Therefore, recruitment will continue until at least 1ml of blood, suitable for analysis, is obtained prior to BCG vaccination and at visit 2.

4.4 Expected duration of study

There are several weekly BCG immunisation clinics upcoming at RCH with up to 12 patients / clinic. Weekly referrals to the ID clinic at RCH for pre-travel vaccination assessment are on average 3 / clinic. Assuming 50% recruitment rate it would take 6 weeks to recruit the children and 3 months to complete follow up. A similar time frame will be feasible for recruitment of 40 medical students (20 to receive BCG vaccine and 20 to receive Influenza vaccine) in light of the numbers travelling on elective who are recommended BCG vaccine and the uptake of Influenza immunisation in this population. The overall duration of the study will be 12 months including data analysis.

5 RECRUITMENT

5.1 Recruitment

Infants and children who are seen at RCH for administration of BCG vaccination will be identified. The Principal Investigator supervises all the immunisation clinics and will identify eligible participants. Clinical members of the research team will form part of the immunisation team. Furthermore clinicians in the immunisation clinic will be asked to refer eligible patients who are happy to consider participation in the study to the research team. All eligible patients will receive a short promotional leaflet with their routine, clinically required pre-clinic checklist. A paediatrician, and member of study team will take over the current role of clinic administrator and call the patients in the week preceding their appointment to resolve any issues with the pre-clinic checklist and to answer any questions relating to the study. Formal informed consent will take place at the clinic visit. Medical students and staff members who are travelling to countries with a high prevalence of TB for their medical elective or for work and those receiving routine Influenza immunisation will be asked to participate. Medical students and staff members who are prepared to receive BCG without a specific indication may also be included. If they agree they will be offered BCG or Influenza immunisation at the RCH clinic. Informed consent and eligibility assessment will be carried out at the time of clinic attendance.

We are in a unique position to undertake this project as we have access to large numbers of children who are being immunised with BCG vaccine. We also have access to medical students at the University of Melbourne who need BCG immunisation prior to travel to high TB prevalence countries on medical elective. We will use posters and advertisements in campus and university media and will speak

at relevant student lectures in order to recruit potential student participants. We have successfully used this model in a previous study (A comparative analysis of polyfunctional T cells and secreted cytokines induced by Bacille Calmette-Guérin immunisation in children and adults (9)).

5.2 Eligibility criteria

5.2.1 Inclusion Criteria

Participants must meet all of the following criteria in order to be eligible for inclusion in the study

- Have been referred to the Immunisation clinic for routine BCG vaccination
- Have an English speaking mother;
- An informed consent form must be signed and dated by their parent(s) or legally acceptable representative (LAR) after the nature of the study has been explained and prior to any study assessments/procedures.
- Able to attend a follow-up appointment within 7 (+/- 4) days after BCG is given

5.2.2 Exclusion Criteria

A patient meeting any of the following criteria will be excluded from study participation

- Referral for BCG was due to direct household exposure to TB
- They are receiving any other travel vaccines at the same date
- They are febrile / have an inter-current infectious illness.

5.3 Consent

All eligible participants will be approached at the time of the clinic by a member of the study team who will give them information about the study and explain what participation involves. If they agree to their infant's participation, the infant's mother (or legally acceptable representative (LAR)) will sign and date an informed consent form prior to any study assessments/procedures being conducted. The consent form will describe the purpose of the study, the procedures to be followed and outline the risks and benefits of participation. The team member will ensure that the infant's parent/LAR understands the information provided and has had the opportunity to discuss their child's participation with their health care provider and/or other family members and to direct any questions to the investigator. Consent will be voluntary and free from coercion. The research nurse that conducted the informed consent discussion will also sign the consent form. A copy of the consent form will be given to the participant's parent/LAR.

5.4 Subject withdrawal

5.4.1 Reasons for withdrawal

The investigator may withdraw a participant from the study if the participant:

- Is in violation of the protocol;

- Experiences a SAE or an AE other a SAE;
- Requires early discontinuation for any reason.

Participants will be free to withdraw from the study at any time upon the request of their parent/LAR. The minimum participation required for analysis in the study is blood sampling prior to BCG vaccination and at visit 2. Patients who fail to attend for a follow up blood sampling appointment will be contacted by telephone to assess their willingness to continue participation and to reschedule their appointment. Patients who withdraw consent or fail to attend visit 2 will be replaced by ongoing recruitment as described in section 4.3.

5.4.2 Handling of withdrawals and losses to follow-up

The investigator or delegate will document the reason for the withdrawal on the relevant page of the case report form (CRF) and make every effort to determine if an AE was associated with the reason for withdrawal.

STUDY VISITS AND PROCEDURES SCHEDULE

For analysis the minimum requirement for sample analysis are the core samples collected at time points 3 and 4. Thus, if participants choose to not provide samples at other time points (travelling, too busy etc.), this will not effect their involvement in the study.

Confirmation of eligibility / Recruitment and consent before BCG / Influenza vaccination

Participants will be recruited and consented as described above. At enrolment they will complete a brief questionnaire with demographic information including vaccination history. This will usually occur with either *time point 1* or *time point 2*.

Time point 2: Pre-Vaccination Blood Sample (OPTIONAL)

(1 week (+/-4 days prior to vaccination)

A blood sample (maximum volume 5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood collection 1 week prior to administration of the BCG vaccine. A convenient appointment for *time point 3* will be established. They will complete a brief questionnaire, which will include information regarding medication usage and any significant inter-current illness.

Time point 3: Pre-Vaccination Blood Sample (CORE)

(within 24hr prior to vaccination)

A blood sample (maximum volume 5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood collection 1 week prior to administration of the BCG vaccine. A convenient appointment for *time point 4* will be established. They will complete a brief questionnaire, which will include information regarding the medication usage and any significant inter-current illness.

Time point 4: Post BCG / Influenza vaccination blood sample (CORE)

7 ± 4 days post vaccination

Participants will attend their recruiting hospital (RCH/RMH) for a follow up blood draw. It will be collected by a qualified member of the research team or by the Phlebotomy Department (max 1-5mL children and 10-20 mL adults). They will complete a brief questionnaire, which will include information regarding the healing of the BCG vaccination site, medication usage and any significant inter-current illness. They will have the opportunity to contact a member of the study team at this point if they have any concerns regarding their vaccination site or participation in the study. If a participant fails to attend their scheduled appointment, a member of the study team will contact them to confirm their ongoing participation or to determine the reason for withdrawal. If appropriate a follow up visit will be scheduled.

Time point 5: post vaccination blood sample (OPTIONAL)

14+/- 4 days post vaccination

A blood sample (maximum volume 1-5 mL from children and 10-20 mL from adults) will be collected by a research nurse, doctor or phlebotomist experienced in paediatric blood at RCH. They will complete a brief questionnaire, which will include information regarding the healing of the BCG vaccination site, medication usage and any significant inter-current illness. A convenient appointment for *time point 6* may be established. A member of the study team will meet study participants at this visit to receive the results of the Vitamin D quantification and arrange any follow up required (details outlined below section 7).

Time point 6: post BCG / Influenza vaccination sample

3 months ± 4 weeks post vaccination

Participants will attend their recruiting hospital (RCH/RMH) for a follow up blood draw at the Phlebotomy Department (max 1-5 mL children and 10-20 mL adults). They will complete a brief questionnaire, which will include information regarding the healing of the BCG vaccination site, medication usage and any significant inter-current illness. If a participant fails to attend their scheduled appointment, a member of the study team will contact them to confirm their ongoing participation or to determine the reason for withdrawal. If appropriate a follow up visit will be scheduled.

6 CLINICAL AND LABORATORY ASSESSMENTS - METHODOLOGY

7.1 Questionnaire

Two brief questionnaires will be administered to the participants (or their parents) to complete during their attendance at visit 1 and visit 3. (Appendix1)

7.2 Laboratory Assessments

Blood samples will be collected from each participant at three time points as described above. Blood will be collected in the laboratory recommended tube for Vitamin D and will be processed in the biochemistry lab at RCH as a clinical sample. Results of the vitamin D level will be reviewed for each patient by a paediatrician involved in the study. Abnormal results in children will be repeated, treated or investigated as appropriate in line with the existing RCH clinical practice guidelines. Results will be fed back to adult participants and with their consent passed on to their GPs for appropriate follow up.

Remaining blood will be incubated for up to sixteen hours *in vitro* in a whole blood assay (that is well established in our laboratory) with different concentrations of stimulatory antigens including killed *S. aureus*, *E. coli*, *S. pneumoniae*, RSV, Rotavirus, group B streptococcus, and *Candida albicans*. Measures of the immune response will include analysis of cell marker and cytokine expression by intracellular flow cytometry, measurement of cytokine production in supernatants by *Luminex*-based multiplex assays (including IFN γ , TNF- α , IL-1 β , IL-6, IL-10, IFN- α , IL-4, IL-12, IL-17, IL-18), and gene expression studies.

7 REPORTING ADVERSE EVENTS

7.1 Definitions

Adverse Event (AE):

Any untoward medical occurrence in a participant enrolled in this study regardless of its causal relationship to study treatment.

Serious Adverse Event

Adverse events re classified as serious or non-serious.

An SAE is defined as any AE that:

- Results in death; or
- Is immediately life-threatening; or
- Requires inpatient hospitalisation; or
- Results in prolongation of existing hospitalisation; or
- Results in persistent or significant disability/incapacity

Significant medical events will be considered an SAE when, based upon appropriate medical judgment, they may jeopardise the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

Suspected Unexpected Serious Adverse Reaction (SUSAR):

A SUSAR is any SAE that is both suspected to be related to the study treatment and is unexpected (that is, not consistent with the applicable product information).

7.2 Eliciting Adverse Event Information

This is a non-interventional study. Serious adverse events are not anticipated. Any adverse event including pain, discomfort or inconvenience resulting from participation in the blood draws and attendance at the hospital will be considered an adverse event and will be elicited from the patients if they choose to withdraw from the study at any point or at completion of the study. Adverse events will be documented on a CRF and submitted to the relevant HREC and reported in the results of the study.

8 STATISTICAL METHODS

8.1 Sample size estimation

The sample size of 20 participants per group is in line with adult studies, which have successfully demonstrated an enhanced immunological response to infective antigens post BCG vaccination. The proposed sample size aims to minimise inconvenience to participants and ensure feasibility in terms of cost.

8.2 Population to be analysed

All patients who are recruited and who have a blood sample taken prior to BCG vaccination and on at least one occasion after which are both suitable for analysis. To assess the persistence of the immune response over time we will require at least 10 participants (50%) with blood samples at two time points.

8.3 Statistical analysis plan

Cytokine expression will be analysed by intracellular flow cytometry, and in supernatants by Luminex-based multiplex assays (IFN- γ , TNF- α , IL-1 β , IL-6, IL-10, IFN- α , IL-4, IL-12, IL-17, IL-18). This will enable us to establish the kinetics of cytokine responses to non-specific infectious antigens in healthy patients and whether these responses vary over time and between age groups. Non-parametric tests will be used to compare cytokine responses at each time point, between each stimulant and between children and adults (Kruskal-Wallis for multiple groups; Mann Whitney U test for two-group comparisons).

9 DATA HANDLING

9.1 Records to be kept / Data Collection

The Principal Investigator or delegate will maintain an individual file and source documentation for every participant enrolled in the study. The file will document the participant eligibility; visit dates as well any adverse experienced and other notes as appropriate. The participant file will be kept up to date so that it reflects the latest observations on the participant. The original, signed Parent/Guardian Information Statement (P/GIS) will be filed with each participant's file.

Study questionnaires will be given to the participant's parents/guardians for the recording of information as described. To ensure consistency of reporting, study personnel will instruct parents/guardians on how to complete these documents.

9.1 Data Management / Quality control

All study information collected by study personnel will be entered into an electronic database. Access to the database will be restricted by unique password and username

9.2 Study Record Retention

Data will be stored securely for a period of 25 years after the last study publication.

10 ADMINISTRATIVE ASPECTS

10.1 Confidentiality

Participant confidentiality will be strictly held in trust by the investigator, study personnel, the Murdoch Children's Research Institute (MCRI) and their agents. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. The study protocol, documentation, data and all other information will be held in strict confidence. No information concerning the study or the data will be released to any unauthorised third party, without prior written approval of the sponsoring institution. Authorised representatives of the MCRI may inspect all documents and records being maintained by the Investigator, including but not limited to, medical records and pharmacy records for the participants in this study. The investigator will permit access to such records. Any laboratory specimens, evaluation forms, reports or other records that leave the study site will be identified only by participant identification number in order to maintain participant confidentiality. Participant information will not be disclosed without written permission of the participant's parent/legal representative, except as necessary for monitoring by the relevant HREC or regulatory authorities.

10.2 Independent HREC Approval

This protocol and the informed consent documentation and any subsequent modifications will be reviewed and approved by the relevant Human Research Ethics Committee (HREC). A letter of protocol approval by the HREC will be obtained prior to the commencement of the study, as well as approval for other study documents subject to HREC review.

10.3 Modifications of the protocol

This study will be conducted in compliance with the current, ethically approved version of the protocol. Any change to the protocol or informed consent documentation that affects the scientific intent, study design, participant safety, or may affect the willingness of a parent/guardian to continue to allow their child's participation in the study will be considered an amendment and will therefore be

prepared and filed as an amendment to this protocol and/or informed consent documentation, all such amendments will be submitted to the relevant HHREC for approval prior to becoming effective.

10.4 Protocol Deviations

The investigator and study personal will exercise due diligence to avoid protocol deviations. However, any protocol deviations will be recorded in the participant's source document and CRF. Any deviations deemed to have a potential impact on the integrity of the study results, participant safety or the ongoing ethical acceptability of the study will be report to the relevant HREC within the specified timelines. All significant protocol deviations will be documented in the Clinical Study Report (CSR). Where deviations to the protocol identify issues for protocol review, the protocol will be amended.

10.5 Participant Reimbursement

Participants will not be offered any monetary reimbursement for participation in this study. They will be reimbursed for the cost of parking or travel to their two follow up appointments.

10.6 Financial Disclosure and obligations

No member of the study team will receive extra remuneration for their participation in the study outside of their existing employment and scholarship agreements. There is no conflict of interest declared by any member of the study team.

USE OF DATA AND PUBLICATIONS POLICY

The results of this study will be published in high impact scientific journals . A letter of thanks outlining the main findings of the study will be sent to all participants upon final publication. Published data will summarise group outcomes only. Individual study participants will not be identified.

REFERENCES

1. Roth A, G.P., Nhaga A, Djana Q, Poulsen A, Garly ML, Jensen H, Sodemann M, Rodrigues A, Aaby P, BCG vaccination scar associated with better childhood survival in Guinea-Bissau. *Int J Epidemiol*, 2005. 34((3)): p. 540-7.
2. Roth A, J.H., Garly M, Lisse IM, Sodemann , Aaby P. , Low birth weight infants and Calmette Guerin bacillus vaccination at birth: community study from Guinea Bissau. *Paediatr Infects dis J* 23(6): p. 544-550.
3. Vaugelade J, P.S., Guiella G, Elguero E, Simndon F., Non-specific effects of vaccination on child survival: prospective cohort study in Burkina Faso. *BMJ*, 2004. 329(7478).
4. Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed *Mycobacterium leprae* vaccine for prevention of leprosy and tuberculosis in Malawi. Karonga Prevention Trial Group. *Lancet*, 1996. 348(9019): p. 17-24.
5. Nakamura, M. and W.R. Cross, Susceptibility of Rabbits Immunized with *Mycobacterium bovis* (BCG) or *Mycobacterium phlei* to *Shigella* Keratoconjunctivitis. *Infection and Immunity*. 6(6): p. 1025-1025.
6. Mathurin, K.S., et al., CD4 T-cell-mediated heterologous immunity between mycobacteria and poxviruses. *Journal of Virology*, 2009. 83(8): p. 3528-3539.
7. Clark, I.A., A.C. Allison, and F.E. Cox, Protection of mice against *Babesia* and *Plasmodium* with BCG. *Nature*, 1976. 259(5541): p. 309-311.
8. Kleinnijenhuis J, Q.J., Preijers F, et al. , Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. . *Proc Natl Acad Sci U S A*, 2012. 109(43): p. 17537-42.
9. Ritz N, Strach M, Yau C, Dutta B, Tebruegge M, Connell TG, Hanekom WA, Britton WJ, Robins-Browne R, Curtis N. A comparative analysis of polyfunctional T cells and secreted cytokines induced by Bacille Calmette-Guérin immunisation in children and adults. *PLoS One*. 2012;7(7):e37535. Epub 2012 Jul 19.

14. APPENDICES

APPENDIX A

Causality of Serious Adverse Events (SAEs)

The relationship of serious adverse events to the BCG vaccine will be assessed as follows:

- **Unrelated:** *There is no association between the BCG vaccine and the reported event. SAEs in this category do not have a reasonable temporal relationship to exposure to the test product, or can be explained by a commonly occurring alternative aetiology.*
- **Possible:** *The BCG vaccine could have caused or contributed to the SAE. SAEs in this category follow a reasonable temporal sequence from the time of exposure to the vaccine and/or follow a known response pattern to the vaccine, but could also have been produced by other factors.*
- **Probable:** *The association of the SAE with the BCG vaccine seems likely. SAEs in this category follow a reasonable temporal sequence from the time of exposure to the vaccine and are consistent with the known mode of action of the vaccine, known or previously reported adverse events to the vaccine, or judgment based on the investigators clinical experience.*
- **Definite:** *The SAE is a consequence of administration of the BCG vaccine. SAEs in this category cannot be explained by concurrent illness, progression of disease state or concurrent medication reaction. Such SAEs may be widely documented as having an association with BCG vaccine or occurring after rechallenge.*

APPENDIX B

Questionnaire Content

Investigating the immunological basis for the non-specific effects of BCG vaccination in children

Questionnaire at enrolment

Part 1 Eligibility assessment

Language barrier? (Y/N)

Have you been referred here for any reason other than travel overseas? (Y/N)

Is your proposed travel date less than 3 months away? (Y/N/DN)

Is your child having any other travel vaccines today? (Y/N)

Has your infant been unwell with a temperature or infection in the last 24 hours? (Y/N)

Is there any other indication for BCG (eg ATSI, TB exposure at home)? (Y/N)

Eligible for trial (Y/N/DN)

Part 2: Contact Details

Personal contact details for adults

Mother

First name

Surname

DOB

Address

Postcode

Email

Mobile phone

Home phone

Partner

Same as mother

Part 3: Demographics

Sex (of child)

Male

Female

DOB

Born in Australia? (Y/N)

If No

Place of birth

Year arrived in Australia

Place of residence before entering Australia

Are all the routine immunizations up to date? (Y/N)

Has your (Have you) child received any extra immunisations? (Y/N)

If Yes which:

Has your child (Have you) suffered from any fever or infection in the last month? (Y/N)

If Yes

Where was it managed?

- Home
- GP
- Hospital

Which of the following best describes the diagnosis which you received?

- Viral infection with rash
- Viral infection without rash
- Cold / upper respiratory tract infection
- Cold sores / mouth ulcers
- Ear infection
- Throat infection
- Chest infection or pneumonia
- Bronchiolitis
- Croup
- Gastroenteritis
- Urinary tract infection
- Septicaemia
- Meningitis
- Infection in a joint (septic arthritis)
- Infection in a bone (osteomyelitis)
- Infection in a muscle (myositis)
- Eye infection (orbital or periorbital cellulitis)
- Skin infection (cellulitis)
- None of the above

Were you prescribed antibiotics? (Y/N)

If Yes?

- Name
- Dose
- Duration

Is your child taking any other routine medications or supplements (including vitamins, iron, probiotics)? (Y/N)

- If yes
- Name
- Dose

Questionnaire at 3 months

Has your (child's) BCG scar fully healed? (Y/N)

Were there any complications associated with the healing process? (Y/N)

If yes which best describes the problem

- Persistent oozing and discharge
- Formation of a sore or an ulcer at the site
- Formation of a lump or an abscess at the site of injection
- Formation of a lump or lymph nodes under the arm

Has your child (Have you) received any other immunisations in the last three months? (Y/N)

If Yes

Which one?

Date:

Has your child (Have you) suffered from any fever or infection in the last 3 months? (Y/N)

If Yes

Where was it managed?

- Home
- GP
- Hospital

Which of the following best describes the diagnosis which you received?

- Viral infection with rash
- Viral infection without rash
- Cold / upper respiratory tract infection
- Cold sores / mouth ulcers
- Ear infection
- Throat infection
- Chest infection or pneumonia
- Bronchiolitis

- Croup
- Gastroenteritis
- Urinary tract infection
- Septicaemia
- Meningitis
- Infection in a joint (septic arthritis)
- Infection in a bone (osteomyelitis)
- Infection in a muscle (myositis)
- Eye infection (orbital or periorbital cellulitis)
- Skin infection (cellulitis)
- None of the above

Were you / your child prescribed antibiotics? (Y/N) If yes?

- Name
- Dose
- Duration

Is your child (Are you) taking any other routine medications or supplements (including vitamins, iron, probiotics)? (Y/N)

- If yes
- Name
- Dose

APPENDIX C



Research study to better understand the beneficial effects of BCG vaccination

As you know your child is coming to RCH before you travel overseas to be given BCG vaccination to protect them from tuberculosis (TB).

But did you know that BCG might have a number of other beneficial effects due to its ability to boost the immunity in general. Studies suggest that BCG may help prevent:

- Chest infections (other than TB)
- Allergy, including asthma and eczema
- some cancers, including melanoma

We are trying to better understand how BCG has this positive effect on the immune system. We want to make sure that these effects would be preserved in any new TB vaccine that is developed.

We would like to invite you to consider allowing your child to participate in one of our studies. To this end, we will be calling you a few your days before you appointment at RCH to review your BCG clinic check list (enclosed) and to talk to you about our study and answer any questions you may have.

Thank you for taking the time to read this leaflet. We look forward to meeting you at you BCG visit.

Information statement and consent form

HREC Project Number: 33128

Research Project Title: Investigating the immunological basis for the non-specific effects of BCG vaccination

Principal Researcher: Prof Nigel Curtis,
Leader of Infectious Diseases & Microbiology Group, Murdoch Children's Research Institute
Professor of Paediatric Infectious Diseases, Department of Paediatrics, The University of Melbourne
Head of Infectious Diseases, The Royal Children's Hospital Melbourne

Version Number: 7 **Version Date:** 28th October 2015

Thank you for taking the time to read this **Parent/Guardian Information Statement and Consent Form**. We would like to invite your child to participate in a research project that is explained below. This document is 6 pages long. Please make sure you have all the pages.

What is an Information Statement?

These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you decide whether or not you would like your child to take part in the research. Please read this Information Statement carefully.

Before you decide if you want your child to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to your child taking part in the project.

We will give you a copy of this information and consent form to keep.

1. What is the purpose of this research?

BCG is a vaccine used to prevent tuberculosis (TB). It is the most widely administered vaccine worldwide.

Studies in developing countries show that BCG vaccine can protect children against infections other than TB, and in some areas has reduced infant mortality by 50% from diseases like pneumonia and diarrhoea. The mechanism by which BCG influences infant immunity to exert this effect is not completely understood.

The aim of this study is to analyse how BCG vaccine affects your child's immunity. We will do this by analysing the immune response in blood samples in our laboratory. By checking blood samples before you have had the BCG vaccine, and again 7 days and 3 months after the vaccine, we will be able to see how the effect of BCG vaccine changes over time.

During the course of the study we will also analyse how the Influenza vaccine affects the immune system. Unlike the BCG vaccine, the Influenza immunisation has not so far been associated with protection against diseases other than Influenza. We will analyse the immune response in blood taken just before, 7 days and 3 months after Influenza immunisation in 40 healthy adults. We will compare this response to the response in people who receive BCG vaccine. This will allow us to determine that the response seen is only attributable to BCG vaccine and does not occur after a different vaccine.

We hope the results from this study will help us to harness these effects for children in Australia and overseas.

This is a new project and will involve 60 children and 40 adults. The adults will be in two groups, 20 who receive BCG vaccine and 20 who will receive Influenza immunisation.

Prof Nigel Curtis, Head of Infectious Diseases at The Royal Children's Hospital Melbourne, has initiated this research.

This research has been funded by the Murdoch Children's Research Institute.

This research is being conducted by a collaboration involving researchers based at The Royal Children's Hospital (RCH), The University of Melbourne and Murdoch Children's Research Institute (MCRI).

No member of the research team will obtain any financial benefit from involvement in this project (other than their ordinary wages).

2. Why am I and my child being asked to participate?

You are invited to take part in this research project because your child is having a BCG vaccination

3. What does participation in this research involve?

When you arrive at the clinic you and your child will be met by one of our researchers and be given a written and verbal description and explanation of this study. You will have time to read the information and discuss it with a member of the research team who will be happy to answer any questions you might have before your immunisation appointment. We will also determine whether your child is eligible for participation in the study. We will ask you to provide your written consent when you have decided you are happy for you and your child to participate.

If you are happy for your child to participate we will ask you to fill out a short questionnaire here in the waiting area and will ask your child to have a blood test taken by an experienced member of staff prior to your child's planned vaccination. We will then ask you and your child to return to the hospital to have further blood tests

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in one week and three months time. These follow up visits are important as they allow us to study the change in your child's immune response after receiving BCG vaccination and how this response is maintained over time. We will determine with you today the dates that are most convenient for you to return for the follow up visits. A member of the research team will be happy to discuss you and your child's participation with you and answer any questions you might have at these follow up visits. You will not be paid for you and your child's participation in this research, but you will be reimbursed for car parking fees that you incur as a result of participating in this research project.

Summary of study involvement

At Immunisation Clinic	Brief questionnaire (5 minutes) Blood sample BCG vaccination
In the next 3-11 days	Blood sample at RCH
At 3 months +/- 2 week	Blood sample at RCH Brief questionnaire (5 minutes)

You are free to withdraw your child from this study at any time. If you and your child do not attend a scheduled appointment a member of the study team will contact you to see if you wish to reschedule your appointment and answer any questions you might have.

4. What will happen to my child's test samples?

Blood samples will be collected in coded form at designated time points for analysis. We will analyse various components of the immune response in blood samples. We will also test the blood sample for your child's vitamin D level. The samples will be collected and labelled with the assigned study allocated number. They will be transported to the Infectious Diseases and Microbiology research laboratory at the MCRI and stored in freezers until further analysis. Only the members of the research team will be able to access these samples and will update reports on their location and processing. The freezers are locked and can only be opened by members of the research team who have access to the key. We will ask your consent to keep the samples for other future studies related to BCG.

5. What are the possible benefits?

We cannot guarantee or promise that you will receive any benefits from this research, however, your child may benefit from contact with the study team in the weeks following the BCG vaccination as they will be available to answer any questions you have on your return visits. If your child's vitamin D level is low, a member of the study team will inform you and any necessary supplementation will be arranged. On a larger scale the results of this will inform study will be relevant to children worldwide who routinely receive BCG vaccination.

6. What are the possible risks?

Adverse effects related to blood collection

Having a blood sample taken may cause some discomfort or bruising. Sometimes, the blood vessel may swell, or blood may clot in the blood vessel. Rarely, there could be a minor infection or bleeding. If this happens, it can be easily treated without any known long-term effects. The discomfort can be minimised by using an appropriate technique with the smallest gauge needle performed by experienced staff. For your child we will employ techniques to decrease pain at the time of blood collection.

7. Can my child have other treatments during this research project?

While your child is participating in this research project, he/she will not be restricted from using any other medication or treatment for conditions which may arise incidentally during the research study period. However, it is important to inform the research staff about any treatments or medications your child may be taking, including over-the-counter medications, vitamins, pre/pro biotic preparations or herbal remedies as these may interfere with the analysis of results.

10. Do my child and I have to take part in this research project?

Participation in a research project is voluntary. Your child does not have to take part in this project if you do not want them to.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect any treatment or care your child gets, or your family's relationship with The Royal Children's Hospital.

11. What if my child and I withdraw from this research project?

If you and your child decide to withdraw from the project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to further discuss any concerns or special requirements linked to withdrawing.

If you decide to leave the project, the researchers would like to keep the personal and health information about your child and the tissue samples that have been collected. This is to help them make sure that the results of the research can be measured properly. If you do not want them to do this, you must tell them before you and your child join the research project.

12. What will happen when my participation in this research project ends?

At the end of the 3 month study period, your child will continue to have routine care as any other child. There will be no further expectations or requirements regarding the study.

14. How will I be informed of the results of this research project?

At the end of the study, the information collected will be analysed and a report of the findings will be prepared. The results may also be published in an appropriate medical journal. This process may take several months. We will send you a letter summarising the study's main findings, although individual results will not be given. Should you have any further questions about the results, we will discuss these with you by telephone or correspondence.

15. What else do I need to know?

What will happen to information about my child and I?

Data will be stored in coded/re-identifiable form which will be password protected.

The principle investigators, co investigators, research nurses and biostatistician will have access to participant information as identified by their allocated study number. No one else will be able to access participant information.

Information will be stored for 5 years from the date of publication of results, in accordance with the Australian Code for Responsible Conduct of Research. After this period, data will be destroyed.

Any information obtained for the purpose of this research project that can identify you will be treated as confidential and securely stored. It will be disclosed only with your permission, or as permitted by law. Information about your child may be obtained from your health records held at this health service for the purposes of this research

Your child's health records and any information obtained during the study are subject to inspection (for the purpose of verifying the procedures and the data) by the relevant authorities, the organisations relevant to this PICF, Royal Children's Hospital Melbourne, or as required by law. By signing the consent section, you authorise release of, or access to, this confidential information to the relevant study personnel and regulatory authorities as noted above.

In any publication and/or presentation, information will be provided in such a way that your child cannot be identified, except with your permission.

Information about your child's participation in this research project may be recorded in their health records.

How can I access my child's information?

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access the information collected and stored by the researchers about your child. You also have the right to request that any information with which you disagree be corrected. Please contact one of the researchers named at the end of this document if you would like to access your child's information.

Is this research project approved?

The ethical aspects of this research project have been approved by the Human Research Ethics Committees the Royal Children's Hospital, Melbourne. This project will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

What happens if my child is injured during the project?

In the unlikely event that your child suffers an injury because of participating in this project, the public health service will provide hospital care and treatment at no cost to you.

16. Who can I contact?

If you have any concerns and/or complaints about the project, the way it is being conducted or your child's rights as a research participant, and would like to speak to someone independent of the project, please contact:

Director, Research Development & Ethics, The Royal Children's Hospital Melbourne on telephone: (03) 9345 5044.

For further information or appointments:

If you want any further information concerning this project or if you have any medical problems which may be related to your involvement in the project (for example, any side effects), you can contact the principal researcher Prof Nigel Curtis via Royal Children's Hospital switchboard 03 9345 5522. If you have any concerns regarding your follow up appointments or commitments to the study please contact a member of the team during office hours on 0410148615.

Consent Form

HREC Project Number: 33128

Research Project Title: Investigating the immunological basis for the non-specific effects of BCG vaccination in children

Version Number: 7

Version Date: 28 Oct 2015

- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my child's involvement in this project.
- I voluntarily consent for my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that this project has been approved by The Royal Children's Hospital Ethics Committee and will be carried out in line with the National Statement on Ethical Conduct in Human Research (2007).
- I understand I will receive a copy of this Information Statement and Consent Form.

OPTIONAL CONSENT

☐ I do	☐ I do not	consent to be contacted about future research related to this project

Child's Name

Parent/Guardian Name

Parent/Guardian Signature

Date

Name of Witness to
Parent/Guardian's Signature

Witness Signature

Date

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child's involvement in this project.

Research Team Member Name

Research Team Member Signature

Date

Note: All parties signing the Consent Form must date their own signature.