The Impact of Maternal Infection with *Mycobacterium tuberculosis* on the Infant Response to BCG Immunisation

AKUSA PATRICE MAWA

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Department of Immunology and Infection
Faculty of Infectious and Tropical Diseases
LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

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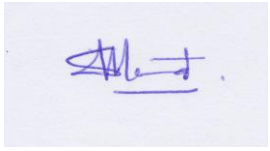
Research group affiliation(s): MRC/UVRI Uganda Research Unit on AIDS, P.O. Box 49, Entebbe, Uganda.

Declaration by Candidate

I, Akusa Patrice Mawa, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



December 6, 2016

Signed……Date……..

Full name: Akusa Patrice Mawa

Abstract

Bacille Calmette Guérin (BCG) immunisation induces variable protection against tuberculosis (TB) in adolescents and adults. More information on how it protects, and when, is needed. The infant response to BCG immunisation in Uganda and the influence of maternal latent *Mycobacterium tuberculosis (M.tuberculosis)* infection (LTBI) and maternal BCG scar on these responses were examined.

Innate responses from 29 mother-infant pairs was measured using a Luminex® assay. Gene expression profiles in unstimulated infant samples collected at 1 (n=42) and 6 (n=51) weeks after birth were also analysed. Frequencies of PPD-specific IFN-γ^+CD4+ T cells after 24-hour stimulation of infant samples were assessed by flow cytometry, and the time course of BCG-induced responses measured using Luminex® assay. Immunoglobulin G to PPD and tetanus toxoid was measured in plasma samples. The impact of maternal LTBI and maternal BCG scar on infant responses was investigated.

Maternal BCG scar was associated with an increased infant pro-inflammatory response. Interferon and inflammation pathways were down-regulated at 1 week, but up-regulated at 6 weeks in infants of mothers with LTBI. In contrast, these pathways were both up-regulated in infants of mothers with a BCG scar at 1 and 6 weeks. PPD-specific IFN-γ^+CD4+ T cells increased at 1 week and decreased at 6 weeks after birth (p=0.031). Maternal LTBI was associated with lower frequencies of IFN-γ^+CD4+ T cells (p=0.015) and IFN-γ^+, TNF-α^+ and IL-2^+ CD4+ T cells, combined (p=0.002), at 1 week after BCG. BCG-induced responses peaked around 24 weeks of age, but were not associated with maternal LTBI. Antibody responses dropped rapidly at 1 week and were not associated with maternal LTBI.

In conclusion, infant responses peaked around 24 weeks of age, and maternal BCG scar was associated with increased infant proinflammatory responses. There was evidence of a shorter-term influence of maternal LTBI on infant responses.

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Above all, I thank GOD.

Statement of work

The work presented in this thesis is part of a larger study, and therefore many people have contributed to it.

The studies were conceived and designed by Prof. Alison M. Elliott, Prof. Hazel M. Dockrell, Dr Stephen Cose, Dr Steven Smith, Prof. Pontiano Kaleebu and myself.

The study was co-ordinated by Dr Stephen Cose, Dr Dorothy Aibo, Dr Joel Serubanja, Prof. Alison M. Elliott and myself.

Dr Dorothy Aibo, study nurses/midwives and field workers coordinated recruitment of participants, phlebotomy and participant management.

I participated in sample processing (WBA and storage of plasma) with the help of Ms. Grace Nabakooza (the study technologist). I performed the Luminex® assays and the antibody ELISAs. I organized the infant samples for gene expression assays for shipment to Dr. Rafick Sekaly's laboratory in Florida.

I performed the entry and cleaning of the Luminex® and antibody data. The MRC/UVRI Statistics/data entry staff under the leadership of Mr Lawrence Muhangi and Mr Lawrence Lubyayi performed the entry and management of other laboratory and clinical data.

All the statistical analysis for the work presented here was performed by myself under the guidance of Dr Emily L. Webb and Dr Stephen Nash, the study statisticians.

Part of the work presented has been published and another paper is in press. These have been included in the appendices.

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List of Acronyms and Abbreviations

AEC	Airway Epithelial Cell
AIDS	Acquired Immunodeficiency Syndrome
ASL	Airway Surface Liquid
BCG	Bacille Calmette-Guérin
CD	Cluster of Differentiation
CiSP	Co-infections Studies Programme
CFP-10	Culture Filtrate Protein-10kDa
CO_2	Carbon dioxide
CyTOF	Cytometry by time of flight
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific ICAM-3-Grabbing Non-intergrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot
EMaBS	Entebbe Mother and Baby Study
EPI	Expanded Programme on Immunisation
ESAT-6	Early Secretory Antigenic Target-6kDa
FCS	Fetal Calf Serum
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
ICS	Intracellular Cytokine Staining
IFN-γ	Interferon-gamma
IL	Interleukin
IP-10	Interferon-inducible Protein-10
LSHTM	London School of Hygiene & Tropical Medicine
LTBI	Latent tuberculosis infection
MCP-1	Monocyte Chemoattractant Protein-1
MIP-1α	Macrophage Inflammatory Protein-1α
MIP-1β	Macrophage Inflammatory Protein-1β
M.tuberculosis	*Mycobacterium tuberculosis*
MRC/UVRI	MRC/UVRI Uganda Research Unit on AIDS
NTM	Non-Tuberculous Mycobacteria

OD	Optical Density
PAMP	Pathogen Associated Molecular Patterns
PPD	Purified Protein Derivative
PRR	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
RNI	Reactive Nitrogen Intermediates
SSI	Statens Serum Institut
TT	Tetanus Toxoid
Th	T-helper
TLR	Toll-Like Receptors
TNF	Tumour Necrosis Factor Alpha
TB	Tuberculosis
TST	Tuberculin Skin Testing
UNCST	Uganda National Council for Science and Technology
UK	United Kingdom
USA	United States of America
UVRI	Uganda Virus Research Institute
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation

Chapter 1
Introduction

1.1. Tuberculosis

1.1.1. The global burden of tuberculosis

Tuberculosis (TB) now ranks above Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) as the leading cause of infectious disease deaths globally. In 2015, 1.4 million HIV-uninfected and 0.4 million HIV-infected people died of TB disease (1). Globally, there were an estimated 10.4 million new TB cases in 2015 (equivalent to 142 cases per 100,000 population), with 5.9 (56%) million among men, 3.5 (34%) million among women and 1.0 (10%) million among children (1). The majority of those infected will remain asymptomatic (latent *M.tuberculosis* infection (LTBI)) (2, 3) and up to 10% will go on to develop active TB disease (4).There was a slow fall in the absolute number of incident cases per capita (1.5% and 2.1% average rates per year for 2000-2014 and 2013-2014, respectively), and 1.5% average rate per year for 2014-2015 (1).

In 2015, the African region contributed 26% (275 cases per every 100,000 population) of cases globally, more than the global estimate. In 2015, 0.86 million and 0.38 million HIV-negative men and women, respectively, died of TB disease (1). Up to 60% of the global burden is borne by India, Indonesia, China, Nigeria, Pakistan and Southern Africa (1).

There is effective drug treatment for TB and there has been good progress since the introduction of the Directly Observed Therapy Strategy (DOTS) of the World Health Organisation (WHO) in 1995 (whose target was treatment of up to 85% of the TB cases globally), the Stop TB Strategy of 2006 and the Millenium Development Goals. Treatment with a combination of four antibiotics (rifampicin, isoniazid, pyrazinamide and ethambutol) for six months forms the current regimen for anti-TB treatment (1). An estimated 35 million HIV-uninfected people were effectively treated between 2000 and 2014 (5). The introduction of a rapid molecular test for TB drug resistance (GeneXpert), and the availability of two new drugs (bedaquiline and delamanid) have added to the efforts being made.

TB treatment efforts have, however, been challenged by the emergence of multidrug resistant TB (MDR-TB) (defined as resistance to two of the common drugs isoniazid and rifampicin), rifampicin-resistant TB (RR-TB), and extensively drug resistant TB (XDR-TB) (defined as resistance to a second-line injectable and fluoroquinolone) strains. This is due, mainly, to wrong regimens and dosages. Globally, there are 580,000 cases of MDR/RR-TB, with MDR-TB alone accounting for 83% of the total. An estimated 3.9% of new cases and 21% of cases previously treated for TB have MDR/RR-TB globally. In 2015 alone, up to 250,000 people reportedly died of MDR/RR-TB. Countries in the WHO regions of Asia (China and India) and eastern Europe (Russian Federation) have the highest MDR-TB burden, accounting for 45% of the global burden (1, 5, 6). In the African region, the Democratic Republic of the Congo, Ethiopia, Kenya, Mozambique, Nigeria and South Africa are among the 30 high MDR-TB burden countries (1, 7). Up to 9.5% of patients with MDR-TB have XDR-TB (1). In 2012, 15 countries in the African region had at least one XDR-TB case notification (6). By 2015, 117 countries had reported XDR-TB (1). Bedaquiline was used to treat patients with drug resistance in up to 70 countries in 2015 and delamanid was used in 39 countries (1).

Another challenge to TB control efforts is the HIV epidemic. HIV infection is the strongest risk factor for TB. The lifetime risk of developing active TB disease is estimated at 5-15% in HIV-infected people per year, whereas the lifetime risk for developing active TB from LTBI in healthy individuals estimated to be 5% to 10% per year (8). Up to 11% of notified cases in 2015 were co-infected with HIV, worldwide. The number of TB pateints co-infected with HIV was highest in the WHO African region (81%) and the Americas (82%) in 2015. An estimated 9.6 million deaths were avereted in TB/HIV co-infected cases between 2000 and 2015 using TB treatment supported by antiretroviral therapy (ART) (1).

Diabetes mellitus, alcohol use and smoking are some of the other factors associated with high risk of progression to active TB, in addition to poor treatment outcomes (9). Compared to those without, patients with diabetes mellitus had up to 3 times higher risk of developing TB disease (10-12). Diabetes has been shown to negatively affect the functions of alveolar macrophages (13) and the initiation of innate and adaptive immune responses in animal studies (14). Alterations in host immune responses are suggested to

be responsible for increased TB susceptibility in persons with diabetes (15). Interventions targeted at these risk factors would greatly reduce the global burden of TB (16, 17).

Responses to challenges to the TB control effort are included in the recent seventeen Sustainable Development Goals (SDGs) launched in January 2016. Target 3.3 of Goal 3 highlights efforts towards ending HIV, TB, malaria and neglected tropical diseases by 2030 (18, 19). The End TB Strategy, aimed at eliminating TB by 2035 in countries with low incidence, has also been established (20, 21). This is a challenging goal with the current progress.

1.1.2. The burden of tuberculosis in Uganda

Previously, Uganda was among the 22 high TB burden countries prioritized globally since 2000. In 2015 alone, these countries contributed to 87% of the global estimate of TB cases (1). Using the new post-2015 criteria, Uganda is among the 30 highest TB/HIV burden countries in the world (1, 7).

The DOT strategy was adopted by the national TB programme to improve adherence by making sure there is regular supply of anti-TB medication that are needed and for monitoring of case detection and treatment outcomes (22, 23). There have been reports of poor access to anti-TB drugs due to drug sockouts and challenges in drug delivery to remote or mobile communities (24, 25). Recent reports have further highlighted poor implementation of community-based DOTS in some communities in Uganda (26).

In 2015, the estimated incidence of TB in Uganda was 202 cases per 100,000 people for all forms of TB (1, 5). The estimated TB incidence in HIV-infected persons alone was 66 cases per 100,000 people (27). Between 2007 and 2013, TB case notification increased from 41, 612 to 47,650 (28). However, in 2015, 43, 736 TB cases were notifed in total (27). TB mortality remains high in Uganda, with annual rates of 14 and 16 cases per 100,000 population for HIV-negative and HIV-positive persons, respectively (1, 27).

A study carried out in an urban setting in Uganda reported the prevalence of LTBI in adults at 49% (29). Among adolescents of 12-18 years of age in a rural setting in eastern Uganda, the prevalence was 16.1% (30).

Challenges of poor health-seeking behaviour, poor adherence to drugs and inadequate funding are some of the reasons for the high TB mortality rates in Uganda. Treatment success rate in Uganda is reported to be low, with a coverage of 53% (27) mainly due to poor case identification and reporting, few trained health workers and noncompliance to treatment leading to emergence of drug resistant strains of *M.tuberculosis*, further complicating control efforts (31). Up to 88% of TB/HIV co-infected patients are on ART (27). In 2015, the estimated incidence of MDR/RR-TB in Uganda was 4.9 cases per 100,000 population (1). Tables 1.1 and 1.2 illustrate the Ugandan and global estimates of TB incidence and mortality, respectively, for 2015.

Table 1.1. Ugandan and global estimates of tuberculosis incidence, 2015

Country/region	Population (millions)	Incidence (including HIV)		Incidence (HIV-positive)		Incidence (MDR/RR-TB)	
		Number (thousands)	Rate	Number (thousands)	Rate	Number (thousands)	Rate
Uganda	39	79 (47-119)	202 (120-304)	26 (16-37)	66 (42-94)	1.9 (1.0-2.8)	4.9 (2.6-7.2)
Global	7323	10400 (8740-12200)	142 (119-166)	1170 (1020-1320)	16 (14-18)	580 (520-640)	7.9 (7.2-8.7)

Table 1.2. Ugandan and global estimates of tuberculosis mortality, 2015

Country/region	Population (millions)	Mortality (HIV-negative people)		Mortality (HIV-positive people)		Mortality (HIV-negative and HIV-positive people)	
		Number (thousands)	Rate	Number (thousands)	Rate	Number (thousands)	Rate
Uganda	39	5.5 (3.3-8.3)	14 (8.5-21)	6.4 (1.7-14)	16 (4.3-36)	12 (6.1-20)	30 (16-50)
Global	7323	1400 (1200-1600)	19 (17-21)	390 (320-460)	5.3 (4.4-6.3)	1800 (1600-2000)	24 (22-27)

Source: Global tuberculosis report, 2016.

1.1.3. *M.tuberculosis* as a pathogen

Mycobacteria are acid-fast gram-positive bacteria with many species, most of which are nontuberculous and abundant in the environment. Human TB is mainly caused by *M.tuberculosis* and *M. africanum*, two members of the *M.tuberculosis* complex (MTBC) (32, 33). *M.bovis*, another member of the MTBC, affects both humans and animals (34). Other animal-adapted members of the MTBC include: *M.microti, M.canetti, M.caprae, M.pinnipedii, M.suricattae and M.mungi* (35). The MTBC lineages include: lineage 1 (East Africa, the Philippines, Indian Ocean rim), lineage 2 (East Asia), lineage 3 (East Africa and Central Asian strain), lineage 4 (Europe, America and Africa), lineage 5 (West African 1), lineage 6 (West African 2), lineage 7 (Ethiopia) (32, 36, 37). TB in Uganda is mainly caused by MTBC Uganda family (a sub-lineage of lineage 4 (Euro-American) lineage) (38-40). Other MTBC lineages found in Uganda include lineage 2 (East Asia), lineage 4 non-Uganda (Euro American lineages other than Uganda family), and lineage 3 (East Africa, India/Central Asian strain). Recently, there has been interest in infections with the *M.avium-intracellulare* complex (MAC) (composed of *M.avium, M.intracellulare* and *M.chimaera*). There have been reports of a rise in the incidence of nontuberculous mycobacteria (NTM) globally, with cases of pulmonary MAC becoming more common (41-43).

1.2. The immune response to *M.tuberculosis*.

1.2.1. Innate immune responses

TB is transmitted when aerosols containing the bacilli are inhaled. The first line of defence against *M. tuberculosis* infection is formed by the mucosa along the respiratory airway (44). The respiratory mucosa is made of the epithelium, which is a layer of airway epithelial cells (AECs). These cells form a barrier that stops initial pathogen invasion. Though not classified as immune cells, these cells are reported to display anti-mycobacterial activities in animal (45) and human studies (46). Other components of the respiratory mucosa include the lamina propria (a layer of immune cells such as lymphocytes and macrophages and connective tissue) and airway surface liquid (ASL), which contains immunoglobulin A, mucus and other innate anti-microbial agents. The bronchial-or nasal-associated lymphoid tissues with anti-mycobacterial roles are also found along the airways (47). The AECs express pattern recognition receptors (PRRs)

that sense pathogen associated molecular patterns (PAMPs) on *M.tuberculosis* (48) and can present antigens to mucosal-associated invariant T cells (46). The AECs are also capable of secreting cytokines and chemokines that influence the functioning of phagocytes (48). There are reports of the presence of antimicrobial peptides such as β-defensin 2, cathelicidin and hepcidin in the ASL whose composition is determined by the AECs (49-51). Type II epithelial cells in the alveoli produce molecules with antimicrobial properties (49) and in particular they secrete hydrolytic enzymes, hydrolases and pulmonary surfactants with anti-mycobacterial roles (52, 53).

The bacilli infect alveolar macrophages and DCs and the result of the encounter with these cells will determine if an individual becomes latently or actively infected (54, 55). Upon infection, the bacteria are contained in a well-defined structure called a granuloma, characterized by a ring of lymphocytes and fibroblasts around multinucleated giant cells and activated macrophages. Other cells such as γδ T cells also get attracted to the granuloma (56). In a study involving a mouse model, the expression of chemokines involved in the formation of the granuloma are dependent upon TNF-α produced by *M.tuberculosis*-infected macrophages and T cells, and this is important in the initiation of granuloma formation (57). Alveolar macrophages can kill *M.tuberculosis* by producing iNOS and RNI after activation by IFN-γ and TNF-α (58). The cells and their products involved in immunity to *M. tuberculosis* infection are summarized in Figure 1.1 below.

There is evidence that the host mounts an effective immune response to the bacilli and stops development of active TB disease in about 90% of individuals infected by *M.tuberculosis*, however in most cases the infection persists in a latent state (LTBI) (59). This latent state can last for a lifetime or be reactivated based on the state of the hosts' immune system (60, 61). *M.tuberculosis* can remain in the lung granulomas and the host immune system is unable to completely clear it (56). Latently infected persons therefore act as reservoirs of new infections and efforts are being made to identify and treat them (62, 63). A dynamic relationship exists between the host and mycobacteria resulting in a broad spectrum of responses and outcomes (64, 65). Latently infected persons therefore may retain the bacilli in an inactive state or the bacilli may be actively replicating without apparent clinical disease (64), a condition referred to as incipient TB (66).

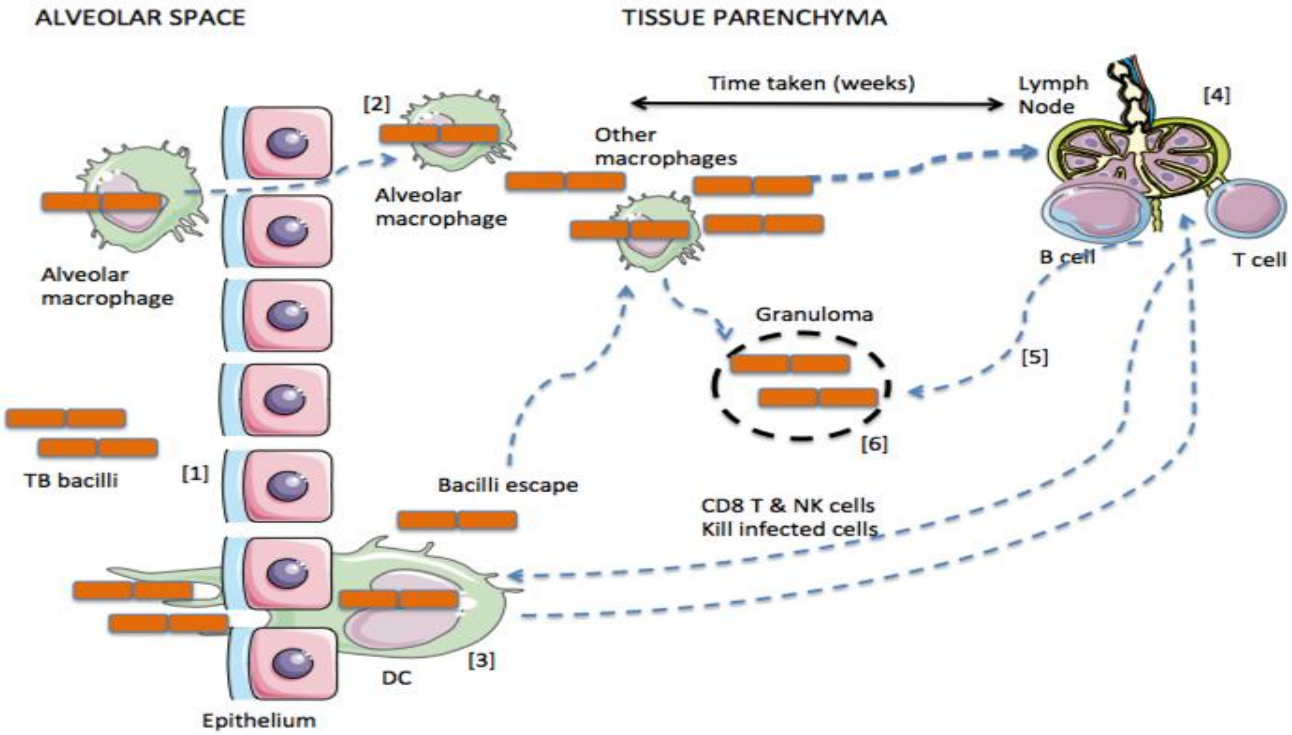


Figure 1.1. The various contributors to immunity to *M.tuberculosis* infection. 1. Production of cytokines (including IL-1β, IL-6 and TNF-α) and anti-mycobacterial peptides by epithelial cells. 2. *M.tuberculosis* bacilli are taken up by alveolar macrophages and killed using reactive oxygen and nitrogen intermediates. 3. *M.tuberculosis* bacilli are taken up by dendritic cells (DCs). 4. T and B cells in the lymph nodes are activated by mycobacterial antigens delivered by macrophages and DCs. 5. The activated T and B cells migrate to the lungs to form granulomas. Activated CD8+ T and NK cells kill infected macrophages and DCs. 6. *M.tuberculosis* bacilli are contained in the granuloma. Source of elements: Servier Medical Art. The *M.tuberculosis bacilli* shown were designed by me.

Innate immune cells control infection by phagocytosis of the microbes and initiation of immune responses, which kills the pathogen. The host elicits anti-microbial responses such as phagosomal maturation, generation of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) after recruitment of phagocytic cells such as macrophages, dendritic cells (DCs) and natural killer (NK) cells.

The *M.tuberculosis* bacilli are engulfed by macrophages into phagosomes that then fuse with lysosomes to create an environment that can disable or kill the mycobacteria (67). This is made possible by PRRs from the macrophages or DCs that interact with PAMPs on *M.tuberculosis*. Toll-like receptors (TLRs) (such as TLR 2, TLR 4 and TLR 9) have been studied widely and play an important role in immunity to mycobacteria (68). DC-SIGN, Dectin 1, mannose receptor and mannose-binding lectin (MBL) are the other receptors engaged during the phagocytosis of *M.tuberculosis* (69).

In humans, ten TLR family members have been identified and each one is associated with a unique signaling cascade upon activation by the PAMPs (55, 70). Mycobacteria have PAMPs such as lipoproteins, lipoglycans and carbohydrates that are targets for TLRs (69). Activation of transcription factors such as NF-κβ and inducible nitric oxide synthase (iNOS) gene expression results in the production of such cytokines as Interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and gamma interferon (IFN)-γ. TLR1/6 polymorphisms in South African infants have been shown to positively influence BCG-induced T helper (Th) 1 responses (71).

M.tuberculosis is capable of evading host immune responses by modulating both innate and adaptive immune responses. Alterations of the phagosomal environment within the macrophages and effects on host cytokine responses, antigen presentation and selective interactions with PRRs are some of the strategies employed by the bacilli (72).

Neutrophils are another set of effector cells with potential for both anti-mycobacterial activity and immunopathology in humans. They can limit the growth of *M. tuberculosis* (73) and once stimulated, they secret chemokines and proinflammatory cytokines that influence the recruitment and activation of cells (74). During the respiratory burst, neutrophils also release collagenase, myeloperoxidase and elastase from their granules and these factors act on both the pathogen and the host. Neutrophils constitute a large

percentage of the *M.tuberculosis*-infected cells in sputum and bronchoalveolar lavage from active TB patients (75), and have been linked to high expression of programmed death ligand 1 (PD-L1) in whole blood, resulting in dysfunctional or exhausted T cells (76).

Studies in animals have shown that DCs make up a large population of cells infected with *M.tuberculosis* (77, 78). Although macrophages are more phagocytic than DCs, DCs are surprisingly efficient at phagocytosing *M.tuberculosis* (79). The engagement of lipoarabinomannan (LAM) from mycobacteria by DC-SIGN serves as the main entry route for *M.tuberculosis* in DCs (80). Alveoli DCs mature on uptake of *M.tuberculosis* bacilli and present processed antigens to T cells (81).The interaction between *M.tuberculosis* LAM and DC-SIGN is also associated with an immunoregulatory immune profile (82).

NK cells also display anti-mycobacterial properties. Various studies have shown *in vitro* lysis of macrophages infected with *M.tuberculosis* by human NK cells (83, 84). The natural cytotoxicity receptor (NCR) NKp44 on NK cells is capable of ligating various PAMPs on *M.tuberculosis* (85). *In vitro,* NK cells are capable of secretion of IFN-γ and IL-22 (86) or initiation of IFN-γ production and lysis of infected cells by CD8+ T cells (87).

A recently described group of hematopoietic cells of the innate immune system are the innate lymphoid cells (ILCs). These cells can be isolated from the lungs, the gut and mucosal surfaces (88-92) and resemble CD4+ T helper cells (88, 91, 93), but do not have rearranged antigen-specific receptors. A variety of PAMPs can activate ILCs (91, 94-96). There are three groups of ILCs: first, NK cells (innate equivalent of CD8+ T cells) and other IFN-γ -producing ILCs (innate equivalent of Th1 cells) (ILC1). These express T-bet as their signature transcription factor. Second, IL-5 and IL-13 producing ILCs express GATA3 (innate equivalent of Th2 cells) (ILC2). Third, IL-17A-, IL-17F and IL-22-producing ILCs express transcription factor RORγt (innate equivalent of Th17 cells) (ILC3) (90, 91, 97). A study in animals has reported the anti-mycobacterial role of ILC1 and ILC3 cells in the lungs of BCG-vaccinated mice (98).

Another innate mechanism employed in the control and elimination of *M.tuberculosis* is autophagy (99). Here, the cytoplasmic contents of the cells are degraded in the autophagosomes (100). When this happens in infected macrophages, the *M.tuberculosis* bacilli are killed in the process.

1.2.2. The adaptive immune response

The host cellular immune response to *M.tuberculosis* is complex and mainly characterized by T helper (Th)-1 cell responses (101-103). The immune responses generated by activated CD4+ and CD8+ T cells are important in the control of the infection (61). CD4+ T cells are activated by *M.tuberculosis* antigens from antigen presenting cells (APCs) in the context of major histocompatibility class II-encoded molecules. The importance of CD4+ T cells in protective immunity to *M.tuberculosis* in humans is shown by increased susceptibility of HIV-infected people to infection with *M.tuberculosis* (104-106). Reactivation of TB infection in mice occured when CD4+ T cells were blocked using antibodies (107) and there was sustained growth of bacilli in the lungs and other organs, and poorer survival in mice without CD4+ T cells (102).

M.tuberculosis-specific CD8+ T cells have been reported in humans (108), and in some cases there were more *M.tuberculosis*-specific CD8+ T cells than *M.tuberculosis*-specific CD4+ T cells in some samples (109, 110). *M.tuberculosis* antigens are processed and presented to CD8+ T cells in the context of MHC class I. The importance of CD8+ T cells in anti-TB immunity is highlighted by susceptibility to TB in mice lacking functional CD8+ T cells due to a deficiency in β_2-microglobulin (111). CD8+ T cells are thought to play a greater role in the latent phase of the infection (112), although other studies report their importance during chronic infection (113). CD8+ T cells produce various cytokines, including IFN-γ and TNF-α (114) and are capable of directly killing infected macrophages (115). Granulysin and perforin produced by cytotoxic CD8+ T cells are effective anti-mycobacterial agents (116). Cytotoxic CD4+ T cells have also been reported, but with a different killing mechanism from CD8+ T cells (117). Differential expression of genes in CD4+ and CD8+ T cells in response to stimulation with *M.tuberculosis* has also been reported by Cliff *et al*. (118). Recently, cytolytic or suppressive HLA-E restricted CD8+ T cells that express GATA3, secrete Th2 cytokines including IL-4, IL-5, IL-13, and are capable of B-cell help have been

identified. These cells were able to inhibit growth of *M.tuberculosis* in infected macrophages (119).

Non-conventional T cells that recognize lipids, modified peptides and small-molecule metabolites have previously been reported. These include CD1-restrcited mucosal-associated invariant T (MAIT), natural killer T (NKT), gamma delta T, and germ-line encoded mycolyl-reactive T cells (120-122). These cells have innate cell-like properties and have been demonstrated to have anti-mycobacterial properties (123-126).

1.2.3. Cytokines and chemokines involved in immunity to *M.tuberculosis*

Cytokines play important roles in immunity to mycobacteria. In addition to TNF-α, IL-1α and IL-1β are required for protective immunity against *M.tuberculosis* infection. Human IL-1β gene polymorphism studies and studies in animal models have been instrumental in unraveling the importance of this cytokine in anti-TB immunity (127-130). The requirement of both IL-1α and IL-1β in immunity to TB has been shown by Mayer-Barber and colleagues (131). There are reports of marked production of IL-1β by *M.tuberculosis*-infected macrophages. IL-1β has also been implicated in boosting of CD4+ T cell responses (132). The recruitment of inflammatory cells to *M.tuberculosis* granulomas is brought about by IL-1β (133-137).

The interleukin-1 receptor antagonist (IL-1Ra) binds competitively to IL-1 receptors thus acting as an anti-inflammatory chemokine. The role of IL-1Ra in impairing of IL-4 and IgE responses (compared to increased IFN-γ and IgG2a) has previously been reported in the mouse (138). IL-1Ra is further reported to play a role in delayed-type hypersensitivity and TB disease in humans (127).

Cytokines produced by activated T cells are essential in the fight against *M.tuberculosis* infection (139), and among these are IFN-γ, IL-12, TNF-α and IL-2. T cells are able to elicit *M.tuberculosis*-specific immune responses, before establishment of memory (140).

One important feature of immunity to TB is the delay in the development of detectable adaptive responses, which in humans takes 5-6 weeks after infection (141, 142), and in

mice it takes up to 12 days (143). *M.tuberculosis* is also known to impair antigen processing and the initial priming of naïve T cells (144). *M.tuberculosis* is able to induce immunomodulation or immunosuppression by inducing production of IL-10 and TGF-β, two cytokines with suppressive effects on T cells (145-147). More work is needed to understand the initiation of adaptive responses to *M.tuberculosis*.
Alveolar macrophages are activated by IFN-γ and TNF-α to induce production of iNOS and RNI that are important for killing *M.tuberculosis* (58). The importance of these two cytokines, including IL-12 and IL-6, in anti-TB immunity was shown in studies where animals were treated with cytokines and then infected; use of anti-cytokine antibodies; and in animals in which key genes were knocked out (58, 139, 148-153). Individuals with defects in the IFN-γ or IL-12 receptor genes were susceptible to *M.tuberculosis* infection (154-158). IL-12p40 secreted by DCs is important for anti-mycobacterial Th1 responses (159, 160).

Th2 immunity, induced by IL-4, is thought to oppose protective Th1 responses to TB (161). Th2 activation of macrophages results in alternatively activated phenotypes that are less potent in their antimicrobial activity (162). Intracellular killing of *M.tuberculosis* through the process of autophagy is reportedly impaired in a Th2 environment (163). However, Th2-like CD8+ T cells with anti-mycobacterial properties have recently been identified (119).

Little is known about the role for B cells in the control of *M.tuberculosis* infection. The roles for B cells and antibodies in anti-TB immune responses has recently been discussed, including calls for further studies to understand the mechanisms involved (164, 165). Antigen-specific antibodies produced by plasma cells can influence activities of phagocytic cells, as well as processes involving antibody-dependent cellular cytotoxicity. The receptors involved in the interaction between the host and pathogen may also be affected, thus influencing the activities of other immune cells (166, 167). In a recent study, antibodies from latently infected persons have been shown to have distinct properties from antibodies from active TB patients, including glycosylation patterns, antibody Fc properties and FcγRIII binding. These antibodies were able to increase antimicrobial activities and reduce survival of bacilli when applied to human macrophages infected with *M.tuberculosis* (168). B cells also play an antigen-presenting role, with specific effects on proliferation of T cells (169). There have been reports of

changes in the local T cell and cytokine responses, increased bacterial load and impaired inflammatory responses in granulomas of nonhuman primates with depleted B cells, highlighting the importance of B cells in anti-mycobacterial immune responses (170). Activated B cells, plasma cells and antibodies within granulomas have previously been reported (171). A study by Ashenafi and colleagues demonstrated that patients with active TB, compared to latently infected or controls, had higher proportions of IgG-expressing plasmablasts, showing differences in antibody production in humans with disease (172). Differential B-cell phenotypes in latent and active *M.tuberculosis* infection, as well as induction, by BCG, of long-lived mycobacteria-specific memory B-cells in healthy individuals has previously been reported in our setting (173, 174). The modulation of genes associated with B-cells has also been reported during TB treatment (175). B cells can also act as a source of pro- and anti-inflammatory cytokines (176). Recently, IFN-γ -expressing B cells have been described, further highlighting the importance of these cells in immune response to infections through activation of macrophages (177). TLRs are also expressed by B cells (178). In a recent study by Fletcher and colleagues, IgG to Ag85A antigen correlated with a reduced risk of TB disease in infants (179).

M.tuberculosis also elicits a variety of chemokines (180). Th1 cells are recruited to the site of infection by inflammatory chemokines such as IP-10 (CXCL10), whereas MIP-1α (CCL3) and MIP-1β (CCL4) are thought to prime cells towards a Th2 phenotype (181, 182). Differential expression in the lungs of active pulmonary TB patients of chemokines and regulatory protein has previously been reported (183). Other chemokines, such as MCP-1, also play important anti-mycobacterial roles. Studies that looked at mutations in MCP-1 and susceptibility to TB have highlighted the importance of this chemokine in TB infection (184).

IL-8 (CXCL8) enhances the phagocytic and killing capabilities of immune cells (185) and acts as a chemoattractant and activator of lymphocytes during granuloma formation. Infected monocytes or macrophages, neutrophils and epithelial cells are thought to be the sources of IL-8 (74, 135, 186-188). Recently, production of IL-8 by neonatal T cells has been demonstrated as an anti-microbial effector mechanism (189).
The growth factor GM-CSF acts to promote T cell responses in *M.tuberculosis* infection (190) and plays a pivotal role in the differentiation of alveolar macrophages ((191-193).

GM-CSF is also important in the initiation of cellular responses and granuloma formation in *M.tuberculosis*-infected mice (194, 195). Invariant NKT (iNKT) cells are among the cells that produce GM-CSF with anti-mycobacterial properties (196)

1.3. Correlates of protective immunity to tuberculosis

Many studies aimed at understanding correlates of protective immunity to TB have been reported, but this knowledge is still limited, yet it is needed for evaluation of new tuberculosis vaccines (197). The production of IFN-γ by activated CD4+ T cells has for long been used as a measure of an effective immune response to TB (58). However, other studies have shown this immunity to be insufficient (198) or not correlated with protection (199, 200), stressing how complex immunity to TB disease is. In a recent study by Fletcher and colleagues, IFN-γ -secreting T cells specific for BCG correlated with a reduced risk of TB disease in infants (179).

Polyfunctional T cells that produce IFN-γ, TNF-α and IL-2 have been considered important T-cell based biomarkers (123). High proportions of polyfunctional T cells have been associated with protection against *M.tuberculosis* infection in vaccinated mice (125, 201, 202). However, such associations have not been demonstrated in humans where the patterns of cytokine production did not correlate with anti-TB protection (199, 200), but a recent report from a study in the UK shows that polyfunctional T cells are associated with inhibition of mycobacterial growth *in vitro* in infants vaccinated with BCG (203). A study of human AdHu5Ag85A in BCG vaccinated adults showed enhanced expression of polyfunctional CD4+ and CD8+ T cells (204). Re-assessment of the use of IFN-γ as a correlate of protection and the role of other cell types in protection against TB has therefore been called for (205). CD4+ T cells that produce IL-17 or IL-22 are thought to also play a role in anti-mycobacterial immune responses (206). Activated CD4+ T cells expressing HLA-DR have also been shown recently to be associated with risk of TB disease (179).

1.4. Infant responses to BCG immunisation

BCG is the only vaccine against TB currently available (207, 208). BCG has been used for almost a century, with its inclusion in the Expanded Programme on Immunisation (EPI) of WHO in 1974. It is the most widely used vaccine with a global annual estimate of more than 120 million doses (209, 210). There are global differences in BCG policies and practices, with some countries vaccinating everyone and others restricting it to groups at high risk for TB (211). However, there are reports of high mortality due to disseminated BCG disease in infants infected with HIV (212-214), and this is the reason why WHO changed its recommendations on use of BCG in infants known to be infected

with HIV, and to delay BCG immunisation for HIV-exposed infants until such a time that their HIV infection status is ascertained (214). In Uganda, BCG vaccine is administered to newborns at birth, in accordance with WHO recommendations for a TB endemic area (214), though a study on the timing and coverage of EPI vaccines in low- and middle-income countries reports that up to 46% of infants do not receive BCG immunisation until after 10 weeks of birth (215).

The infant immune system is "immature" and this would be disadvantageous for vaccines given at birth since the induced immune response would not be adequate. However, BCG is efficacious against the disseminated form of TB disease in children (216-218). Age-related changes in infant immune responses have previously been reported (219-222); for example infants have been shown to have poor immune responses to polysaccharide antigens (223).

Several studies show that BCG immunisation of infants produces a measurable immune response. Infants have been shown to generate BCG-induced cytokine-expressing T cells of the same magnitude as adults, though with a bias towards polyfunctional cells (224). Infants have been shown to generate Th1 responses to mycobacterial antigens following BCG immunisation (225).

Studies in humans and mice have shown induction of effector T cells following BCG immunisation (226-228), and effector T cells, compared to memory T cells, are short-lived (229). However, the induction of memory T cells in infants and adults after BCG immunisation has also been reported (230, 231). Memory T cells are of two main types: central memory T cells that are found in the secondary lymphoid tissues and blood; and effector memory T cells found in peripheral tissues and the blood and the spleen (229, 232, 233), although there are also distinct tissue-resident memory T cells (233, 234).

In a recent study, clearly different patterns of response to BCG were observed in infants immunised with BCG, though there was no evidence of correlates of risk of TB disease (235). The induction of granulysin and perforin expression at 10 weeks of age in infants immunised with BCG at birth has been demonstrated (236). In a study in South African children, T-cell responses to mycobacterial antigens were reported following BCG immunisation (237). The complexity of BCG-induced immunity has further been

demonstrated by the differential expression of immune genes following BCG immunisation of infants (238). In a recent study in Copenhagen, BCG vaccine-related severe adverse reactions and BCG-osis were not observed (239).

The sequence in which infant vaccines are given has been a hot topic for discussion recently. Several studies have been conducted, each giving contrasting results. Th1 and Th2 responses to unrelated childhood vaccines have been shown to be enhanced by BCG immunisation (225, 240). Antibody responses to OPV and HBV were also increased in BCG immunised infants (240). Fewer deaths and hospital admissions were registered when OPV alone was given to children in Bissau, compared to children who received both OPV and DPT at the same time (241). Impaired Th1 responses, but enhanced humoral responses, have been reported when neonates were administred oral polio vaccine (242). This has implications for the control of pathogens where Th1 responses are required. In a Danish study, OPV was associated with fewer hospital admissions (243). In another study in Bissau, co-administration of OPV and BCG was associated with reduced infant responses to PPD (244, 245). Girls, compared to boys, have a reported high mortality when OPV was missed at birth (246).

Several studies have looked at differences in infant responses when BCG is administered at birth or when BCG vaccination is delayed, with contradictory results. A study in Uganda demonstrated reduced capacity to elicit IFN-γ-producing T cells and polyfunctional cells producing IFN-γ, TNF-α and IL-2 in infants where BCG immunisation was delayed to 6 weeks, compared to those that were vaccinated at birth (247). In a study in the Gambia, there was a report of reduced BCG-specific cytokine responses (IFN-γ, IL-6, IL-17) *in vitro* in infants where BCG immunisation was delayed (248). However, a study by Kagina and colleagues demonstrated increased proportions of BCG-induced T cells when BCG immunisation is delayed (226). A recent study by Ritz *et al*. in Australia did not show any differences in responses between infants vaccinated at birth and those where BCG immunisation was delayed (249). In another study, antibody responses to infant vaccine antigens were not affected by the timing of BCG immunisation (250).

There is little information about the peak immune response in infants following BCG immunisation, yet this knowledge is important for the design and use of vaccines aimed

at boosting immunity primed by BCG (251). An established memory population (after the peak effector phases) would be suitable to boost in a prime-boost vaccine strategy. This has been highlighted by studies on viral infection models involving T cells (252-254). It is thought that T cells may be less effective or prone to cell death if boosted during the primary effector phase (252, 255, 256). A peak in response between 6 and 10 weeks has been shown for CD4+ T cell responses following BCG immunisation (220).

Several strains of BCG vaccine have been in use globally and these include genetically diverse strains such as Danish 1331, Pasteur 1173, Tokyo 172, Russian and Moreau (257-259). UNICEF supplies the most common strains in use in developing countries and these include BCG-Denmark, BCG-Japan and BCG-Bulgaria. The influence of BCG strains on infant responses is discussed below.

1.5. Heterologous effects of BCG immunisation

Evidence that BCG immunisation may influence innate responses includes findings in both observational studies and randomized controlled trials that have highlighted the heterologous effects of BCG on childhood survival in both low- and high-income countries (260-264). This has been suggested to be due to BCG-induced increases in function of the innate immune system, a phenomenon now termed 'trained immunity' (265-269). This is an observation of great global health significance, since mortality due to infectious agents other than TB is high in developing tropical countries (270). However, a recent study did not observe beneficial heterologous effects of BCG immunisation on childhood infection (271). Also, a systematic review of available literature did not find sufficient evidence of non-specific immunological effects following BCG immunisation (272). The data showing heterologous effects mostly has come from West Africa, with potential biases in the observation that could affect the findings, and there were methodological and epidemiological issues with some of them, with infants receiving vaccine or no vaccine based on vaccine availability, and thus not a proper randomised trial, and with the possibility of confounding. Many of the effects were also seen in low birth weight infants. If non-specific effects of some vaccines truly exist, these would be heterogeneous, and may differ between populations (273).

1.6. BCG immunisation scars

The presence or absence of a scar has for long been used as a sign of previous immunisation with BCG, in place of or in addition to vaccination records (274-276). From 52% to 97% of newborns immunized with BCG develop a scar, with strain of BCG vaccine used, the administrator and age of administration accounting for the differences observed (274, 277-280). However, not all BCG vaccinated babies will scar. Previous studies have shown a correlation between the presence of a scar and protection against TB (281, 282), and infants with a BCG scar have been shown to have better survival with fewer respiratory infections (278, 283, 284), fewer skin infections and sepsis (285).

Little is known about the link between the development of a BCG scar in mothers and immune responses in infants. Maternal BCG scar has been shown to be associated with lower T helper (Th) 2 responses to crude culture filtrate proteins of mycobacteria in the infants in one of our studies (286). Effects of maternal BCG scar on innate immune responses in infants are not known.

1.7. Latent *M.tuberculosis* infection

The infected host and mycobacteria are in a dynamic relationship resulting in a broad spectrum of responses and outcomes (64, 65). The baccili may therefore be in an inactive form in latently infected individuals or are actively replicating without resulting in clinical disease (64).

We therefore proposed the hypothesis that maternal latent *M. tuberculosis* infection (LTBI) influences the neonatal response to BCG (and to *M. tuberculosis*), rendering the response to BCG less effective, and susceptibility to tuberculosis greater. Maternal latent infection with *M. tuberculosis* might lead to exposure to mycobacterial antigens *in utero* and the development of a modified profile of response to mycobacteria after birth – involving either sensitisation (287), or the induction of tolerance (288, 289) in the fetus.

In Uganda, where the annual incidence of infection is estimated at 3%, up to 60% of young women of childbearing age are likely to be infected with *M. tuberculosis*. Latent maternal infection with *M. tuberculosis* is likely to have a more important effect on the

infant than for example a remote history of maternal immunisation with the BCG vaccine.

1.8. Clinical trials and efficacy of BCG vaccination

There have been a number of efficacy trials and epidemiological studies carried out since BCG was introduced in the 1920s, with variable results. The results for severe extrapulmonary forms of TB in children (TB meningitis and miliary disease) show that BCG provides between 60-80% protective efficacy (216-218). For pulmonary TB in adults and adolescents, the efficacy of BCG vaccine ranges between 0-80% (290). Several hypotheses have been put forward to explain the variability in efficacy of BCG vaccine and these include the following: first, the genetically diverse strains of BCG vaccine used in the different trial sites. BCG strains have been passaged many times and sub-strains prepared resulting in the use of different strains. Human and animal studies have demonstrated differences in BCG-induced responses with strains of the BCG vaccine used (277, 280, 291, 292). However, it is difficult to relate the observed differences in immune responses to the variability in protection from BCG. Meta-analyses of current research evidence shows that BCG strains are not associated with lack of efficacy of BCG vaccine (293, 294). It is not possible for strain differences alone to account for the observed differences in efficacy, though it may contribute somewhat to the differences observed (290, 293, 295). Second, there have been differences in dose and route of administration of BCG vaccine with time and trial locations. Initially administered as an oral vaccine, BCG is now given through the intradermal route. However, no differences in efficacy were reported for studies where BCG was administered intradermally versus percutaneously in a study in South Africa (296). Third, meta-analyses of trials of BCG immunisation indicate that latitude is a significant factor in the protection achieved in adolescents and adults (290, 293, 294, 297). One suggested explanation for the variability in BCG efficacy, and its relationship to latitude, is that of helminth infection, which is widespread in the tropics and has an influence on immune responses, both impairing the response to BCG immunisation and increasing susceptibility to TB itself (298). An extension of this hypothesis is that *in utero* exposure to maternal helminth infection may influence the neonatal response to BCG given at birth. This idea is supported by a finding in Kenya where infant responses to BCG were skewed towards a Th2 profile if they were sensitised *in utero* to

schistosome or filarial antigens (299). This hypothesis has been further explored in a study in Uganda (300), with results showing that maternal helminth infections do not have a major effect on responses to BCG immunisation (301). Another hypothesis is that sensitisation to NTM, which is more common in lower latitudes (302), alters the protection induced by BCG (303). Induction of a protective effect by BCG might be blocked by exposure to NTM, or an equivalent protection to BCG might be generated, obscuring or masking the benefit provided by BCG (304-306). A recent meta-analysis of the literature shows that previous exposure to NTM or to *M.tuberculosis* is associated with lower efficacy of BCG (294). A study in mice showed that oral exposure to NTM (*M. avium*) after BCG immunisation also reduced BCG-induced protective immunity (307). The neonatal response to BCG given at birth may be influenced by *in utero* exposure to maternal *M.tuberculosis* or NTM infection. Maternal BCG immunisation or exposure to NTM, or exposure to *M. tuberculosis* are possible ways through which mothers can get exposed to mycobacteria, resulting in infant exposure to mycobacterial antigens *in utero.* This may be associated with sensitisation (287), or the induction of tolerance (as reported for maternal helminths) (288, 289). However, in a recent study, there were no associations between maternal *M.tuberculosis* and infant responses (308), although the study design was unable to conclusively rule in or out *M.tuberculosis* exposure, and so the conclusions of the study are not as powerful as they otherwise might have been.

1.9. Tuberculosis vacines under development

New and effective vaccines against TB are urgently needed due to the slow reduction in the incidence of TB worldwide and the emergence of drug-resistant strains of the bacilli. The current TB candidate vaccines being developed (Table 1.3) (1, 309) are targeted at boosting responses to BCG (subunit vaccines and whole-mycobacterial-cell) or replacing BCG (whole-mycobacterial-cell). These vaccines are whole-mycobacterial-cell-derived and viral vectored or adjuvanted protein subunit vaccines aimed at improved protection by preventing infection, progression to disease at the initial stages, or latent TB reactivation (5, 309) and include: The subunit vaccine, MVA85A, is a live attenuated vaccinia vectored vaccine candidate expressing an immunogenic antigen (Ag85A) of *M.tuberculosis.* It has recently been intensively evaluated in infants and adults. A study by McShane *et al.* showed that this vaccine was able to boost responses

in volunteers who had previously received BCG (310). In other trials, the vaccine showed no efficacy against *M.tuberculosis* infection or TB disease, but was immunogenic and well tolerated in infants (200) and adults (311). The failure to show protective efficacy was associated with a slight increase in incidence of TB disease, and the vaccine did induce memory T cell responses, although these may not have been sufficient in frequency. MVA85A has been shown to induce increased inflammatory responses in South African children a day after immunisation (312). The induction of long-lasting T cell responses by MVA85A in persons infected with HIV has been shown in another study in South Africa (313). Further studies are now investigating delivering MVA85A by the aerosol route. More data from the MVA85A trial shows potential benefit of antibodies and negative effect of activated CD4+ T cells (179), and that QuantFERON conversion rates with high cytokine levels suggest increased susceptibility to disease (314). Other viral vectored vaccines include Ad5Ag85A, ChAdOx185A and TB/FLU-04L all expressing antigen 85A (Ag85A) from *M.tuberculosis*. Another vaccine that shows promise in terms of safety and immunogenicity is the AERAS-402 vaccine. This is an adenovirus 35-vectored infant TB vaccine candidate that is administered as a boost following a prime with BCG. There was a dose-dependent response in BCG immunised infants when AERAS-402 was given to them (315). Adjuvanted protein subunit vaccines developed with the aim of boosting BCG-primed immune responses include M72: AS01E, H4: IC31, H1: IC31, H56: IC31 and ID93: GLA-SE. Another vaccine candidate designed to replace BCG in infants and to prevent recurrent TB disease in adults following successful completion of treatment for active pulmonary TB is VPM1002. It is the only recombinant BCG vaccine candidate currently in clinical trials with an insertion of a gene for listeriolysin and the deletion of urease gene in the BCG DNA. MTBVAC, another candidate vaccine, is the only whole cell candidate derived from *M.tuberculosis.* It is being developed as a BCG replacement vaccine in infants and is in phase II trials. Another vaccine candidate DAR-901, like *M. vaccae* (*Vaccae*™, AnHui Longcom Biologic Pharmacy Co., Ltd [Longcom], Beijing, China), is a heat-inactivated whole cell, NTM. RUTI® (Archivel Farma, Barcelona, Spain) has been developed for immunotherapeutic use in active TB patients.

Table 1.3. Tuberculosis vaccines in the pipeline

Phase I	Phase IIa	Phase IIb	Phase III
MTBVAC TBVI, Zaragoza, Biofabri	DAR-901 Dartmouth	VPM1002 Max Planck, VPM, TBVI, SII	*Vaccae*™ Anhui Zhifel Longcom
Ad5Ag85A McMaster, CanSino	RUTI Archivel Farma, S.L	M72+ AS01E GSK, Aeras	
ChAdOx1.85A/ MVA85A Oxford, Birmingham	H1/H56: IC31 SSI, Valneva, Aeras		
MVA85A/ MVA85A (ID, Aerosol) Oxford	H4: IC31 SSI, Sanof Pasteur, Aeras		
TB/FLU-04L RIBSP	ID93+GLA-SE IDRI, Aeras, Wellcome Trust		

Source: Global tuberculosis report, 2016

We still do not know when the peak of the response to BCG immunisation is, and we still have no correlate of protection against TB. We also do not know the influence NTM or maternal LTBI have on effectiveness of BCG.

1.10. Hypothesis and objectives of the study

The work in this thesis was designed to test the hypothesis that maternal infection with *M.tuberculosis* is associated with an impaired infant response to BCG immunisation. The objectives of the study included:

1. To investigate innate immune responses and gene expression profiles in infants.
2. To assess the time course of BCG-induced priming of the infant response, and the establishment of the peak in response after BCG immunisation.
3. To determine whether maternal LTBI and maternal BCG scar influence the response to BCG immunisation in infancy.

Chapter 2
Materials and Methods

2.1. The pilot infant BCG study

2.1.1. Study design

The pilot study was an exploratory investigation in a relatively small number of subjects. The number of infants included in the study was chosen to be feasible within the time frame and resources available, and analyses were restricted to infants who had relevant results at all time points. This study was designed to generate pilot data for the hypothesis that maternal infection with *M.tuberculosis* influences infant responses to BCG immunisation.

2.1.2. Study setting

The study was conducted at Entebbe General Hospital where the Co-infection Studies Programme (CiSP) has a long-established collaboration with the maternity department. Mothers residing within Entebbe Municipality and Katabi sub-county were recruited (Figure 2.1).

2.1.3. The Co-infection Studies Programme

The CiSP developed from an interest in the immunomodulating effects of chronic helminth infection, and the impact of such effects on major infectious diseases including TB, malaria and HIV infection. It has evolved to encompass interactions between infectious and non-communicable diseases (NCDs). The aim is to investigate the epidemiological and immunological interactions between chronic, immunomodulating infections and both NCDs and infectious disease outcomes. This pilot study was embedded within the Programme as a separate study.

2.1.4. Ethical considerations

Ethical approval was given for this study by the Uganda Virus Research Institute (UVRI) Research Ethics Commitee (Appendix A), Uganda National Council for Science and Technology (UNCST) (Appendix B) and the London Schoool of Hygiene & Tropical Medicine (LSHTM) (Appendix C).

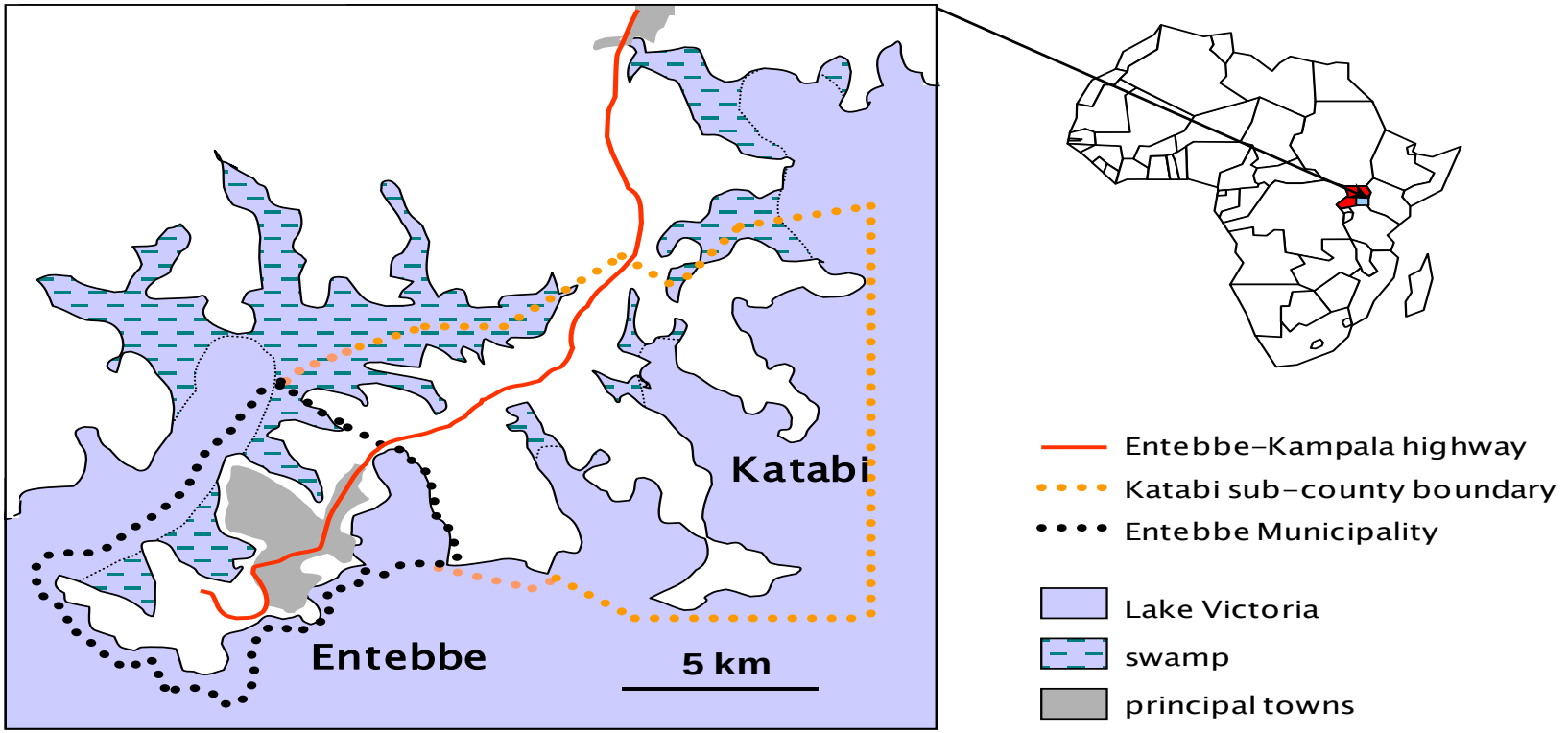


Figure 2.1. Map showing the study setting.

2.1.5. Recruitment procedures

2.1.5.1. Consent process

Information about the study was made available at the antenatal clinics, so that mothers were aware of the study before they came for delivery (Appendices D and M). Women were counseled regarding the study by a maternity ward midwife. On presentation in early labour, mothers were given a detailed explanation of the study and asked to provide consent for participation, collection of cord blood and follow up procedures for their infants and for access to their clinical records (Appendices E and N). The timing of the consent procedure was considered carefully. Consent at antenatal clinics was not feasible for this study because of the likely time delay and loss to follow up. A two-stage procedure with limited consent during labour (for collection of cord blood) and further consent for follow up procedures after delivery was considered. Advice was sought from colleagues at Mulago Hospital, Kampala, who were undertaking a study requiring placental blood and cord sampling, and we were advised that they had been able to obtain full consent satisfactorily in early labour. This was therefore the procedure used for this study. At follow up visits (and especially on the day after delivery and at the one week visit) study staff confirmed that the mother had understood the study and its requirements, before further procedures were conducted.

2.1.5.2. Inclusion and exclusion criteria

Women delivering in the hospital were eligible for inclusion, and approached for consent if they were willing to participate in the study, they had a normal singleton pregnancy, they resided in Entebbe Municipality and Katabi sub-county, and they were HIV negative (based on records available for HIV testing performed during antenatal care for this pregnancy).

Neonates were excluded if cord blood was not obtained, the delivery was not normal, the mother was unwilling to undergo a repeat HIV test or was found to be HIV positive on repeat testing, birth weight was below 2500g, the neonate was clinically unwell, as judged by the midwife, the mother had an indeterminate TB infection status (as described below), or the neonate presented with significant congenital abnormalities likely to impair the child's general health, growth and development, as judged by the attending midwife. Minor abnormalities such as birthmarks and extra digits, did not

constitute exclusion from the study. Copies of eligibility forms are in Appendices F and G.

2.1.5.3. Procedures at the maternity ward

A cord blood sample was collected from the umbilical vein by needle and syringe, after the cord had been clamped, from the placental side of the cord.

A questionnaire was completed regarding demographic and socio-economic characteristics and clinical history (including personal or family history of TB, and current symptoms) (Appendix H). Characteristics during pregnancy were obtained from the antenatal and delivery records. BCG immunisation was given to the neonate at birth or soon afterwards. A single batch of the BCG vaccine, BCG-Russia (BCG-1 Moscow strain, Serum Institute of India, India) was administered intradermally for all infants.

2.1.6. Procedures at the CiSP clinic

Mothers were asked to return to the clinic one week after delivery. At this time, maternal blood (15 ml) was drawn for immunological studies and for investigation of LTBI (see below) and participants were evaluated by the clinical team.

2.1.6.1. Tests for latent TB infection

2.1.6.2. Tuberculin skin testing

Women were investigated for LTBI using the tuberculin skin test (TST) or Mantoux test involving injection of 0.1 ml (2 TU) of *M. tuberculosis* PPD RT 23 (Statens Serum Institut, Copenhagen, Denmark) intradermally on the volar aspect of the forearm, midway between the elbow and wrist joints using a 27G syringe. The injection site was marked with a waterproof pen, and the date and time noted. Mothers were advised to neither scratch nor apply any soap or chemical solution to the site. Trained research nurses interpreted results 48 to 72 hours later using the ballpoint pen method. Mothers with a TST induration equal to, or more than, 10mm were classed as positive as induration of this size is less likely to be due to exposure to NTM (316). Tuberculin skin testing was performed in the mother after the mother's blood sample had been obtained.

2.1.6.3. T-SPOT.TB assay

In addition to the TST test, maternal LTBI was further investigated using the T-SPOT.TB assay (Oxford Immunotec, Abingdon, UK). This modification of the ELISpot assay examines IFN-γ responses to peptide pools from *M.tuberculosis* -specific antigens (6 kDa Early Secreted Antigenic Target of *M.tuberculosis* (ESAT-6) and 10 kDa culture filtrate protein (CFP10)) as is described in detail elsewhere (317). Briefly, PBMCs purified from the mother's blood collected one week after delivery were stimulated for 16-20 hours at 37°C in 5% CO_2. Medium was used as a negative control and phytohaemagglutinin (PHA) served as a positive control. The release of IFN-γ by stimulated cells was counted as spots using an ELISpot plate reader (AID, Strasberg, Germany). A positive response to ESAT-6 and CFP-10 in the T-SPOT.TB was considered likely to represent *M. tuberculosis* infection in this setting, although a small number of other mycobacterial species do express these antigens (318-320).
A test was regarded as positive if the negative control (medium alone) had five or less spot forming units (SFUs), and the wells containing ESAT-6 (panel A) or CFP-10 (panel B) peptides had six or more SFUs. The positive control was expected to have more than 20 SFUs. If the positive control well had less than 20 SFUs or the negative control well had 10 or more spots, the test was regarded as indeterminate.

2.1.6.4. Definition of LTBI

Because the TSPOT.TB assay is a commercial kit, positivity is assessed according to the manufacturer's instructions. According to the manufacturer, the specificity of the TSPOT.TB assay is 100% with sensitivity of 98.8%, but in previous studies in Uganda we have observed discordance between TST and T-SPOT.TB (317). For this reason, and to minimise possible effects of exposure to NTM, we carried out both tests and enrolled participants who were either positive on both tests or negative on both tests. When mothers returned for their TST reading, all mothers with LTBI and a systematic sample (the second or the third) of uninfected mothers and their infants, were asked to continue in the study. Mothers with LTBI were investigated for active tuberculosis based on symptoms, sputum examination (if available) and chest x-ray.

A repeat HIV test was also performed using the standard rapid test algorithm (usually Determine (Inverness Medical, Tokyo, Japan) confirmed by HIV 1/2 STAT-PAK Dipstick test (Chembio Diagnostic Systems, Medford, NY, USA) with Uni-Gold HIV

test (Trinity Biotech plc, Bray, Ireland) as a tie-breaker. Mother-baby pairs were excluded if the mother was found to be HIV-positive.

2.1.6.5. Tests for helminth infection

Two stool samples from mothers were obtained on separate days. Stool samples were examined by trained staff of the Clinical Diagnostic Laboratory Services (CDLS) of MRC/UVRI Unit using the Kato-Katz method (321). Two Kato-Katz slides were prepared from each sample, each examined within 30 minutes for hookworm, and the following day for other parasites. Blood from mothers obtained at one week after delivery was examined for *Mansonella perstans* by a modified Knott's method (322). Intensity of infection was assessed by microfilaria counts in blood.

2.1.6.6. Sampling strategy

The original plan for the pilot study was to identify approximately 40 mothers with and 40 without LTBI, but 21 mothers with and 50 without LTBI were recruited because there were fewer infected mothers than expected and the study had a short recruitment period. The infants born to these mothers were followed up at one and six weeks after BCG immunisation. Two milliliters (2 ml) of blood was collected at each time point and processed within four hours. The follow up visits coincided with the EPI vaccine schedules of the Ministry of Health. Mothers were therefore advised to first bring the babies to the research clinic for study procedures before taking them to the immunisation clinic. The flow of the participants in the pilot infant BCG study and the numbers included for the various assays are illustrated in Figures 2.2 to 2.4.

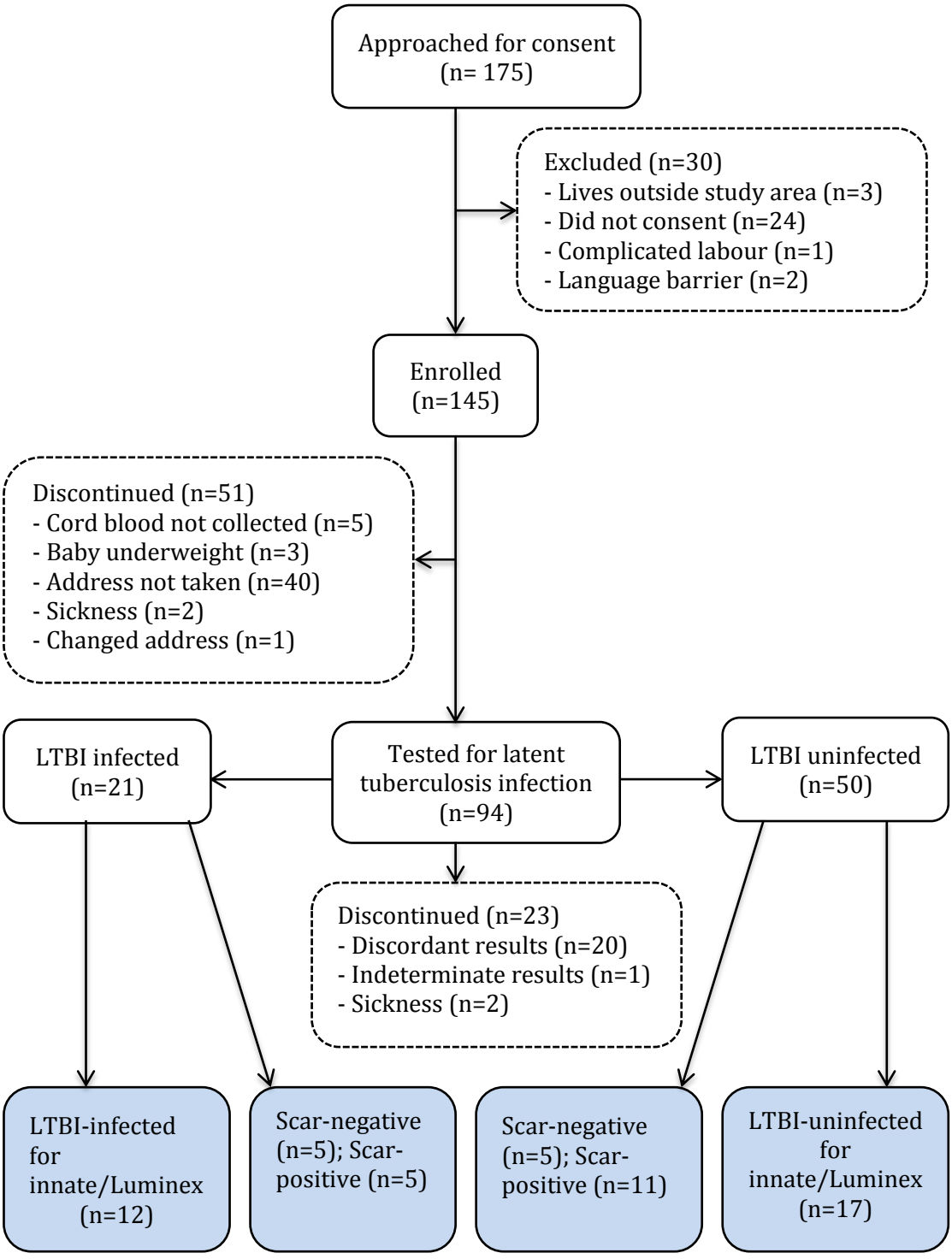


Figure 2.2. Flow of participants through the pilot infant BCG study for Luminex assays for innate responses

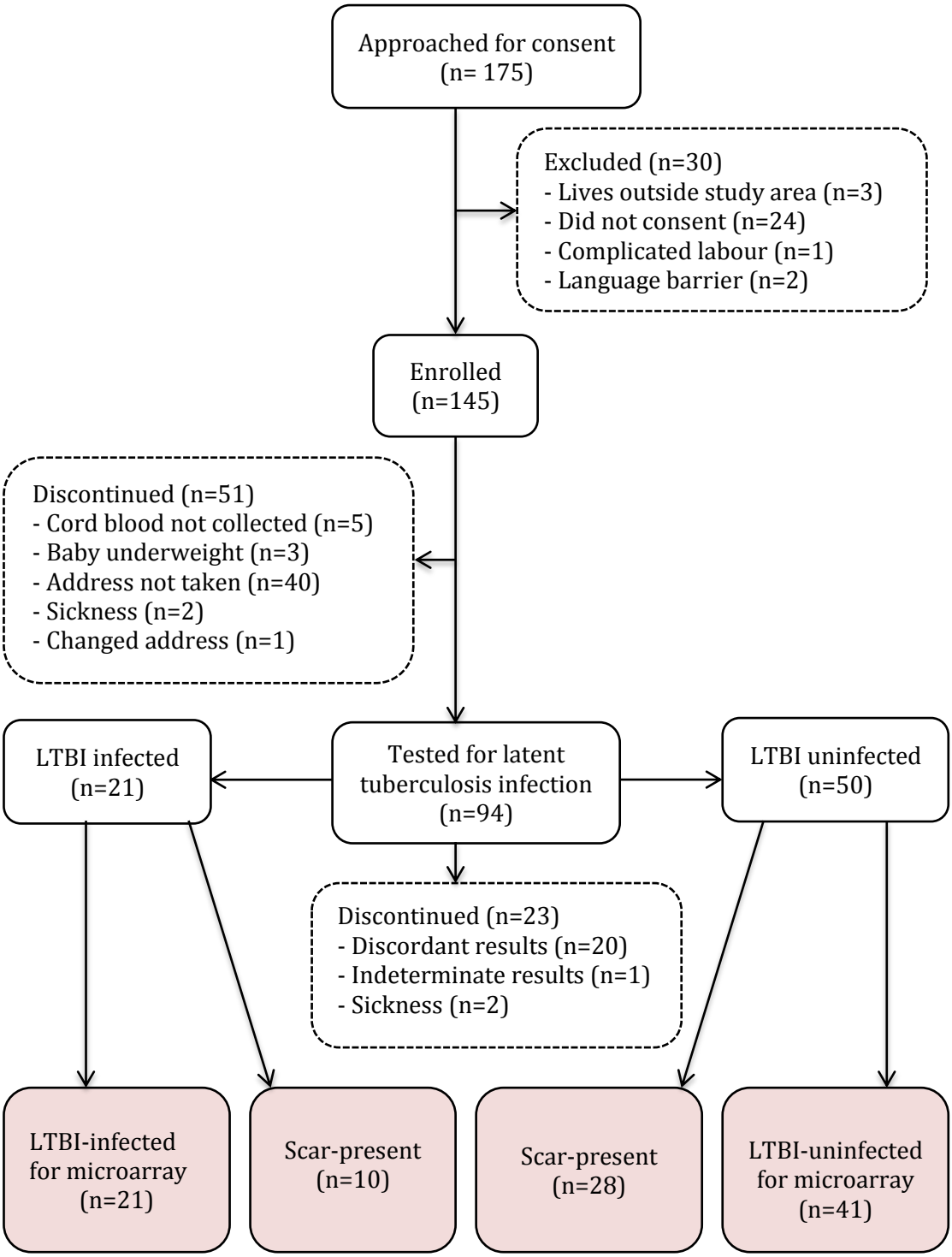


Figure 2.3. Flow of participants through the pilot infant BCG study for gene expression microarray.

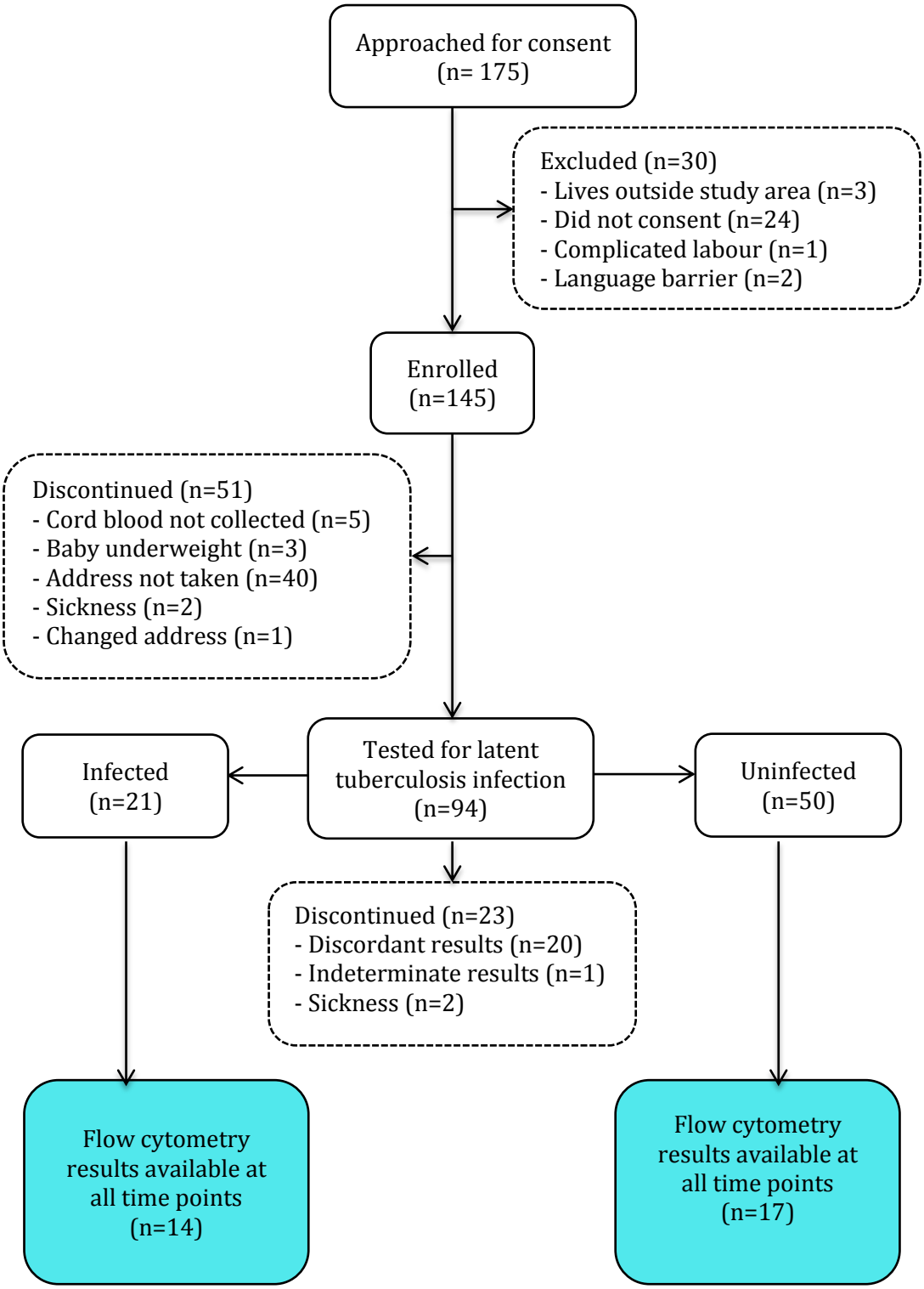


Figure 2.4. Flow of participants through the pilot infant BCG study for flow cytometry assay.

2.1.7. Laboratory procedures

2.1.7.1. Major equipment, supplies and reagents

The list of equipment, supplies and reagents is shown in Appendix I.

2.1.7.2. Antigens for 24-hour whole blood assay (WBA) for innate responses

- Lipopolysaccharide (LPS) (toll-like receptor (TLR) 4 agonist, 100ng/ml), the mycoplasma lipopeptide FSL-1 (TLR2/6 agonist, 50ng/ml), and the oligodeoxynucleotide CpG-ODN2006 (TLR9 agonist, 5μg/ml) all came from InvivoGen, San Diego, CA, USA.
- The synthetic triacylated lipopeptide PAM3Cys-Ser (TLR1/2 agonist; ECM Microcollections GmbH, Tubingen, Germany; 100ng/ml), Mannan (DC-SIGN agonist; Sigma-Aldrich; 100μg/ml), Curdlan (Dectin-1 agonist; Wako Chemicals GmbH, Neuss, Germany; 100μg/ml and Phytohaemaglutinin (PHA, Sigma-Aldrich, MO, USA) at 10μg/ml.

2.1.7.3. Antigens for 24-hour PBMC stimulation for flow cytometry

- RPMI 1640 medium (Life Technologies Corporation, NY, USA).
- PPD (RT 50 (for *in vitro* use), Statens Serum Institut (SSI), Copenhagen, Denmark) at 20μg/ml final concentration.
- Staphylococcal Enterotoxin B (SEB, Sigma-Aldrich, MO, USA) at 200ng/ml final concentration.

2.1.7.4. Antigens for antibody assays

- PPD (RT 50, SSI, Copenhagen, Denmark) at 10μg/ml final concentration.
- Tetanus toxoid (TT) (T155-1, SSI, Copenhagen, Denmark) at 12.12 Lf/ml final concentration.

2.1.7.5. Immunological techniques

- Luminex® assay for innate responses.
- Intracellular cytokine staining and flow cytometry for adaptive responses.
- ELISA for anti-PPD and anti-TT total IgG antibodies.
- Microarray on unstimulated infant blood samples.

2.1.7.6. Main outcome measures

- Cytokine and chemokine responses in 24-hour WBA stimulated with a panel of

Toll-like receptor ligands and analysed by 17-plex Luminex® assays.

- PPD-specific intracellular cytokine responses measured by flow cytometry.
- PPD-and TT-specific anti-TB antibody responses measured by ELISA.
- Gene expression profiles measured by microarray.

2.1.7.7. Innate immune responses measured using Luminex® assay

The flow of cord blood and maternal samples are shown in Figures 2.5 and 2.6.

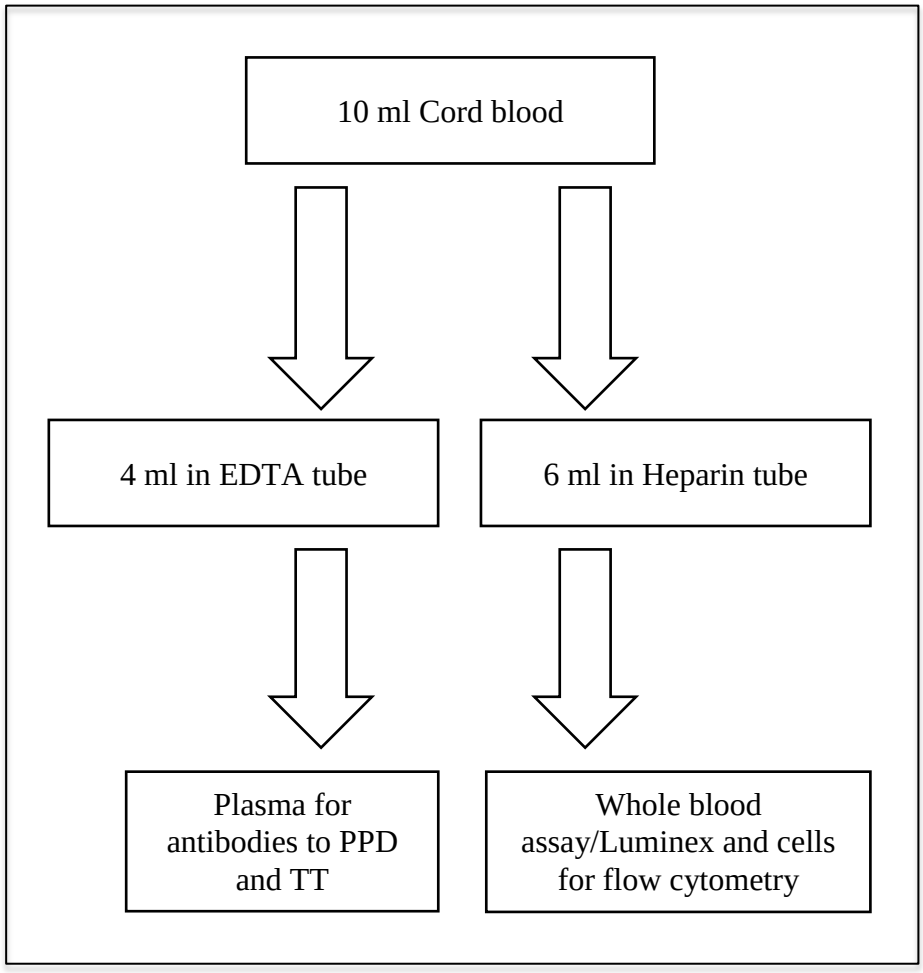


Figure 2.5. Flow of cord blood sample for separating plasma and for whole blood cultures.

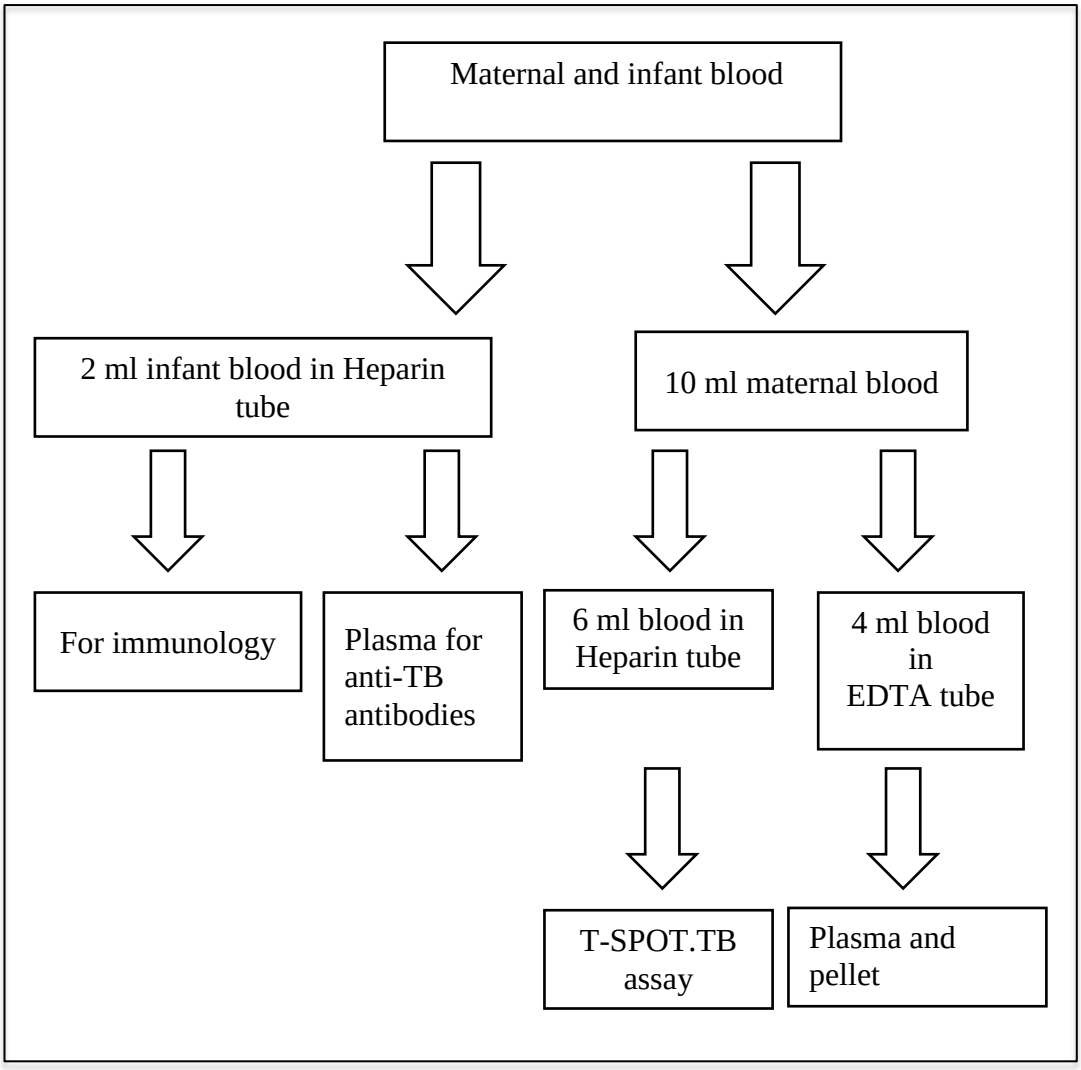


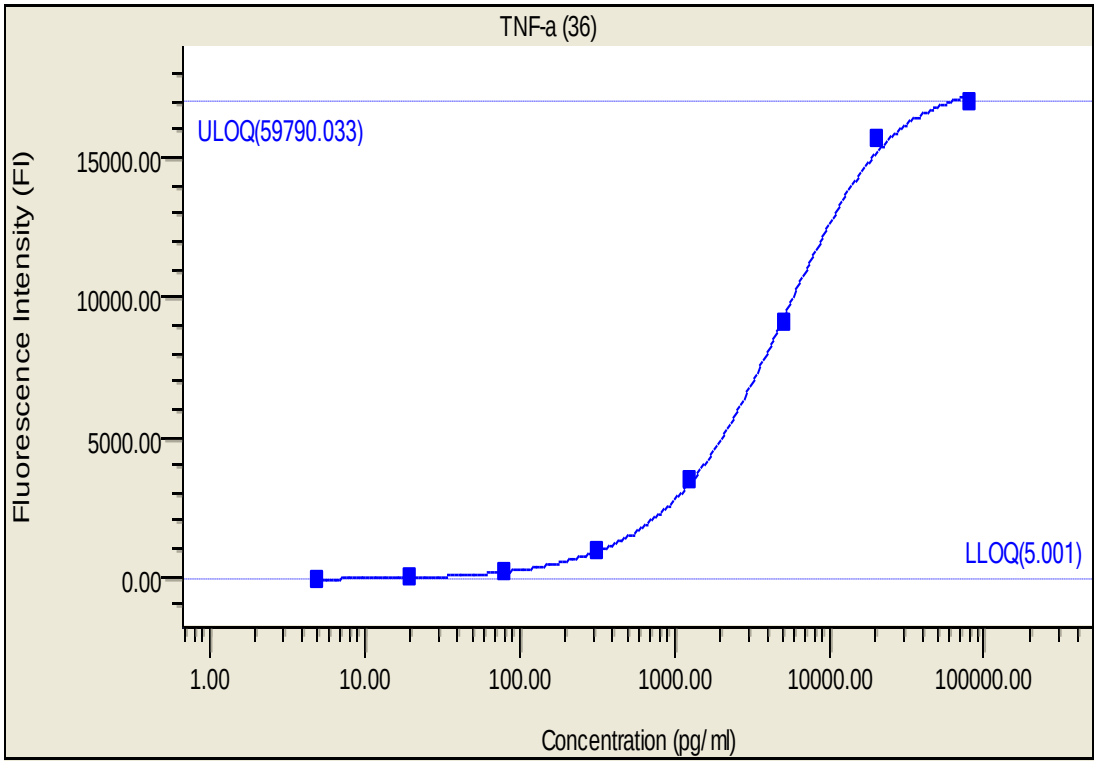
Figure 2.6. Flow of maternal and infant blood for T-SPOT.TB test on maternal blood, plasma separation for IgG measurement by ELISA and whole blood cultures for cytokine and chemokine measurement by Luminex® assay.

2.1.7.8. Stimulation of whole blood with TLR ligand

Heparinized blood from 29 mother-cord blood pairs were diluted 1:1 with RPMI 1640 medium and stimulated with LPS, FSL-1, PAM3Cys-Ser, Mannan, Curdlan and CpG-ODN2006. An unstimulated well was included to act as a negative control, and PHA served as a positive control. After 24 hours of incubation at 37°C in 5% CO_2, culture supernatants were harvested and stored at -80°C for analysis of cytokines and chemokines.

2.1.8. Measurement of cytokines and chemokines by multiplex assay system

The concentrations of analytes in the culture supernatants were measured using a Bioplex multiplex cytokine assay system (Bio-Rad Laboratories, Hercules, CA, USA), following instructions from the manufacturer. Briefly, 50µl of standard and the test samples along with 50µl of mixed beads were added in the wells of a 96-well microtitre plate. The plates were incubated for 1 hour on a rocker and washed; 25µl of detection antibody mixture was then added per well and the plates incubated for 30 minutes on a rocker. After washing with wash buffer, 50µl of streptavidin-PE was added per well and plates incubated for 10 minutes, washed and beads suspended in 75µl of assay buffer per well. The beads were analysed using a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) and the Bio-Plex Manager software (version 6.0; Bio-Rad Laboratories, Hercules, CA, USA) was used. According to the manufacturer's instructions, a curve fit was applied to standard curves (illustrated in Figure 2.7) that were then used to extract sample concentrations. Limits of the assay working range (lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ)) quoted by the manufacturer for each cytokine and chemokine were used to clean the data. For values below the acceptable range, half of the LLOQ was used and for values above the ULOQ, the ULOQ value for that particular analyte was used. The following analytes were assayed: interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, interferon (IFN)-γ, IFN-γ-induced protein-10 (IP-10/CXCL10), monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), tumour necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF).



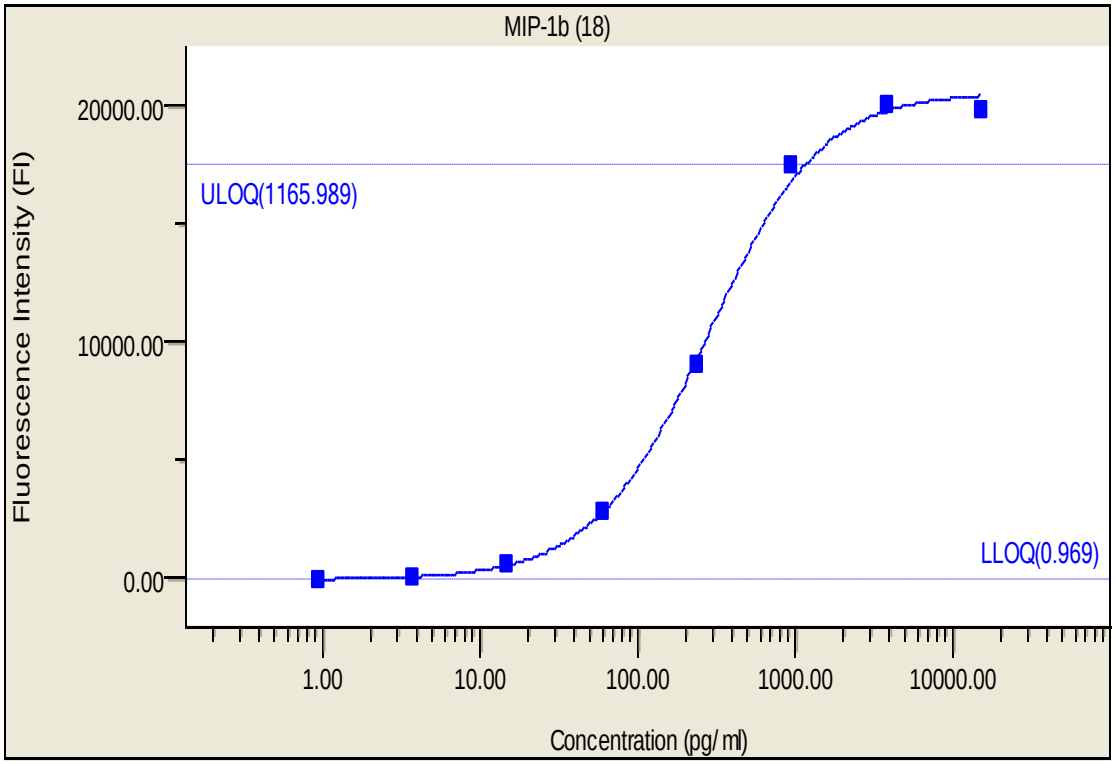


Figure 2.7. Example of standard curves from 17-Plex Luminex®. Curves for TNF-α (top panel) and MIP-1β (bottom panel) are shown. A 5PL curve fit was used.

2.1.9. Intracellular cytokine staining and flow cytometry

2.1.9.1. Separation of mononuclear cells

Mononuclear cells were isolated from cord blood (n=31), and from infant blood obtained at one (n=31) and six (n=31) weeks after birth by standard Ficoll-Paque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation, and cryopreserved in 50% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO), 40% RPMI 1640 medium (Life Technologies Corporation, NY, USA) containing 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate and HEPES and 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) according to standard protocols.

2.1.9.2. Intracellular cytokine staining assay

The cells were thawed and rested for 24 h at 37°C in 5% CO_2, viability and numbers were checked using a Cellometer Vision Cell Profiler (Nexcelom Bioscience, LLC-Lawrence, MA, USA). All thawed cells were stimulated with PPD (20 mg/ml batch RT 50 for *in vitro* use, Statens Serum Institut, Copenhagen, Denmark). Medium alone was used as a negative control, and Staphylococcal Enterotoxin B (SEB, 200 ng/ml, Sigma-Aldrich) served as a positive control. The co-stimulatory antibodies anti-CD28 and anti-CD49d (1 mg/ml, BD Biosciences, San Jose, CA, USA) were included in all conditions. The cells were incubated at 37°C in 5% CO_2 for a total of 24 h. Brefeldin A (10 mg/ml, Sigma-Aldrich) was added to SEB wells after 2 h, and to PPD and negative control wells after 20 h. The cells were then stained with aqua viability dye (Life Technologies, OR, USA), and antibodies directed against the following human molecules: CD3 (QDot655, S4.1) and CD4 (QDot605, S3.50), both obtained from Life Technologies, OR, USA; CD8 (BV570, RPAT8, BioLegend, San Diego, CA, USA); and IFN-γ (APC, B27), TNF-α (PE-Cy7, Mab11) and IL-2 (FITC, 5344.111), all obtained from BD Biosciences, NJ, USA. Stained cells were acquired on an LSRII flow cytometer (BD Biosciences, NJ, USA) and the gating strategy used for analysis is shown in Figure 2.8. Flow cytometry was not performed blinded to the maternal LTBI status, but the infants' samples were tested in a randomized sequence to limit the possibility of bias in the results due to day-to-day variation in the assay.

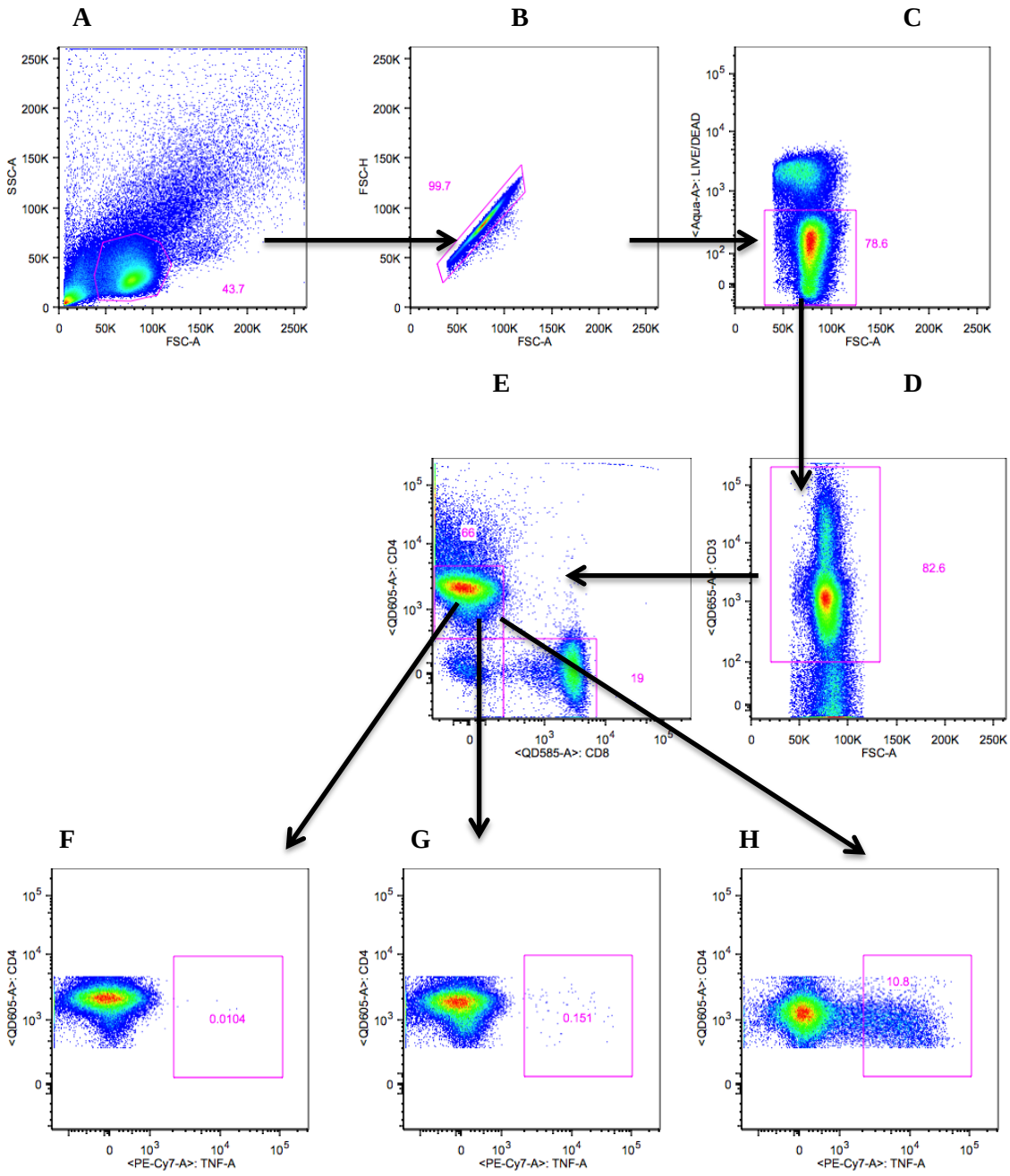


Figure 2.8. Gating strategy for ICS analysis. Gating of Lymphocytes (**A**), Singlet cells (**B**), Live cells (**C**), CD3+ T-cells (**D**) and $CD3^{+}CD4^{+}$/$CD3^{+}CD8^{+}$ T-cells (**E**) was sequentially performed for each sample. Cytokine gates set on unstimulated tubes (**F**) was applied to results obtained with PPD (**G**) and SEB (**H**) tubes.

2.1.10. Humoral responses in BCG-immunised infants

2.1.10.1. Optimisation of IgG ELISA protocol

The reagents, antigens and samples used in the IgG ELISA were titrated to obtain suitable concentrations for the assay. Figure 2.9 is an illustration of this.
The IgG standard concentrations that gave the best curve fit ranged from 0.01-0.625µg/ml (Figure 2.9A). For PPD, the concentration chosen was 5-10µg/ml (Figure 2.9B). A sample dilution of 1/100 was suitable for testing IgG in the maternal and infant samples (Figure 2.9B).

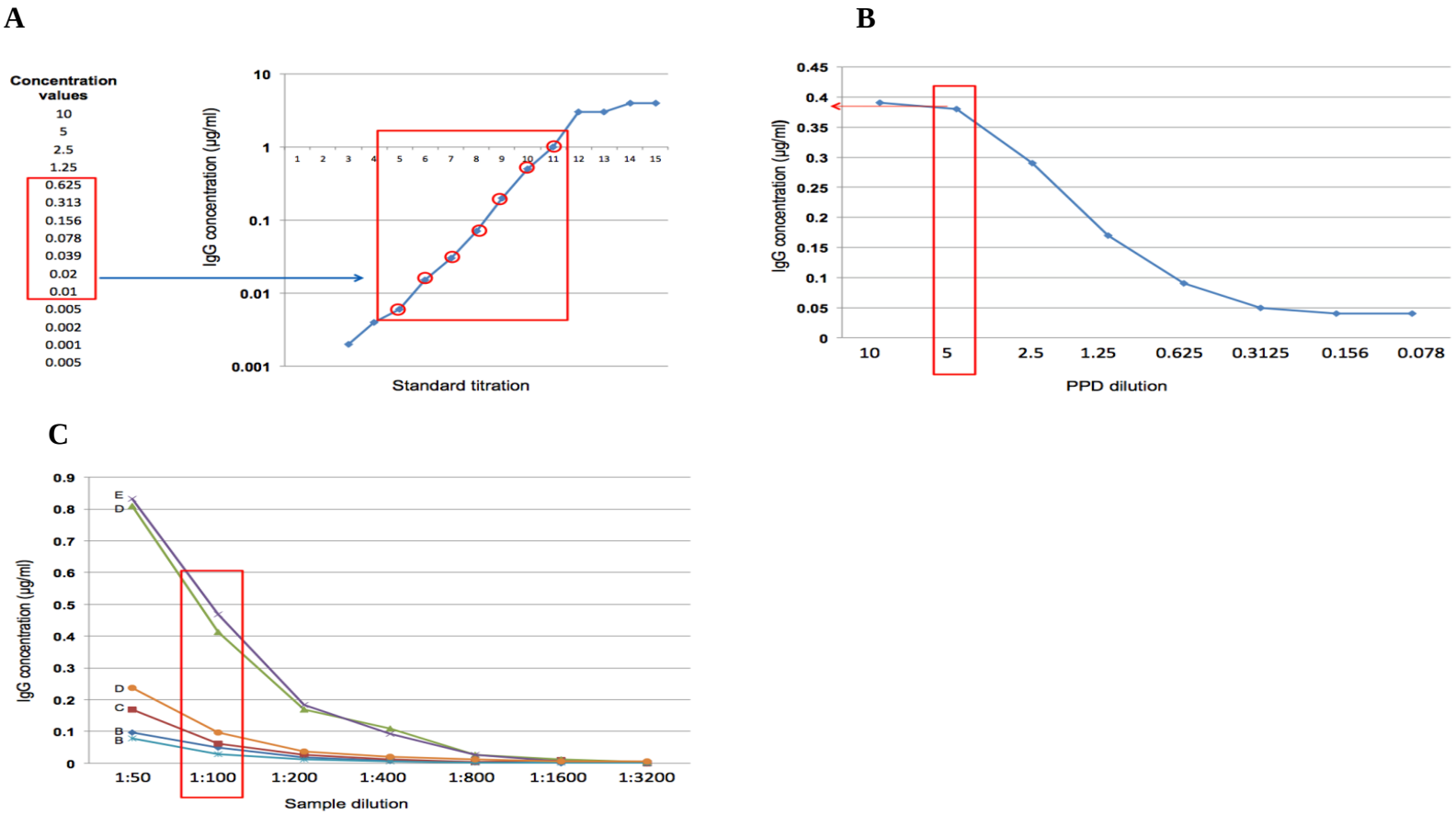
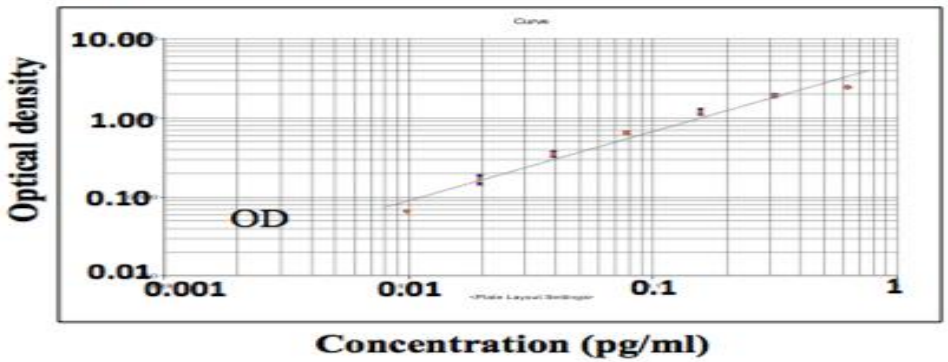
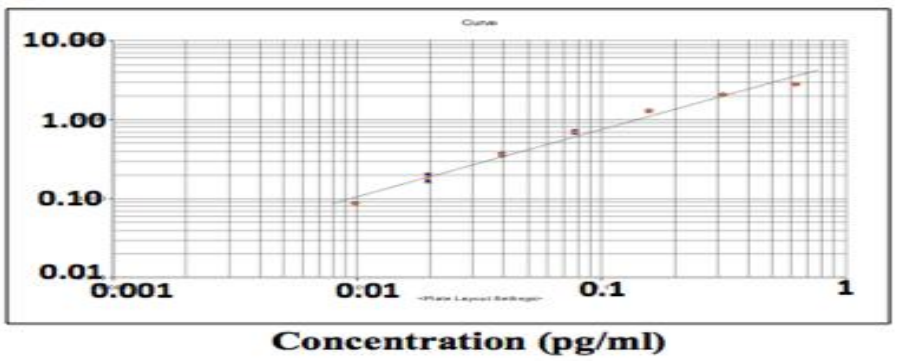


Figure 2.9. Optimisation of human purified IgG standard, PPD and samples for antibody ELISA. IgG standard was titrated to determine the points for the best curve fit (A); PPD was titrated to determine the suitable concentration for the assay (B) and samples titrated to determine the suitable dilution for maternal and infant samples (C).

A



B



C

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.444	0.43	0.11	0.043	0.267	0.267	0.476	0.238	0.217	0.044	0.082	<0.008
B	0.343	0.321	0.226	0.078	0.246	0.041	0.402	0.296	0.337	0.041	0.015	<0.008
C	0.207	0.178	0.103	0.046	0.311	0.327	0.385	0.488	0.421	0.067	0.136	0.045
D	0.098	0.093	0.054	0.183	0.416	0.196	0.398	0.371	0.042	0.062	0.092	0.038
E	0.051	0.043	0.179	0.128	0.381	0.346	0.362	0.38	0.044	0.043	0.05	0.087
F	0.023	0.018	0.26	?????	0.2	0.188	0.281	0.414	?????	0.295	0.052	0.037
G	<0.008	<0.008	0.046	?????	0.187	0.286	0.263	0.182	?????	0.024	0.03	0.024
H	<0.008	?????	0.145	?????	0.373	0.289	0.39	0.421	?????	0.335	<0.008	0.413

D

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.473	0.462	0.063	0.112	0.415	0.408	0.451	0.332	0.335	0.084	0.168	0.116
B	0.337	0.329	0.133	0.13	0.421	0.233	0.339	0.205	0.259	0.066	0.089	0.205
C	0.194	0.185	0.192	0.064	0.344	0.371	0.293	0.363	0.296	0.138	0.078	0.075
D	0.096	0.088	0.063	0.161	0.309	0.384	0.483	0.289	0.585	0.084	0.163	0.147
E	0.045	0.041	0.301	0.063	0.405	0.419	0.326	0.278	0.284	0.035	0.267	0.324
F	0.017	0.021	0.067	0.32	0.375	0.266	0.387	0.439	0.178	0.085	0.126	0.108
G	0.008	0.008	0.114	0.371	0.585	0.389	0.421	0.423	0.327	0.261	0.083	0.137
H	?????	<0.008	0.054	0.292	0.44	0.195	0.282	0.176	0.172	0.297	0.182	0.071

Figure 2.10. Representative standard curves for the IgG standard for the PPD and TT ELISAs and sample IgG concentrations. IgG standard curves showing OD versus concentration (A and B for pilot and infant BCG studies, respectively), and sample IgG concentrations (pg/ml) (C and D for pilot and infant BCG studies, respectively). Columns 1 and 2 show concentrations for serially diluted IgG standard, columns 3-7 show responses to PPD and columns 8-12 show response to TT. Values shown by ???? are where the OD is negative and <0.008 is for values less than the lowest standard concentration of 0.01 pg/ml.

2.1.10.2. ELISA for anti-PPD and anti-TT total IgG antibodies

Plasma samples were collected from heparinized cord blood (n=53), and from infant blood samples (n=53) at the time of PBMC purification. Total plasma immunoglobulin (Ig) G specific for PPD and TT was assayed using an "in-house" indirect Enzyme-linked Immunosorbent Assay (ELISA). Briefly, flat-bottomed 96-well Microlon plates (Greiner Bio-One, Germany) were coated with either purified IgG standard (GenScript, NJ, USA) in bicarbonate coating buffer at a maximum concentration of 0.625 μg/ml and minimum concentration of 0.01 μg/ml, (10μg/ml, RT 50, Statens Serum Institut, Copenhagen, Denmark) or TT (12.12 Lf/ml, T155-1, Statens Serum Institut, Copenhagen, Denmark). Each PPD and TT well had a control comprising 0.1% Marvel milk powder (Premier International Foods, UK) in coating buffer. After overnight incubation, the plates were blocked with 150μl/well of 1% milk powder/PBS for one hour at room temperature. Samples diluted 1 in 100 in 0.1% milk powder/PBS were added to the plates and left overnight at 4 ^{o}C. Polyclonal anti-human IgG Horse Radish Peroxidase (Poly HRP, 0.5μg/ml, Dako, Denmark) was added at 50μL/well and plates incubated for one hour at room temperature. A total of 100μl/well of o-phenylenediamine (OPD, Sigma-Aldrich, MO, USA) substrate mixture (3 mg OPD, 0.1 M citric acid, 0.2 M Na2HPO4, 3 ml 30% hydrogen peroxide in distilled water) was added for 15 min at room temperature (in the dark). The reaction was stopped with 25μl/well 2M Sulphuric acid and the plates were read at test wavelength 490nm and reference wavelength 630nm using an MRX1.1 plate reader and Gen5 1.07 software (BioTek Instruments, Inc., VT, USA). The sensitivity of the test was determined as the lowest standard concentration above which antibody concentrations were detectable (0.01 μg/ml).

2.1.11. RNA amplification and microarray

The original plan for performing gene expression microarray was to acquire equipment and train personnel to perform assays at the Uganda Virus Research Institute. However, we lacked equipment and expertise to perform these assays in Uganda. As a result, the samples collected were sent to our collaborating partner at the Vaccine and Gene Therapy Institute (VGTI) in Florida (Dr. Rafick Pierre Sakaly's group) where RNA extraction and microarray were performed. I was shown how the analysis and interpretation of the generated data was done.

Gene expression microarrays were undertaken using unstimulated whole blood samples obtained from 42 and 51 infants at one and six weeks, respectively, to assess gene expression profiles after BCG immunisation (Figure 2.11). The Illumina RNA Amplification Kit (Ambion, Austin, TX, USA) was used to amplify a median of 124 ng (range 63-174 ng) of the extracted RNA. A Biotin-16-UTP label was incorporated into amplified RNA during the *in vitro* transcription process (Perkin Elmer Life and Analytical Sciences, Woodbridge, Ontario, Canada). Amplification gave yields ranging from 1 µg to 25 µg. Amplified RNA (1000 ng per array) was hybridized to the IlluminaHumanHT-12_V4 BeadChip according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The IlluminaHumanHT-12_V4 bead chip comprises 42,000 sequences representing 31,000 annotated genes from the curated portion of the NIH Reference Sequence Database (http://www.ncbi.nlm.nih.gov/RefSeq/). Each sequence is represented at least 30 times on the array. Arrays were scanned with an Illumina bead array confocal scanner, according to the manufacturer's instructions. Array data processing and analysis was performed using Illumina BeadStudio software. I participated in sample collection and shipment to VGTI where gene arrays were performed.

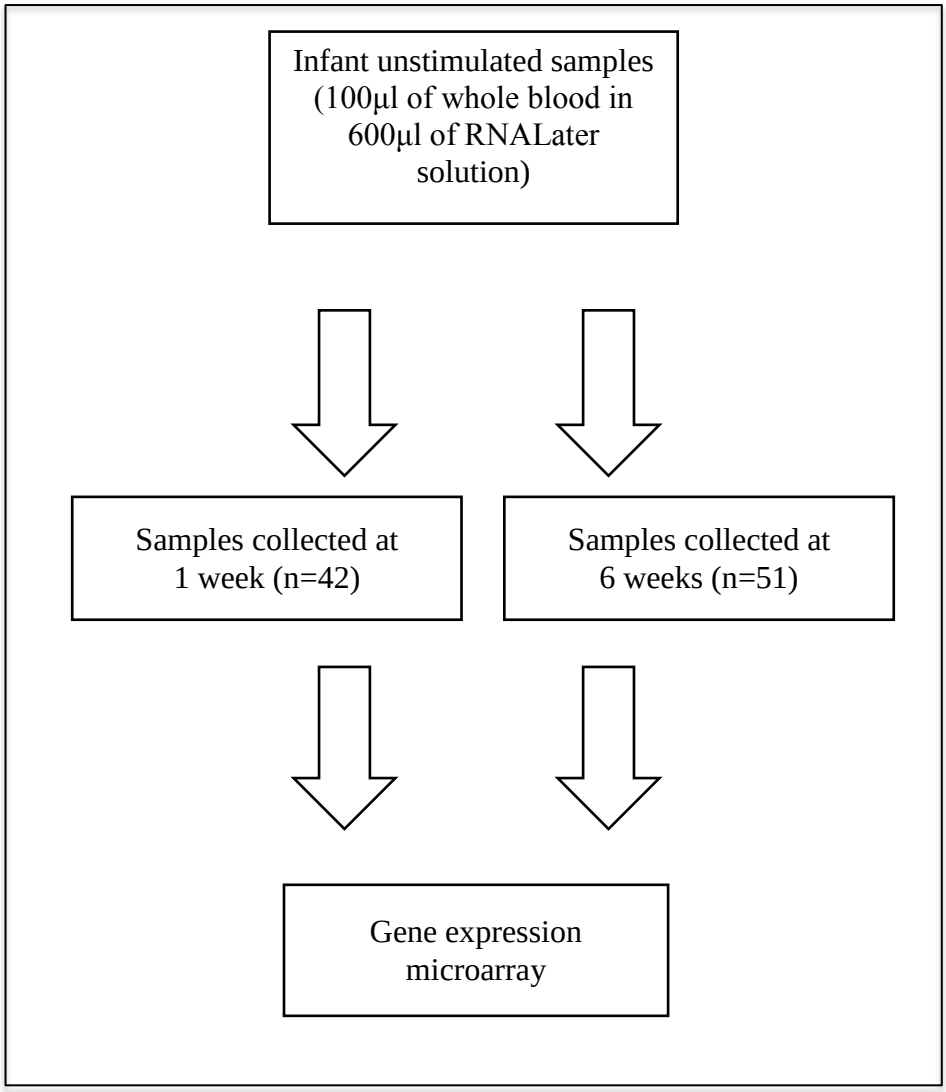


Figure 2.11. Flow of infant samples for gene expression microarray.

2.2. The main infant BCG Study

2.2.1. Cohort and immunisation schedule

A cohort of 284 pregnant women was recruited at the Maternity Wards of Entebbe Hospital. The experiences from the pilot study showed that it would take 18 months to recruit 150 mothers with and 150 without *M.tuberculosis* infection. Because there was another study involving infant recruitment also ongoing at the same time as our study, enrolment was staggered between the two studies, to ensure both studies fulfilled their recruitment numbers. Based on the pilot study it was anticipated that, to maintain a balanced rate of recruitment to the two arms, it was appropriate to invite every second uninfected mother to continue in the study, but this was adjusted according to the recruitment rates achieved. During the pilot study, it was also found that mothers actually returned for their "one-week" check between 4 and 14 days post delivery. Thus, infant blood was collected from day 6 post delivery, up to day 17. Further follow up samples were obtained at 4, 6, 10, 14, 24 weeks (2 ml), and at 1 year (5 ml). It was hoped that these time points would provide a clear picture of the evolution of the infant response, while fitting in with visits required for additional immunisations. Additional infant immunisations comprised Oral Polio Virus (OPV) at birth, Diphtheria, Pertussis, Tetanus (DPT), *Haemophilus influenzae* type B (HiB), hepatitis B (HBV) and OPV at 6, 10 and 14 weeks of age, and measles at 9 months (Table 2.1). At each visit, estimates of prior malaria episodes in the infants were recorded using a questionnaire.

Other factors may also have important effects on the infant response to BCG immunisation. In Uganda, we have shown that BCG strain is an important determinant of the response (277) and a single strain (BCG Denmark) and a single batch (113033C) was used. This strain of BCG has been used before in Uganda (277), and was also selected so that comparisons with infants vaccinated in the UK would be possible.

Other factors that we, or others, have found to be associated with the infant response to BCG and that are potential confounders of an association with maternal *M.tuberculosis* infection included HIV infection (HIV-positive mothers were excluded) and a range of factors which were measured, and accounted for in multivariate analyses including maternal age, parity, nutritional status, BCG scar, *Mansonella perstans* infection, infant weight and length for age, and placental malaria infection (286, 308, 323, 324). We also

collected stool samples from mothers and from one year olds to examine for presence of intestinal helminths (by Kato Katz and by PCR), and also blood for *M. perstans*.

Table 2.1. Summary of sample collection time-points and amount of blood collected

Timelines (weeks)	**0**	**1**	**4**	**6**	**10**	**14**	**24**	**36**	**52**
Time window (days)			± 3	± 3	± 3	± 3	± 3	± 3	± 3
Vaccination									
BCG	✔								
Oral polio virus (OPV)	✔					✔			
Diphtheria, Pertussis and Tetanus (DPT)				✔	✔	✔			
Haemophilus influenzae type B (HIB)				✔					
Hepatitis B (HBV)					✔				
Measles								✔	
Samples collected									
Cord blood	✔								
Whole blood (ml)		2	2	2	2	2	2	2	5
Stool		✔ (Mother)							✔ (Infant)
Assays performed									
T-SPOT.TB assay		✔ (Mother)							
Whole blood assay for Luminex®	✔	✔	✔	✔	✔	✔	✔		✔
ELISA for antibodies	✔	✔	✔	✔	✔	✔	✔		✔

2.2.2. Sampling Strategy

This is an ongoing study, and data was collected from early time points and available samples from infants who have completed follow up. Because the sampling procedure was frequent, it was considered unreasonable to request samples from all babies at each time point. Infants were randomly assigned into two separate sampling strategies: 150 infants (75 from *M.tuberculosis* infected, and 75 from uninfected, mothers) gave blood at the first routine visit (approximately 1 week) and at week 6 and 14, and the remaining infants gave blood at week 4, 10 and 24. All infants were requested to give blood at age one year. Overall, there were 134 mothers who were infected with *M.tuberculosis* and 150 uninfected with *M.tuberculosis* (Figure 2.12) according to the test criteria described in section 2.1.6.1. For this analysis, 55 *M.tuberculosis*-infected and 85 uninfected mothers were considered for Luminex assays for T cell responses (Figure 2.12) and 100 *M.tuberculosis*-infected and 122 uninfected mothers were considered for antibody ELISA (Figure 2.13). The numbers of infants considered for analysis of Luminex® results after follow up to 52 weeks are shown in Table 2.2. There were very few mothers (three) who returned at the 14th week time point and their infants were not included in the analysis.

By sampling both early and late time points, we aimed to accurately define the timing of the peak immune response in infants following BCG immunisation and to determine whether maternal *M.tuberculosis* status affects the priming and/or recall response to BCG antigens in infants.

Table 2.2. Details of samples for analysis of Luminex® results.

	Age (weeks)						
LTBI groups	0	1	4	6	10	24	52
LTBI-negative	85	36	42	32	37	9	65
LTBI-positive	55	27	23	25	25	6	38
Total	140	63	65	57	62	15	103

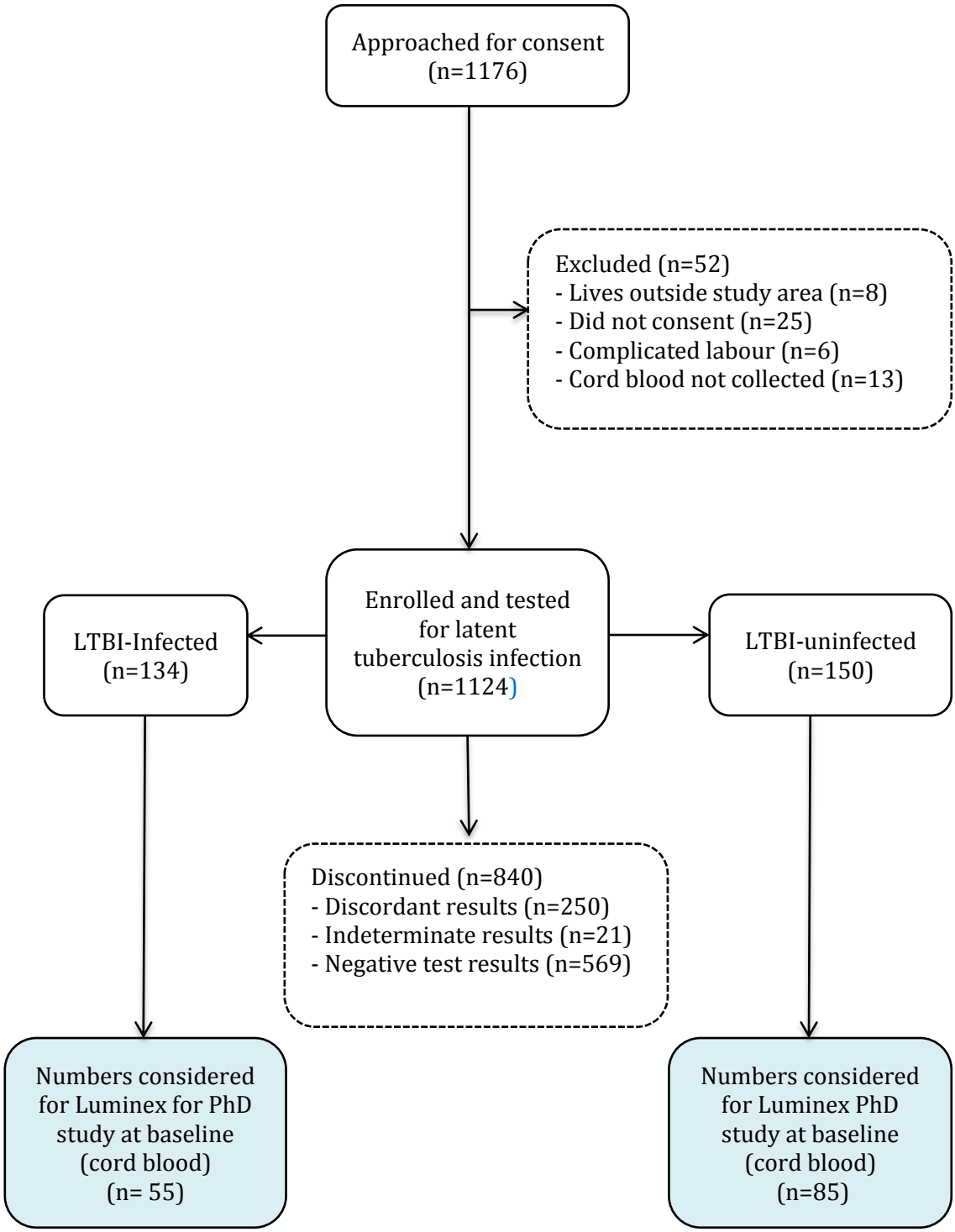


Figure 2.12. Flow of participants through the main infant BCG study whole blood assay/Luminex for T cell responses.

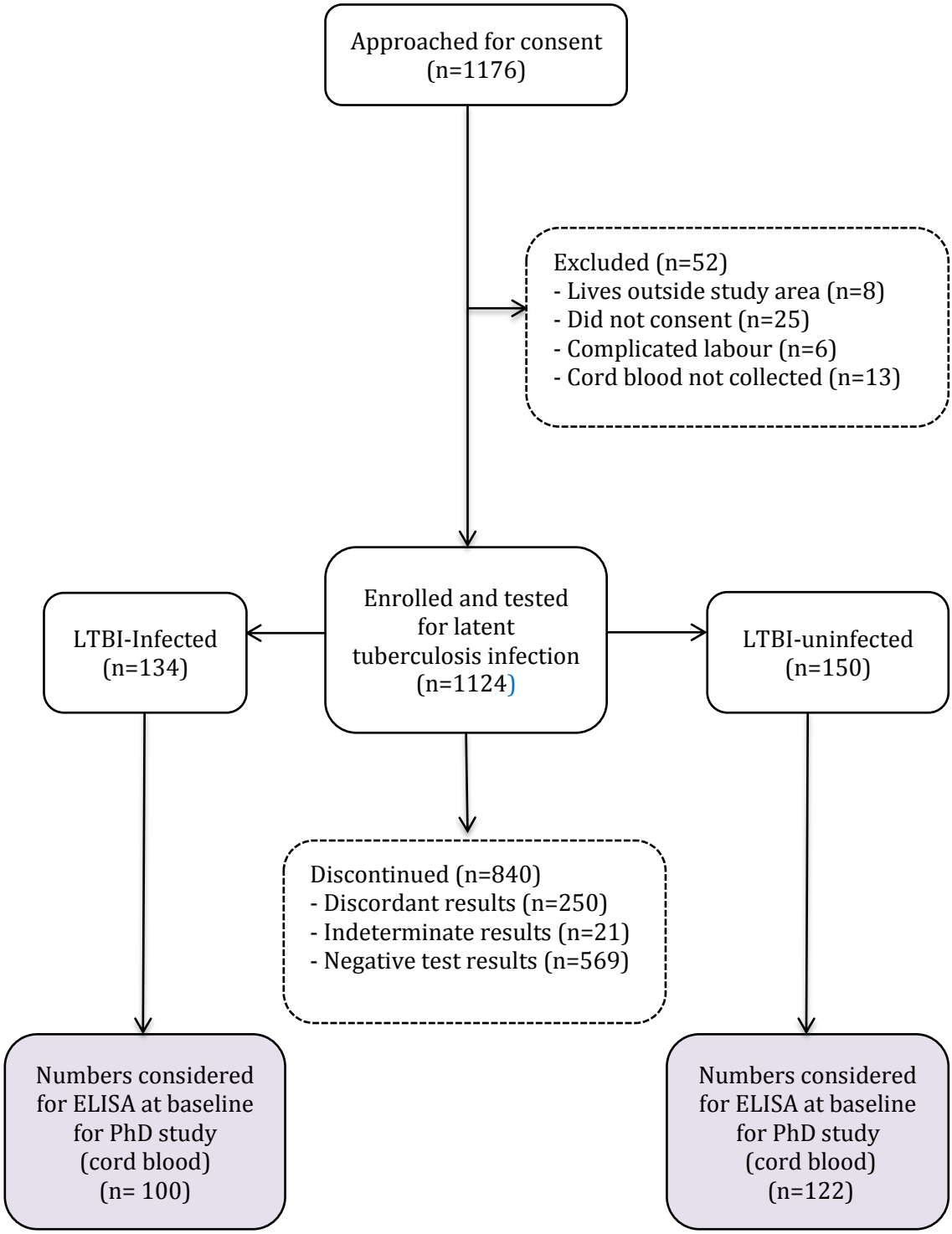


Figure 2.13. Flow of participants through the main infant BCG study ELISA for antibody responses.

2.2.3. Recruitment procedures

The consent process and the inclusion and exclusion criteria used in the pilot infant BCG study were used in the main infant BCG study (see section 2.1.5.2 above).

2.2.3.1. Sample size determinations

Previous studies have shown extreme differences in cytokine responses between African and UK infants, of the order of >0.7log10 (230, 325). However, we expected differences between infants of infected and uninfected Ugandan mothers to be smaller. Using a significance level of 1% to allow for the multiple testing arising from the fact that we examined many cytokines (since many of the cytokine responses were expected to be correlated, a Bonferroni adjustment which assumes independence would be too stringent), and assuming the standard deviation of log10 cytokine responses in Uganda to be approximately 0.9 (15), we had 80% power to detect a difference of 0.35log10 between 150 infants from *M.tuberculosis*-infected mothers and 150 from uninfected mothers in cord blood, and at one year of age. For all other time points, 75 children from each group were sampled, allowing us 80% power to detect a difference of 0.5log10 between the two groups.

2.2.3.2. Ethical considerations for main infant BCG study

This was an exploratory observational study without the use of investigational products. TST is a standard procedure that may occasionally cause some discomfort and scarring. Mothers with a positive TST result were followed up, had a chest X-ray performed and were seen by a physician at the CiSP clinic. Further investigations (such as sputum examination) were conducted if clinically indicated. If found to have active TB, mothers were referred for treatment at the Entebbe Grade B Hospital TB clinic. If a mother was found to be sputum smear positive, isoniazid preventive therapy was provided for the infant and, although the infant was retained in the study, the results were considered separately as a case study, since isoniazid may itself influence the response to BCG immunisation. Mothers with LTBI were not offered prophylactic isoniazid treatment. Current policy in Uganda recommends this only for children under five years of age and people with HIV infection, and the benefit-risk balance in favour of treatment versus side effects decreases with increasing age. If a mother was found to be HIV positive on analysis of the post delivery blood sample, a CD4+ T cell count was performed and she was referred to an HIV care provider. Anti-retroviral therapy for prevention of mother-to-child HIV transmission (PMTCT) was offered for the infant within the PMTCT

programme at Entebbe Hospital. Nurses from the CiSP team with appropriate experience and expertise performed bleeding of very young infants. Small blood volumes were collected and so this procedure was not detrimental to the infants. Mothers found to be infected with helminths were treated when results were available, at the four or six week post-delivery visit. Two-step consent was taken where mothers gave verbal consent during labour and written consent before discharge. Mothers were free to withdraw from the study, with their infants, at any time. Fathers were encouraged to fully participate with their child and family in this study. To this end, after consent had been obtained from the mother, we sent home to the father an information sheet for him to read, and to encourage him to turn up at routine visits to ask the study staff any questions he had. Ethical approval was sought from UVRI (Appendix J), UNCST (Appendix K), and LSHTM (Appendix L).

2.2.4. Laboratory methods

The details of cord blood, maternal and infant blood samples are shown in Figures 2.14 and 2.15.

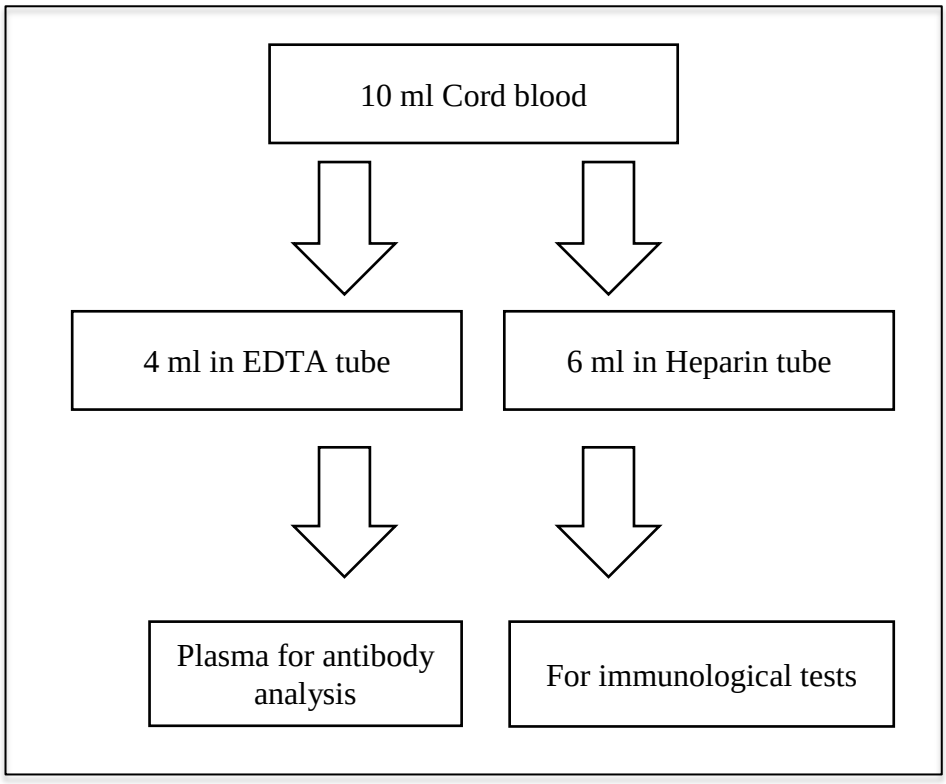


Figure 2.14. Flow of cord blood sample for separating plasma and for whole blood cultures**.**

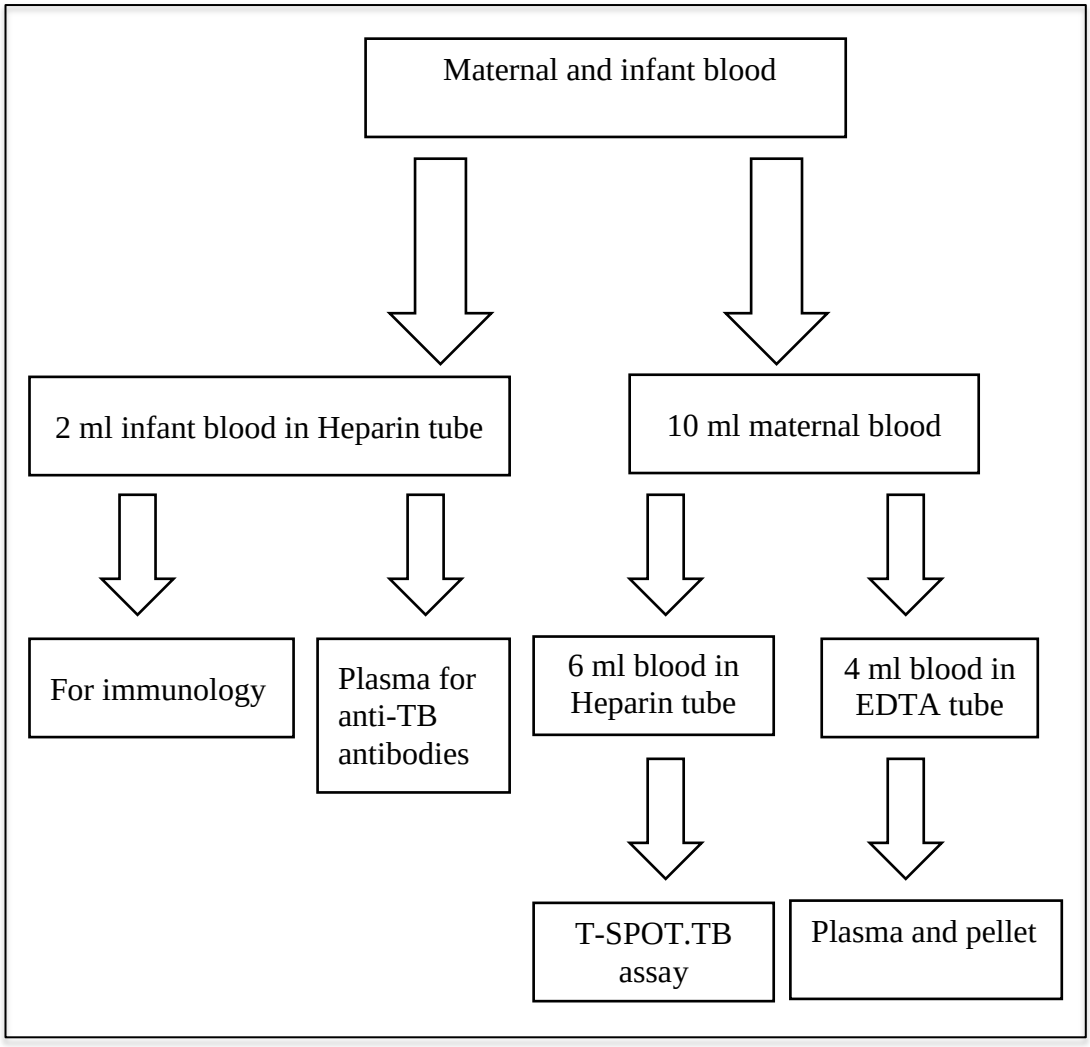


Figure 2.15. Flow of maternal and infant blood for T-SPOT.TB test (maternal), plasma separation and whole blood cultures.

2.2.4.1. Outcome measures

- Time course of PPD-specific responses at 1, 4, 6, 10, 14, 24 and 52 weeks after. BCG immunisation measured using Luminex® (for cytokines and chemokines) and ELISA (for anti-TB antibodies).
- The influence of maternal infection with *M.tuberculosis* on the infant responses.

2.2.4.2. Immunological techniques

- Six-day WBA for Luminex® assay.
- ELISA for anti-PPD and anti-TT total IgG antibodies.

2.2.4.3. Antigens for 6-day WBA

- RPMI 1640 medium (Life Technologies Corporation, NY, USA).
- *M.tuberculosis* PPD (RT 50, Statens Serum Institut (SSI), Copenhagen, Denmark) at 10µg/ml final concentration.
- ESAT-6 (BEI Resources, Manassas, VA, USA) at 5µg/ml final concentration (recombinant protein).
- CFP-10 (BEI Resources, Manassas, VA, USA) at 5µg/ml final concentration (recombinant protein).
- PHA (Sigma-Aldrich, MO, USA) at 10µg/ml final concentration.

2.2.4.4. Antigens for ELISA

- PPD at 5µg/ml final concentration.
- TT at 2µg/ml final concentration.

2.2.4.5. Stimulation of infant whole blood

Supplemented RPMI 1640 was pre-warmed at room temperature for 15 minutes.
Up to 450µl of heparinized venous blood was diluted with 1350µl of supplemented RPMI to make a 1:4 dilution. Up to 200µl of diluted blood was added to each well, in duplicate. To give a final concentration of 10µg/ml, 2µl of a 1mg/ml PPD stock was added to 200µl of diluted blood per well. Both ESAT-6 and CFP-10 antigens were prepared into 1mg/ml stock solutions. These were pooled during stimulation and 1µl of each was added to 200µl of diluted blood to give a final concentration of 5µg/ml. PHA was reconstituted to a 1mg/ml solution. To give a final concentration of 10µg/ml, 2µl of the solution was added to 200µl of diluted blood. PHA served as a positive control and RPMI alone was used as a negative control. Testing was done to identify the optimum

concentration for these stimulants (data not shown). Plates were incubated at 37 °C in 5% CO_2 for 6 days.

2.2.4.6. Harvesting supernatants

Culture plates were removed from the incubator and spun at 800g for 5 minutes to attain a cleaner supernatant. Microtubes for storage of supernatants were labeled with study participant laboratory number and antigen code. Up to 150µl of supernatant was removed from the culture plates and transferred into the labeled microtubes. Supernatants from duplicate wells were pooled in a single microtube, mixed gently by pipetting up and down and freezing until analysis.

2.2.5. Measurement of cytokines and chemokines by multiplex assay system

2.2.5.1. Quality control

In order to assess inter and intra plate variability, two quality control samples (Control 1 and Control 2) supplied by the manufacturer were added in duplicate wells to the plates. Figure 2.16 illustrates the values for the 23 plates that were run, A for absolute values in pg/ml and B for $\log_{10}$ values.

For Control 1, the concentrations for most cytokines and chemokines are between 100-250pg/ml, except for MIP-1α which had a higher concentration (greater than 250pg/ml) overall (Figure 2.16A, left panel).

Control 2 values are higher than for Control 1 for all plates, ranging between 550-1100pg/ml. As for Control 1, MIP-1α values were higher than for other analytes (>11000pg/ml). IP-10 showed inconsistent values across plates (Figure 2.16A, right panel).

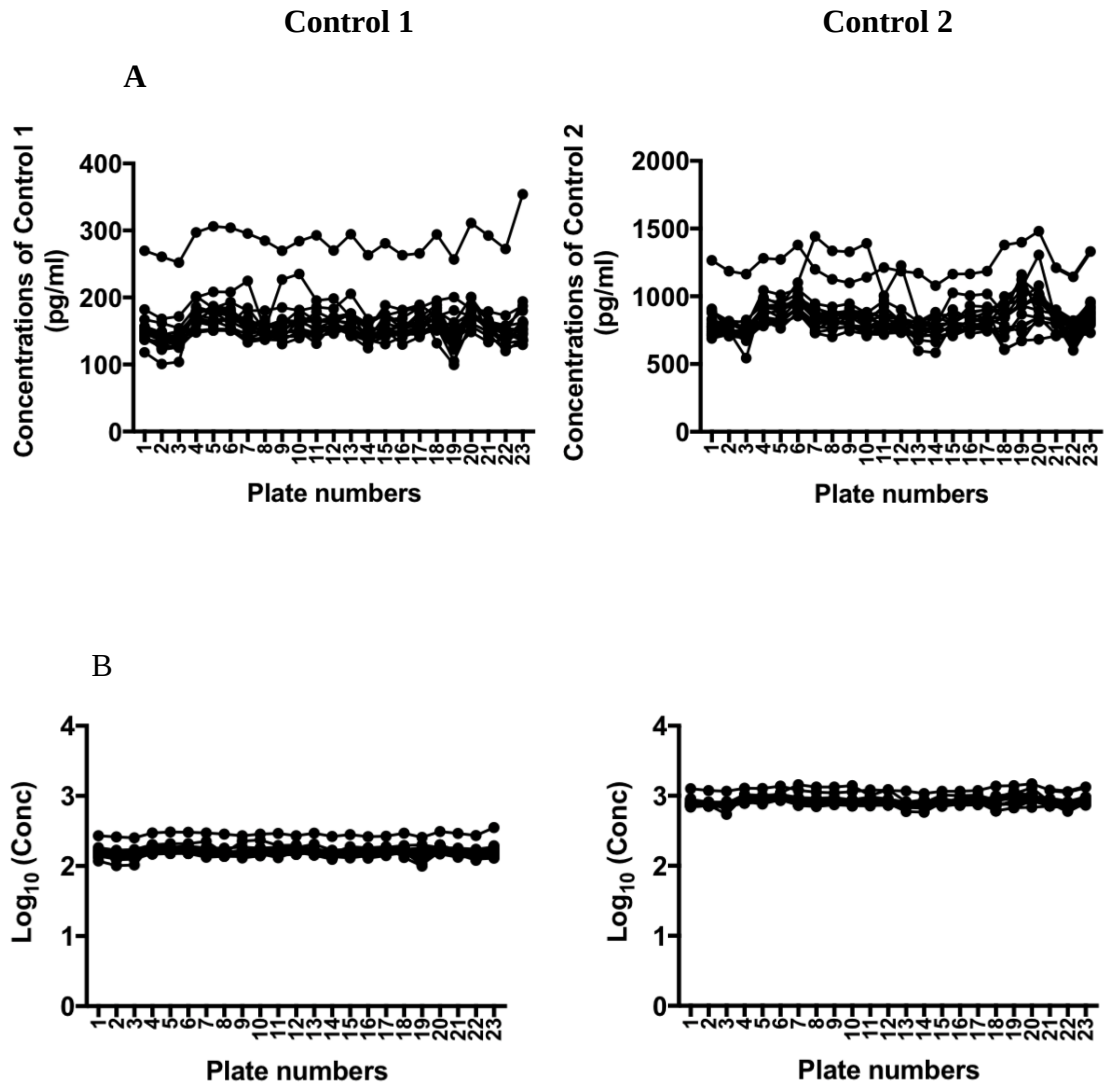


Figure 2.16. Luminex® quality control results for the 17 cytokines and chemokines measured. To control for inter and intra plate variability, two positive controls supplied by the manufacturers were used on each plate. The results are shown above for Control 1 (left panel) and Control 2 (right panel). The top panels (A) show values in pg/ml and the bottom panels (B) are values in $\log_{10}$.

2.2.5.2. The Luminex® assay

The concentrations of analytes in the culture supernatants were measured using a Millipore Milliplex assay system (Merck Millipore, Abingdon, United Kingdom) following instructions from the manufacturer. Briefly, working standard solutions were prepared by double-diluting them serially. The bead mixture, controls and wash buffer were also prepared. Up to 200 µL of wash buffer was added into each well of the plate. Plates were sealed and mixed on a plate shaker for 10 minutes at room temperature (20-25^{0}C). Wash buffer was decanted and the residual amount removed from all wells by inverting the plate and tapping it smartly onto paper towels several times. Next, 25 µL of each Standard or Control was added into the appropriate wells. Assay buffer was used for 0 pg/mL standard (background). Up to 25 µL of Assay buffer was added to the sample wells, background, standards, and control wells. The mixing bottle containing the prepared beads was vortexed and 25 µL of the mixed beads was added to each well. (Note: During addition of the beads, the bead bottle was shaken intermittently to avoid settling of the beads). Plates were sealed with a plate sealer and wrapped with foil and incubated with agitation on a plate shaker (speed 600rpm) for 2 hours at room temperature (20- 25^{0}C). After the 2 hours of incubation, the plates were removed from the plate shaker, unsealed one at a time and placed on the plate washer magnet for 60 sec, then washed 2 times using the MAG2X protocol. Then 25 µL of detection antibody was added into each well. Plates were sealed, covered with foil and incubated with agitation on a plate shaker at 600rpm for 1 hour at room temperature (20- 25^{0}C). After the 1 hour incubation, 25 µL of the Streptavidin-Phycoerythrin conjugate was added to each well containing the 25 µL of detection antibody. Plates were sealed, covered with foil and incubated with agitation on a plate shaker (at 600rpm) for 30 minutes at room temperature (20-25^{0}C). Plates were washed 2 times with wash buffer and 50 µL of sheath fluid was added to all wells. Beads were resuspended on a plate shaker (at 600rpm) for 5 minutes and plates were run on Bio-plex 200™ Luminex® machine, and the median fluorescent intensity (MFI) data were saved and analyzed using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. The following analytes were assayed: IL-1β, IL-Rα, IL-2, IL-5, IL-8, IL-10, IL-12p40, IL-13, IL-17, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α and GM-CSF.

2.2.5.3. ELISA for anti-PPD and anti-TT total IgG antibodies

The antibody concentrations were measured using the ELISA protocol for the pilot study described in section 2.1.10.2.

2.2.6. Statistical and data collection methods

Datasets were generated from questionnaires and from immunological and molecular assays including Luminex®, ELISA and gene expression microarray.
Data were entered using Microsoft Access and Excel. Analysis was done using Flowjo v. 9.5.2 (Tree Star Inc., Ashland, OR, USA), Stata version 13.1 (College Station, Texas, USA) and GraphPad Prism v7.0a (GraphPad software, Inc., La Jolla, CA, USA). All of these formats allowed for sharing and long-term validity of stored data. Dr Emily L. Webb, the study statistician, as well as the MRC Statistics Unit were consulted for the correct statistical analysis of the data.

2.2.7. Analysis of innate responses and gene expression profiles in BCG-vaccinated infants

The objective of this analysis was to investigate the effects of maternal LTBI and helminth infection on infant innate immune responses. In the event, helminth infections were rare in this study group (326), so the principal exposures considered were maternal LTBI and maternal BCG scar. In the multivariate analysis, the effects of maternal LTBI and maternal BCG scar were adjusted for. Maternal and infant factors such as maternal age, gravidity status, infant birth weight and gender were not crudely associated with infant innate responses and were not adjusted for, and the numbers involved were generally small.

Cytokine and chemokine concentrations showed skewed distributions. Results were transformed to $\log_{10}$ (cytokine concentration+1) for graphical representation using GraphPad Prism v7.0a (GraphPad software, Inc., La Jolla, CA, USA) and for analysis by linear regression using bootstrapping (327) using STATA v. 13.1 (College Station, TX, USA). Results from regression analyses are presented as adjusted geometric mean ratios (aGMR) [95% confidence interval (CI)]. Multiplex data values below the lowest concentration were assigned as 1.6 pg/mL. Unstimulated responses were subtracted from antigen-stimulated results and negative values were set to zero. The Mann–Whitney U-test was used to compare responses between infants of mothers with and without LTBI and those with and without a BCG scar and correlation between two

continuous variables was assessed using the Spearman rho test. For the different stimuli, the median maternal and cord blood responses, as well as the associations of infant responses with maternal LTBI and maternal BCG scar were analysed. In addition to looking at single cytokines and chemokines, Principal Component Analysis (PCA) (328) was performed on the cytokine and chemokine variables to summarize them. For this, an average cytokine or chemokine response was worked out for each infant by calculating the mean concentration obtained from the seven different stimuli (after subtracting unstimulated responses). The R programme (v3.2.2. R Foundation for Statistical Computing, Vienna, Austria) was used for further assessment of the associations.

For microarray, raw Illumina probe data were exported from BeadStudio and screened for quality. Pre-processing and statistical analysis was conducted using the R statistical language and various software packages from Bioconductor (329). Quantile normalization was applied, followed by a $\log_2$ transformation. The LIMMA package was used to fit a linear model to each probe and (moderated) *t* tests or *F* tests were performed on the groups being compared. To control the expected proportions of false positives, the FDR for each unadjusted *p* value was calculated using the Benjamini and Hockberg method implemented in LIMMA. The microarray data are available through the National Center for Biotechnology Information Gene Expression Omnibus (GSE87801). Pathway analysis was performed using Gene Set Enrichment Analysis (GSEA), a non-parametric annotation-driven statistical analysis method (330), to assess which biological processes are associated with the different LTBI and BCG scar groups. We tested gene sets from the Molecular signature Database (MsigDB, http://www.broad.mit.edu/gsea/msigdb Hallmark collection (h.all.v5.0.symbols.gmt) which summarise and represent specific well-defined biological states or processes displaying coherent expression. Statistical significance was set for *p* value below 0.05. This analysis was done by Dr. Rafick Pierre Sekaly's group at the VGTI (now at Case Western Reserve University School of Medicine, Cleveland, Ohio, United Satates). I was later shown how the analysis was done and results interpreted.

2.2.8. Analysis of cellular immune responses in BCG-vaccinated infants

Antibody concentrations were summarised using medians and interquartile ranges

(IQR) and compared between different time points using paired student's t-test. Antibody levels showed a skewed distribution with large numbers of undetectable results, therefore results were transformed to $\log_{10}$ (antibody concentration+1) for graphical presentation.

Flow cytometry data were analysed using FlowJo v9.5.2 (Tree Star Inc., Ashland, OR, USA). Results were expressed as the frequency of positive events above the negative control. Prism v6.0e (GraphPad software, Inc., La Jolla, CA, USA) was used for crude analyses and data presentation. Characteristics of mothers with and without LTBI were compared using the Mann-Whitney U test. Responses at different time points were compared using the Wilcoxon signed rank test. Differences in responses between LTBI exposed and unexposed infants were analysed using the Mann-Whitney test. Stata version 13.0 (College Station, Texas, USA) was used for multivariate linear regression to adjust for potential confounders (maternal age, gravidity status and infant gender) (illustrated in causal diagram in Figure 2.17), with 95% CI estimated by bootstrapping. Results from regression analyses were presented as crude and adjusted mean difference (95% CI). P-values <0.05 were considered statistically significant.

Multiplex data values below the lowest concentration were assigned as 1.6 pg/mL. Unstimulated responses were subtracted from antigen-stimulated results and negative values were set to zero. The Mann–Whitney U-test was used to compare responses between infants of mothers with and without LTBI and correlation between two continuous variables was assessed using the Spearman rho test. Linear regression with random effects were used to compare responses between infants of mothers with and without LTBI.

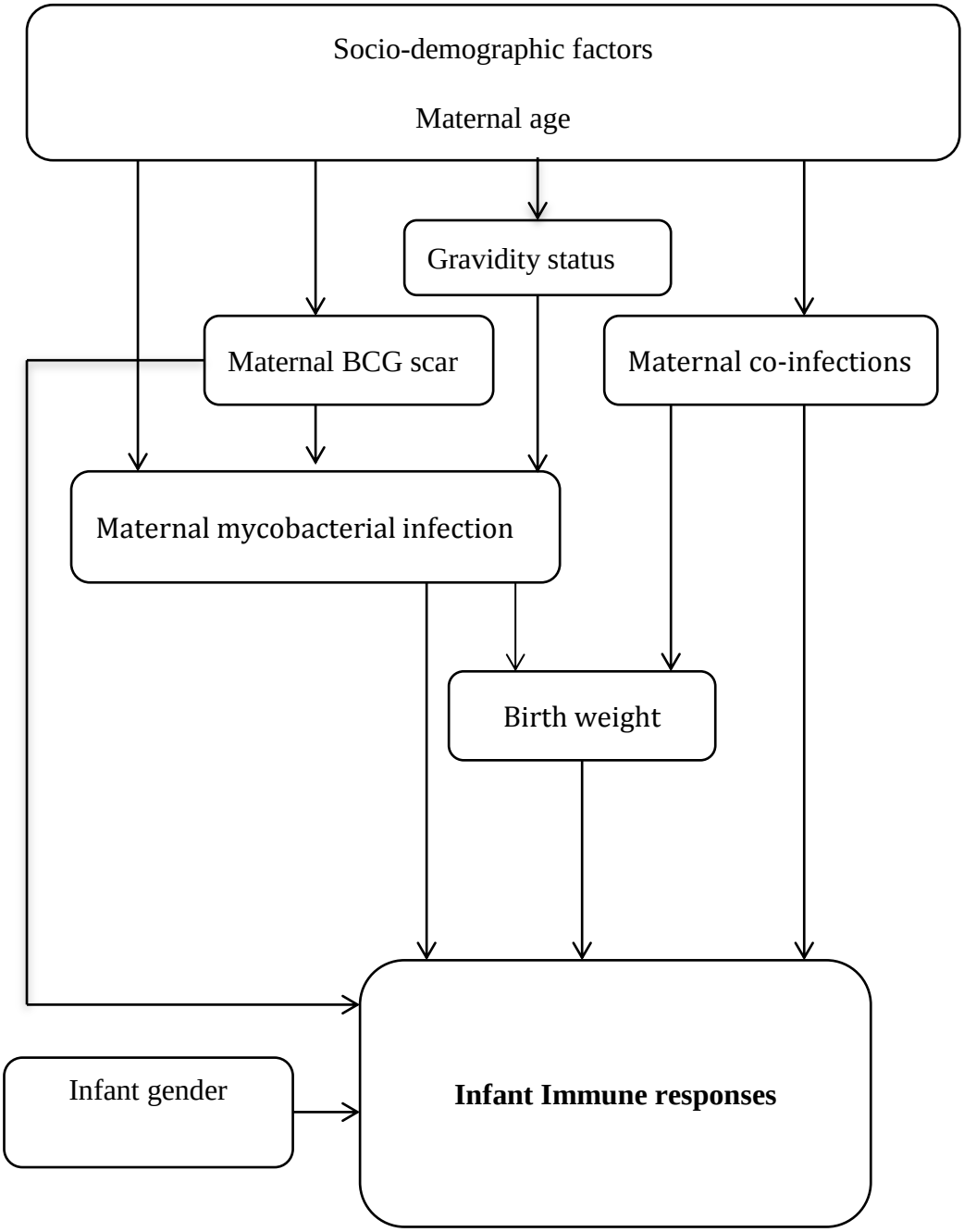


Figure 2.17. Causal diagram

Chapter 3

Investigation of immune responses in mothers and infants: the pilot infant BCG study

3.1. Introduction

Innate cells such as APCs take up microbes and initiate direct killing of the microbes or lysis of microbe-infected cells. DCs and macrophages have sensors (PRRs) that interact with microbial PAMPs, resulting in initiation of immune responses. Neonates have been shown to have reduced responses to innate stimuli (331-333), though concentrations of IL-6 have been reported to be high at the time of birth (334, 335). The assessment of infant responses to innate stimuli and their associations with maternal and infant factors has not yet been extensively studied, and further research in this area is required. A recent study conducted in South Africa showed that maternal LTBI had no association with adaptive immune responses in the infants following BCG immunisation (308). In this study, the impact of maternal LTBI on innate responses in infancy was not assessed.

Innate immune responses are now known to directly affect the quality of the adaptive response. Immunisation with BCG, for example, has been suggested to prime heterologous immunity through the innate immune system, through a process termed innate training (265-269). In addition, maternal genetics and response to BCG may play a role. Evidence from observational studies and randomized controlled trials have shown this priming to be associated with beneficial effects on childhood survival in both low- and high-income countries (260-264). The association between maternal BCG scar and lower T helper (Th) 2 responses to mycobacterial antigens has previously been demonstrated in infants (286), although the effects of maternal BCG scar on innate immune responses in infancy were not examined in this study.

Differential expression of immune genes following BCG immunisation of infants has previously been demonstrated (238), but again the effects of maternal and infant factors were not assessed.

In this Chapter, the associations between maternal BCG scar and immune response profiles in the offspring was evaluated in a study designed to investigate the effects of maternal infections, including LTBI, on infant immune responses.

This chapter is based on a manuscript in press in Vaccine and titled “Maternal BCG scar is associated with increased infant proinflammatory immune responses**.** Mawa, P.A, Webb, E.L., Filali-Mouhim, A., Sekaly, R.P., Nkurunungi, G., Lule, S.A., Prentice, S., Nash, S., Dockrell, H.M., Elliott, A.M., and Cose, C” (Appendix R) with additional unpublished data. After a description of the socio-demographic and clinical characteristics of the study population, the concentrations of innate stimulus-induced cytokines and chemokines in maternal post-delivery blood and in infant cord blood (measured by Luminex® assay), as well as the associations between the innate responses and maternal and infant factors are presented. Gene expression profiles following BCG immunisation in infancy and their associations with maternal LTBI and maternal BCG scar was also assessed.

The specific objectives for this Chapter were:

1. To measure the concentrations of cytokines and chemokines in maternal post-delivery blood and infant cord blood after stimulation with innate stimuli.
2. To assess the correlations between concentrations of cytokines and chemokines measured.
3. To analyse the associations between maternal and infant factors, and innate responses in the mothers and their infants.
4. To investigate the effect of maternal LTBI and maternal BCG scar on gene expression profiles in BCG-immunised infants.

3.2. Results

3.2.1. Characteristics of participants in the pilot infant BCG study

Between February and May 2012, 175 women were approached to participate. Of these, 145 were enrolled in the study (Figures 2.2 to 2.4, Chapter 2). Fifty-one mothers who were enrolled into the study were excluded, mainly because their contact details were not taken and they could not be traced when they defaulted from further follow-up. Of the remaining 94 mothers who were tested for LTBI, 23 had discordant LTBI test results and were excluded. Twenty-one mothers were identified as LTBI-positive by both T-SPOT.TB and TST testing and 50 as LTBI-negative. On repeat testing, none of the mothers was found to be infected with HIV.

Twenty-nine mothers were included for the analysis of infant innate responses, and of these, 12 had LTBI and 16 had a BCG scar. Information on BCG scar was missing from three mothers, and these women were excluded from analysis. Mothers with and without a BCG scar were comparable in terms of LTBI (31% versus 50%, p=0.42), age (25 years versus 26 years, p=0.78) and gravidity status (37% versus 50% primigravida, p=0.70). Their infants were comparable in terms of male gender (19% versus 40%, p=0.38) and birth weight (3.09 Kg versus 3.22 Kg, p=0.47) (Table 3.1).

Table 3.1. Characteristics of participants by maternal BCG scar status.

Characteristics of mothers and infants	**BCG Scar present (n=16)**	**BCG Scar absent (n=10)**	***P*-value**
Mothers			
Age, mean (years)	25	26	0.78
Latent TBI status, Present, no (%)	5 (31)	5 (50)	0.42
Gravidity, Primigravida, no (%)	6 (37)	5 (50)	0.70
Infants			
Sex, Male, no (%)	3 (19)	4 (40)	0.38
Mean birth weight (Kg)	3.09	3.22	0.47

The figures are given as numbers with percentage (%) in brackets, or as mean values. *P* value is based on an unmatched *t* test for differences in maternal age and infant birth weight, and a two-sided Fisher's exact test for differences in maternal LTBI, gravidity status and infant gender between scar-positive and scar-negative groups.

3.2.2. Responses to innate stimuli

3.2.2.1. Combined and individual innate immune responses

Maternal blood collected at approximately one week after delivery and infant cord blood samples (collected at delivery) were stimulated with innate stimuli for 24 hours and the cytokines and chemokines in culture supernatants were measured by Luminex® assay.

The median cytokine and chemokine responses to the different stimuli and for combined responses were analysed (after subtraction of background unstimulated responses). These are illustrated in Figure 3.1A for mothers and Figure 3.1B for infants (for combined responses) and Table 3.2 for mothers and Table 3.3 for infants (for responses to individual stimuli). There were overall low to moderate concentrations of cytokines, chemokines and growth factors in both maternal and cord blood samples, except for IL-6, IL-8, MCP-1, MIP-1α, MIP-1β and CL097-specific IP-10 responses, where concentrations were high for the different stimuli (Figure 3.1 and Tables 3.2 and 3.3). Infant responses, in the combined analyses, mirrored those of maternal responses.

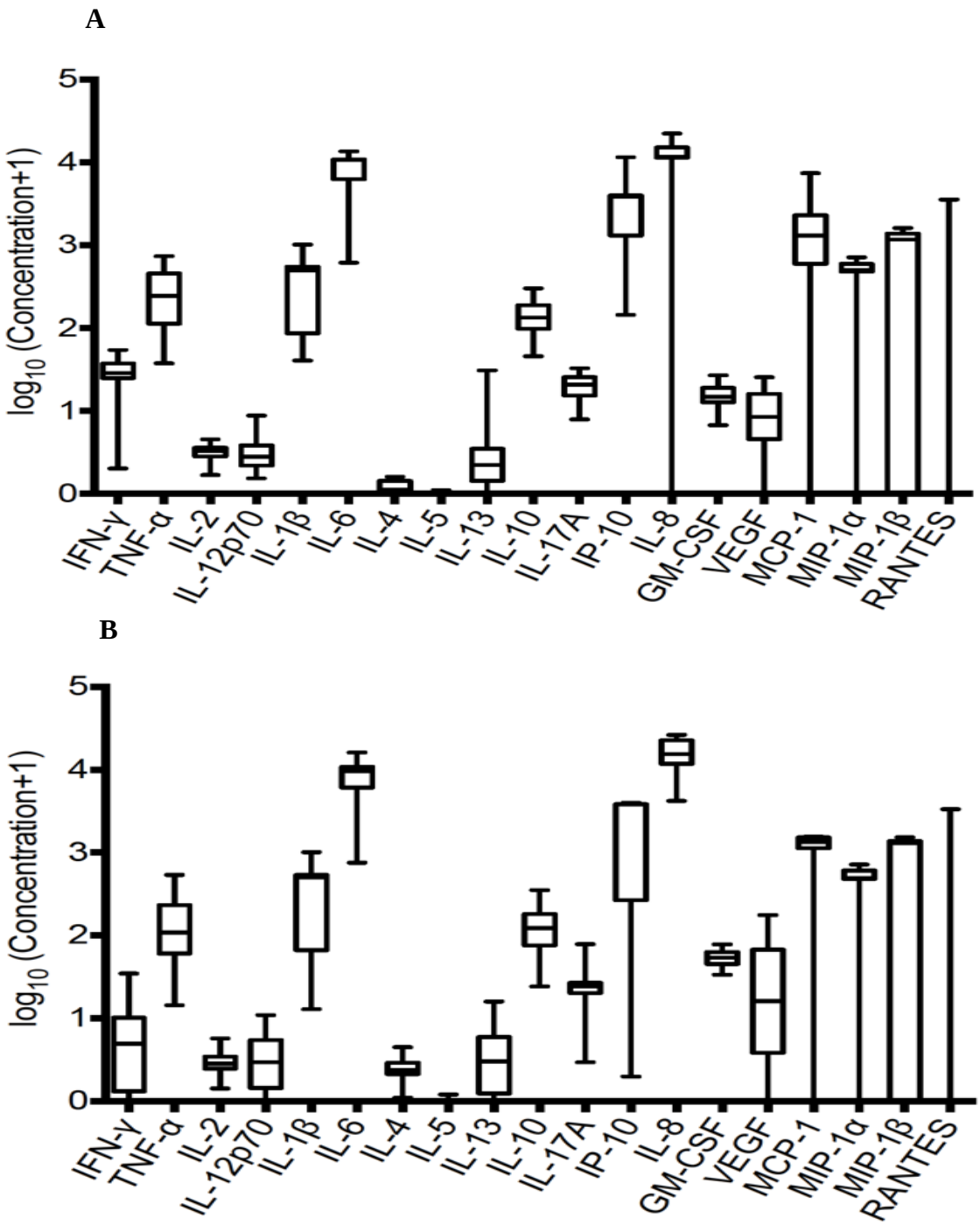


Figure 3.1. Concentrations of cytokines and chemokines in culture supernatants of maternal and cord blood samples measured using Luminex® assay. Panels A and B show concentrations in maternal blood and cord blood, respectively. Cytokine concentrations are expressed as $\log_{10}$ (concentration+1)**.** The mean response was calculated per cytokine and chemokine for the seven different stimuli that were used in the WBA. Box plots represent median and interquartile ranges, and the whiskers represent minimal and maximal concentrations. Samples from 26 mother-infant pairs were tested.

Table 3.2. Cytokine and chemokine responses to individual innate stimuli, showing concentrations in culture supernatants from mothers' stimulated post-delivery blood.

Cytokine/ Chemokine	**PAM3Cys-Ser (TLR 1/2 agonist)**	**FSL-1 (TLR 2/6 agonist)**	**LPS (TLR 4 agonist)**	**CL097 (TLR 7/8 agonist)**	**CpG-ODN2006 (TLR 9 agonist)**	**Mannan (DC-SIGN agonist)**	**Curdlan (Dectin-1 agonist)**
IFN-γ	33.29	15.22	43.17	42.75	1.1399	10.31	49.81
TNF-α	51.22	5.15	350.52	262.39	0	3.24	854.78
IL-2	2.86	0.95	4.04	3.07	0	0.75	4.21
IL-12p70	0.45	0	2.27	2.98	0	0.929	2.61
IL-1β	17.32	0.82	149.47	121.94	0	0	3255.72
IL-6	188342.53	183.94	18871.69	18861.84	0.67	18.43	18875.71
IL-4	0.08	0	0.26	0	0	0	0.33
IL-5	1.55	1.55	1.55	1.55	1.55	1.55	1.55
IL-13	0.49	0	0.69	0	0	0	4.09
IL-10	75.53	16.22	206.31	156.46	0	1.44	454.08
IL-17A	21.76	15.03	26.85	23.84	1	9.98	31.54
IP-10	5.50	39.13	231.29	26710.98	0	26.39	378.16
IL-8	26403	981.56	26403	890.59	203.7	1389.14	26403
GM-CSF	33.34	23.9	45.34	35.74	18.17	22.61	43.9
VEGF	0	0	9.84	1.09	1.80	3.92	21.75
MCP-1	1445.21	1445.21	1445.15	1339.21	0	1401.2	1059.21
MIP-1α	831.82	47.91	831.83	831.82	1.78	25.84	831.83
MIP-1β	1533.27	1533.27	1533.27	1529.29	0	100	1533.27
RANTES	0	0	0	0	0	0	0

The values are median responses in pg/ml, measured by Luminex® assay, on blood samples from 26 mothers.

Table 3.3. Cytokine and chemokine responses to individual innate stimuli, showing concentrations in culture supernatants from stimulated cord blood.

Cytokine/ Chemokine	**PAM3Cys-Ser (TLR 1/2 agonist)**	**FSL-1 (TLR 2/6 agonist)**	**LPS (TLR 4 agonist)**	**CL097 (TLR 7/8 agonist)**	**CpGODN2006 (TLR 9 agonist)**	**Mannan (DC-SIGN agonist)**	**Curdlan (Dectin-1 agonist)**
IFN-γ	0	0	5	5	0	0	10
TNF-α	51.76	7.05	245.18	142.02	0	3.61	396.22
IL-2	2	1	3	3	0	0	3
IL-12p70	2	1	2	0	0	1	4
IL-1β	31	4	130	125	0	2	3257
IL-6	16025	338	18873	18765	0	44	18873
IL-4	0	0	0	0	0	0	0
IL-5	1	1	1	1	1	1	1
IL-13	0.63	0.23	0	2	0	0	4
IL-10	67	11	108	156	0	2	483
IL-17A	27	20	31	29	2	15	33
IP-10	5	9	15	26756	0	4	14
IL-8	26403	1532.49	26403	26403	181.31	2712.5	26403
GM-CSF	55	45	62.36	55	39	42	64
VEGF	37.17	9.69	6.60	0.91	0.23	13.41	62.42
MCP-1	1797	1597	1794	1797	0	1597	1798
MIP-1α	831	73	832	832	0	18	832
MIP-1β	1545	1544.98	1545	1544.98	0	1516.97	1544.98
RANTES	0	0	0	0	0	0	0

The values are median responses in pg/ml, measured by Luminex® assay. Cord blood samples from 26 infants were tested.

3.2.2.2. Correlations between concentrations of innate cytokines and chemokines in maternal post-delivery blood and in cord blood

The data above showed that the combined innate cytokine data in infants was similar to that of their mothers. To assess the responses in each group (mothers or infants), correlations between concentrations of cytokines and chemokines measured were assessed using Spearman rank correlation. This enabled the strength and direction of relationships between the cytokines and chemokines measured to be examined.

The results for mothers and infants are summarized in Tables 3.4 and 3.5, respectively. Values with statistical evidence of correlation are shown in bold.

For mothers, there were strong correlations observed between proinflammatory cytokines (IL-6 versus IL-1β (r=0.72)), and between proinflammatory cytokines and IL-10: (IL-1β versus IL-10 (r=0.71), IL-6 versus IL-10 (r= 0.75). There were more cytokines and chemokines correlated with TNF-α (IL-1β, IL-2, IL-6 and IL-10) than IFN-γ (IL-2 and IL-12p70) (Table 3.4).

In the infants, 5 cytokines and chemokines showed statistical evidence of correlation with both IFN-γ and TNF-α, and these included IL-1β, IL-2, IL-4, IL-8 and IL-10. The cytokines and chemokines that were strongly correlated included TNF-α versus IL-4 (r=0.70), TNF-α versus IL-8 (r=0.80), IL-2 versus IL-1β (r=0.75), IL-4 versus IL-1β (r=0.79), IL-4 versus IL-2 (r=0.98), IL-8 versus IL-2 (r=0.75), IL-8 versus IL-4 (R=0.72), IL-10 versus IL-1β (r=0.71) and MIP-1α versus IL-4 (r=0.73) (Table 3.5).

Table 3.4. Pairwise Spearman rank correlation coefficients for concentrations of cytokines and chemokines in maternal blood measured by Luminex®assay.

	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IL-13	IL-17A	GMCSF	IFN-γ	IP-10	MCP-1	MIP-1α	MIP-1β	RANTES	TNF-α
IL-2	0.22	1.00															
IL-4	0.30	**0.53**	1.00														
IL-6	**0.72**	0.38	**0.44**	1.00													
IL-8	**0.43**	0.38	**0.42**	**0.53**	1.00												
IL-10	**0.71**	**0.40**	0.38	**0.75**	**0.47**	1.00											
IL-12p70	0.26	0.23	0.08	0.06	0.17	0.15	1.00										
IL-13	0.28	0.22	0.26	0.31	0.15	**0.57**	0.06	1.00									
IL-17A	-0.23	0.37	-0.09	-0.02	0.12	-0.19	-0.01	-0.08	1.00								
GMCSF	0.21	0.37	0.11	0.20	0.10	0.12	0.16	-0.04	**0.50**	1.00							
IFN-γ	-0.03	**0.56**	0.10	0.00	0.19	0.04	**0.41**	-0.23	0.36	0.33	1.00						
IP-10	0.37	0.12	0.13	0.25	0.25	0.30	**0.53**	0.24	-0.23	-0.03	0.23	1.00					
MCP-1	-0.29	-0.10	**-0.54**	-0.23	-0.10	-0.27	-0.10	-0.17	**0.44**	0.02	0.37	-0.01	1.00				
MIP-1α	0.23	0.16	0.15	**0.50**	**0.40**	0.31	-0.20	0.10	0.32	**0.50**	-0.08	-0.22	-0.01	1.00			
MIP-1β	**-0.41**	0.18	-0.31	-0.23	-0.01	-0.24	-0.36	-0.24	**0.52**	-0.01	0.23	-0.22	**0.49**	0.07	1.00		
RANTES	-0.09	-0.19	0.13	0.03	-0.32	-0.31	-0.10	-0.21	-0.01	0.15	-0.13	-0.23	-0.09	0.20	-0.18	1.00	
TNF-α	**0.52**	**0.46**	0.30	**0.39**	0.33	**0.48**	0.01	0.06	0.04	0.32	0.34	-0.13	0.00	0.13	-0.11	-0.10	1.00
VEGF	0.14	0.01	-0.15	0.05	0.20	-0.01	0.03	0.19	0.17	-0.21	-0.35	-0.05	0.18	0.09	0.10	**-0.44**	-0.04

Correlation between concentrations of cytokines and chemokines from stimulated samples from 26 mothers was assessed using the Spearman rho test. *P* values less than 0.05 are shown in bold.

Table 3.5. Pairwise Spearman rank correlation coefficients for concentrations of cytokines and chemokines in cord blood measured by Luminex®assay.

	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IL-13	IL-17A	GMCSF	IFN-γ	IP-10	MCP-1	MIP-1α	MIP-1β	RANTES	TNF-α
IL-2	**0.75**	1.00															
IL-4	**0.79**	**0.98**	1.00														
IL-6	**0.62**	**0.61**	**0.56**	1.00													
IL-8	**0.56**	**0.75**	**0.72**	0.36	1.00												
IL-10	**0.71**	**0.61**	**0.67**	**0.43**	**0.53**	1.00											
IL-12p70	0.38	0.33	0.36	0.22	0.34	0.23	1.00										
IL-13	0.31	**0.56**	**0.52**	0.23	0.22	0.14	0.11	1.00									
IL-17A	0.26	**0.55**	**0.53**	0.21	**0.47**	0.33	0.32	0.03	1.00								
GMCSF	**0.41**	**0.42**	**0.40**	0.18	0.27	0.28	**0.55**	**0.41**	0.11	1.00							
IFN-γ	**0.47**	**0.68**	**0.69**	0.19	**0.67**	**0.46**	0.37	**0.46**	**0.44**	**0.53**	1.00						
IP-10	0.12	-0.05	-0.01	0.21	-0.22	0.14	-0.07	-0.11	0.01	0.20	0.01	1.00					
MCP-1	0.20	0.34	0.36	0.00	0.39	0.25	-0.10	0.11	**0.43**	0.15	0.48	0.20	1.00				
MIP-1α	**0.58**	**0.66**	**0.73**	**0.48**	**0.46**	**0.58**	0.18	**0.40**	0.31	0.25	0.35	-0.12	0.36	1.00			
MIP-1β	-0.14	0.04	0.01	-0.02	0.05	0.01	0.10	-0.06	**0.42**	-0.05	0.10	-0.17	0.19	-0.17	1.00		
RANTES	0.09	-0.15	-0.10	0.06	**-0.40**	0.13	-0.07	0.04	**-0.48**	0.07	-0.05	0.19	-0.13	0.02	-0.19	1.00	
TNF-α	**0.61**	**0.68**	**0.70**	0.28	**0.80**	**0.58**	0.16	0.22	0.32	0.19	0.64	-0.10	0.34	**0.48**	-0.15	-0.02	1.00
VEGF	**0.46**	0.34	0.38	0.24	0.34	0.30	**0.93**	0.15	0.30	**0.45**	0.39	-0.08	-0.10	0.21	0.08	0.04	0.22

Correlation between concentrations of cytokines and chemokines from stimulated cord blood samples from 26 infants was assessed using the Spearman rho test. *P* values less than 0.05 are shown in bold.

3.2.2.3. Principle Component Analysis of maternal and infant innate immune responses

Having shown correlations for cytokines and chemokines, a PCA was performed to better visualize the data and see whether the data grouped according to particular characteristics – for example whether the pro-inflammatory cytokines grouped together. These are illustrated in Tables 3.6 (for mothers) and 3.7 (for infants).
For the mothers, two principle components (PCs) were identified that summarized the cytokines and chemokines measured. Together, these accounted for 43% of the variance in the dataset. The first PC (PC1) explained 25% of the total variance and was characterized by IFN-γ, TNF-α, IL-12p70, IL-1β, IL-6, IL-4, IL-10, IL-13 and the second PC (PC2) explained a further 18% of the total variance and was characterized by MCP-1, MIP-1α, MIP-1β, IL-8, and IL-17A based on factor loadings > 0.1 (Table 3.6, Figure 3.2A).

In the mothers, most cytokines and chemokines measured were positively loaded in PC1, except for IL-17A, VEGF, MCP-1, MIP-1β and RANTES which were negatively loaded, meaning an increase of PC1 would represent an increase in the first set of cytokines and chemokines and a simultaneous decrease in the second set of cytokines and chemokines (Table 3.6, right panel).

For the infants, two PCs identified accounted for 53% of the variance in the dataset. PC1 explained 39% of the total variance and was characterized by most of the cytokines measured (IFN-γ, TNF-α, IL2, IL-12p70, IL-4, IL-13, IL-10, IL-1β, IL-6, IL-8, VEGF and GM-CSF) (Table 3.7, Figure 3.2C). PC2 explained a further 14% of the total variance and was characterized by MCP-1 and MIP-1β. In the infants, most cytokines and chemokines measured were positively loaded in PC1, except for MIP-1β and RANTES which were negatively loaded, meaning an increase of PC1 would represent an increase in the first set of cytokines and chemokines and a simultaneous decrease of MIP-1β and RANTES (Table 3.7, right panel).

Mothers with and without a BCG scar were scattered all over the two PCs (Figure 3.2B), whereas infants of mothers with a BCG scar, compared to those without, grouped towards PC1 (Figure 3.2D).

Table 3.6. Principal Component Analysis of cytokine concentrations in maternal post-delivery blood.

Component	Eigenvalue	Difference	Proportion	Cumulative
Component 1	**4.36**	**1.31**	**0.25**	**0.25**
Component 2	**3.05**	**1.03**	**0.18**	**0.43**
Component 3	2.02	0.36	0.11	0.54
Component 4	1.67	0.29	0.09	0.63
Component 5	1.37	0.15	0.08	0.69
Component 6	1.22	0.27	0.07	0.71
Component 7	0.96	0.14	0.06	0.77
Component 8	0.82	0.10	0.05	0.82
Component 9	0.72	0.24	0.04	0.86
Component 10	0.48	0.14	0.03	0.89
Component 11	0.35	0.06	0.02	0.91
Component 12	0.28	0.07	0.02	0.92
Component 13	0.21	0.02	0.02	0.94
Component 14	0.19	0.04	0.01	0.95
Component 15	0.15	0.06	0.01	0.96
Component 16	0.09	0.06	0.01	0.97
Component 17	0.35	0.03	0	1.00
Component 18	0	-	0	1.00

Variable	Component 1	Component 2
IFN-γ	**0.31**	0.13
TNF-α	**0.28**	-0.01
IL-2	**0.37**	0.18
IL-12p70	**0.23**	0.09
IL-1β	**0.31**	-0.17
IL-6	**0.26**	-0.02
IL4	**0.24**	-0.10
IL-13	**0.25**	-0.03
IL-10	**0.39**	-0.11
IL-17A	-0.06	**0.47**
IP-10	**0.32**	0.14
IL-8	0.07	**0.48**
GM-CSF	0.13	**0.22**
VEGF	-0.05	0.05
MCP-1	-0.09	0.16
MIP-1α	0.01	**0.47**
MIP-1β	**-0.20**	**0.33**
RANTES	-0.15	-0.07

Principal components analysis of 18 cytokines from supernatants from 26 maternal post-delivery blood samples stimulated with innate stimuli. In bold are cytokines and chemokines with important contributions (≥0.20) in the component. Linear combination of the variables listed in Table 10 (right panel) constitute component 1. For example Component 1= 0.31 log IFN-γ + 0.28 log TNF-α+ 0.37 log IL-2 +0.23 log IL-12p70 +0.31 log IL-1β + 0.26 log IL-6 + 0.24 log IL-4 + 0.25 log IL-13 + 0.39 log IL-10 + -0.06 log IL-17A + 0.32 log IP-10 + 0.07 log IL-8+ 0.13 log GM-CSF + -0.05 log VEGF + -0.09 log MCP-1 + 0.01 log MIP-1α + -0.20 log MIP-1β + -0.15 log RANTES.

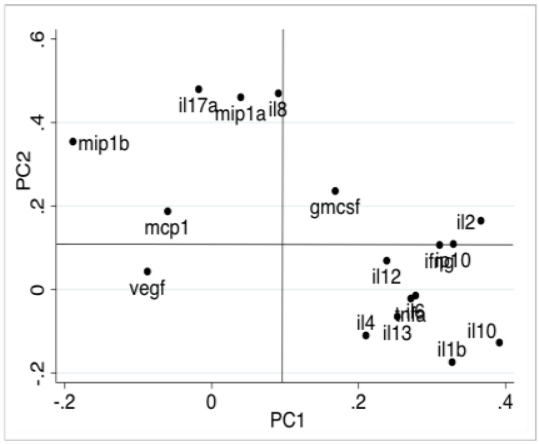
Table 3.7. Principal Component Analysis of cytokine and chemokine concentrations in cord blood

Component	Eigenvalue	Difference	Proportion	Cumulative
Component 1	**6.90**	**4.51**	**0.39**	**0.39**
Component 2	**2.39**	**0.54**	**0.14**	**0.53**
Component 3	1.85	0.41	0.12	0.65
Component 4	1.43	0.45	0.08	0.73
Component 5	0.98	0.18	0.07	0.80
Component 6	0.80	0.07	0.05	0.85
Component 7	0.74	0.28	0.04	0.89
Component 8	0.46	0.08	0.03	0.92
Component 9	0.37	0.08	0.02	0.94
Component 10	0.29	0.03	0.02	0.96
Component 11	0.26	0.09	0.01	0.97
Component 12	0.17	0.02	0.01	0.98
Component 13	0.15	0.03	0.01	0.99
Component 14	0.12	0.06	0.01	1.00
Component 15	0.05	0.02	0.	1.00
Component 16	0.03	0.03	0	1.00
Component 17	0		0	1.00

Variable	Component 1	Component 2
IFN-γ	**0.29**	-0.13
TNF-α	**0.27**	-0.05
IL-2	**0.36**	-0.01
IL-12p70	**0.25**	-0.18
IL-1β	**0.30**	-0.01
IL-6	0.17	-0.01
IL4	**0.36**	0.04
IL-13	0.16	**-0.42**
IL-10	**0.30**	0.11
IL-17A	**0.24**	**0.41**
IL-8	**0.30**	-0.05
GM-CSF	**0.22**	-0.19
VEGF	**0.23**	-0.15
MCP-1	0.07	**0.51**
MIP-1α	**0.20**	**0.47**
MIP-1β	-0.01	**0.21**
RANTES	-0.05	0.05

Principal components analysis of 17 cytokines from supernatants from cord blood from 26 infants stimulated with innate stimuli. In bold are cytokines and chemokines with important contributions (≥0.20) in the component. Linear combination of the variables listed in Table 11 (right panel) constitute component 1. For example Component 1= 0.29 log IFN-γ + 0.27 log TNF-α+ 0.36 log IL-2 +0.25 log IL-12p70 +0.30 log IL-1β + 0.17 log IL-6 + 0.36 log IL-4 + 0.16 log IL-13 + 0.30 log IL-10 + 0.24 log IL-17A + 0.30 log IL-8+ 0.22 log GM-CSF + 0.23 log VEGF + 0.07 log MCP-1 + 0.20 log MIP-1α + -0.01 log MIP-1β + -0.05 log RANTES.

A



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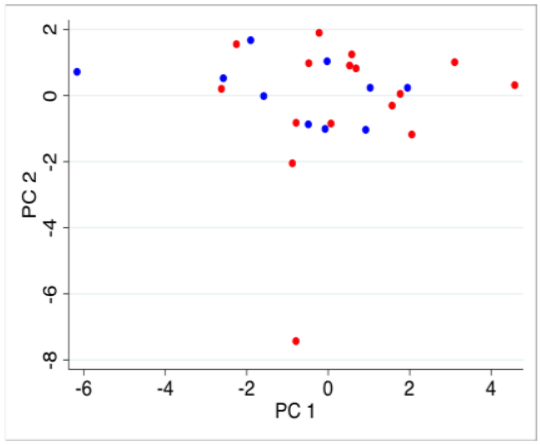

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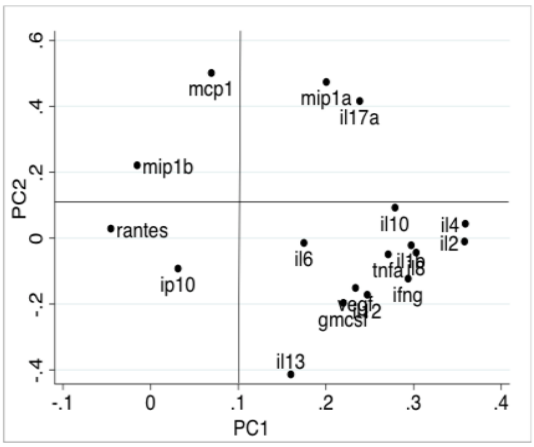

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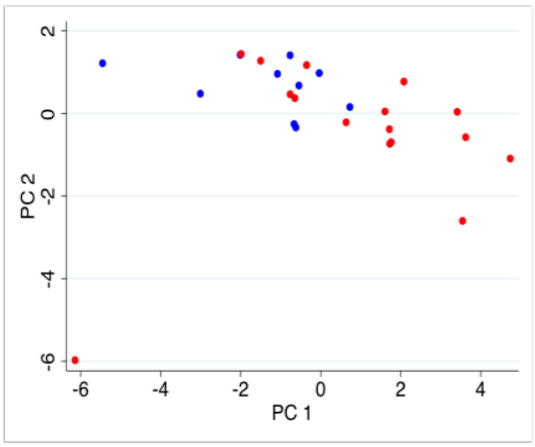


Figure 3.2. Scatterplots of first and second factor loadings for mothers' post-delivery blood and infant cord blood, derived from Principal Component Analysis of cytokines and chemokines measured. The graphs show cytokines and chemokines for mothers (A) and infants (C), with individual mothers (B) and neonates (D) represented as discrete plots. For mothers, the first principal component (PC) was characterized by a mixture of cytokines and the second PC consisted of chemokines. For neonates, the first PC was characterized by proinflammatory cytokines and the second PC consisted of chemokines, based on factor loadings >0.1. Red circles represent BCG scar-positive (Scar+) mothers or their infants. BCG scar-negative (Scar-) mothers or their infants are represented by blue circles. Samples from 26 mother-infant pairs were tested.

3.2.2.4. Analysis of clusters of concentrations of cytokines and chemokines

The finding that infants of mothers with a BCG scar, compared to those without, grouped towards PC1 was an unexpected finding. To further examine sets of cytokines and chemokines that might be expressed coordinately in infants of mothers with and without a BCG scar, a hierarchical bicluster analysis of the stimulated innate cytokine and chemokine responses was performed. This was done to determine whether the cytokines and chemokines grouped together in a particular pattern. As illustrated in Figure 3.3, three cytokine and chemokine clusters were identified: Cluster (C) 1 was characterized by MCP-1, MIP-1α, MIP-1β, IL-17A; C2 by VEGF, GM-CSF, IL-12p70 and C3 by IL-1β, IL-8, TNF-α, IFN-γ, IL-2, IL-4, IL-10. There was an additional cluster (C4) that contained high concentrations of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β, IL-6 and IL-8, mainly produced by infants of mothers with a BCG scar.

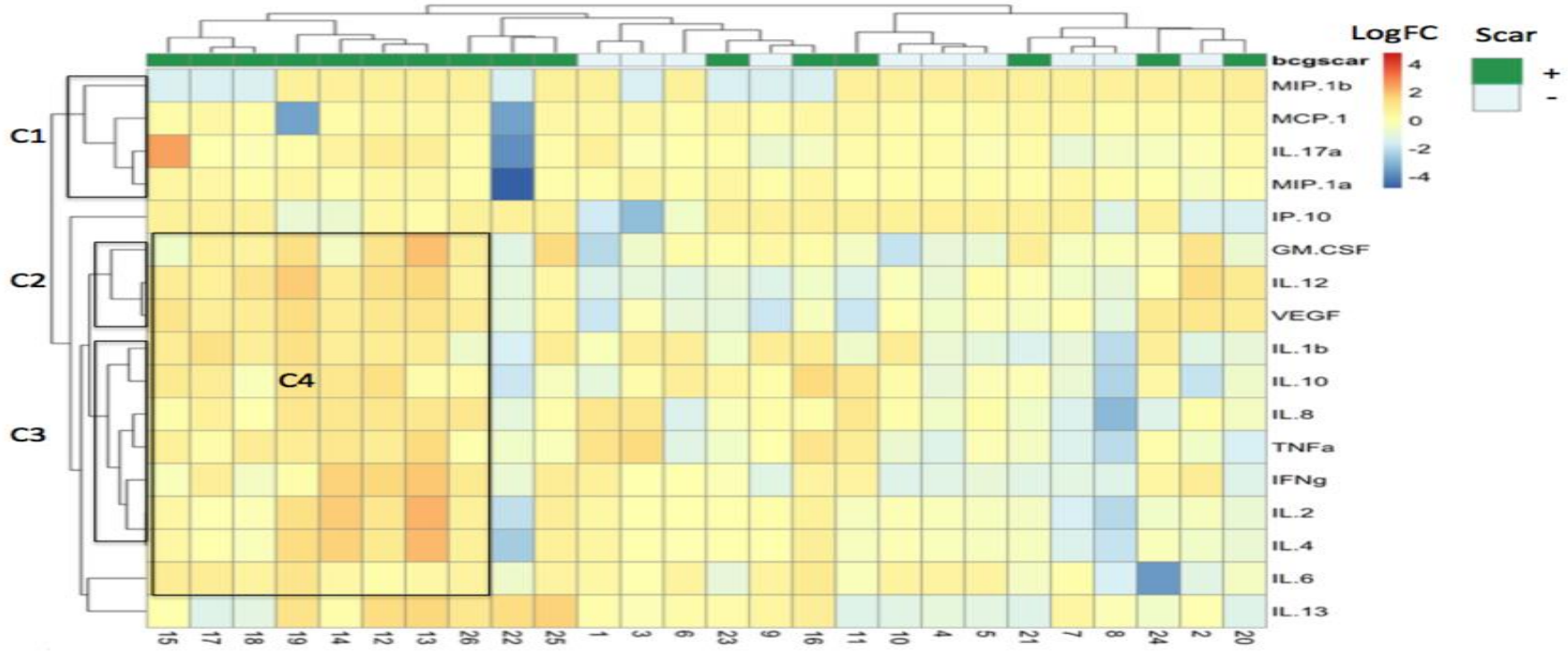


Figure 3.3. Cluster analysis of the stimulated innate cytokine and chemokine responses using the average linkage distance between clusters using R. Clusters go from root to leaf node for each cytokine and for the individual infants. Clusters in between are based on their agglomeration value. The branch shows the similarity; the shorter the branch, the higher the similarity. Expression levels of individual cytokines (log_{10} [pg/ml]) are represented by shades of blue to red based on their correlations according to the dendrogram on the left, with highest values in dark red and the lowest in dark blue. Three distinct sets of correlated cytokines "clusters" (C) are indicated as C1, C2 and C3 on the left. In addition, eleven cytokines (C4) formed a cluster that had mainly inflammatory cytokines. Most infants of mothers with a BCG scar (top, green) clustered together in one discrete group, distinct from infants of mothers without a BCG scar (top, light blue).

3.2.3. Factors associated with maternal and infant innate immune responses

3.2.3.1. Maternal latent *M. tuberculosis* infection

Having shown that the cytokines and chemokines measured formed clusters, the associations between innate infant and maternal responses and maternal LTBI was assessed. This is important because if infant responses differed based on maternal LTBI, then this may argue for treatment of LTBI in pregnant mothers and/or may be a contributing factor to the poor efficacy of BCG in this setting.

The median cytokine and chemokine responses in mothers without and with LTBI, and in their infants, based on the innate stimulated cytokine data, are shown in Table 3.8, and the associations between maternal LTBI and maternal responses are illustrated in Figure 3.4. For the combined responses, maternal responses were not associated with their own LTBI, except for IL-13 where mothers without LTBI had higher median responses than those with LTBI.

Figure 3.5 illustrates the associations between maternal LTBI and infant innate responses. Maternal LTBI was not associated with innate infant responses in the cord blood, except for IP-10 where mothers with LTBI, compared to those without, had overall higher responses, with an aGMR [95% CI] of 5.10 [1.21, 21.48] (Table 3.9). This was after adjusting for the effect of maternal BCG scar on infant responses.

Table 3.8. Cytokine and chemokine responses in mothers without and with LTBI, and in their infants, measured by Luminex® assay.

Cytokine/ Chemokine	Mothers without LTBI (n=16)	Mothers with LTBI (n=10)	*p* value	Cytokine/ Chemokine	Infants of mothers without LTBI (n=17)	Infants of mothers with LTBI (n=12)	*p* value
IFN-γ	26 (24, 32)	35 (20, 37)	0.370	IFN-γ	4 (1, 9)	6 (0, 9)	0.790
TNF-α	343 (138, 485)	150 (60, 369)	0.171	TNF-α	114 (59, 223)	89 (54, 331)	0.658
IL-2	2 (2, 3)	2 (2,3)	0.268	IL-2	2 (1, 2)	2 (1, 2)	0.579
IL-12p70	1 (1, 3)	2 (1, 3)	0.399	IL-12p70	3 (0, 5)	1 (0, 3)	0.658
IL-1β	312 (73, 560)	497 (77, 520)	0.544	IL-1β	499 (70, 533)	338 (63, 540)	0.859
IL-6	10796 (6383, 10880)	10809 (5588, 10846)	0.493	IL-6	9475 (4805, 10857)	10350 (8336, 10828)	0.400
IL-4	0 (0, 0)	0 (0, 0)	0.09	IL-4	1 (1, 2)	1 (1, 2)	0.707
IL-13	2 (1, 3)	1 (0, 1)	**0.03**	IL-13	2 (1, 4)	2 (0, 8)	0.790
IL-10	135 (99, 216)	123 (61, 141)	0.188	IL-10	130 (78, 180)	107 (64, 181)	0.757
IL-17A	18 (12, 20)	24 (17, 28)	0.082	IL-17A	22 (18, 26)	24 (20, 29)	0.376
IP-10	3878 (3843, 4041)	2683 (818, 4004)	0.343	IP-10	2063 (123, 3849)	3838 (3826, 3852)	0.215
IL-8	12112 (11447, 17370)	11607 (9661, 15039)	0.399	IL-8	15448 (10811, 22658)	15433 (12532, 22669)	0.757
GM-CSF	13 (10, 18)	14 (13, 16)	0.673	GM-CSF	53 (45, 59)	51 (42, 66)	1.00
VEGF	7 (2, 15)	8 (3, 16)	0.874	VEGF	54 (3, 67)	13 (6, 76)	0.790
MCP-1	1093 (74, 1905)	1417 (774, 2918)	0.225	MCP-1	1329 (1076, 1529)	1397 (1222, 1524)	0.215
MIP-1α	505 (479, 596)	495 (480, 627)	0.916	MIP-1α	481 (442, 652)	505 (480, 600)	0.425
MIP-1β	0 (0, 1377)	1273 (0, 1407)	0.233	MIP-1β	1204 (0, 1366)	1328 (1222, 1391)	0.163

The values are shown as medians in pg/ml with the interquartile range in brackets. The p values shown were obtained using Mann-Whitney test.

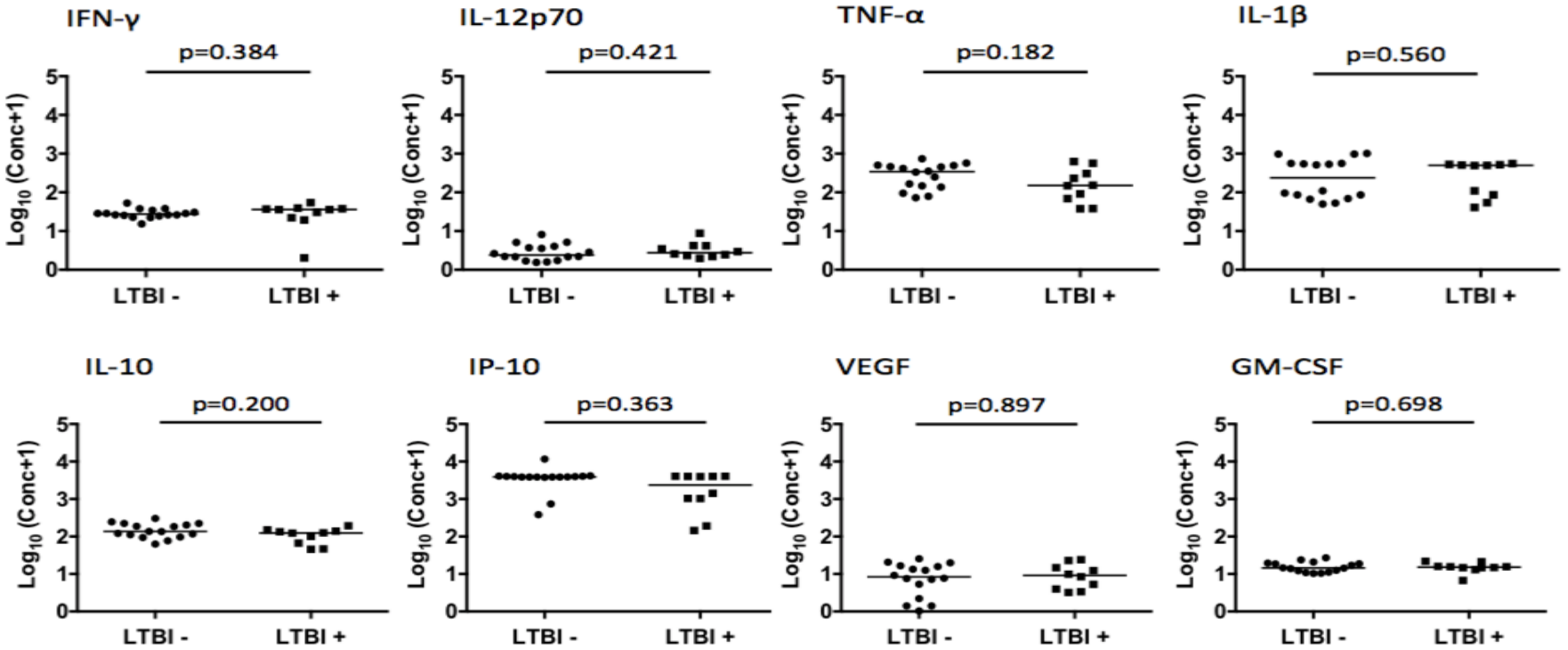


Figure 3.4. The association between maternal LTBI and maternal innate immune responses. Median cytokine and chemokine production following overnight stimulation with LPS (TLR 4 agonist), FSL-1 (TLR2/6 agonist), CpG-ODN2006 (TLR9 agonist), PAM3Cys-Ser (TLR1/2 agonist), CL097 (TLR7/8 agonist), Mannan (DC-SIGN agonist) and Curdlan (Dectin-1 agonist) are shown. Cytokines representing Th1/proinflammatory responses (IFN-γ, IL-12p70, TNF-α and IL-1β), immunoregulatory responses (IL-10) and chemokines/growth factors (IP-10, VEGF and GM-CSF) measured by Luminex® assay are shown for mothers without (n=17) and with (n=12) LTBI. Data presentation was performed using GraphPad Prism.

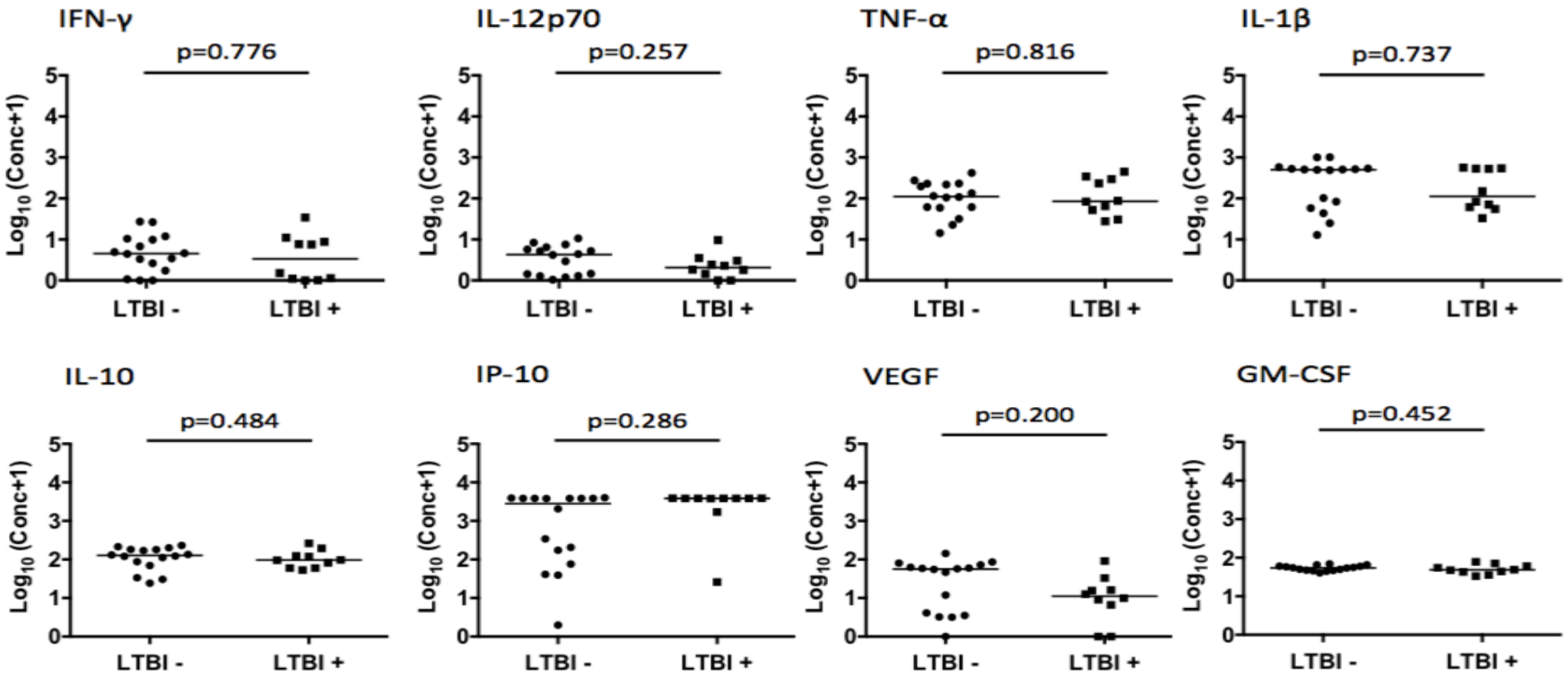


Figure 3.5. The association between maternal LTBI and infant innate immune responses. Median cytokine and chemokine production following overnight stimulation with LPS (TLR 4 agonist), FSL-1 (TLR2/6 agonist), CpG-ODN2006 (TLR9 agonist), PAM3Cys-Ser (TLR1/2 agonist), CL097 (TLR7/8 agonist), Mannan (DC-SIGN agonist) and Curdlan (Dectin-1 agonist) are shown. Cytokines representing Th1/proinflammatory responses (IFN-γ, IL-12p70, TNF-α and IL-1β), immunoregulatory responses (IL-10) and chemokines/growth factors (IP-10, VEGF and GM-CSF) measured by Luminex® assay are shown for cord blood samples from infants of mothers without (n=17) and with (n=12) LTBI. Data presentation was performed using GraphPad Prism.

Table 3.9. The association between maternal LTBI and infant immune responses.

Cytokine/ Chemokine	**Crude GMR, 95% CI**	**Adjusted GMR, (95% CI)** [a]
IFN-γ	0.99 (0.41, 2.45)	0.95 (0.41, 2.24)
TNF-α	1.17 (0.57, 2.40)	1.23 (0.60, 2.57)
IL-2	1.07 (0.85, 1.35)	1.07 (0.85, 1.38)
IL-12p70	0.85 (0.48, 1.55)	0.78 (0.43, 1.38)
IL-1β	0.91 (0.37, 2.34)	0.79 (0.27, 2.24)
IL-6	1.35 (0.91, 1.95)	1.38 (0.89, 2.09)
IL-4	1.10 (0.89, 1.35)	1.10 (0.89, 1.38)
IL-13	1.10 (0.57, 2.04)	0.87 (0.44, 1.74)
IL-10	1.07 (0.68, 1.70)	1.07 (0.69, 1.66)
IL-17A	1.12 (0.81, 1.55)	1.12 (0.85, 1.51)
IP-10	**3.80 (1.02, 14.45)**	**5.10 (1.21, 21.38)**
IL-8	1.15 (0.85, 1.55)	1.12 (0.83, 1.55)
GM-CSF	0.99 (0.83, 1.17)	0.95 (0.81, 1.15)
VEGF	0.74 (0.22, 2.51)	0.54 (0.16, 1.78)
MCP-1	2.40 (0.81, 7.08)	2.24 (0.79, 6.46)
MIP-1α	1.51 (0.72, 3.09)	1.44 (0.74, 2.75)
MIP-1β	5.89 (0.63, 54.95)	9.77 (0.83, 114.81)

The values are shown as crude and adjusted geometric mean ratios (GMR) with 95% CI for 29 infant samples. [a] adjusted for maternal BCG scar.

3.2.3.2. Associations between maternal LTBI and infant responses to the individual stimuli

Having shown little association with maternal LTBI in the combined cytokine and chemokine response analysis, it was possible that there were subtle differences between individual stimuli that were lost due to the averaging process. Infant responses to individual innate stimuli were therefore analysed to assess their associations with maternal LTBI. Figures 3.6A and 3.6B illustrate these analyses. The following CpG-specific cytokines and chemokines were positively associated with maternal LTBI: IL-12p70 ($p=0.014$), MCP-1 ($p=0.011$) and MIP-1β ($p=0.007$) (Figure 3.6B). No other analyte measured, or stimulus, was associated with maternal LTBI.

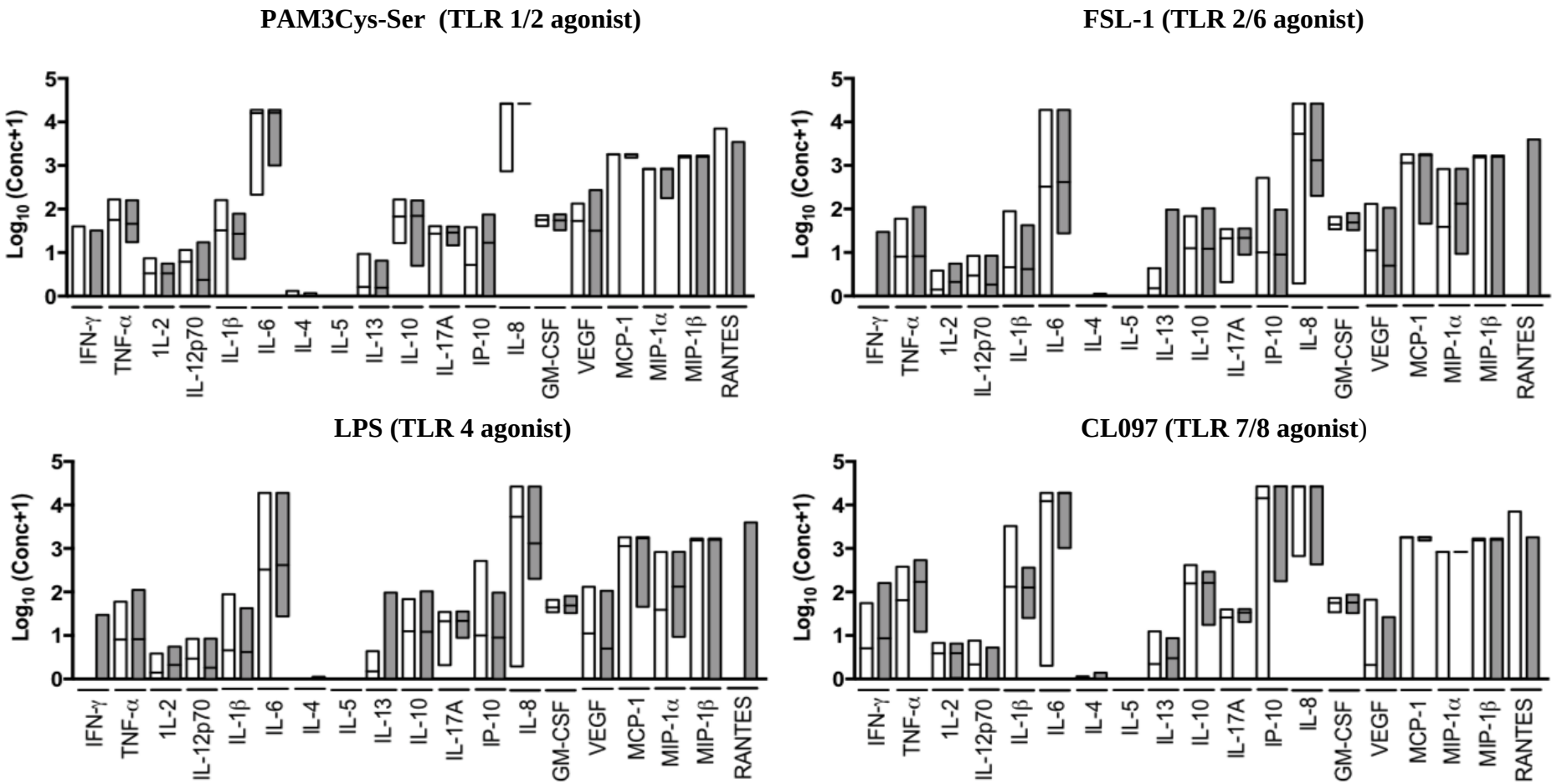


Figure 3.6A. **Cytokine and chemokine responses to TLR1/2, TLR2/6, TLR4 and TLR7/8 agonists**. Concentrations in cord blood are shown. Clear and grey bars represent infants of mothers without (n=17) and with LTBI (n=12), respectively. The horizontal lines represent the median. Statistically significant differences are shown by (*). Kruskal-Wallis test was used to compare the two groups of infants.

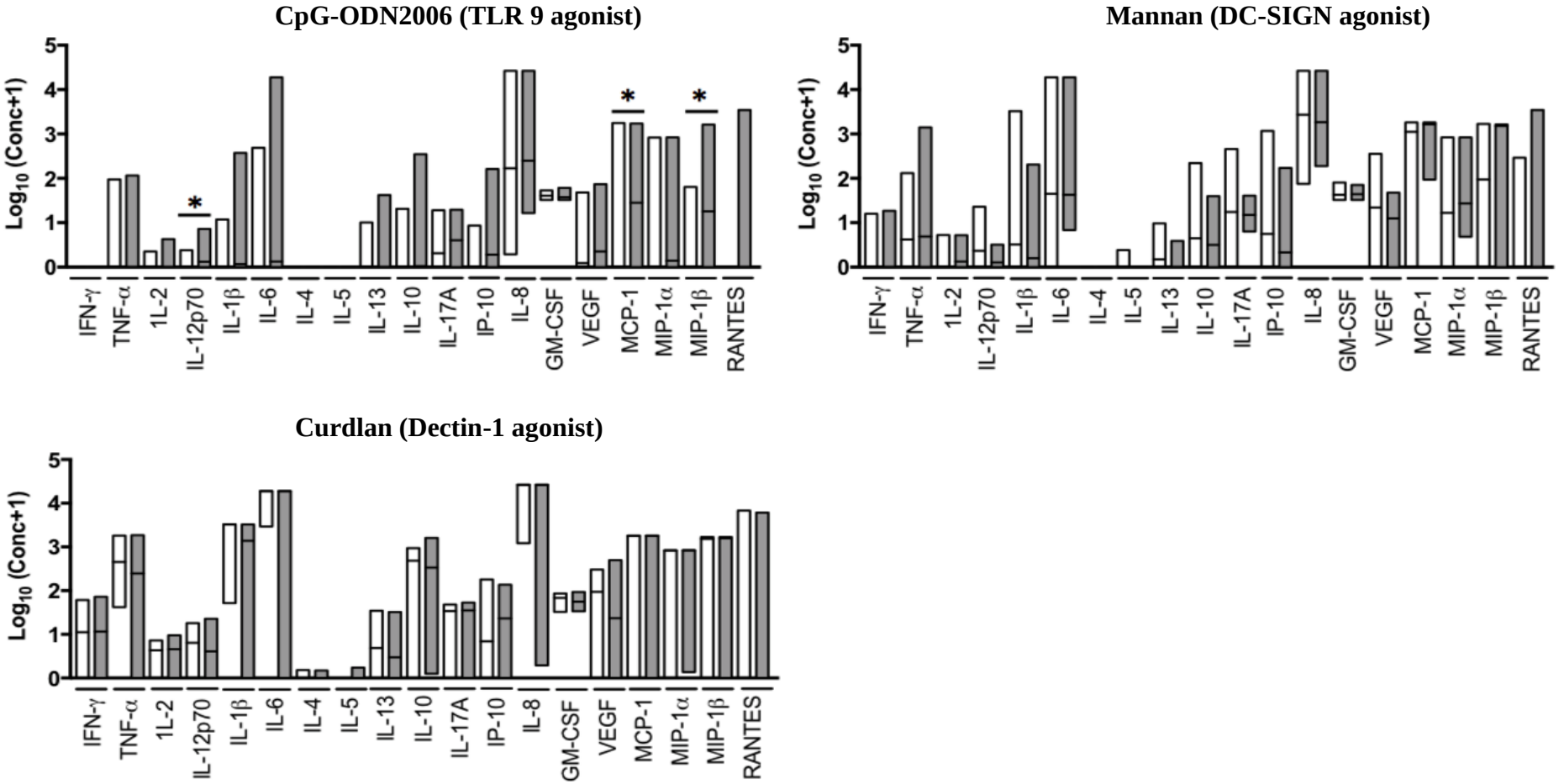


Figure 3.6B. Cytokine and chemokine responses to TLR 9, DC-SIGN and Dectin-1 agonists. Concentrations in cord blood are shown. Clear and grey bars represent infants of mothers without (n=17) and with (n=12) LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by (*). Kruskal-Wallis test was used to compare the two groups of infants.

3.2.3.3. Maternal BCG scar

Having shown that there was little association between maternal LTBI and the innate infant response to BCG immunisation, and that infants of mothers with a BCG scar clustered together, the effect of maternal BCG scar on infant innate responses was examined. This was important to query because maternal BCG scar has been associated with differential infant T cell responses, and we know that a good innate immune response leads to a better quality adaptive immune response (336).

For the combined responses, maternal responses were not associated with their own BCG scar, except for VEGF where mothers without a BCG scar, compared to those with, had higher concentrations (p=0.031, Figure 3.7). For IL-4, mothers with a BCG scar, compared to those without, had higher responses (p=0.012, Table 3.10). Compared to those without a BCG scar, cord blood samples obtained from infants of mothers with a BCG scar had overall higher responses to innate stimuli for the following analytes: IFN-γ (aGMR 2.69 [1.15, 6.17]), IL-12p70 (1.95 [1.10, 3.55]), IL-10 (1.82 [1.07, 3.09]), VEGF (3.55 [1.07, 11.48]) and IP-10 (6.76 [1.17, 38.02] (Figure 3.8, and Tables 3.10 and 3.11) after adjusting for the effect of maternal LTBI.

Table 3.10. Cytokine and chemokine responses in mothers without and with a BCG scar, and in their infants, measured by Luminex® assay.

Cytokine/ Chemokine	Mothers without a BCG scar (n=10)	Mothers with a BCG scar (n=16)	p value	Cytokine/ Chemokine	Neonates of mothers without a BCG scar (n=10)	Neonates of mothers with a BCG scar (n=16)	p value
IFN-γ	24 (19, 36)	29 (25, 36)	0.215	**IFN-γ**	0 (0, 5)	6 (2, 11)	**0.035**
TNF-α	197 (60, 391)	328 (107, 486)	0.327	TNF-α	55 (29, 166)	164 (69, 232)	0.076
IL-2	2 (2, 2)	2 (2, 3)	0.215	IL-2	1.2 (1, 2)	2 (1, 3)	0.146
IL-12p70	2 (1, 3)	1 (1, 2)	0.333	**IL-12p70**	0 (0, 1)	3 (1, 5)	**0.017**
IL-1β	497 (62, 556)	309 (85, 590)	0.989	IL-1β	110.04 (52, 505)	512 (82, 557)	0.076
IL-6	10792 (5181, 10843)	10814 (6235, 10877)	0.504	IL-6	10069 (7045, 10795)	9694 (5730, 12844)	0.643
IL-4	0.00 (0, 0)	0.147 (0, 2)	**0.012**	IL-4	1.23 (1, 2)	1 (1, 2)	0.070
IL-13	1 (0, 2)	1 (1, 3)	0.219	IL-13	2 (0.25, 2)	2 (0, 7)	0.443
IL-10	105 (58, 160)	135 (113, 197)	0.085	**IL-10**	77 (47, 119)	132 (89, 198)	**0.010**
IL-17A	19 (14, 24)	19 (12, 26)	0.89	IL-17A	19 (16, 23)	24 (20, 27)	0.085
IP-10	3950 (866, 4028)	3863 (2007, 3998)	0.89	IP-10	2085 (35, 3842)	3847 (1796, 3854)	0.076
IL-8	11587 (9634, 12750)	13423 (11466, 9634)	0.256	IL-8	15289 (8122, 17340)	15499 (12230, 22671)	0.215
GM-CSF	13 (10, 16)	15 (12, 18)	0.328	**GM-CSF**	46 (40, 54)	56 (46, 63)	**0.046**
VEGF	16 (7, 22)	6 (2, 11)	**0.031**	**VEGF**	8 (2, 15)	55 (8, 75)	**0.035**
MCP-1	1399 (1092, 2407)	1007 (118, 2110)	0.391	MCP-1	1166 (1084, 1480)	1359 (1247, 1541)	0.196
MIP-1α	486 (478, 597)	506 (482, 601)	0.328	MIP-1α	480.10 (478, 533)	503 (449, 677)	0.382
MIP-1β	1260 (0, 1437)	587 (0, 1381)	0.521	MIP-1β	1317 (850, 1381)	1304 (0, 1377)	0.683

The values are shown as medians in pg/ml with the interquartile range in brackets. The p values shown were obtained by Mann-Whitney test.

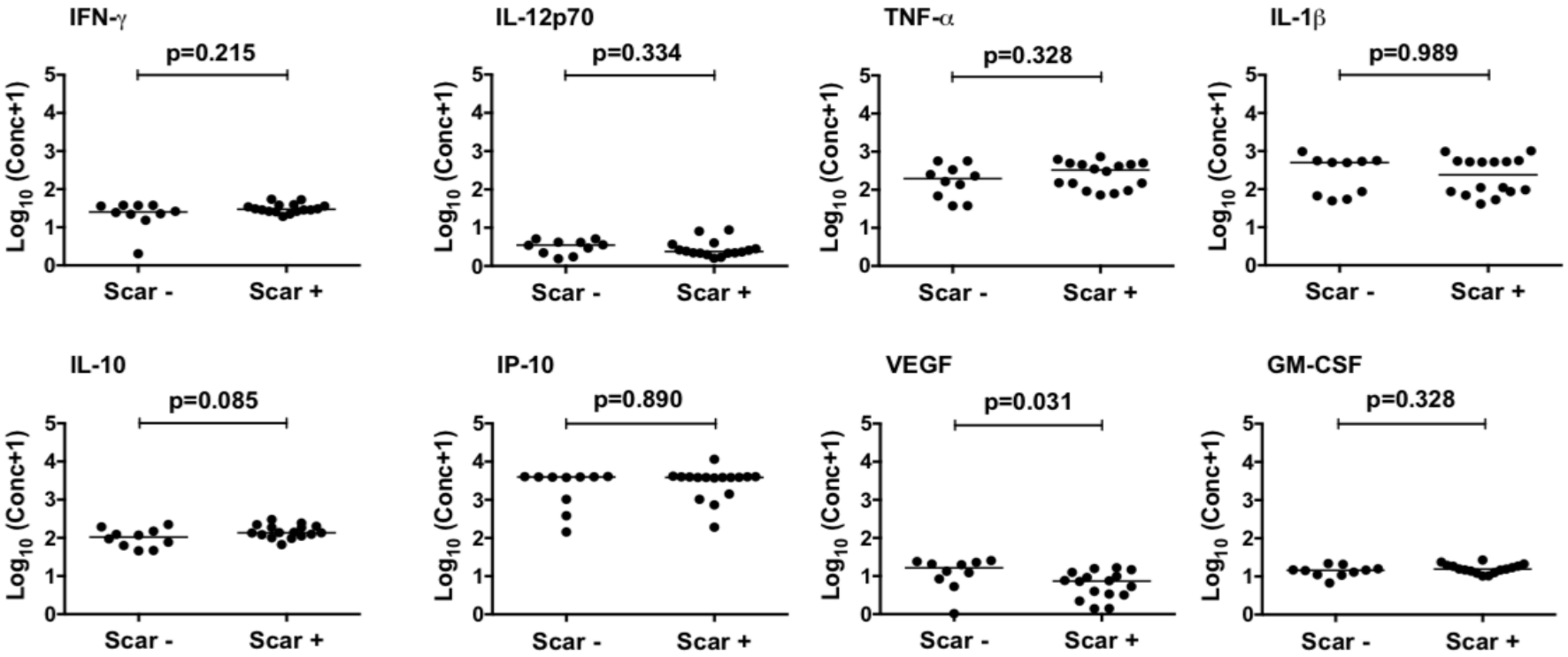


Figure 3.7. The association between maternal BCG scar and maternal innate immune responses. Median cytokine and chemokine production following overnight stimulation of maternal blood with LPS TLR) 4 agonist), FSL-1 (TLR2/6 agonist), CpG-ODN2006 (TLR9 agonist), PAM3Cys-Ser (TLR1/2 agonist), CL097 (TLR7/8 agonist), Mannan (DC-SIGN agonist) and Curdlan (Dectin-1 agonist) are shown. Cytokines representing Th1/proinflammatory responses (IFN-γ, IL-12p70, TNF-α and IL-1β), immunoregulatory responses (IL-10) and chemokines/growth factors (IP-10, VEGF and GM-CSF) measured by Luminex® assay are shown for the scar-negative (n=10) or scar-positive (n=16) mothers' blood. Data presentation was performed using GraphPad Prism.

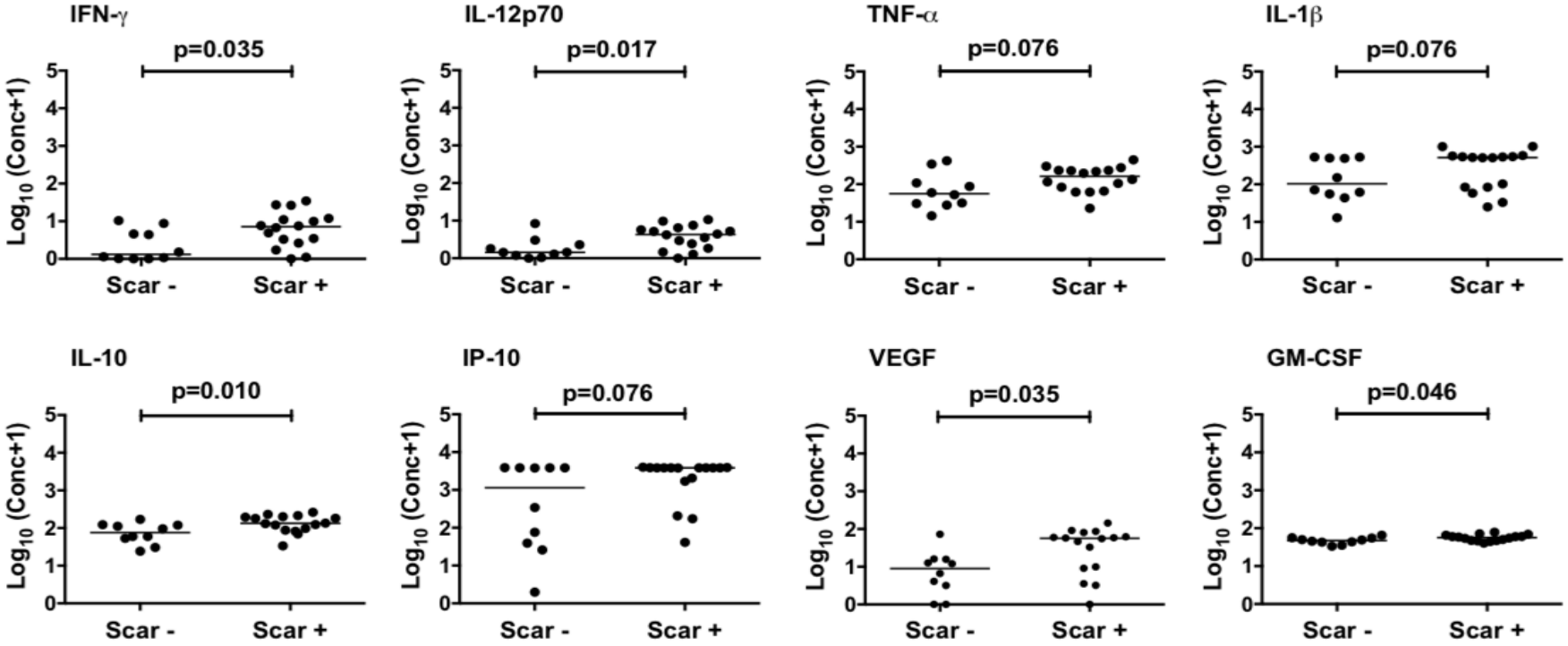


Figure 3.8. The association between maternal BCG scar and infant innate immune responses. Median cytokine and chemokine production following overnight stimulation with LPS (TLR 4 agonist), FSL-1 (TLR2/6 agonist), CpG-ODN2006 (TLR9 agonist), PAM3Cys-Ser (TLR1/2 agonist), CL097 (TLR7/8 agonist), Mannan (DC-SIGN agonist) and Curdlan (Dectin-1 agonist) are shown. Cytokines representing Th1/proinflammatory responses (IFN-γ, IL-12p70, TNF-α and IL-1β), immunoregulatory responses (IL-10) and chemokines/growth factors (IP-10, VEGF and GM-CSF) measured by Luminex® assay are shown for cord blood from infants of mother without (n=10) and with (n=16) a BCG scar. Data presentation was performed using GraphPad Prism.

Table 3.11. The association between maternal BCG scar and infant responses.

Cytokine/ Chemokine	Crude GMR, 95% CI	Adjusted GMR, (95% CI) [a]
IFN-γ	**2.69 (1.24, 5.83)**	**2.69 (1.15, 6.17)**
TNF-α	2.04 (0.98, 4.36)	1.99 (0.69, 5.89)
IL-2	1.25 (1.00, 1.57)	1.23 (0.89, 1.70)
IL-12p70	**2.06 (1.24, 3.43)**	**1.95 (1.10, 3.55)**
IL-1β	1.90 (0.71, 5.25)	1.55 (0.37, 6.61)
IL-6	0.98 (0.62, 1.55)	0.89 (0.45, 1.74)
IL-4	1.23 (1.00, 1.51)	1.20 (0.89, 1.62)
IL-13	1.35 (0.79, 2.34)	1.17 (0.63, 2.24)
IL-10	**1.82 (1.14, 2.88)**	**1.82 (1.07, 3.09)**
IL-17A	1.12 (0.81, 1.55)	1.10 (0.71, 1.70)
IP-10	5.01 (0.79, 30.90)	**6.76 (1.17, 38.02)**
IL-8	1.29 (0.91, 1.82)	1.35 (0.78, 2.29)
GM-CSF	**1.19 (1.02, 1.39)**	1.15 (0.95, 1.38)
VEGF	**3.97 (1.38, 11.40)**	**3.55 (1.07, 11.48)**
MCP-1	0.46 (0.14, 1.44)	0.49 (0.10, 2.34)
MIP-1α	0.76 (0.35, 1.62)	0.68 (0.22, 2.09)
MIP-1β	0.29 (0.02, 3.80)	0.69 (0.05, 9.77)

The values are shown as crude and adjusted geometric means ratios (GMR) with 95% CI for 26 infant samples. [a] adjusted for maternal LTBI.

3.2.3.4. Associations between maternal BCG scar and infant responses to the individual stimuli

Although these results showed that there was an association with maternal BCG scar in the combined cytokine and chemokine response analysis, it was also possible that averaging of infant responses to innate stimuli led to a loss of other, perhaps more subtle, changes between individual stimuli. Infant responses to individual innate stimuli were therefore analysed to assess their associations with maternal BCG scar.

The associations between infant responses to the different stimuli and maternal BCG scar were analysed. The following cytokines and chemokines were positively associated with maternal BCG scar: IL-10 (p=0.017) and GM-CSF (p=0.042) to PAM3Cys-Ser; TNF-α (p=0.044), IL-2 (p=0.019), IL-1β (0.005), IL-6 (p=0.017), IL-10 (p=0.001), GM-CSF (p=0.014) and VEGF (p=0.048) to FSL-1; TNF-α (0.017) to LPS; IFN-γ (p=0.018), IL-12p70 (p=0.023), GM-CSF (p=0.047) to CL097 (Figure 3.9A); IL-2 (p=0.048), IL-1β (0.017), IL-10 (p=0.040), IL-8 (p=0.011), GM-CSF (p=0.027) to Mannan; TNF-α (p=0.027), IL-12p70 (P=0.012) and VEGF (p=0.003) to Curdlan (Figure 3.9B). This data showed that different innate stimuli elicited different responses in the infants, and that such small differences might be lost when responses to individual stimuli are combined.

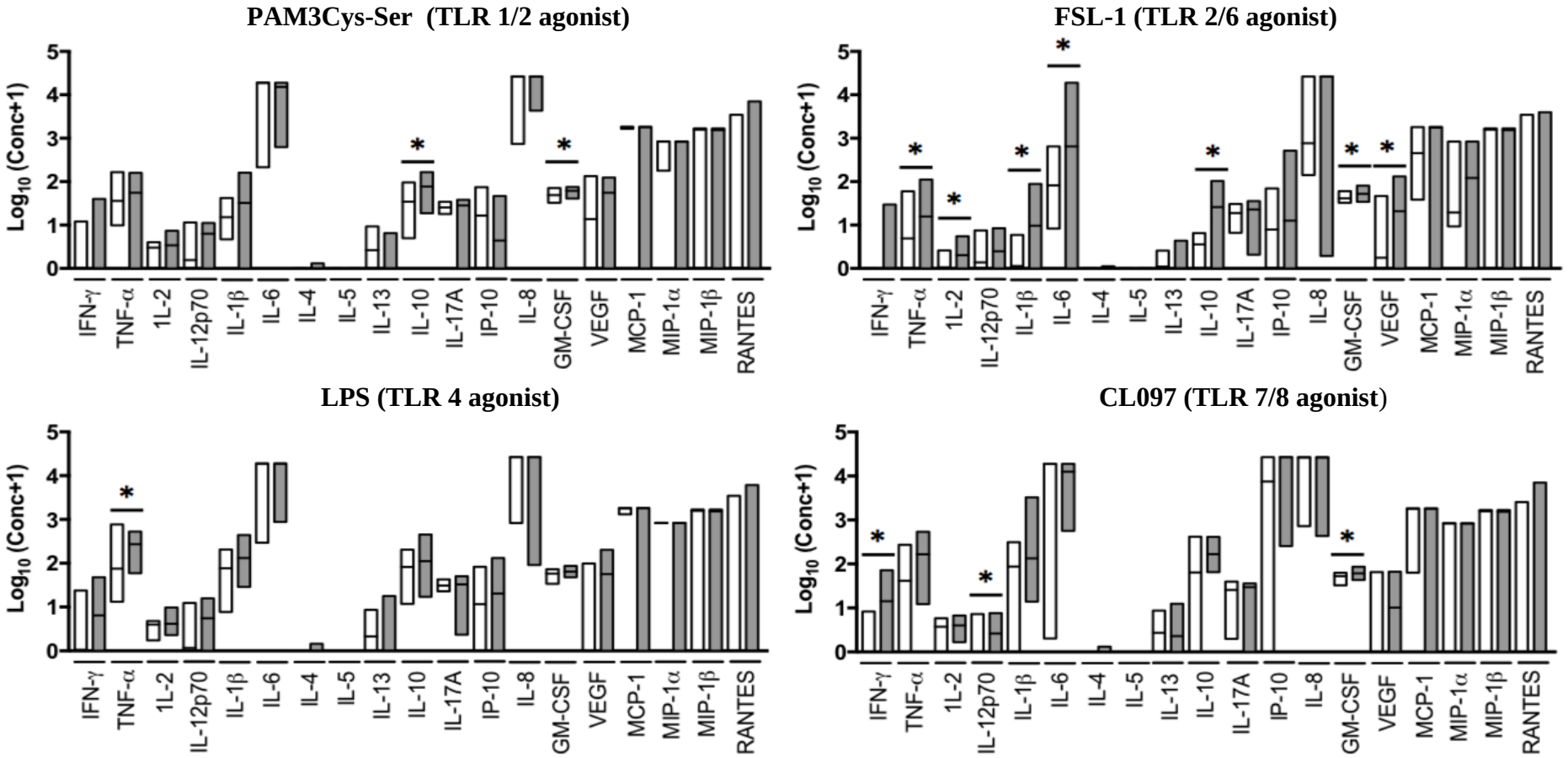


Figure 3.9A. Cytokine and chemokine responses to TLR1/2, TLR2/6, TLR4 and TLR7/8 agonists. Concentrations in cord blood are shown. Clear and grey bars represent infants of mothers without (n=17) and with (n=12) LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by (*). Kruskal-Wallis test was used to compare the two groups of infants.

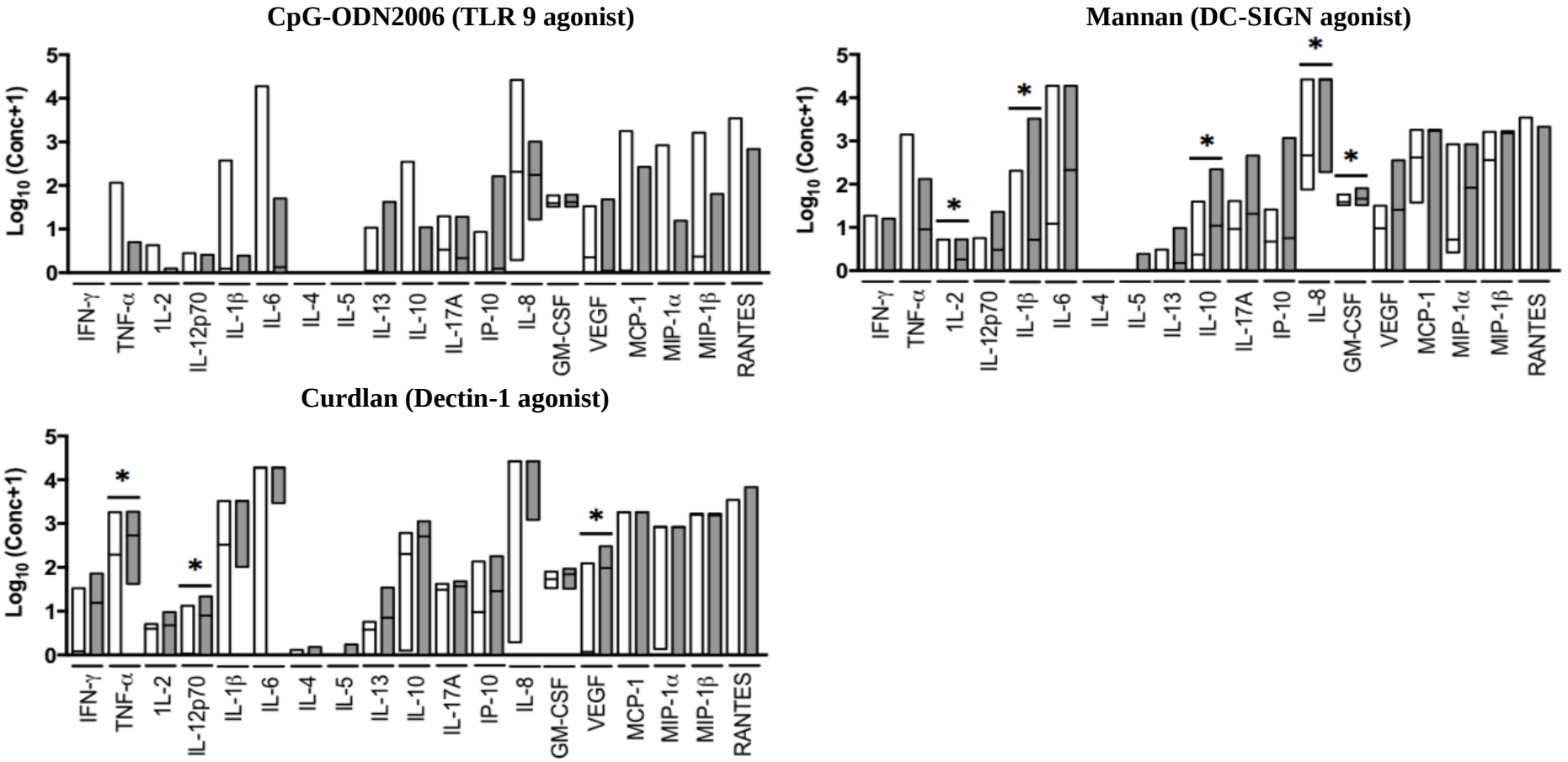


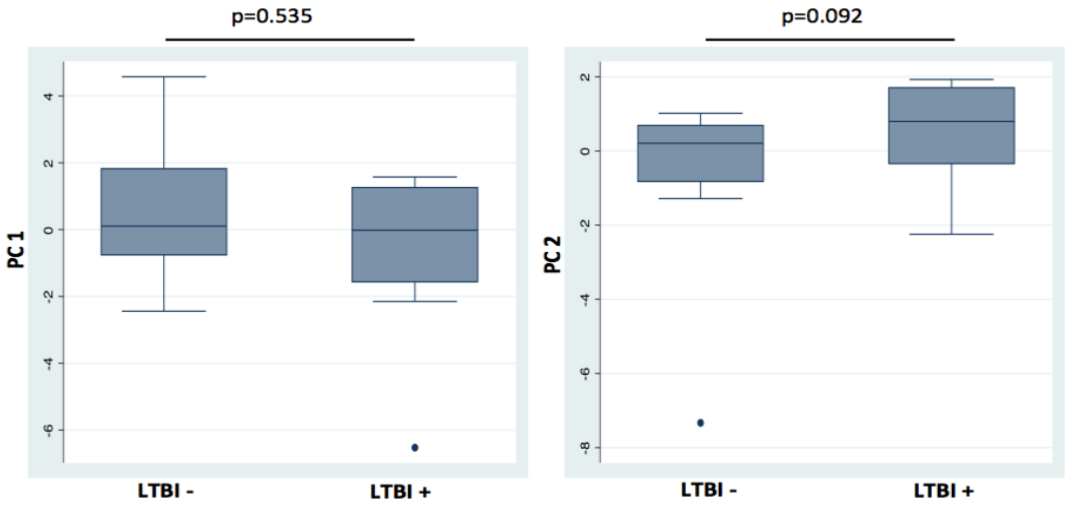
Figure 3.9B. Cytokine and chemokine responses to TLR 9, DC-SIGN and Dectin-1 agonists. Concentrations in cord blood are shown. Clear and grey bars represent infants of mothers without (n=17) and with (n=12) LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by (*). Kruskal-Wallis test was used to compare the two groups of infants.

3.2.4. Principal Component Analysis of the associations between maternal and infant factors, and innate responses in the mothers and their infants

Having examined the associations between maternal LTBI and maternal BCG scar and infant responses, a PCA was performed to better visualize the data and see whether the data grouped according to particular patterns. In addition to maternal LTBI and maternal BCG scar, other maternal and infant factors were also analysed.

Figures 3.10 to 3.13 illustrate the associations between maternal and infant factors, and the innate responses in the mothers and their infants. Neither maternal LTBI (p=0.535 for PC1 versus p=0.092 for PC2, Figure 3.10A) nor maternal BCG scar (p=0.225 for PC1 versus p=0.673 for PC2, Figure 3.10B) was associated with mothers' own PC scores. There were no associations between maternal LTBI and levels of PCs in the infants (p=0.859 for PC1 versus p=0.425 for PC2, Figure 3.11A). Maternal BCG scar was, however, associated with high levels of PC1 in the infants (median level of scores: 1.44 in scar-positive versus -0.94 in scar-negative, p=0.020, Figure 3.11B). There was no association between maternal BCG scar and levels of PC2 in the infants (median level of scores: -0.002 in scar-positive versus 0.754 in scar-negative, p=0.065, Figure 3.11B). Maternal age (p=0.463 for PC1 versus p=0.183 for PC2, Figure 3.12A), maternal gravidity status (p=0.071 for PC1 versus p=0.961 for PC2, Figure 3.12B), infant gender (p=0.542 for PC1 versus p=0.309 for PC2, Figure 3.13A) and infant birth weight (p=0.688 for PC1 versus p=0.789 for PC2, Figure 3.13B) were not associated with infant responses.

A



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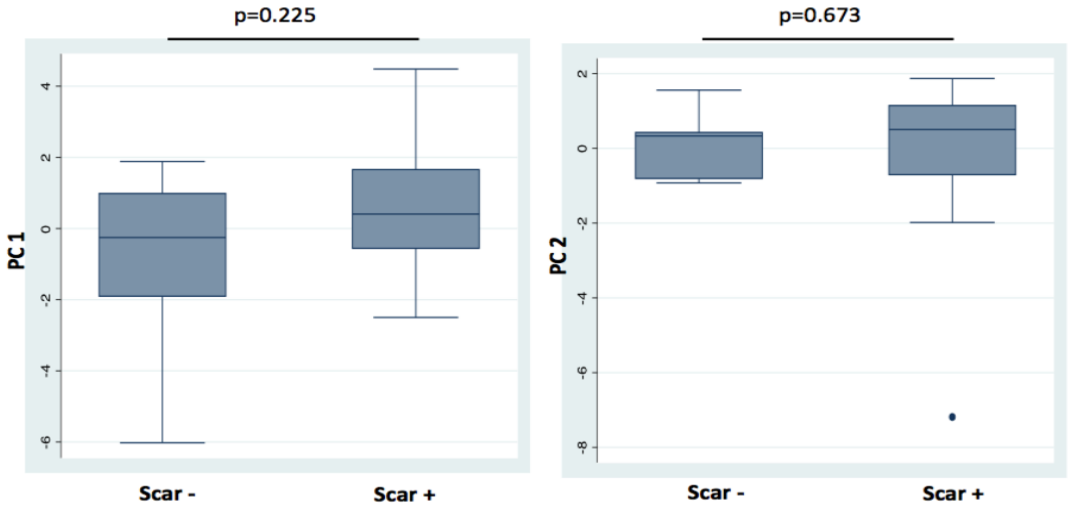
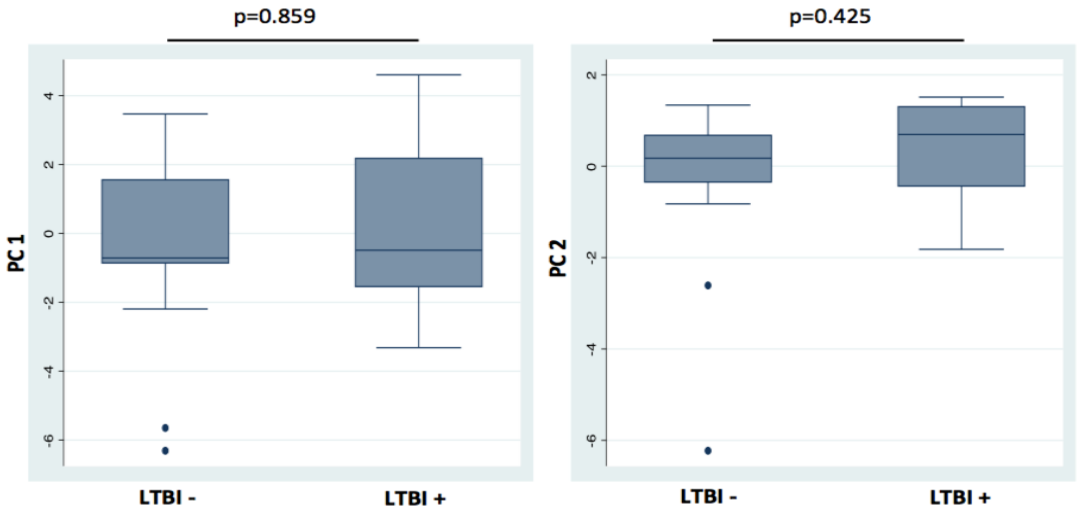


Figure 3.10. The association between maternal LTBI, maternal BCG scar and the innate immune responses in mother's post-delivery blood. PCA was used to assess the association between maternal LTBI, maternal BCG scar and maternal responses. The association between maternal LTBI and maternal responses (A), and between maternal BCG scar and maternal responses (B) are shown. Two PCs that explained 43% of the variance in the dataset were identified. The box plots represent the median and the interquartile range of the levels of the two PCs. The whiskers show the minimum and maximum values. *P* values are from Wilcoxon rank sum test.

A



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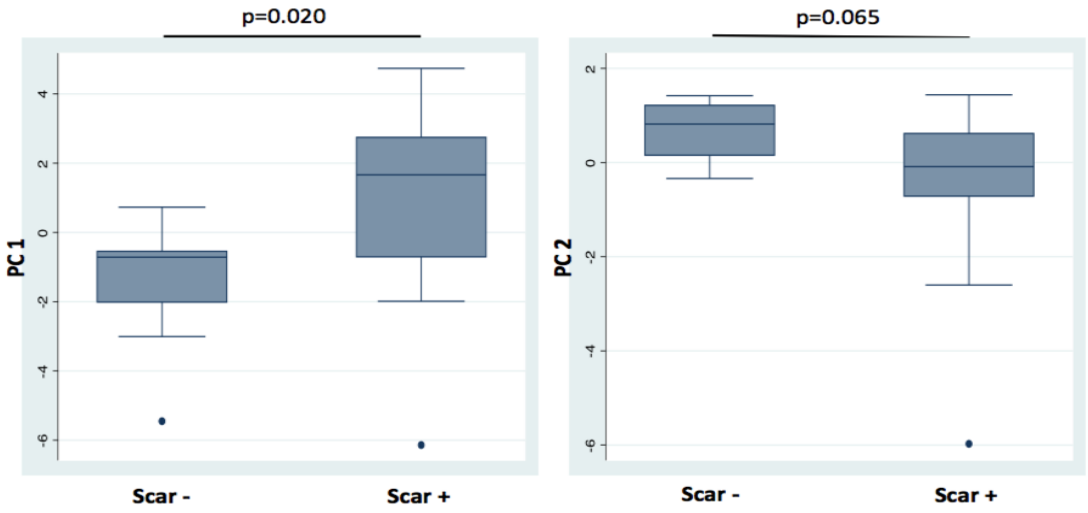
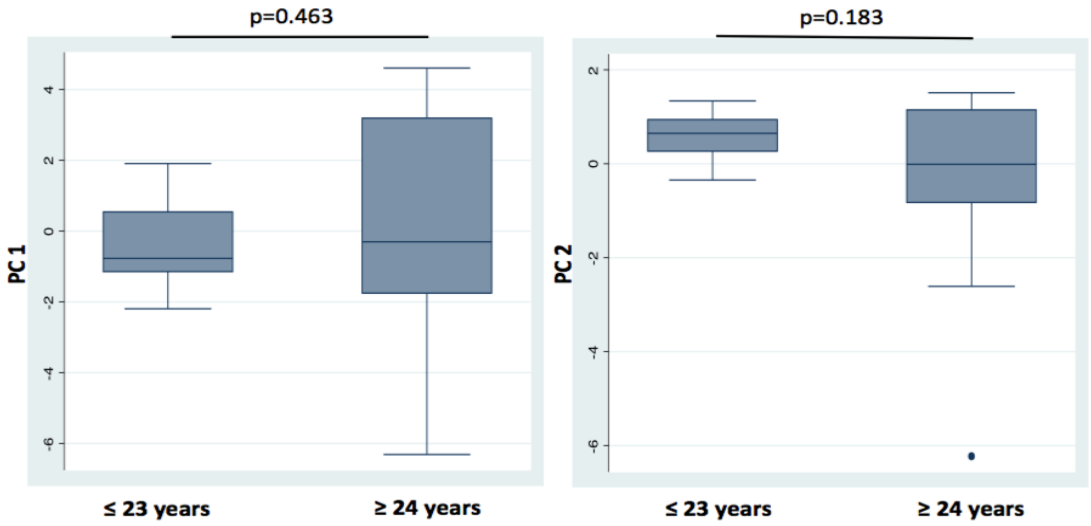


Figure 3.11. The association between maternal LTBI, maternal BCG scar and the innate immune responses in infant cord blood. PCA was used to assess the association between maternal LTBI, maternal BCG scar and infant responses. The association between maternal LTBI and infant responses (A), and between maternal BCG scar and infant responses (B) are shown. Two PCs that explained 53% of the variance in the dataset were identified. The box plots represent the median and the interquartile range of the levels of the two PCs. The whiskers show the minimum and maximum values. *P* values are from Wilcoxon rank sum test.

A



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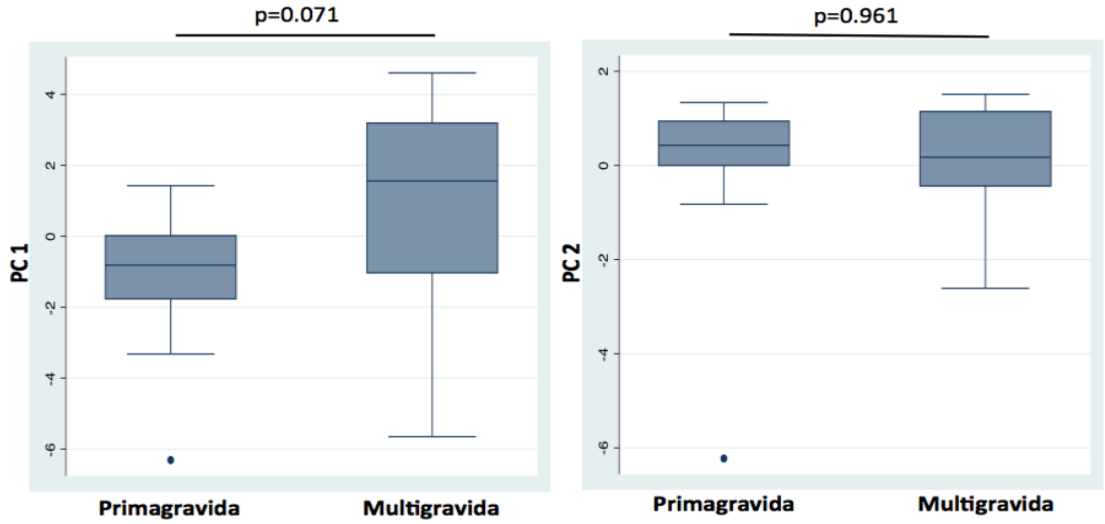
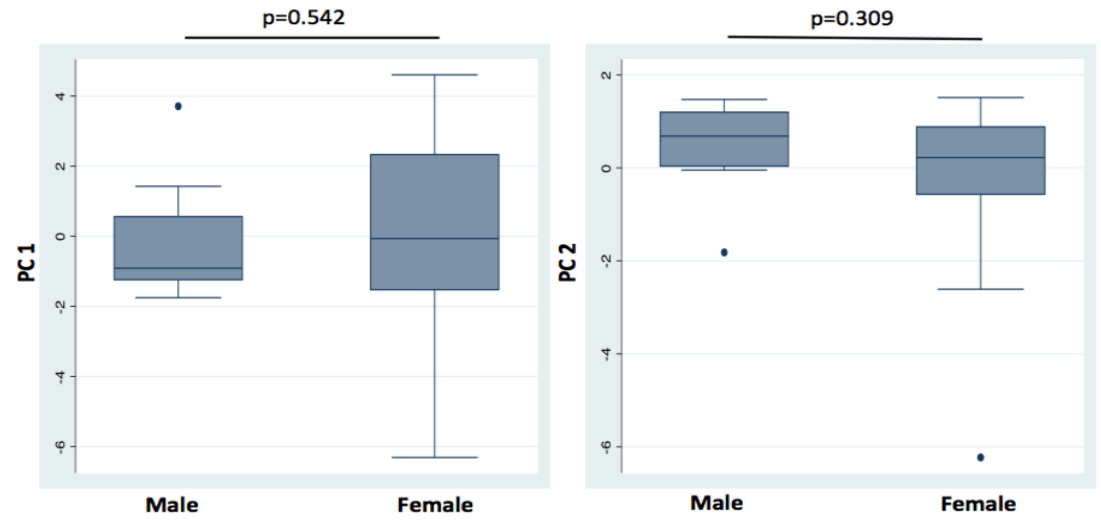


Figure 3.12. The association between maternal age, maternal gravidity status and the innate immune responses in infant cord blood. PCA was used to assess the association between maternal age (in years), maternal gravidity status and infant responses. The association between maternal age and infant responses (A), and the association between maternal gravidity status and infant responses (B) are shown. Two PCs that explained 53% of the variance in the dataset were identified. The box plots represent the median and the interquartile range of the levels of the two PCs. The whiskers show the minimum and maximum values. *P* values are from Wilcoxon rank sum test

A



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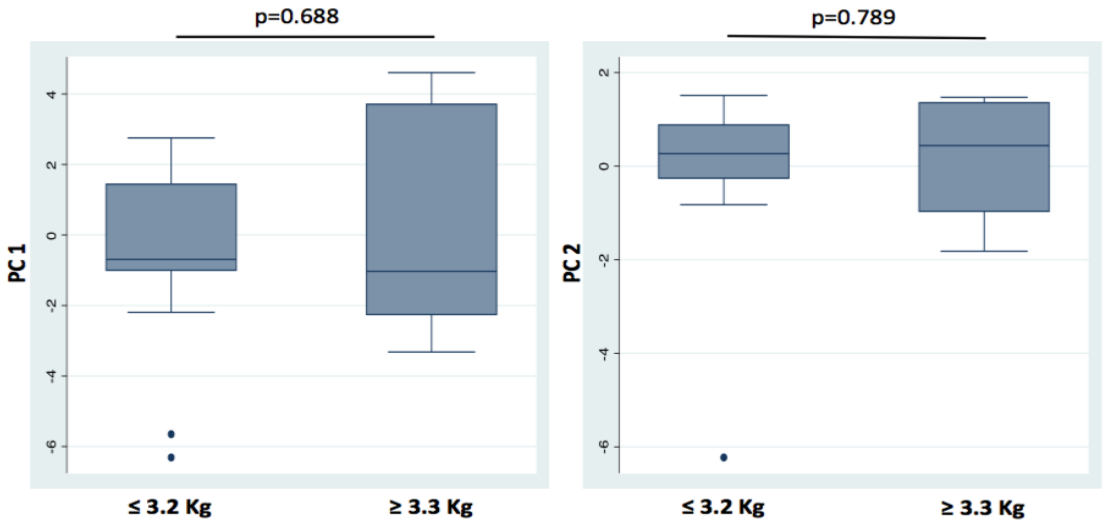


Figure 3.13. The association between infant gender, infant birth weight and the innate immune responses in infant cord blood. PCA was used to assess the association between infant gender, infant birth weight (in kilograms) and infant responses. The association between infant gender and infant responses (A), and between infant birth weight and infant responses (B) are shown. Two PCs that explained 53% of the variance in the dataset were identified. The box plots represent the median and the interquartile range of the levels of the two PCs. The whiskers show the minimum and maximum values. *P* values are from Wilcoxon rank sum test.

3.2.5. Gene expression profiles in BCG-immunised infants, and associations with maternal LTBI and maternal BCG scar.

To assess the associations between maternal LTBI and maternal BCG scar and their infants' immune responses in a more unbiased way, gene expression analysis by microarray was used. Unstimulated whole blood obtained from 42 and 51 infants at one and six weeks post-BCG immunisation, respectively, were sent to VGTI for RNA extraction and gene expression microarray.

This analysis aimed to further examine the associations observed (and perhaps pathways involved) with the innate immune responses using the Luminex® assay. Gene expression profiles in the infants of mothers with and without LTBI, and those of mothers with and without a BCG scar were compared.

3.2.5.1. Participant characteristics

Of the mothers considered for gene expression microarray, 21 had a LTBI and 38 had a BCG scar. Mothers with and without a BCG scar were comparable in terms of LTBI (26% versus 41%, p=0.26), age (24 years versus 25 years, p=0.34), gravidity status (39% versus 45% primigravida, p=0.78). Their infants were comparable in terms of birth weight (3.24 versus 3.21, p=0.77) and male gender (40% versus 47%, p=0.77) (Table 3.12).

Table 3.12. Characteristics of participants for gene expression profiling, by maternal BCG scar status.

Characteristics of mothers and infants	**BCG Scar present (n=38)**	**BCG Scar absent (n=22)**	***P*-value**
Mothers			
Age, mean (years)	24	25	0.39
Latent TBI status, Present, no (%)	10 (26)	9 (41)	0.26
Gravidity, Primigravida, no (%)	14 (39)	10 (45)	0.78
Infants			
Sex, Male, no (%)	14 (40)	8 (47)	0.77
Mean birth weight (Kg)	3.24	3.21	0.77

The figures are given as numbers with percentage (%) in brackets, or as mean values. *P* value is based on an unmatched *t* test for differences in maternal age and infant birth weight, and a two-sided Fisher's exact test for differences in maternal LTBI, gravidity status and infant gender between scar-positive and scar-negative groups.

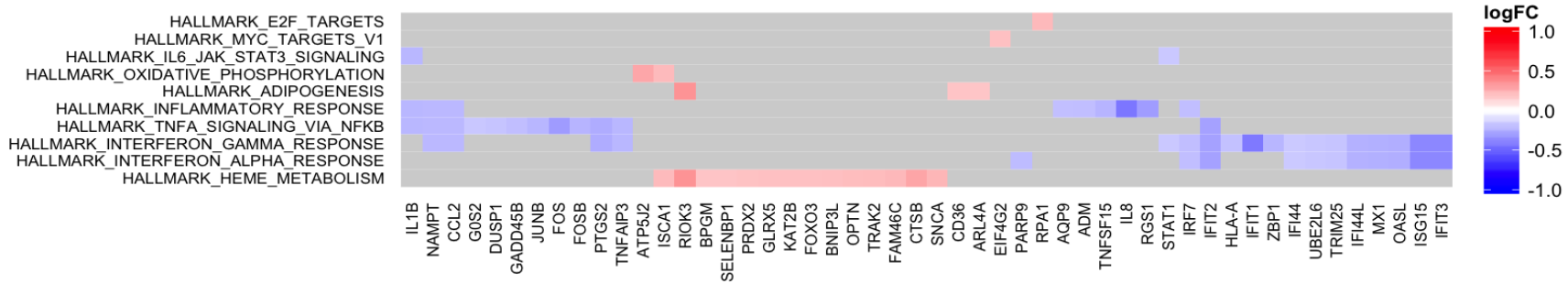
3.2.5.2. Associations between maternal LTBI and maternal BCG and gene expression profiles in infancy

Infants of mothers with LTBI, compared to those of mothers without LTBI, had down-regulated interferon (IFN-α, IFN-γ), and inflammatory response (TNF-α, IL-6 JAK/STAT3) pathways one week after BCG immunisation. In total, the following additional genes were down-regulated: IL-1β, NAMPT, CCL2, GOS2, DUSP1, GADD45B, STAT1, JUNB, FOS, FOSB, PTGS2, TNFA1P3, PARP9, AQP9, ADM, TNFSF15, IL-8, RGS1, IRF7, IFIT2, HLA-A, IFIT1, ZBP1, IFI44, UBE2L6, TRIM25, IFI44L, MX1, OASL, ISG15, IFIT3 (Figure 3.14A). Six weeks post immunisation, interferons (IFN-α, IFN-γ), IL-2/STAT5, MTORC1 and inflammatory response (TNF-α, IL-6 JAK/STAT3) pathways were up-regulated in infants of mothers with LTBI compared to infants of mothers without LTBI. In total, the following additional genes were up-regulated: IRF1, CXCL10, PLEK, MARCKS, IL-13RA1, LY6E, EIF2AK2, IFITM1, PARP9, OAS1, STAT2, IFITM3, SAMD9L, EPST11, IFITM2, IFI27, UBE2L6, IFI44, IFI35, OASL, PRIC285, RSAD2, IFIT3, ISG15, MX1, IFI44L, ZBP1, FCGR1A, MT2A, OAS2, IFIT1, MX2, TNFA1P6, IFIT2, TAP1, IRF7, PSMB9, SERPING1, HLA-DQA1, STAT1, WARS, GBP2 (Figure 3.14B).

In contrast, the interferons (IFN-α, IFN-γ) and inflammatory response (IL-6 JAK/STAT3) pathways were both up-regulated in infants of mothers with a BCG scar at one (Figure 3.15A and Figure 3.16) and six (Figure 3.15B and Figure 3.17) weeks after BCG immunisation. DNA replication (E2F targets) and cell cycle progression (G2M checkpoint) pathways were also up-regulated in infants of mothers with a BCG scar at one week (Figure 3.15A). The following additional genes were up-regulated at one week after BCG: RPAI, PNN, RANBP1, ABCE1, RPS6, HSPD1, EEF1B2, HNRNPA261, B2M, IFI27, IFI44, SP110, IFI44L, ISG15, PNPT1, OAS3, HLA-G, CCL2, XAF1, CD69, PTGS2, HMGN2 (Figure 3.15A). At six weeks after BCG immunisation, the following genes were up-regulated: SAMD9, NUB1, FAM46A, PARP9, OAS1, IFITM1, LAMP3, STA2, STAT1, CD38, SERPING1, FCGR1A, ZBP1, XAF, MX2, OAS2, MT2A, OAS3, IFIT1, HLA-DRB1, FTSJD2, TAP1, TRAFD1, ISG20, ADAR, UBE2L6, WARS, DDX60, HERC6, IFI35, PRIC285, SAMD9L, PARP14, LAP3, EPST11, OASL, MX1, RSAD2, IFI44, IFI44L, IFIT2, ISG15, IFIT3,

CASP3, PLSCR1, IFITM3, GBP4, TNFSF10, AHR, CYBB, CXCL10, IRF1, EIF2AK2, BST2, LY6E, IRF7 (Figure 3.15B).

A



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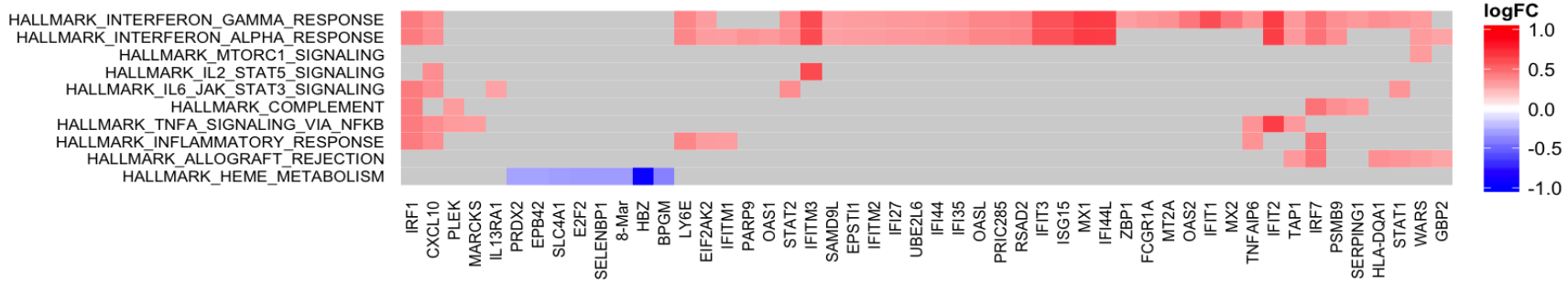
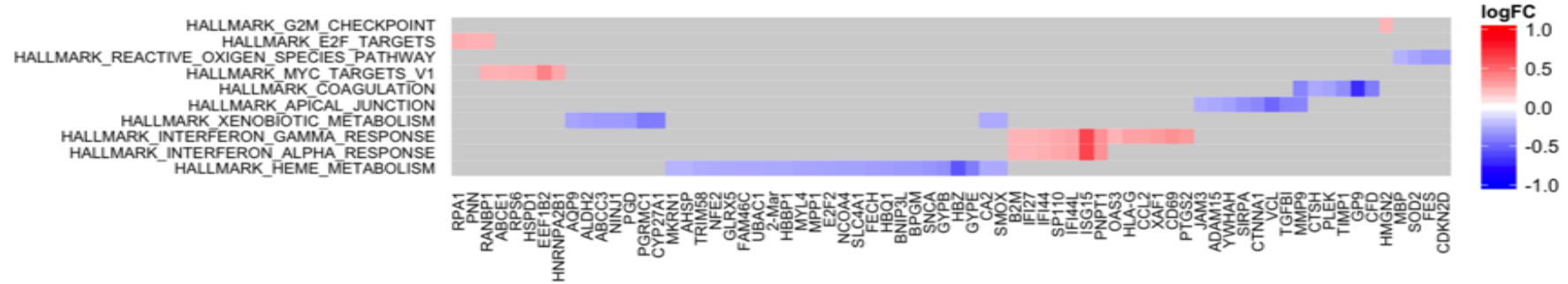


Figure 3.14. Gene Set Enrichment Analysis for the comparison of gene expression in infants of mothers with and without LTBI. The checkerboard maps show the top enriched pathways on the y-axis and top leading edge genes (gene members contributing most to the enrichment score) on the x-axis for infant samples collected at one (A) and six (B) weeks after BCG immunisation. The scale at the right represents the gene expression fold change ($\log_2$ (exposed/unexposed)). Red and blue indicate genes that are up-and down-regulated, respectively, among infants of mothers with LTBI. Interferon and inflammation response pathways were up regulated in infants of mothers with LTBI at six weeks. FDR adjusted *p*-value cut off of < 0.25 was applied for pathways significance.

A



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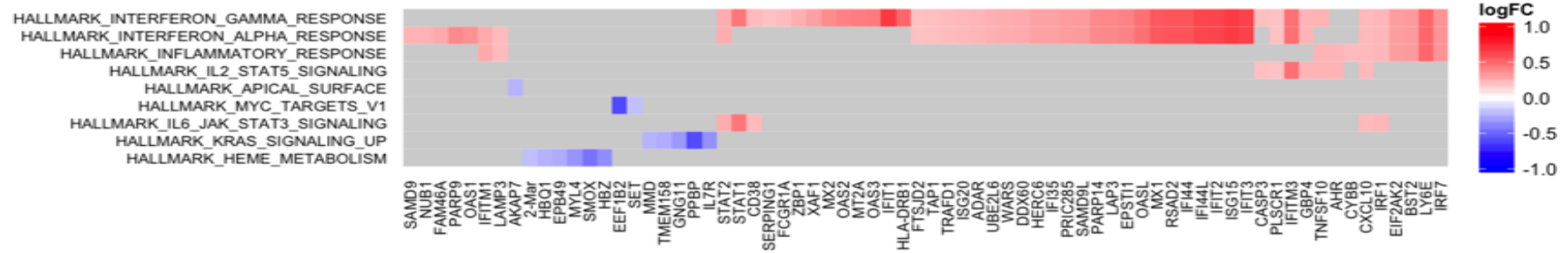

Figure 3.15. Gene Set Enrichment Analysis for the comparison of gene expression in infants of mothers with and without a BCG scar. The checkerboard maps show top enriched pathways on the y-axis and top leading edge genes (gene members contributing most to the enrichment score) on the x-axis for infant samples collected at one (A) and six (B) weeks after BCG immunisation. The scale at the right represents the gene expression fold change (log2 (scar+/scar-). Red and blue indicate genes that are up-and down-regulated, respectively, among infants of scar-positive mothers. Interferon and inflammation response pathways are up regulated in infants of mothers with a BCG scar at one and six weeks after BCG immunisation. FDR adjusted p-value cut off of < 0.25 was applied for pathways significance.

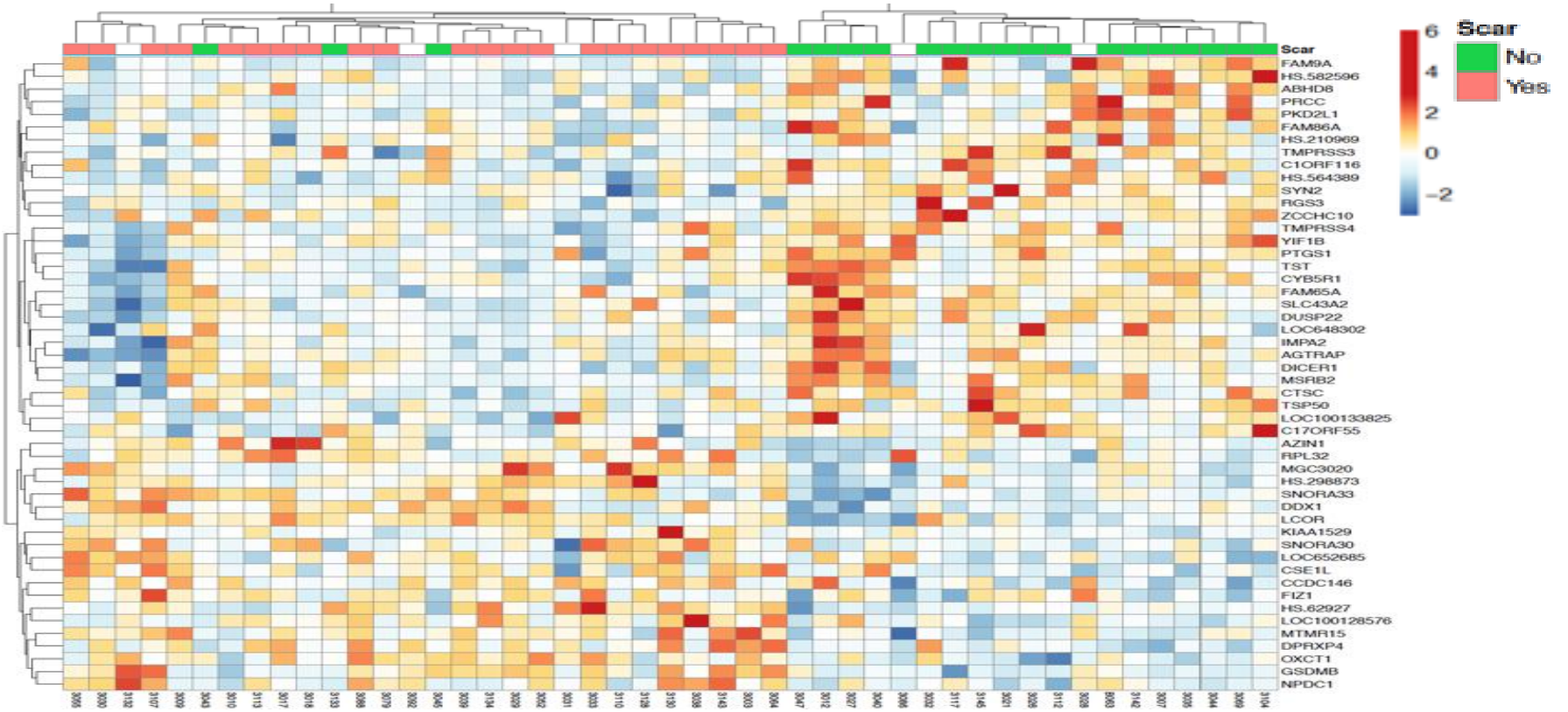

Figure 3.16. Heatmap showing the level of expression of the differentially expressed genes between infants of mothers with and without a BCG scar measured at one week post-BCG immunisation. The genes selected are the top significantly differentially expressed genes selected on fold change (1.3 fold up- or down- regulation) and *p* value ($p<0.05$) basis. The colour scale shows the level of gene expression (scaled across infants) using *z* score, where red and green correspond to up- and down-regulation, respectively.

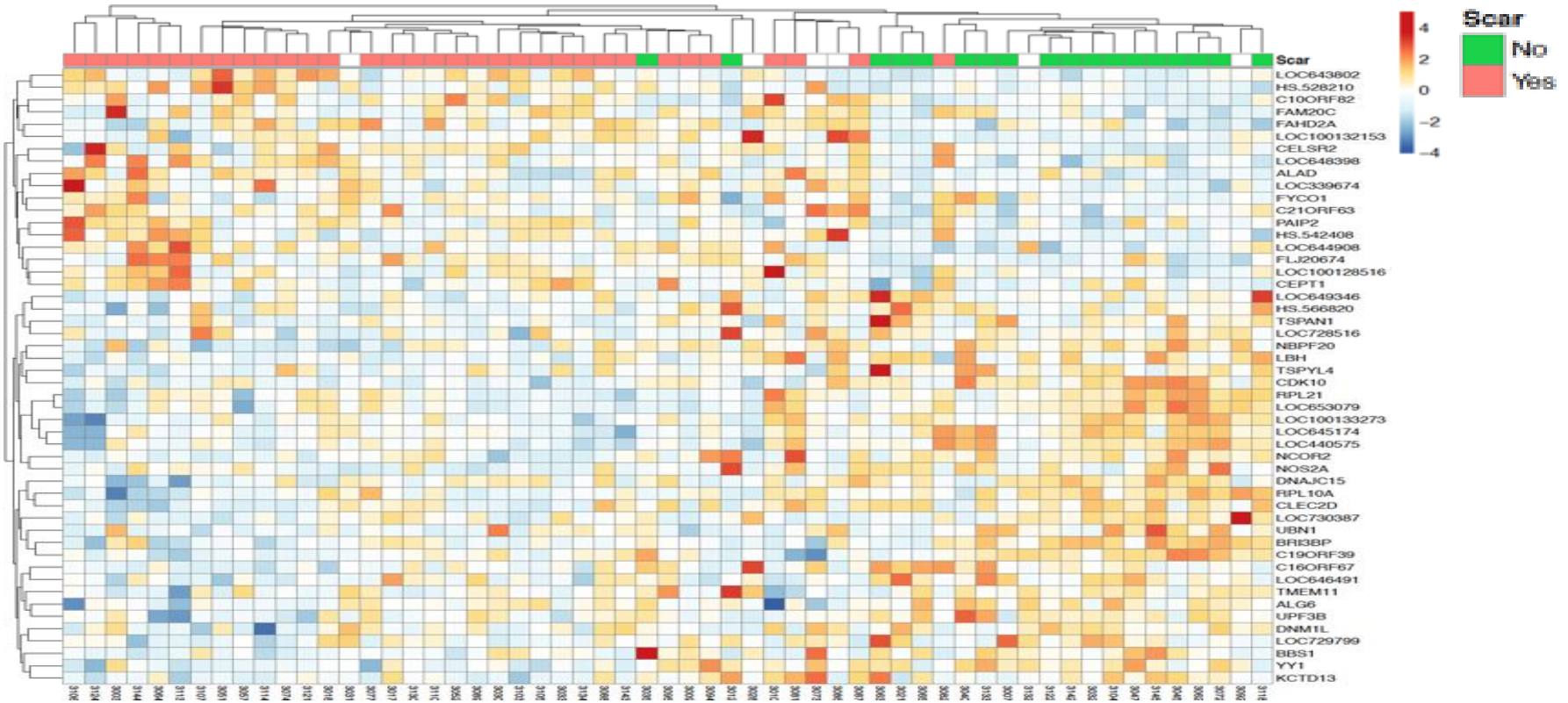

Figure 3.17. Heatmap showing the level of expression of the differentially expressed genes between infants of mothers with and without a BCG scar measured at six weeks post-BCG immunisation. The genes selected are the top significantly differentially expressed genes selected on fold change (1.3 fold up- or down- regulation) and *p* value ($p<0.05$) basis. The colour scale shows the level of gene expression (scaled across infants) using *z* score, where red and green correspond to up- and down-regulation, respectively.

3.3. Discussion

This work was part of a larger consortium to dissect the immunological interplay between poverty-related diseases and helminth infections. The panel of cytokines and chemokines studied was not selected specifically for this piece of work, but the focus was on innate responses (including IFN-γ responses) (expected to be detectable at 24 hours of stimulation) using stimuli known to interact with innate receptors. Innate cells (such as innate lymphoid cells (group 1 and group 2)) are known to produce these cytokines and would certainly contribute within the 24-hour period.

It was hypothesized that maternal LTBI would affect the innate infant responses pre- and post-BCG immunisation, but this was not found to be the case for innate responses measured in culture supernatants after stimulation of cord blood (pre-BCG). However, maternal BCG scar did show a stronger association with infant responses in cord blood, with an increased proinflammatory immune profile.

Post-BCG immunisation, both maternal LTBI and maternal BCG scar showed associations with gene expression profiles in the infants.

Overall, the concentrations of pro-inflammatory and immunoregulatory cytokines, and chemokines were high in both the stimulated maternal post-delivery blood collected on average one week post-delivery, and the stimulated infant cord blood. Neonates have been shown to demonstrate adult-like concentrations of certain cytokines and chemokines soon after birth (337). This is attributed to the effect of the birth process (such as increased acute phase responses in normal delivery process) (334) or due to *in utero* sensitization to maternal infections (287).

The concentrations of proinflammatory cytokines measured in cord blood in response to stimulation with innate stimuli using the Luminex® assay were increased in infants of mothers with a BCG scar. The expression of genes in the interferon and inflammation response pathways measured using gene transcription microarray was also increased in infants of mothers with LTBI at six weeks post BCG immunisation, and in infants of mothers with a BCG scar at one and six weeks after BCG immunisation. This unbiased approach showed the same general trend as the infant cytokine data where pro-

inflammatory responses were increased in infants of mothers with a BCG scar, and that infants of mothers with a BCG scar, in general, clustered together and those of mothers without clustered differently.

Innate immune responses may determine how effective adaptive responses are (336) and lead to either biased (338) or regulatory immune profiles (299, 301, 339). The increased responses reported here may therefore impact on immune responses to vaccines given at birth and on the course of infection and disease in childhood. There were no associations between the mothers' own innate immune responses and maternal BCG scar; associations were only observed in the infants. The presence of a maternal BCG scar was used as an indicator of BCG immunisation of a mother during infancy. Positive associations between IFN-γ responses and reactions at the site of BCG immunisation have previously been reported (340, 341), and in other studies the presence of a scar has been shown to be associated with protection against LTBI (281, 282). Scar might therefore be a good measure of protective immune responses. However, it is difficult to reconcile how a response to a vaccine administered to mothers in their infancy would exert its effects decades later in the offspring. It is possible that there may be common genetic factors between the mothers and their infants that determine scar formation and subsequent responses in the infants, or that the factors associated with scar formation in the mothers are transmitted to the infants. The lack of association between maternal BCG scar and the mother's own responses could be attributed to cumulative life-time exposures that alter the initial maternal innate immune responses after BCG immunisation. We did not collect data on scarring in these infants, but the ongoing main infant BCG study (Chapter 4.0) with a longer follow up provides the opportunity to assess relationships between scarring and immune responses in mothers and their infants.

The development of a scar is also dependent upon the strain, dose and method of administration of the BCG vaccine (342). The Danish strain of BCG vaccine, compared to BCG Russia, has been shown to elicit stronger responses in infants one year later and to cause more scarring (244, 277-279, 343), and the intradermal route of administration is associated with the formation of distinctive scars (344, 345). We were unable to ascertain the strain of the vaccine the women received in infancy, although the most common strain currently in use in Uganda is BCG Russia. Since BCG immunisation is

administered in the neonatal period, it is difficult to obtain information about BCG immunisation status of adults in a country where hospitals do not routinely record vaccine strain. There is therefore the possibility of misclassification of women based on the presence or absence of a scar. It is possible that the scar-negative women may have been BCG vaccinated without developing a scar, or that scars were lost with time. Scar formation has also been reported to depend on the way the vaccine is handled and delivered, and the period within which the vaccine is administered (better scar formation when the vaccine is given over 3 months of age, versus within a month after birth) (274). In infants, scars have been reported to be poor indicators of BCG immunisation (346). Our observed differences in infant response may therefore relate either to the mother's BCG immunisation status or to the quality of the mother's response to BCG immunisation.

Previous studies have reported the presence (347-349) or absence (350) of maternal cells in cord blood samples. It is therefore possible that the high proinflammatory response observed in cord blood could be due to responses from maternal cells in cord blood, but the method we used for collecting cord blood (by needle and syringe, with no "milking" of the cord, coupled with the use of trained midwives, see section 2.1.5.3) should have minimized contamination. Previous tests carried out on maternal and cord blood samples in our studies (comparing levels of β-human chorionic gonadotropin) showed that contamination of cord blood by maternal blood was rare (unpublished data).

Interferon and inflammatory pathways were down-regulated in infants of mothers with LTBI at one week, but up-regulated at six weeks after BCG immunisation; this offers some support to the hypothesis that prenatal exposure to maternal LTBI modifies the infant response to BCG, but the change in direction of effect as the immune response matured was unexpected, and these findings would need to be confirmed in a larger study. Up- and down -regulation of genes following BCG immunisation of infants has previously been reported (238). A recent study by Zak and colleagues identified tuberculosis risk signature genes (351), some of which have also been identified in the pilot infant BCG study (GBP4, STAT1, TAP1, TRAFD1, GBP2, FCGRIA and SERPINGI). Some of the interferon response genes such as GBP1, STAT1, and TAP1

have protective roles in TB disease, highlighting the need to further understand the role of interferon and inflammation responses in immunity to TB.

Limitations of the study were its observational and explorative nature, and its small sample size relative to the many outcomes assessed. Maternal and infant factors such as maternal age, gravidity status, infant birth weight and gender were not adjusted for since these were not crudely associated with infant responses, and the numbers involved were generally small. Given the large number of statistical tests performed (or comparisons made), there is a chance of false positive findings, and statistically significant results should be interpreted with caution.

In summary, maternal BCG scar had a stronger association with innate infant responses than maternal LTBI, with an increased pro-inflammatory profile of immune responses. The mechanisms that underlie this association need to be further examined in a larger study.

Having shown that maternal LTBI is not associated with infant innate immune responses pre-BCG immunisation, but that there is some association with gene expression profiles in the infants post-BCG immunisation, and that maternal BCG scar is associated with infant responses pre-and post-BCG immunisation, the next chapter reports results for T cell responses in infancy and the effect of maternal LTBI on infant responses to BCG immunisation.

The pilot infant BCG study generated interesting results and many lessons were learnt. The study proceedures (for example cord blood collection), including appointment visits, used in the main infant BCG study were optimized in the pilot infant BCG study. Learning from the pilot infant BCG study, infant samples were collected at all time points in RNALater solution for further investigation of gene expression profiles.

Further analysis of the innate immune responses in infancy was not investigated in the main infant BCG study whose results are presented later, but in a “sister” project that investivated the nonspecific effects of BCG immunisation (352). Analysis of the results of this study is still on hold as the principal investigator is on maternity leave.

Figure 3.14. illustrates one of the preliminary results from the pilot infant BCG study that was used in the grant application for the main infant BCG study. The results showed that infants of mothers with a LTBI produced more TNF-α than infants of mothers without such exposures, highlighting the importance of investigating, in a larger study, the impact of maternal LTBI on the infant responses to BCG immunisation. The flow cytometry assays for the main infant BCG study have been completed and analysis is underway.

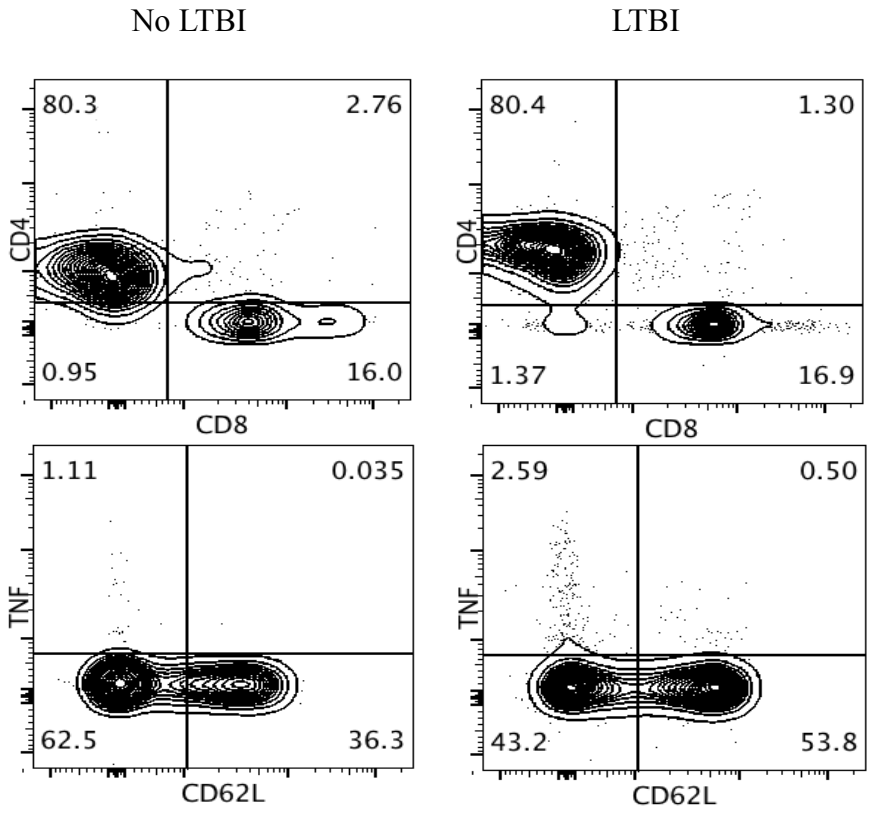

Figure 3.18. Flow cytometry of SEB-stimulated cells from the blood of infants one week post birth. Panels on left hand side are from an infant of a mother without LTBI, panels on right hand side are from an infant of a mother with LTBI. Infants of mothers with LTBI produced more TNF-α.

Chapter 4

T cell immune responses in BCG vaccinated infants

4.1. Introduction

BCG is the only vaccine against TB currently available. There is little information about the peak immune response in infants following BCG immunisation, yet this knowledge is needed for the design and use of vaccines aimed at prime-boost strategies (251). It was proposed that maternal infection with *M. tuberculosis*, which is endemic in this setting, may result in the poor effectiveness of BCG in the tropics, If maternal *M. tuberculosis* infection impacts on the infant response to BCG, the timing, magnitude, or quality of the BCG-indiced immune response may be modified in infants of *M. tuberculosis*-infected women compared to those without. This may result in a delayed peak in response to BCG immunisation, or a lower overall recruitment of cells in the priming phase following BCG immunisation, leading to a lower set point of BCG-specific immunological memory, and as differences in the effector response profile. The timing and magnitude of the peak immune response in BCG vaccinated infants was investigated. Previous studies had looked at the earliest sampling time point only up to three months post-immunisation (353). Other published studies have examined early post-immunisation time points in adolescents and adults (354, 355), but not the earliest stages of the immune response to BCG in human infants. This is important since the size of the pool of cells recruited into the primary immune response directly affects the resulting pool of memory T-cells capable of responding to subsequent infection (356). Thus, understanding the time course and peak response to BCG immunisation in infants is important in understanding the poor efficacy of BCG in the tropics.

The protective efficacy of BCG against TB varies between populations, and latitude has been suggested to explain this variation for adolescents and adults (290, 293, 297). It is thought that sensitisation to NTM, which is more common in lower latitudes (302) modifies the protection induced by BCG (303).

In Uganda, up to 49% of adults are reported to have LTBI (29). Maternal LTBI might, in the same way as NTM, lead to exposure to mycobacterial antigens *in utero* and the development of a modified profile of response to mycobacteria after birth (287). For example the passive transfer of maternal anti-mycobacterial antibodies might interfere with development of the BCG "infection" required to elicit protective immunity. Or, maternal infection could influence the maternal and placental immunological milieu, and hence the fetal and neonatal response on exposure to immunisation (357).

It has therefore been proposed that maternal LTBI influences the neonatal response to BCG (and to *M. tuberculosis*), resulting in less effective response to BCG.
Here I report T-cell response results from a published article titled "The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to bacille Calmette-Guérin immunisation". Mawa, P.A., Nkurunungi, G., Egesa, M., Webb, E.L., Smith, S.G., Kizindo, R., Akello, M., Lule, S.A., Muwanga, M., Dockrell, H.M., Cose, S., Elliott, A.M. Philos Trans R Soc Lond B Biol Sci. 2015 Jun 19; 370 (1671)" (326) (Appendix S), and results from additional work from a larger ongoing infant BCG study.

After describing the socio-demographic and clinical characteristics of the study participants in the pilot infant BCG study, results from PPD-specific CD4+ and CD8+ T cell responses, measured by intracellular cytokine staining and flow cytometry, are presented. These experiments were designed to identify optimal time points for the main infant BCG study (whose results will be presented later in this Chapter, section 4.3), and to investigate whether LTBI infection in the mothers would influence how their infants' T cells respond to BCG vaccination.
The specific objectives of this include:

1. To analyse the longitudinal changes in frequencies of PPD-specific CD4+ and CD8+ T cells in infancy.
2. To examine the impact of maternal infection with *M. tuberculosis* on PPD-specific CD4+ and CD8+ T cell responses in infancy.

4.2. Results for the pilot infant BCG study

4.2.1. Demographic and clinical characteristics of the participants

The socio-demographic and clinical characteristics of the study participants have been described in Chapter 3.0, section 3.2.1. For the participants included in this analysis, mothers with LTBI, compared to those without LTBI, were older (mean age 27.5 versus 23 years, p=0.002), less likely to have a BCG scar (47% versus 70%, p =0.001) and less likely to be primigravida (32% versus 48%, p=0.001), and their children were more likely to be of male gender (53% versus 35%, p=0.001) (Table 4.1).

Table 4.1. Demographic and clinical characteristics of the mothers and their infants used for analysis of T cell responses in the pilot infant BCG study.

Characteristics of mothers and infants	**Mothers without LTBI (n=50)**	**Mothers with LTBI (n=21)**	***p*-value**
Maternal factors			
BCG scar present, no (%)	35 (70)	9 (47)	<0.001
Maternal age, years, mean	23.0	27	0.002
Primigravida, no (%)	24 (48)	6 (32)	<0.001
Infant factors			
Male gender, no (%)	17 (35)	10 (53)	<0.001
Birth weight (Kg)	3.2	3.4	0.09

The figures are given as numbers with percentage (%) in brackets, or as mean values. *P* value is based on unmatched *t* test for differences in maternal age and infant birth weight, and a two-sided Fisher's exact test for differences in maternal LTBI, gravidity and infant gender between LTBI-positive and LTBI-negative groups.

4.2.2. Longitudinal changes in frequencies of cytokine-expressing PPD-specific CD4+ and CD8+ T cells in the pilot infant BCG study

To assess changes in frequencies of PPD-specific CD4+ and CD8+ T cells expressing cytokines, mononuclear cells from cord blood, and from infant samples obtained at one and six weeks after BCG immunisation were stimulated overnight with PPD, and cytokine expression was measured by intracellular cytokine staining and flow cytometry. Thirty-one samples were assayed at each time point, 17 from infants of mothers without LTBI and 14 from infants of mothers with LTBI. The gating strategy is illustrated in Figure 2.8 in the Methods Chapter.

Compared to responses in cord blood, the frequency of PPD-specific IFN-γ+ CD4+ T cells increased at one week and decreased at six weeks after birth. There was a statistically significant difference between frequencies at one and six weeks after birth ($p=0.031$). The frequencies of cells expressing IL-2 and TNF-α were, by contrast, higher at six weeks compared to one week ($p=0.018$ and $p=0.009$, respectively; Figure 4.1A).

The frequencies of PPD-specific CD8+ T cells making any of the three cytokines assessed were higher in cord blood compared to six weeks ($p=0.044$), and higher at one week compared to six weeks ($p=0.003$; Figure 4.1B).

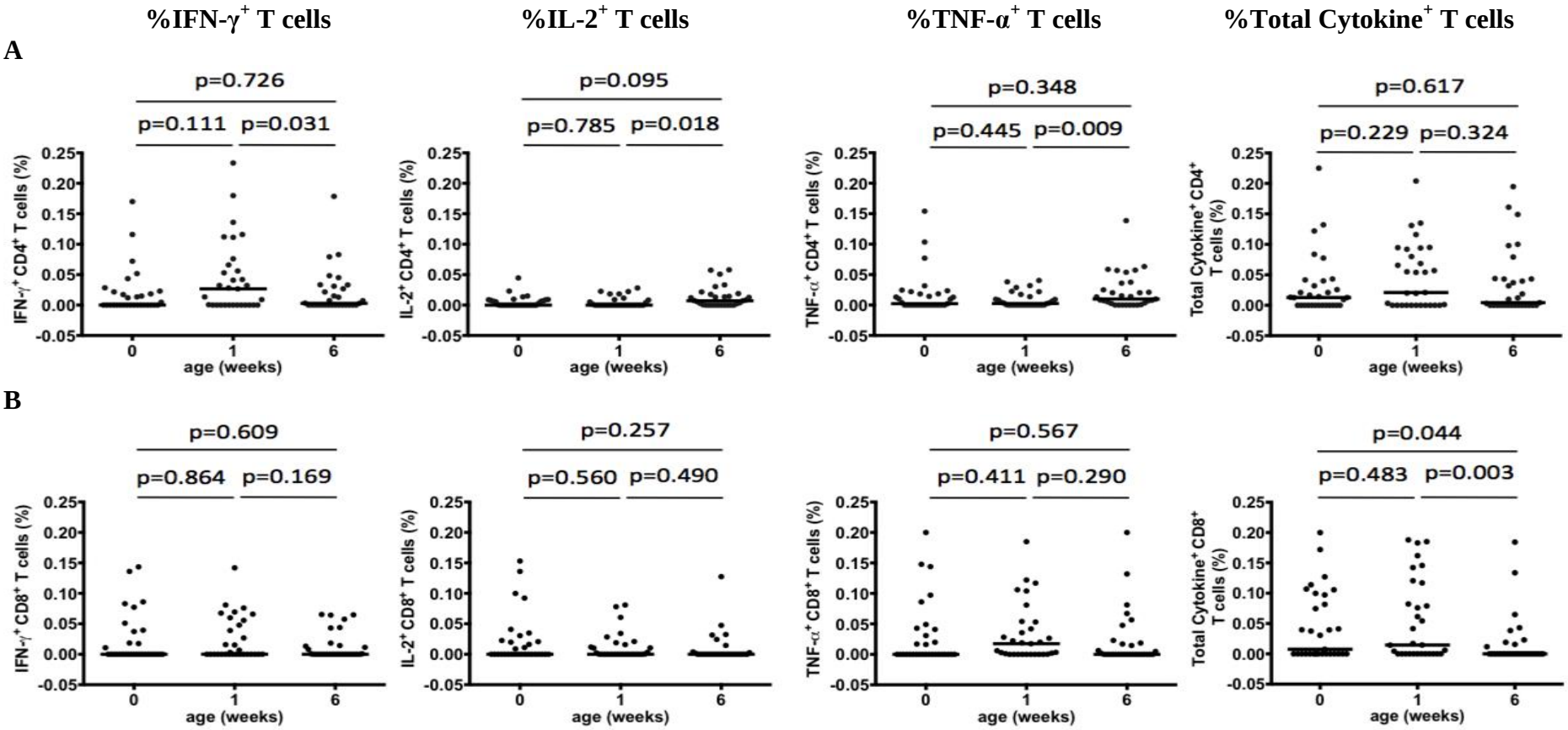

Figure 4.1. Longitudinal changes in frequencies of PPD-specific cytokine expressing T-cells during the first six weeks of life measured by intracellular cytokine staining and flow cytometry. Each symbol represents an individual, and for each plot the horizontal line represents the median. Frequencies of PPD-specific, total IFN-γ^+, total IL-2^+, total TNF-α^+, or total cytokine+ (IFN-γ^+ or IL-2^+ or TNF-α^+) CD4+ (A) and CD8+ (B) T cells are shown. Statistical analysis was performed using Wilcoxon matched-pairs signed rank test. n=31 for all time points.

4.2.3. Impact of maternal LTBI on PPD-specific immune responses in infancy

In the previous section, longitudinal infant response to PPD over time was shown. Here I now show whether these responses differ according to maternal LTBI status. Cytokine expression in cord blood and in infant blood samples obtained at one and six weeks after birth were tested to assess the effect of maternal LTBI. There were no differences in frequencies of T cells expressing any cytokines in cord blood in samples from infants of mothers with and without LTBI (Table 4.2).

In a crude analysis, maternal LTBI was associated with lower frequencies of CD4+ T cells expressing PPD-specific IFN-γ (crude geometric mean ratio (cGMR) (95% confidence interval (CI)) 0.89 (0.83, 0.98), Table 4.2, Figure 4.2A), TNF-α (cGMR) (95% CI 0.98 (0.95, 0.99) and of CD4+ T cells expressing any of the three cytokines assessed, combined (cGMR) (95% CI 0.89 (0.81, 0.95), Table 4.2, Figure 4.2D) at one week after BCG immunisation.

Seven infants had samples with less than 5000 events acquired (12 out of 93 samples that were analysed), and were subsequently excluded. The exclusion of these infant samples from the analysis weakened the association between maternal LTBI and lower frequencies of CD4+ T cells expressing PPD-specific IFN-γ at one week after birth (p=0.068), but that of TNF-α was strengthened (p=0.045) (data not shown). There was therefore an overall maintenance of the evidence for an association between maternal LTBI and infant Th1 responses.

After adjusting for maternal age, maternal gravidity and infant gender in multivariate analyses (incorporating all samples), the association between maternal LTBI and frequencies of CD4+ T cells expressing IFN-γ one week after BCG immunisation was weaker than in the crude analysis (adjusted geometric mean ratio (aGMR) (95% confidence interval (CI)) 0.94 (0.85, 1.04), but the association with a reduced frequency of CD4+ T cells expressing TNF-α became stronger (0.97 (0.95, 0.99)), and the association with reduced frequencies of PPD-specific CD4+ T cells expressing any of the three cytokines, combined, remained strong (aGMR, 95% CI 0.91 (0.83, 0.99)) Table 4.4A.

For CD8+ T cells, cord blood samples obtained from infants of mothers with LTBI, compared to those without LTBI, showed a weak trend towards higher T-cell responses for TNF-α (p=0.067, Figure 4.3C), and for all cytokines, combined. At one week after BCG immunisation, in univariate analyses, maternal LTBI was weakly associated with lower frequencies of CD8+ T cells expressing PPD-specific IFN-γ (p=0.073, Figure 4.3A).

In multivariate analyses incorporating all samples, after adjusting for maternal and infant factors mentioned above, there was a weak association between maternal LTBI and low frequencies of PPD-specific CD8+ T cells at one week (aGMR , 95% CI 0.96 (0.91, 1.01)) Table 4.4B.

Table 4.2. Crude associations between maternal and infant factors and infant CD4+ T cell response to PPD

Cord blood

Factor	IFN-γ	IL-2	TNF-α	Total cytokines
Maternal LTBI	0.98 (0.93, 1.02)	1.00 (0.99, 1.02)	1.02 (0.95, 1.07)	0.99 (0.91, 1.07)
Maternal age	1.05 (1.02, 1.10)	1.02 (1.01, 1.02)	1.05 (0.99, 1.07)	1.10 (1.02, 1.15)
BCG Scar	1.00 (0.93, 1.07)	1.01 (0.99, 1.02)	0.98 (0.93, 1.01)	0.98 (0.91, 1.07)
Gravidity	0.99 (0.93, 1.05)	0.99 (0.98, 1.01)	1.02 (0.98, 1.07)	1.01 (0.93, 1.10)
Infant gender	1.02 (0.95, 1.07)	0.99 (0.98, 1.00)	1.00 (0.95, 1.07)	1.00 (0.91, 1.07)
Birth weight	0.98 (0.93, 1.02)	1.00 (0.98, 1.02)	1.02 (0.91, 1.05)	1.02 (0.89, 1.17)

1 week after BCG immunisation.

Factor	IFN-γ	IL-2	TNF-α	Total cytokines
Maternal LTBI	**0.89 (0.83, 0.98)**	1.00 (0.98, 1.01)	**0.98 (0.95, 0.99)**	**0.89 (0.81, 0.95)**
Maternal age	0.91 (0.81, 1.05)	1.01 (1.00, 1.02)	1.01 (0.98, 1.02)	0.95 (0.85, 1.07)
BCG Scar	0.98 (0.89, 1.05)	1.00 (0.99, 1.02)	1.00 (0.98, 1.02)	0.95 (0.89, 1.02)
Gravidity	1.02 (0.95, 1.10)	1.00 (0.98, 1.01)	1.00 (0.98, 1.02)	1.02 (0.95, 1.10)
Infant gender	0.98 (0.87, 1.07)	1.00 (0.99, 1.02)	1.01 (0.99, 1.02)	0.98 (0.89, 1.07)
Birth weight	1.05 (0.91, 1.17)	0.99 (0.98, 1.00)	0.98 (0.95, 1.00)	0.98 (0.87, 1.10)

6 weeks after BCG immunisation**.**

Factor	IFN-γ	IL-2	TNF-α	Total cytokines
Maternal LTBI	0.99 (0.93, 1.07)	1.02 (0.99, 1.05)	1.02 (0.98, 1.07)	1.02 (0.93, 1.10)
Maternal age	0.98 (0.93, 1.05)	1.02 (0.98, 1.05)	1.02 (0.98, 1.07)	1.05 (0.95, 1.12)
BCG Scar	1.02 (0.98, 1.05)	1.00 (0.98, 1.02)	0.99 (0.93, 1.05)	1.00 (0.93, 1.07)
Gravidity	1.02 (0.98, 1.07)	0.98 (0.95, 1.02)	0.95 (0.91, 1.01)	0.98 (0.89, 1.07)
Infant gender	0.99 (0.95, 1.02)	0.99 (0.95, 1.02)	1.00 (0.95, 1.05)	1.01 (0.93, 1.10)
Birth weight	0.99 (0.91, 1.07)	1.00 (0.95, 1.05)	0.98 (0.91, 1.05)	0.95 (0.87, 1.07)

Data are crude GMR and 95% CI for 31 infants at all the time points. Linear regression with bootstrapping was used for analysis. Confidence intervals not including one (1) are highlighted in bold.

Table 4.3. Crude associations between maternal and infant factors and infant CD8+ T cell response to PPD

Cord blood

Factor	IFN-γ	IL-2	TNF-α	Total cytokines
Maternal LTBI	1.00 (0.93, 1.07)	1.00 (0.93, 1.07)	1.05 (0.95, 1.15)	1.05 (0.95, 1.15)
Maternal age	0.95 (0.87, 1.05)	1.05 (1.01, 1.10)	1.05 (1.00, 1.10)	1.05 (0.95, 1.15)
BCG Scar	1.07 (0.98, 1.15)	1.05 (0.95, 1.12)	1.02 (0.93, 1.15)	1.10 (0.98, 1.20)
Gravidity	0.93 (0.89, 1.00)	0.98 (0.91, 1.05)	0.98 (0.89, 1.07)	0.98 (0.89, 1.07)
Infant gender	0.95 (0.89, 1.02)	1.05 (0.98, 1.10)	0.98 (0.91, 1.05)	0.98 (0.89, 1.07)
Birth weight	0.95 (0.89, 1.05)	0.99 (0.89, 1.10)	0.98 (0.89, 1.05)	0.98 (0.85, 1.10)

1 week after BCG immunisation.

Factor	IFN-γ	IL-2	TNF-α	Total cytokines
Maternal LTBI	0.95 (0.89, 1.00)	1.01 (0.98, 1.05)	0.99 (0.91, 1.07)	0.98 (0.87, 1.07)
Maternal age	1.00 (0.95, 1.07)	1.02 (1.00, 1.05)	1.07 (1.01, 1.15)	1.07 (0.98, 1.17)
BCG Scar	0.98 (0.93, 1.05)	0.98 (0.95, 1.00)	0.95 (0.89, 1.00)	0.91 (0.83, 1.00)
Gravidity	0.95 (0.91, 1.02)	1.02 (0.99, 1.07)	1.07 (1.00, 1.15)	1.07 (0.98, 1.20)
Infant gender	0.98 (0.91, 1.05)	1.01 (0.98, 1.05)	1.02 (0.95, 1.10)	1.01 (0.91, 1.12)
Birth weight	1.02 (0.95, 1.07)	1.01 (0.98, 1.05)	1.02 (0.95, 1.12)	1.10 (0.99, 1.23)

6 weeks after BCG immunisation**.**

Factor	IFN-γ	IL-2	TNF-α	Total cytokines
Maternal LTBI	0.99 (0.95, 1.02)	0.98 (0.93, 1.01)	1.02 (0.93, 1.10)	0.98 (0.91, 1.05)
Maternal age	0.99 (0.93, 1.05)	1.00 (0.95, 1.05)	1.05 (0.98, 1.12)	1.05 (0.99, 1.10)
BCG Scar	1.00 (0.98, 1.05)	1.05 (0.99, 1.10)	0.99 (0.93, 1.05)	1.02 (0.95, 1.10)
Gravidity	1.00 (0.98, 1.05)	0.98 (0.93, 1.02)	1.05 (0.98, 1.12)	1.01 (0.93, 1.10)
Infant gender	1.01 (0.98, 1.05)	1.01 (0.98, 1.05)	1.07 (1.00, 1.05)	1.07 (1.01, 1.12)
Birth weight	0.98 (0.93, 1.02)	0.99 (0.95, 1.05)	0.93 (0.85, 1.00)	0.95 (0.87, 1.02)

Data are crude GMR and 95% CI for 31 infants at all the time points. Linear regression with bootstrapping was used for analysis. Confidence intervals not including one (1) are highlighted in bold.

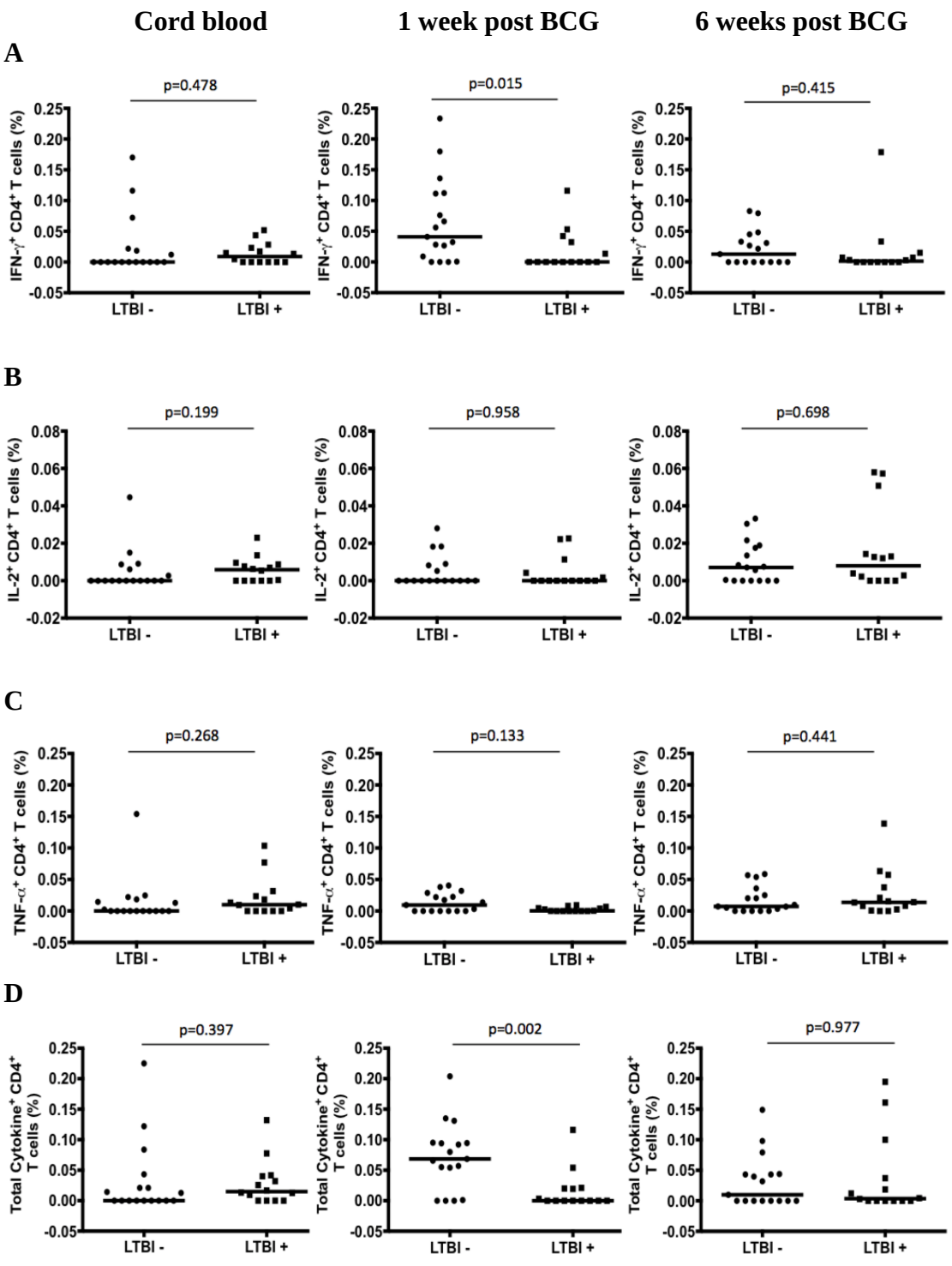

Figure 4.2. The effect of maternal latent *M. tuberculosis* infection on frequencies of CD4+ T cells. Frequencies of PPD-specific IFN-γ^+ (A), IL-2^+ (B), TNF-α^+ (C) and total cytokine$^+$ (D) cells in cord blood and infant samples obtained at one and six weeks after BCG immunisation, comparing infants of mothers with and without LTBI. Statistical analysis was performed using Mann-Whitney test. n=14 and 17 for infants of mothers with and without LTBI, respectively.

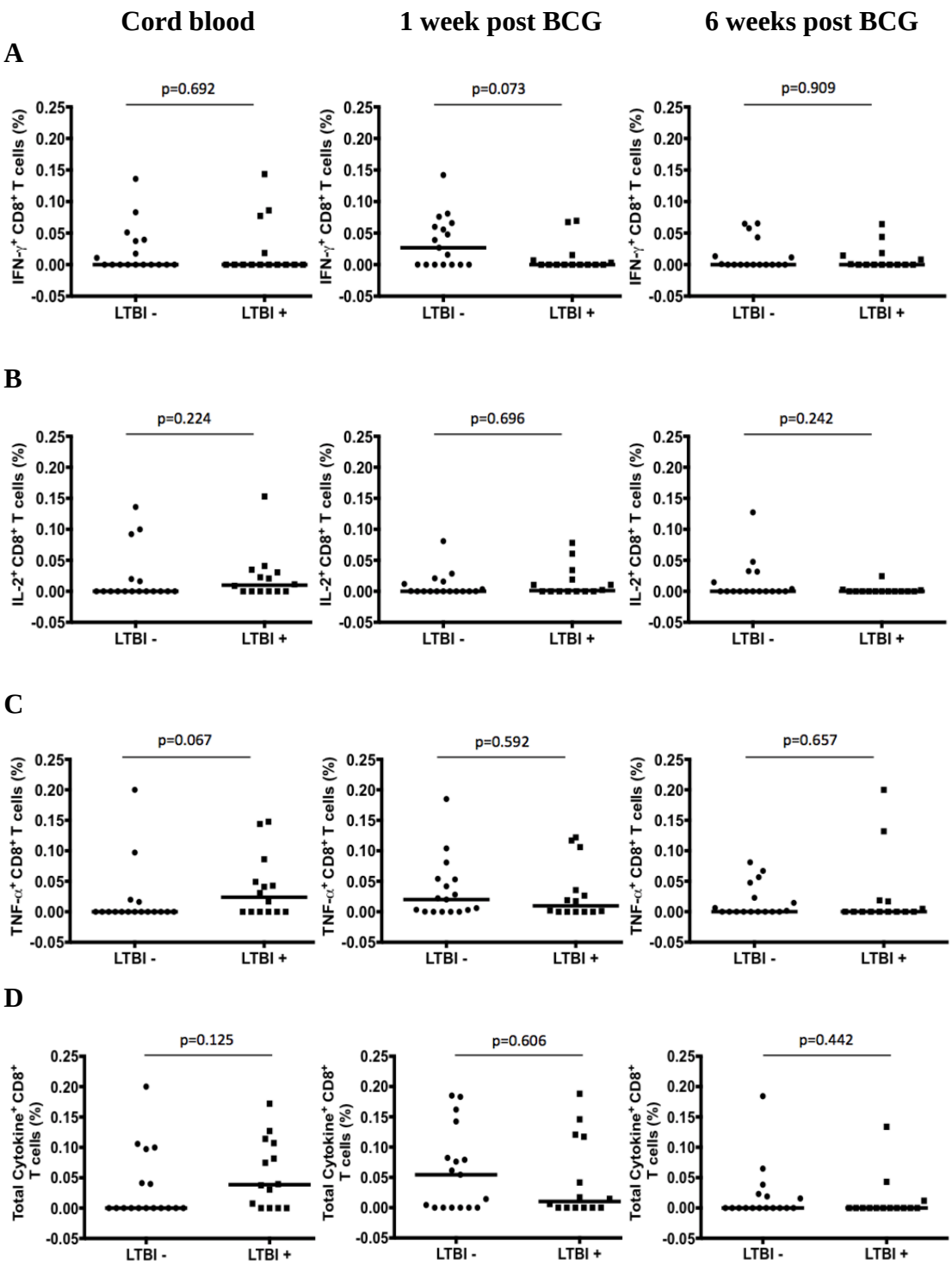

Figure 4.3. The effect of maternal latent *M. tuberculosis* infection on frequencies of CD8+ T cells. Frequencies of PPD-specific IFN-γ^+ (A), IL-2^+ (B), TNF-α^+ (C) and total cytokine$^+$ (D) cells in cord blood, and infant samples obtained at 1 and 6 weeks post BCG immunisation, comparing infants of mothers with and without LTBI. Statistical analysis was performed using Mann-Whitney test. n=14 and 17 for infants of mothers with and without LTBI, respectively.

Table 4.4. Associations between frequencies of cytokine-expressing T cells and maternal LTBI.

A. CD4+ T cells

Cytokine/time point	***Adjusted GMR**
Cord blood	
IFN-γ	0.96 (0.89, 1.05)
TNF-α	0.99 (0.92, 1.07)
IL-2	1.01 (0.99, 1.02)
Total	0.97 (0.88, 1.07)
1 week after BCG immunisation	
IFN-γ	0.94 (0.85, 1.04)
TNF-α	**0.97 (0.95, 0.99)**
IL-2	1.00 (0.98, 1.02)
Total	**0.91 (0.83, 0.99)**
6 weeks after BCG immunisation	
IFN-γ	1.02 (0.92, 1.13)
TNF-α	1.04 (0.97, 1.11)
IL-2	1.03 (0.99, 1.07)
Total	1.04 (0.90, 1.20)

B. CD8+ T cells

Cytokine/time point	***Adjusted GMR**
Cord blood	
IFN-γ	1.03 (0.97, 1.10)
TNF-α	1.02 (0.96, 1.08)
IL-2	1.02 (0.93, 1.11)
Total	1.05 (0.94, 1.18)
1 week after BCG immunisation	
IFN-γ	0.96 (0.91, 1.01)
TNF-α	0.97 (0.88, 1.07)
IL-2	1.02 (0.97, 1.07)
Total	0.95 (0.83, 1.09)
6 weeks after BCG immunisation	
IFN-γ	1.00 (0.95, 1.05)
TNF-α	1.05 (0.93, 1.17)
IL-2	0.97 (0.91, 1.03)
Total	0.98 (0.88, 1.08)

Data are aGMR and 95% CI for 31 infants at all the time points. Linear regression with bootstrapping was used for analysis. Confidence intervals not including one (1) are highlighted in bold. *Adjusted for maternal age, parity and infant gender.

4.3. Results for the main infant BCG study

The pilot infant BCG study gave some very interesting data on innate and (short term) adaptive responses, but was limited in scope and follow up. Nevertheless, this data was used to apply for, and successfully obtain, funding to conduct a larger study (described here and in Chapter 5.0) with a longer follow up, principally to identify the peak response to BCG immunisation and the influence of maternal LTBI on the infant response to BCG. Infants were followed up from birth to one year of age.

The timing and magnitude of the initial response to BCG immunisation, as well as comparison of responses in infants of mothers with and without LTBI are presented. The longitudinal changes in infant responses to PPD and ESAT-6/CFP-10 after 6-day stimulation of cord blood and infant samples obtained at 1, 4, 6, 10, 24 and 52 weeks post-BCG immunisation are presented. After a descriptive presentation of the socio-demographic and clinical characteristics of the participants, results from the analysis of responses to PPD and ESAT-6/CFP-10 are presented. The specific objectives were:

1. To analyse cytokines and chemokines in unstimulated samples, and in samples stimulated with PPD, ESAT-6/CFP-10 and PHA.
2. To assess longitudinal infant responses to PPD and ESAT-6/CFP-10 with age.
3. To examine the correlations in concentrations of individual cytokines and chemokines.

4.3.1. Participant characteristics for the main infant BCG study

Between June 2014 and September 2015, 1176 women were approached to participate in the study and 1124 were screened for LTBI. Of these, 840 were excluded, in most cases for discordant T-SPOT.TB and TST test results and since there were more mothers who tested negative on both tests than those who tested positive, a systematic number of LTBI-negative mothers were recruited to balance numbers recruited and to avoid bias. The selection criteria are included in the Methods Chapter (section 2.1.6.1). 284 mothers were enrolled and their infants were followed up to age one year. Of these, 134 mothers were identified as LTBI-positive and 150 as LTBI-negative. A number of mothers and their infants (182 in total) missed some study appointments (4 at one week, 10 at 4 weeks, 11 at 6 weeks, 20 at 10 weeks, 71 at 14 weeks and 66 at 24 weeks of age). The flow of participants through the study and details of samples for Luminex and ELISA assays are shown in Figures 2.12 and 2.13.

For this analysis, infants who had completed follow up to age one year were considered, with 55 of them born to mothers with LTBI and 85 born to mothers without LTBI. Mothers with and without LTBI were comparable in terms of BCG scar (28.95% versus 26.10, p=0.836), age (24 years versus 25 years, p=0.100), and gravidity status (33% versus 26% primigravida, p=0.750). Their infants were comparable in terms of male gender (58% versus 58%, p=1.000) and birth weight (3.19 versus 3.05, p=0.113) (Table 4.5).

Table 4.5. Demographic and clinical characteristics of participants

Characteristics of mothers and infants	**Mothers without LTBI (n=85)**	**Mothers with LTBI (n=55)**	***p*-value**
Maternal factors			
BCG scar present, no (%)	22 (28.95)	12 (26.10)	0.836
Maternal age, years, mean	24	25	0.100
Primigravida, no (%)	9 (33.33)	5 (26.32)	0.750
Infant factors			
Male gender, no (%)	38 (57.58)	24 (58.54)	1.000
Birth weight (Kg)	3.19	3.05	0.113

The figures are given as numbers with percentage (%) in brackets, or as mean values. *P* value is based on unmatched *t* test for differences in maternal age and infant birth weight, and a two-sided Fisher's exact test for differences in maternal LTBI, gravidity status and infant gender between scar-positive and scar-negative groups.

4.3.2. Comparison of cytokine and chemokine concentrations in unstimulated and stimulated samples

The cytokine and chemokine responses were measured by a 17plex Luminex® assay, based on data from our collaborators at the LSHTM (Prof. Hazel M. Dockrell's group) (358). Although all of the 17-cytokine datasets were analysed, only a selected few are graphically represented for the raw cytokine/chemokine data (without subtraction of background responses in unstimulated samples. (Figures 4.4 to 4.11).

The complete dataset for all cytokines/chemokines (after subtraction of background) are shown in and Figures 4.12 to 4.14 for responses to PPD and Table 4.7 and Figures 4.15 to 4.17 for responses to ESAT-6/CFP-10.

In order to assess spontaneous production in unstimulated cultures, compared to that in stimulated cultures, cytokine and chemokine concentrations were measured in unstimulated samples (medium) (S1), and in samples stimulated with PPD (S2), ESAT-6/CFP-10 protein (S3) and PHA (S4) for 6 days. The results are presented as raw median concentrations (before subtraction of negative control values) and illustrated in Figures 4.4 to 4.11. Concentrations of proinflammatory cytokines (represented by IFN-γ, TNF-α, IL-1α), Th2 (IL-5 and IL-13), chemokines (represented by IP-10 and MIP-1α) and a growth factor (GM-CSF) are shown. Overall, the median cytokine and chemokine concentrations in unstimulated samples were low, except for IL-8, IP-10 and MCP-1, where background concentrations were high.

The raw median IFN-γ production was higher in PPD-stimulated samples than in ESAT-6/CFP-10-stimulated samples at all time points (Figure 4.4). For TNF-α, the raw median responses were overall lower in PPD-stimulated samples than in ESAT-6/CFP-10-stimulated samples (Figure 4.5). The same pattern was observed for IL-1α (Figure 4.6) and MIP-1α (Figure 4.10). For Th2 responses, PPD-stimulated cultures produced more IL-5 and IL-13 than ESAT-6/CFP-10-stimulated samples (Figures 4.7 and 4.8). As expected, the positive control (PHA) showed high responses, indicating that the cells in the whole blood cultures were functional and active.

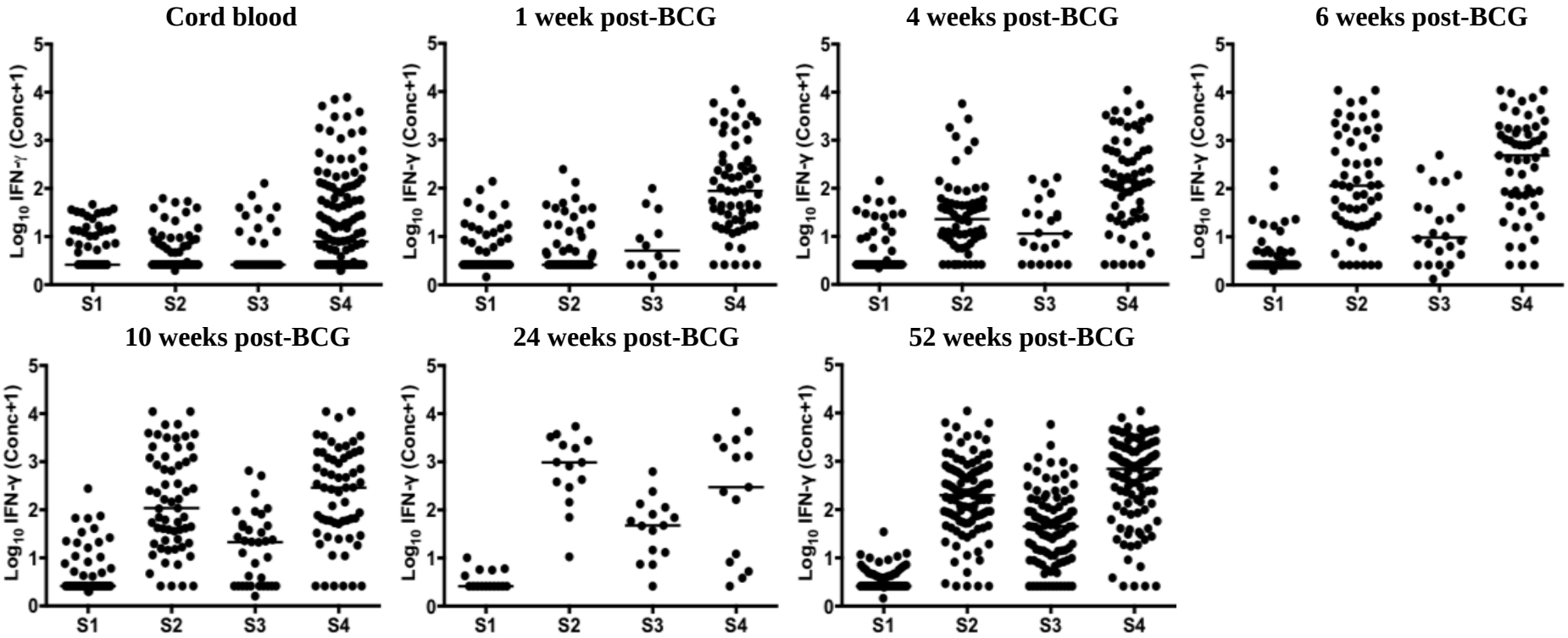

Figure 4.4. Changes in concentrations of IFN-γ in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

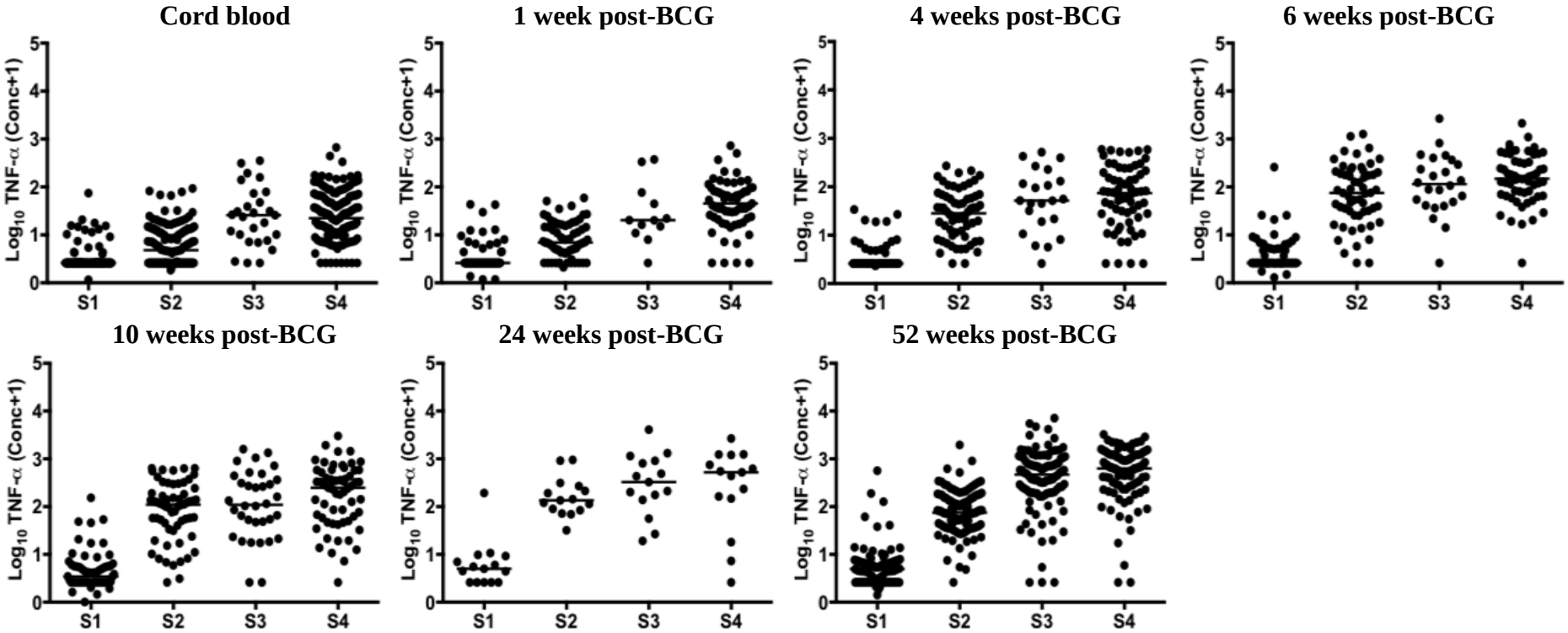

Figure 4.5. Changes in concentrations of TNF-α in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

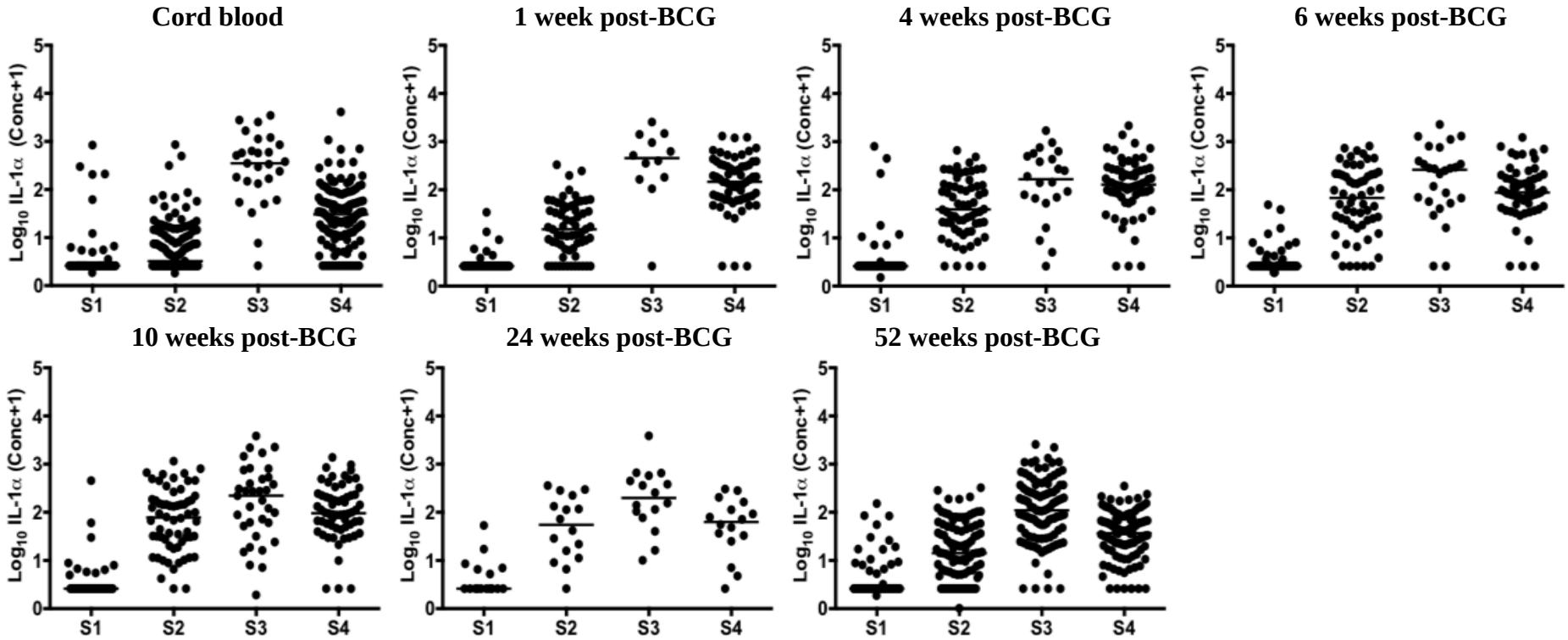

Figure 4.6. Changes in concentrations of IL-1α in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

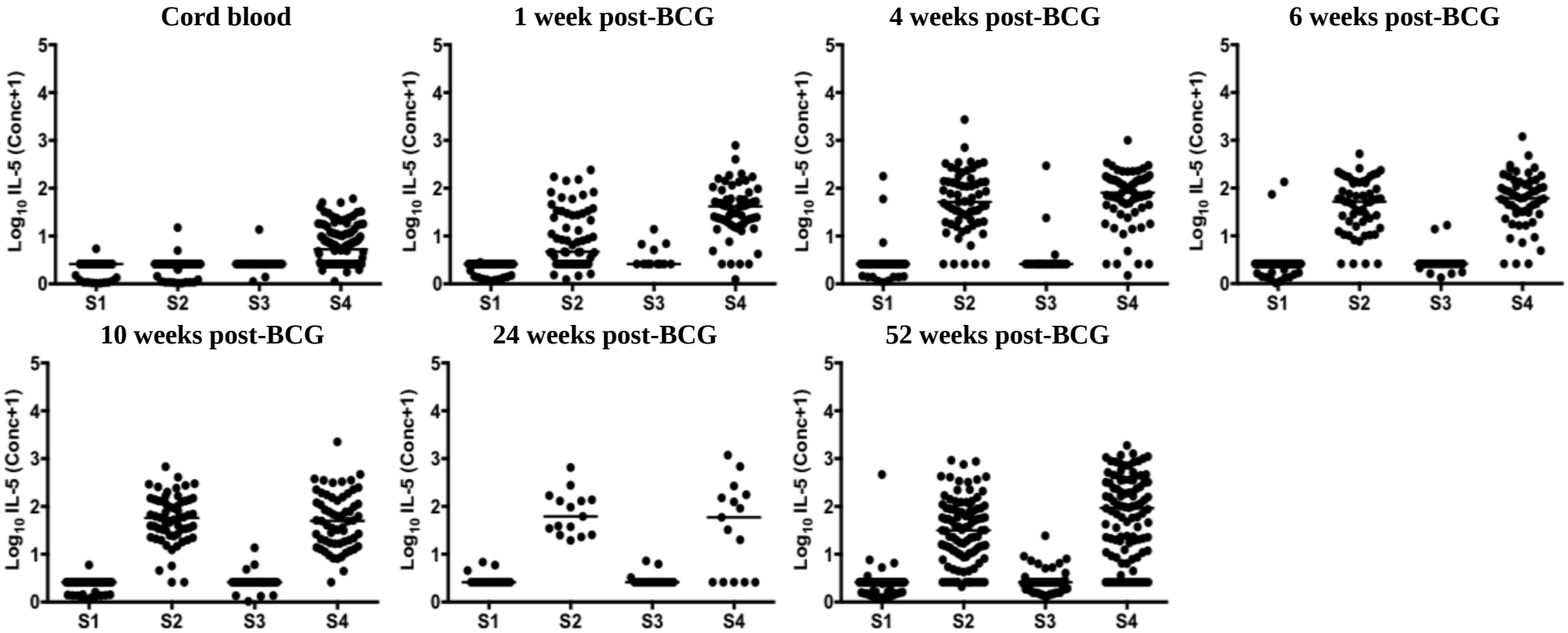

Figure 4.7. Changes in concentrations of IL-5 in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

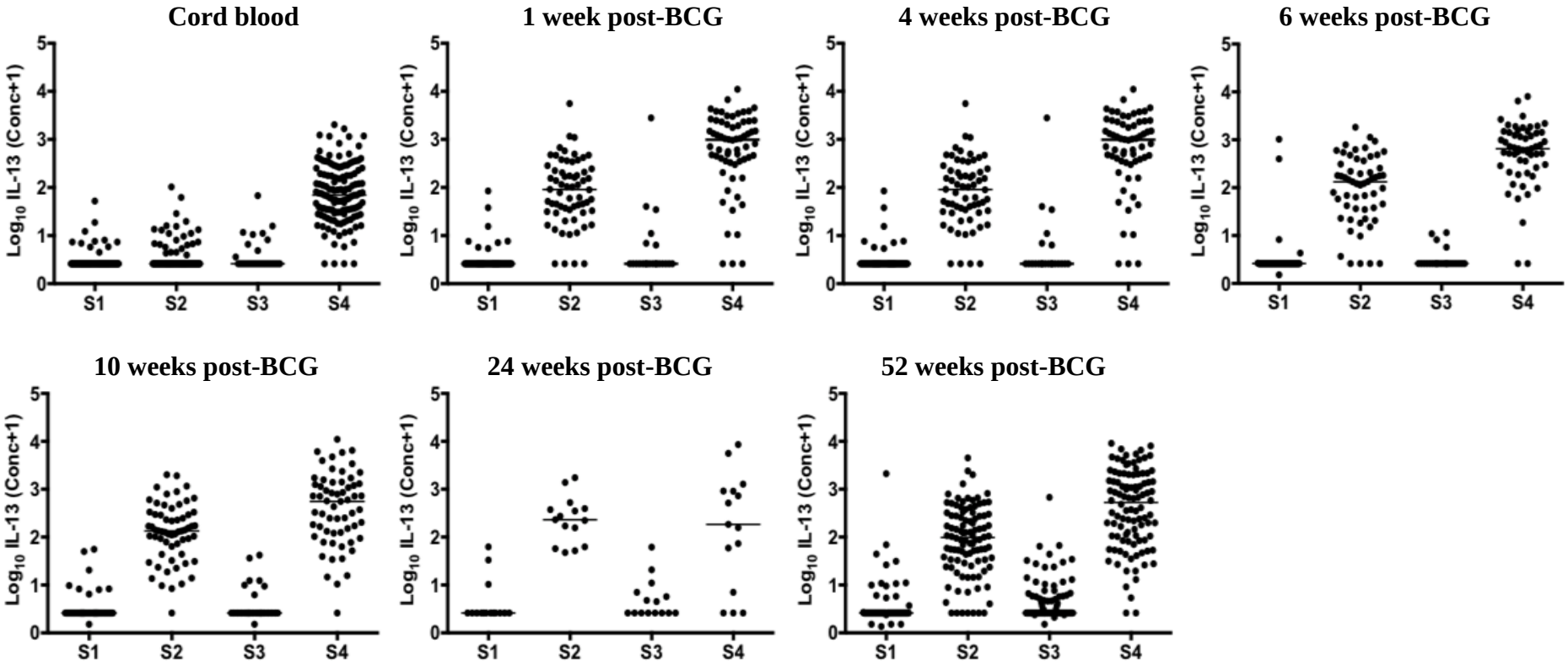

Figure 4.8. Changes in concentrations of IL-13 in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

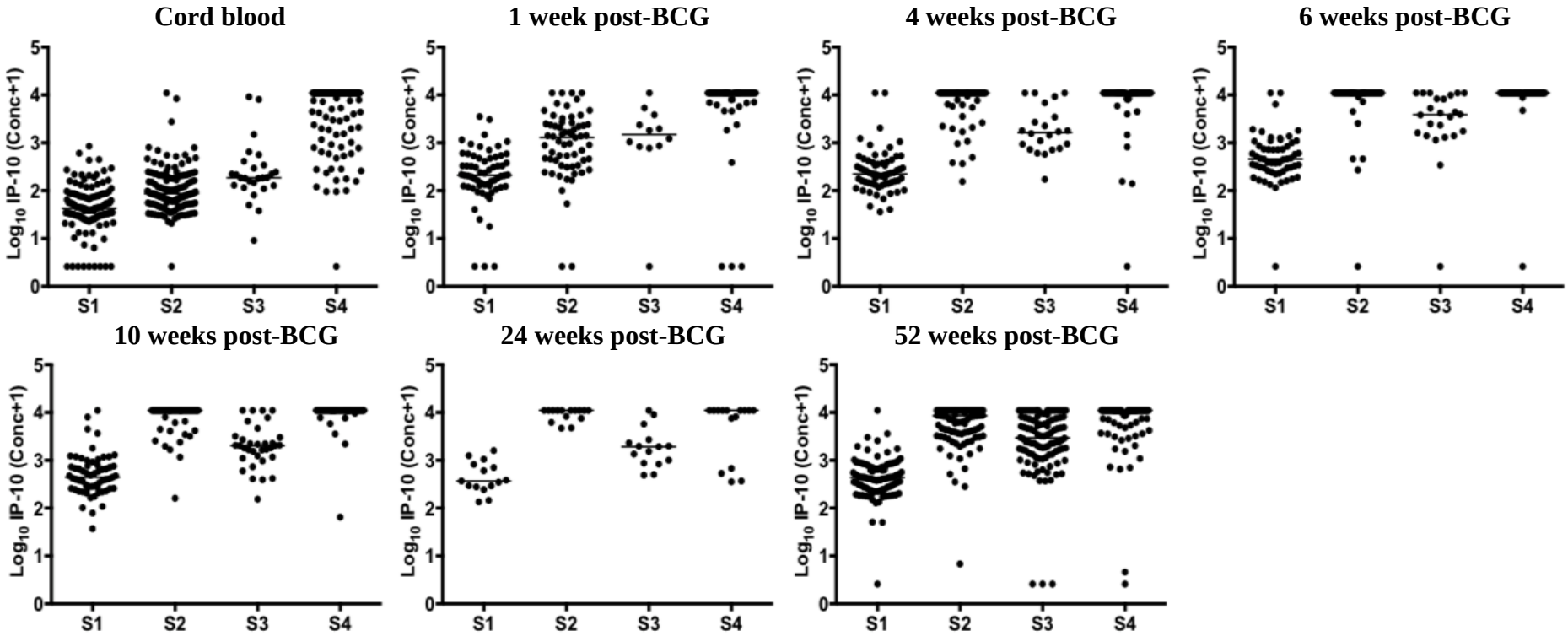

Figure 4.9. Changes in concentrations of IP-10 in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

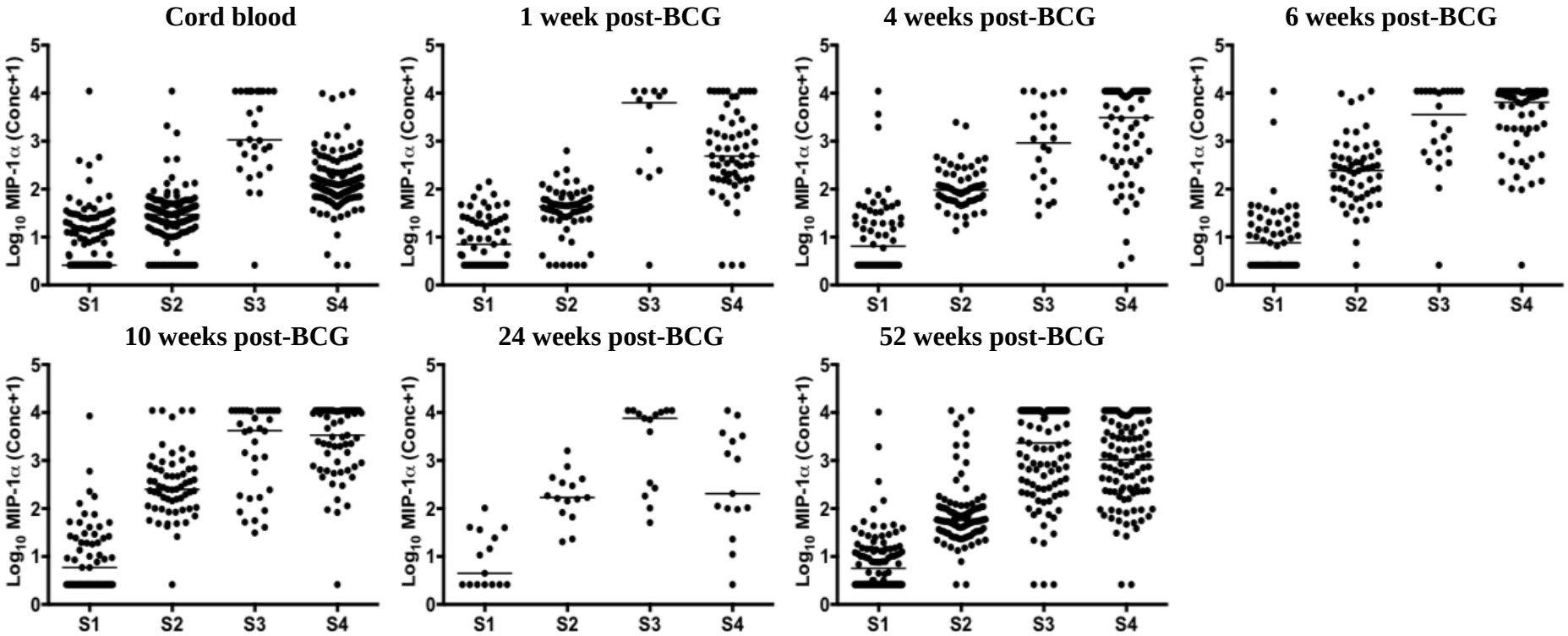

Figure 4.10. Changes in concentrations of MIP-1α in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

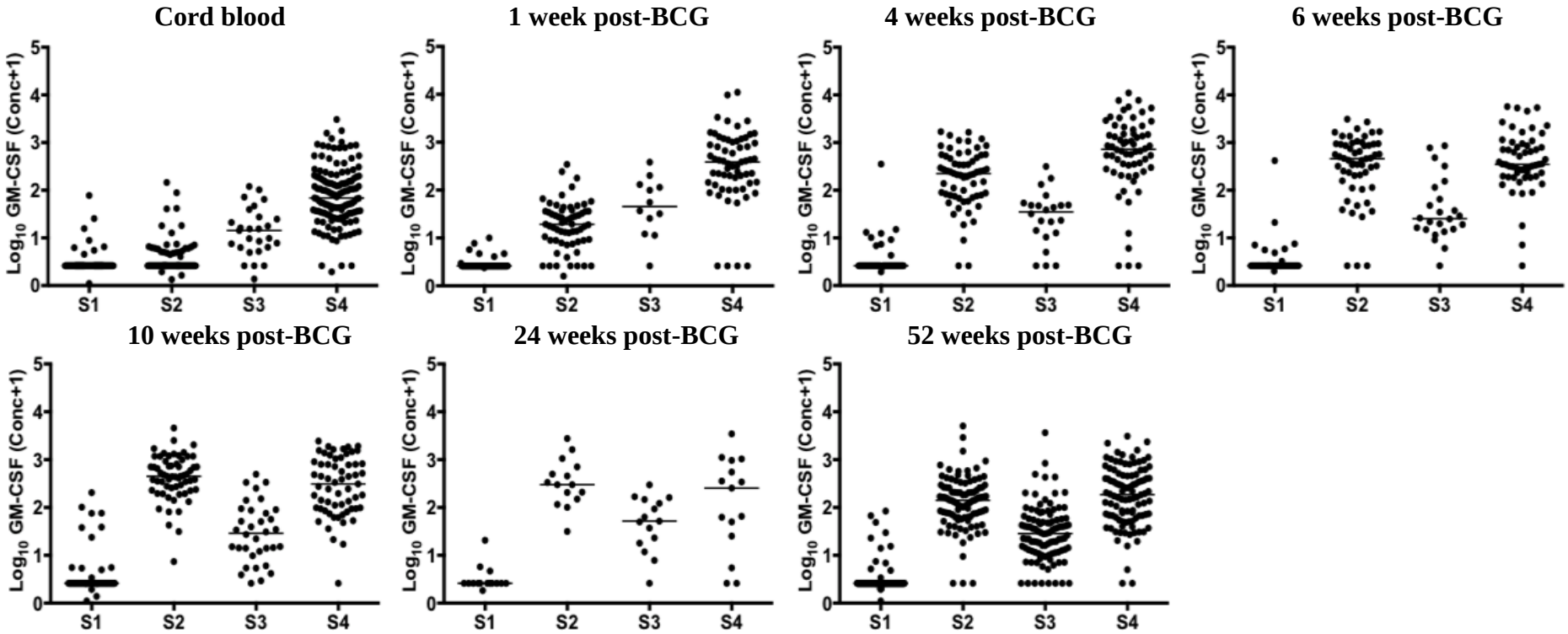

Figure 4.11. Changes in concentrations of GM-CSF in unstimulated and stimulated samples with age. Cytokines and chemokines were measured in supernatants obtained after 6-day stimulation of cultures with medium alone (unstimulated) (S1), PPD (S2), ESAT-6/CFP-10- (S3) and PHA (S4) using a 17-plex assay. For responses to PPD, n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit. For responses to ESAT-6/CFP-10, n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

4.3.3. Longitudinal infant responses to BCG immunisation

Having looked at the raw cytokine and chemokine responses (without subtraction of responses in unstimulated samples) in supernatants of infant samples stimulated with PPD and ESAT-6/CFP-10, and in the negative and positive controls to assess spontaneous and antigen/mitogen-stimulated production of cytokines and chemokines, the time course of the BCG-induced priming of the immune response, and the establishment of the peak in response after BCG immunisation in infancy was then assessed. This is important because little is known about the peak response following BCG immunisation, yet this peak should be targeted for boosting of responses primed by BCG immunisation.

The median cytokine and chemokine concentrations (after subtracting the background) are shown in Table 4.6 and Figures 4.12 to 4.14 for responses to PPD, Table 4.7 and Figures 4.15 to 4.17 for responses to ESAT-6/CFP-10. For most cytokines and chemokines measured, the peak of the responses to PPD was between 6 to 24 weeks of age, and by 52 weeks responses waned to levels at 4 or 6 weeks of age. This is the first time the peak of response has been demonstrated in any study. Figure 4.18 illustrates the kinetics of the individual infant responses to PPD for IFN-γ and TNF-α. Individual infant responses peaked at different times.

IL-2 responses continued to increase to 52 weeks of age, though with a lower magnitude than IFN-γ and TNF-α. Some chemokines responses developed early and were maintained at high concentrations over time (for example, IL-8 and IP-10). The concentrations of IL-8 and MCP-1 in the cord blood were higher than for the other cytokines and chemokines measured, and over time the concentrations were above the measurable range. Th2 responses (IL-5 and IL-13) appeared earlier (at one week) than for IFN-γ (at four weeks) for PPD-stimulated samples, and median IL-13 responses were higher than for TNF-α from one week of age.

For ESAT-6/CFP-10, it was interesting that Th1 responses (IFN-γ and TNF-α) increased over time (Figures 4.15 and 4.19), indicating exposure to mycobacteria expressing these antigens after birth. There was more TNF-α produced at all time points

than IFN-γ, and there was overall low production of IL-2, IL-5, IL-10, IL-13 and IL-17A cytokines, but high concentrations of IP-10, MCP-1, MIP-1α, MIP-1β chemokines.

Table 4.6. Cytokine and chemokine responses to PPD in infancy measured by Luminex® assay.

Cytokine/ Chemokine	**Cord blood (n=132)**	**1 week post-BCG (n=63)**	**4 weeks post-BCG (n=65)**	**6 weeks post-BCG (n=57)**	**10 weeks post-BCG (n=62)**	**24 weeks post-BCG (n=15)**	**52 weeks post-BCG (n=103)**
IFN-γ	0	0	8.52	110.84	105.54	967.89	196.20
TNF-α	2	3	24	66	107	133	70
IL-2	0	0	4	2	7	13	16
IL-1α	0	13	34	72	78	70	11
IL-1β	32	49	48	70	87	30	33
IL-8	1852	9059	9894	9570.31	10082	9790	9507
IL-1Ra	243	209	224	279	183	95	99
IL-12p40	0	0	0	0	0	4.32	0
IL-10	0	0	0	0	0	0	0
IL-5	0	3	47	48	55	59	29
IL-13	0	7	84	120	132	220	84
IL-17A	0	2	31	21	16	34	13
GM-CSF	0	17	220	455	441	298	134
IP-10	36.44	700.11	10591.33	10427.04	10359.72	10175.99	7905.36
MCP-1	7779.65	8638.64	7135.33	5489.61	2872.78	4716.32	6139.65
MIP-1α	16.60	23.76	71	223.10	250.23	153.91	47.05
MIP-1β	15.22	22.21	53.80	270.19	309.59	123.55	44.08

The values are shown as medians in pg/ml. The individual results are illustrated below in Figures 4.12 – 4.14

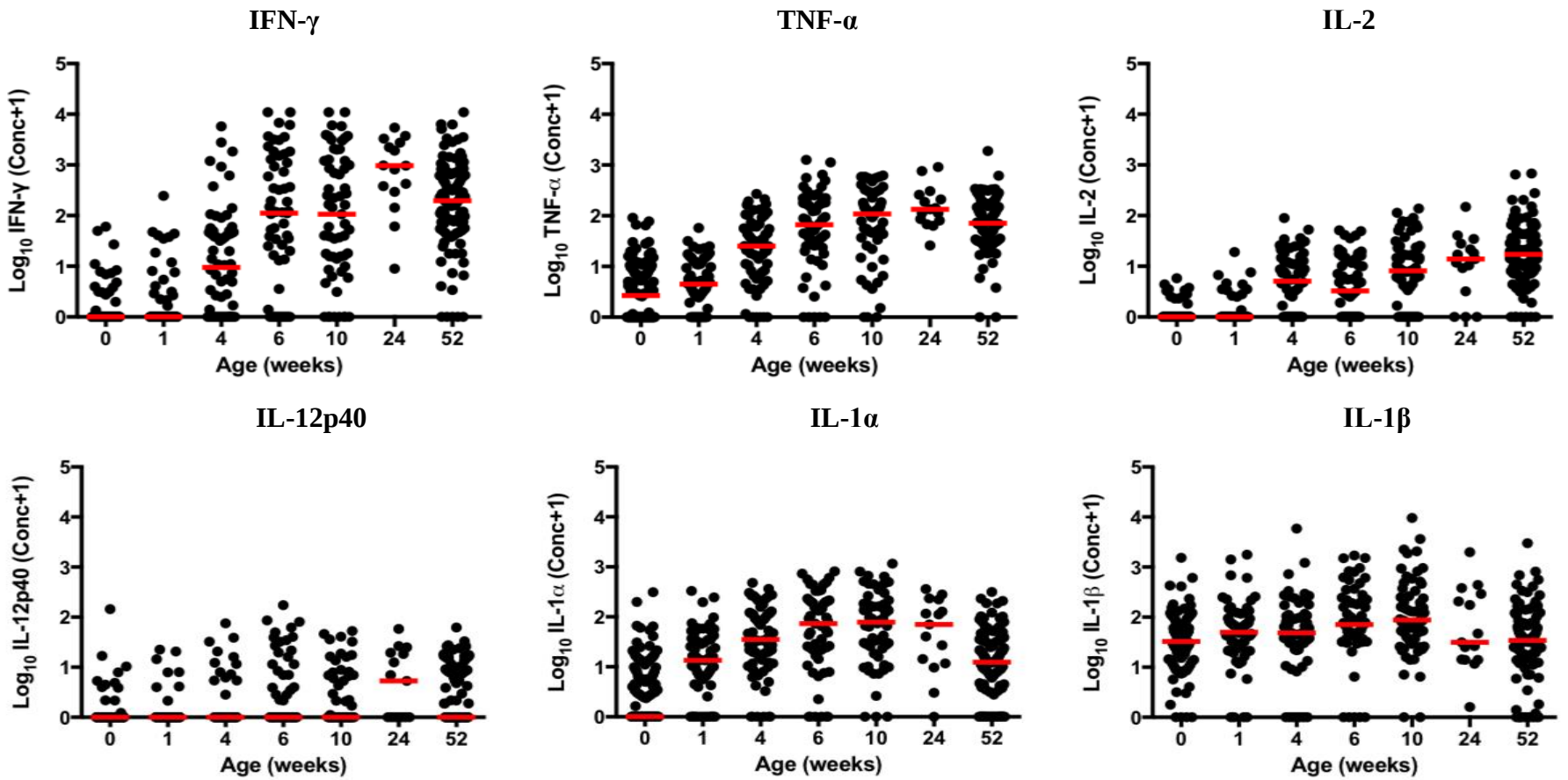

Figure 4.12. Longitudinal changes in concentrations of PPD-specific cytokines and chemokines during the first year of life measured by Luminex® assay. Each symbol represents an individual, and for each plot the horizontal line represents the median. Concentrations of IFN-γ, TNF-α, IL-2, IL-12p40, IL-1α and IL-1β are shown after subtraction of responses in unstimulated cultures. The numbers of infants tested at each time point are shown in Table 4.6.

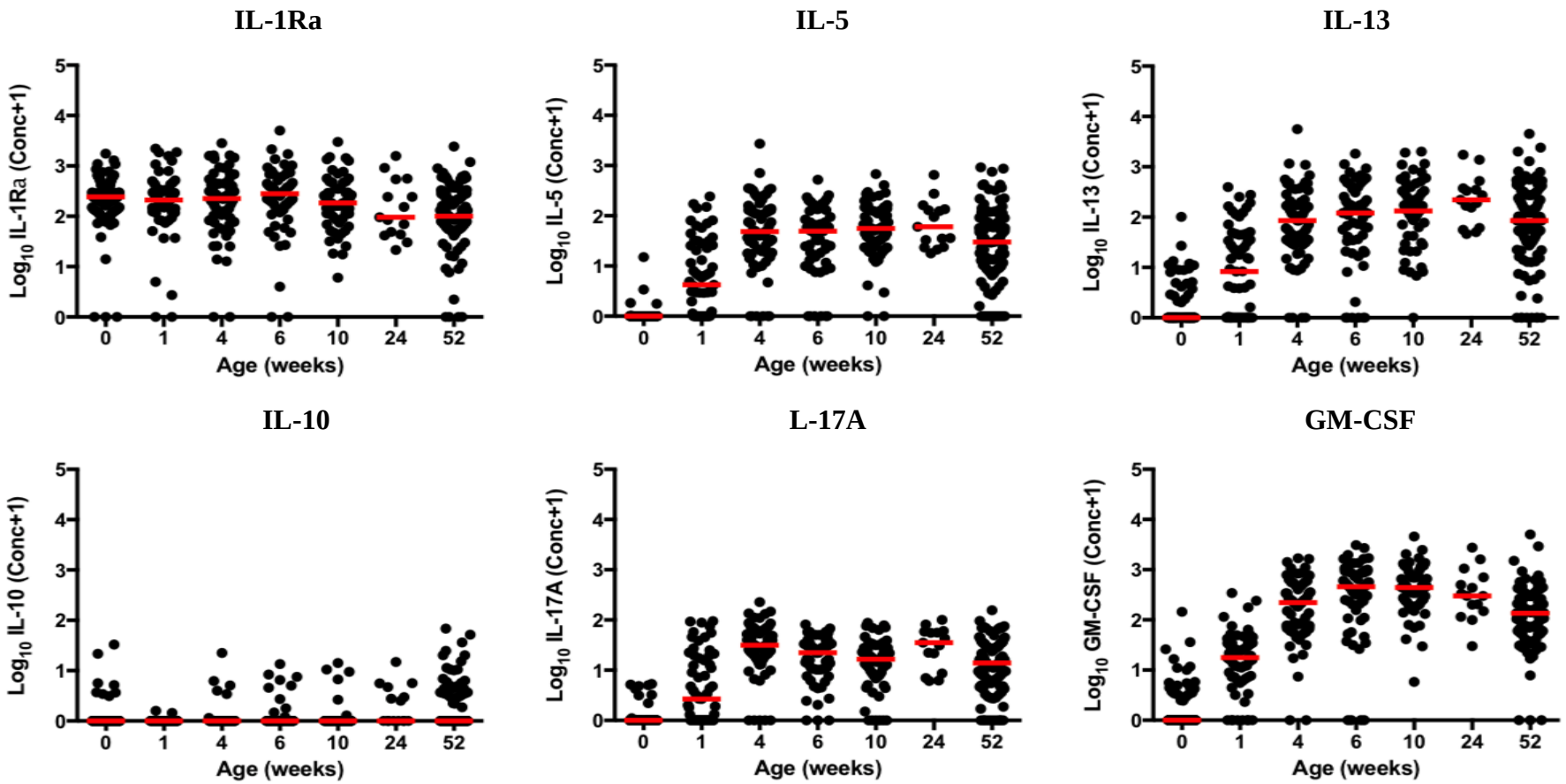

Figure 4.13. Longitudinal changes in concentrations of PPD-specific cytokines and chemokines during the first year of life measured by assay. Each symbol represents an individual, and for each plot the horizontal line represents the median. Concentrations of IL-1Ra, IL-5, IL-13, IL-10, IL-13, IL-17A and GM-CSF are shown after subtraction of responses in unstimulated cultures. The numbers of infants tested at each time point are shown in Table 4.6.

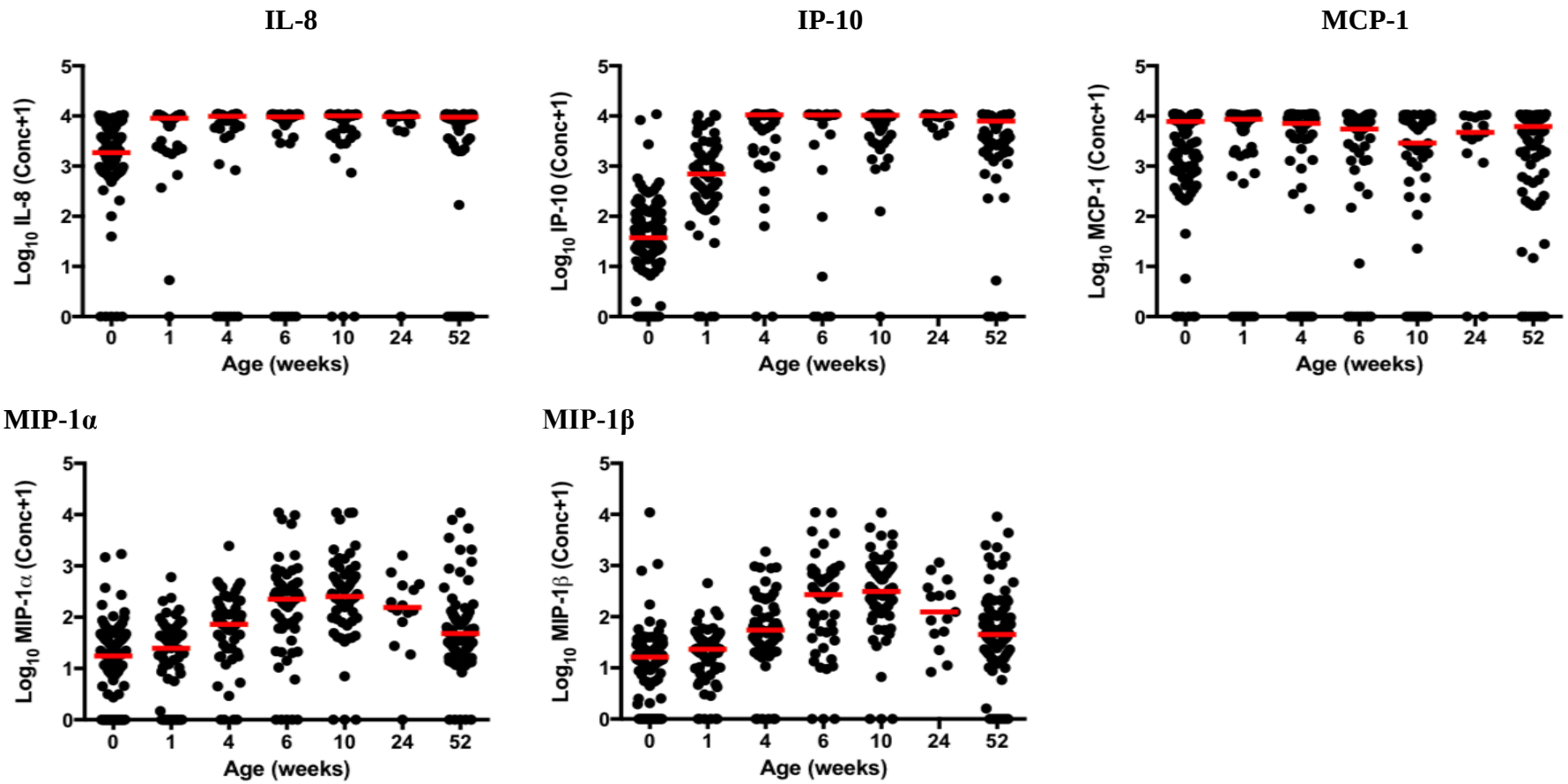

Figure 4.14. Longitudinal changes in concentrations of PPD-specific cytokines and chemokines during the first year of life measured by Luminex® assay. Each symbol represents an individual, and for each plot the horizontal line represents the median. Concentrations of IL-8, IP-10, MCP-1, MIP1α and MIP-1β are shown, after subtraction of responses in unstimulated cultures. The numbers of infants tested at each time point are shown in Table 4.6.

Table 4.7. Cytokine and chemokine responses to ESAT-6/CFP-10 in infancy measured by Luminex® assay.

Cytokine/ Chemokine	**Cord blood (n=27)**	**1 week post-BCG (n=12)**	**4 weeks post-BCG (n=22)**	**6 weeks post-BCG (n=24)**	**10 weeks post-BCG (n=34)**	**24 weeks post-BCG (n=15)**	**52 weeks post-BCG (n=103)**
IFN-γ	0	0	3.37	4.90	8.14	43.99	41.68
TNF-α	23.17	17.67	46.50	110.14	103.04	320.34	465.06
IL-2	0	0	0	0	0	0	0
IL-1α	349.15	456.24	162.39	271.33	243.24	252.27	107.61
IL-1β	2811.96	2096.78	1384.24	1913.95	1422.79	1950.13	1709.88
IL-8	10265.17	9238.75	9980.43	9524.42	9807.6	9790.12	10357.29
IL-1Ra	946.67	621.21	281.36	225.34	146.83	98.08	168.49
IL-12p40	19.36	78.82	22.4	54.22	32.30	25.97	40.90
IL-10	0	0	0	0	0	5.85	2.38
IL-5	0	0	0	0	0	0	0
IL-13	0	1.04	0	0	0	0	0
IL-17A	0	0	0.54	0	0	0	2.3
GM-CSF	11.66	43.55	29.06	22.8	16.12	49.29	24.50
IP-10	145.99	1244.72	1368.98	3006.54	1364.27	1148.45	2256.56
MCP-1	9636.26	8129.11	6831.08	2256.03	2424.33	3857.69	5778.31
MIP-1α	1053.05	6388.59	929.03	3847.71	3931.72	7579.76	2214.36
MIP-1β	1426.77	3771.77	1864.06	4296.67	3105.35	2746.07	2333.22

The values are medians in pg/ml. The individual results are illustrated below in Figures 4.15 – 4.17.

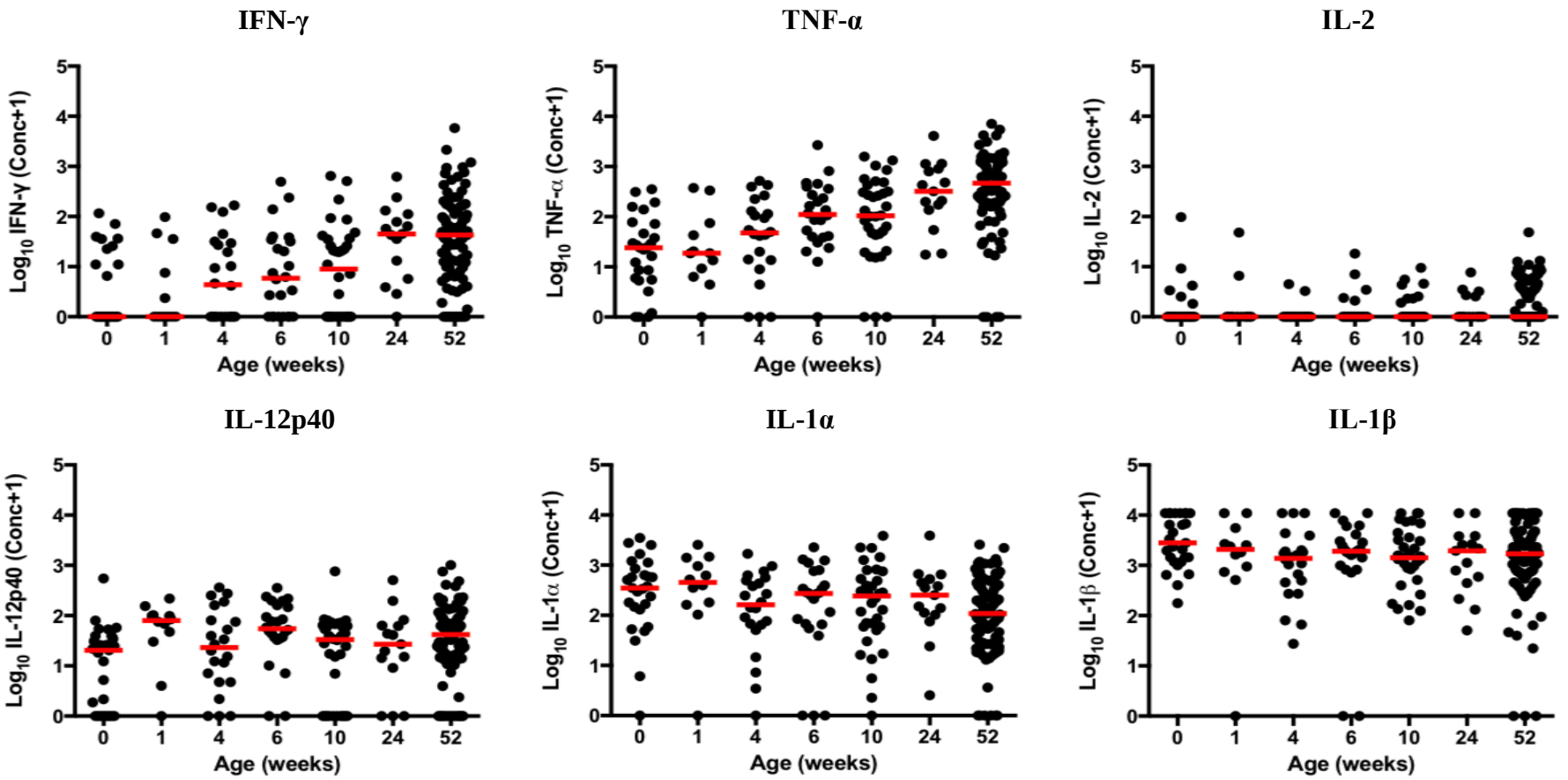

Figure 4.15. Longitudinal changes in concentrations of ESAT-6/CFP-10-specific cytokines and chemokines during the first year of life measured by Luminex® assay. Each symbol represents an individual, and for each plot the horizontal line represents the median. Concentrations of IFN-γ, TNF-α, IL-2, IL-12p40, IL-1α and IL-1β are shown after subtraction of responses in unstimulated cultures. The numbers of infants tested at each time point are shown in Table 4.7.

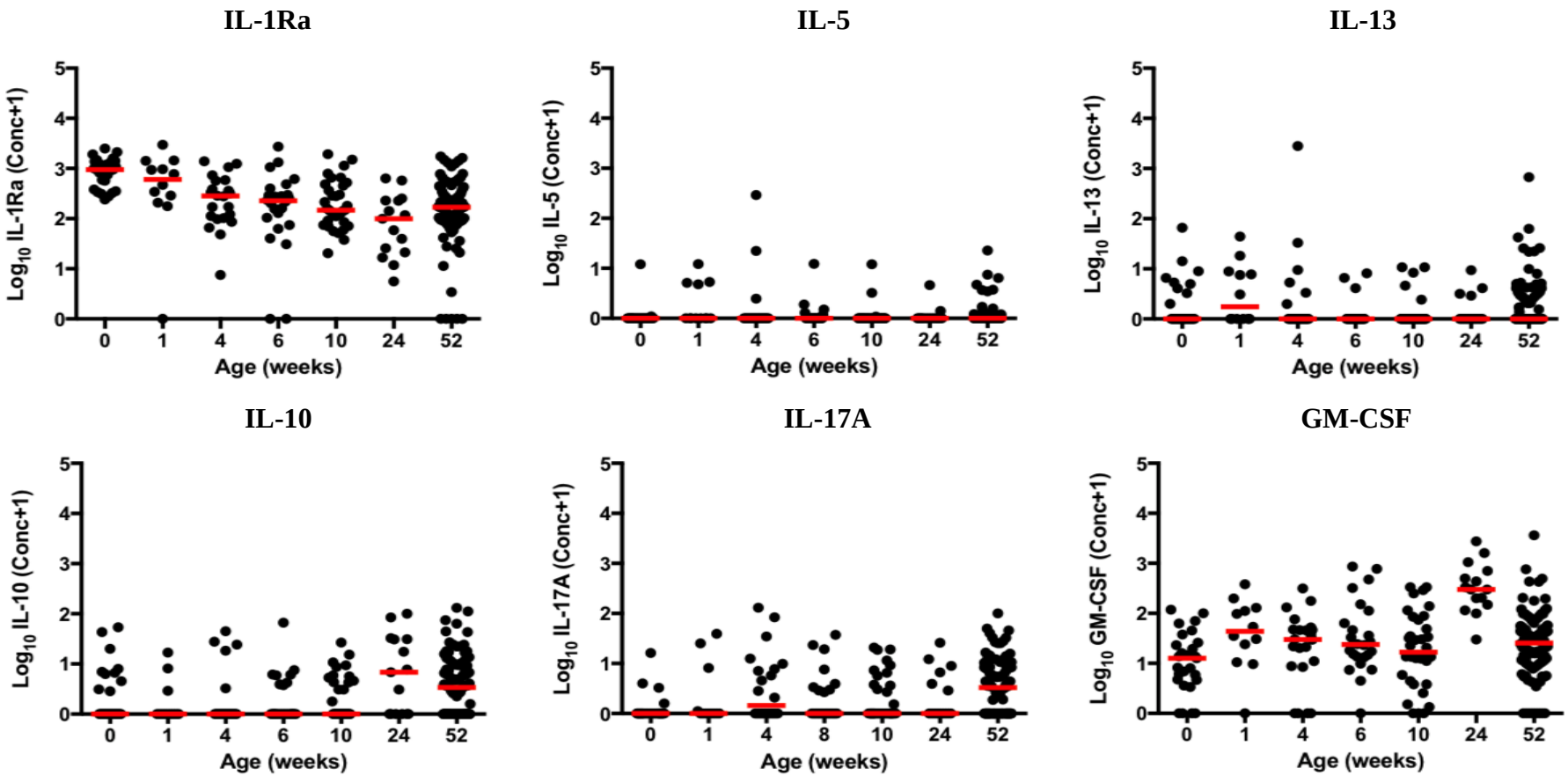

Figure 4.16. Longitudinal changes in concentrations of ESAT-6/CFP-10-specific cytokines and chemokines during the first year of life measured by Luminex® assay. Each symbol represents an individual, and for each plot the horizontal line represents the median. Concentrations of IL-1Ra, IL-5, IL-13, IL-10, IL-13, IL-17A and GM-CSF are shown after subtraction of responses in unstimulated cultures. The numbers of infants tested at each time point are shown in Table 4.7.

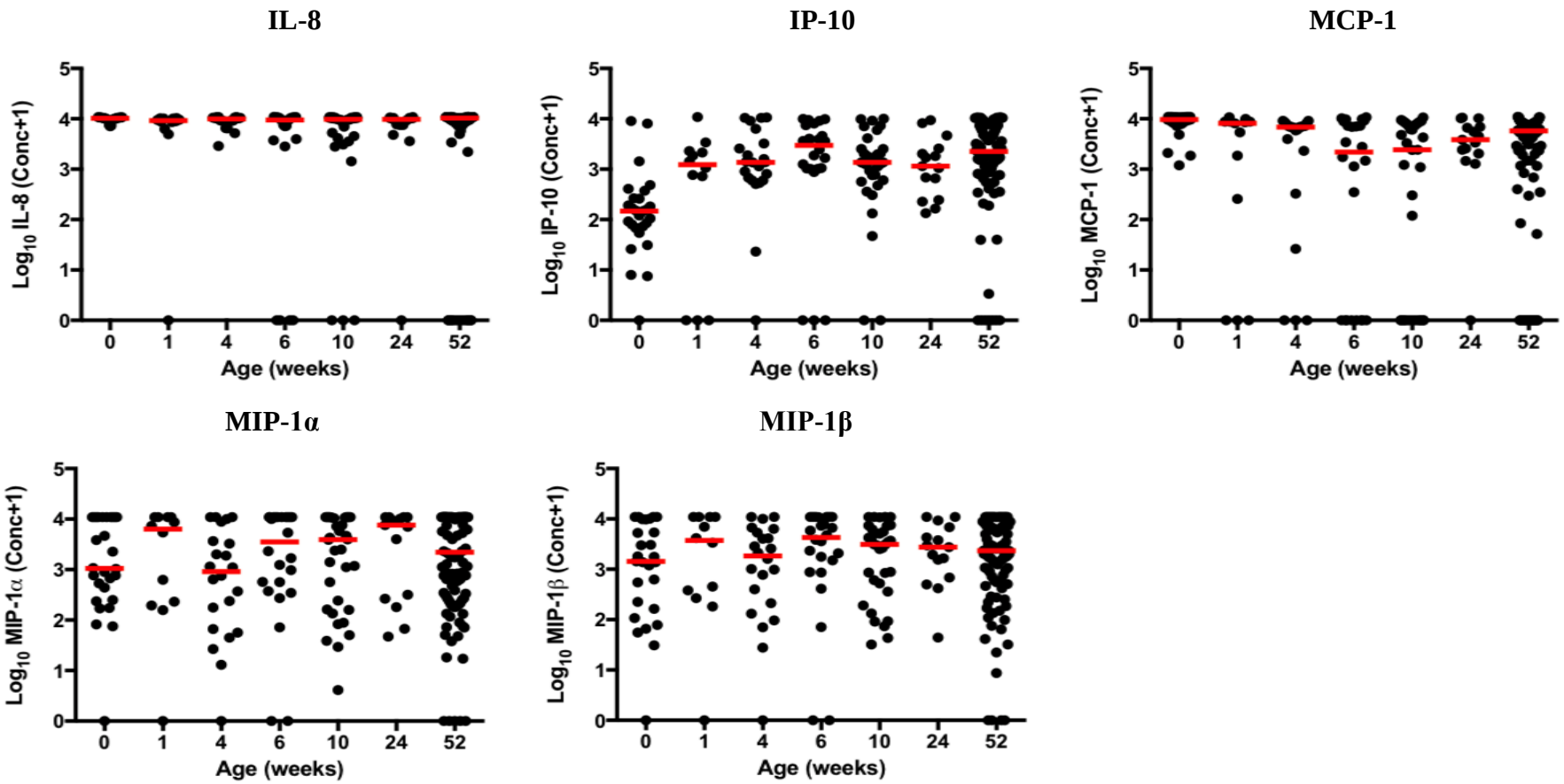

Figure 4.17. Longitudinal changes in concentrations of ESAT-6/CFP-10-specific cytokines and chemokines during the first year of life measured by Luminex® assay. Each symbol represents an individual, and for each plot the horizontal line represents the median. Concentrations of IL-8, IP-10, MCP-1, MIP1α and MIP-1β are shown after subtraction of responses in unstimulated cultures. The numbers of infants tested at each time point are shown in Table 4.7.

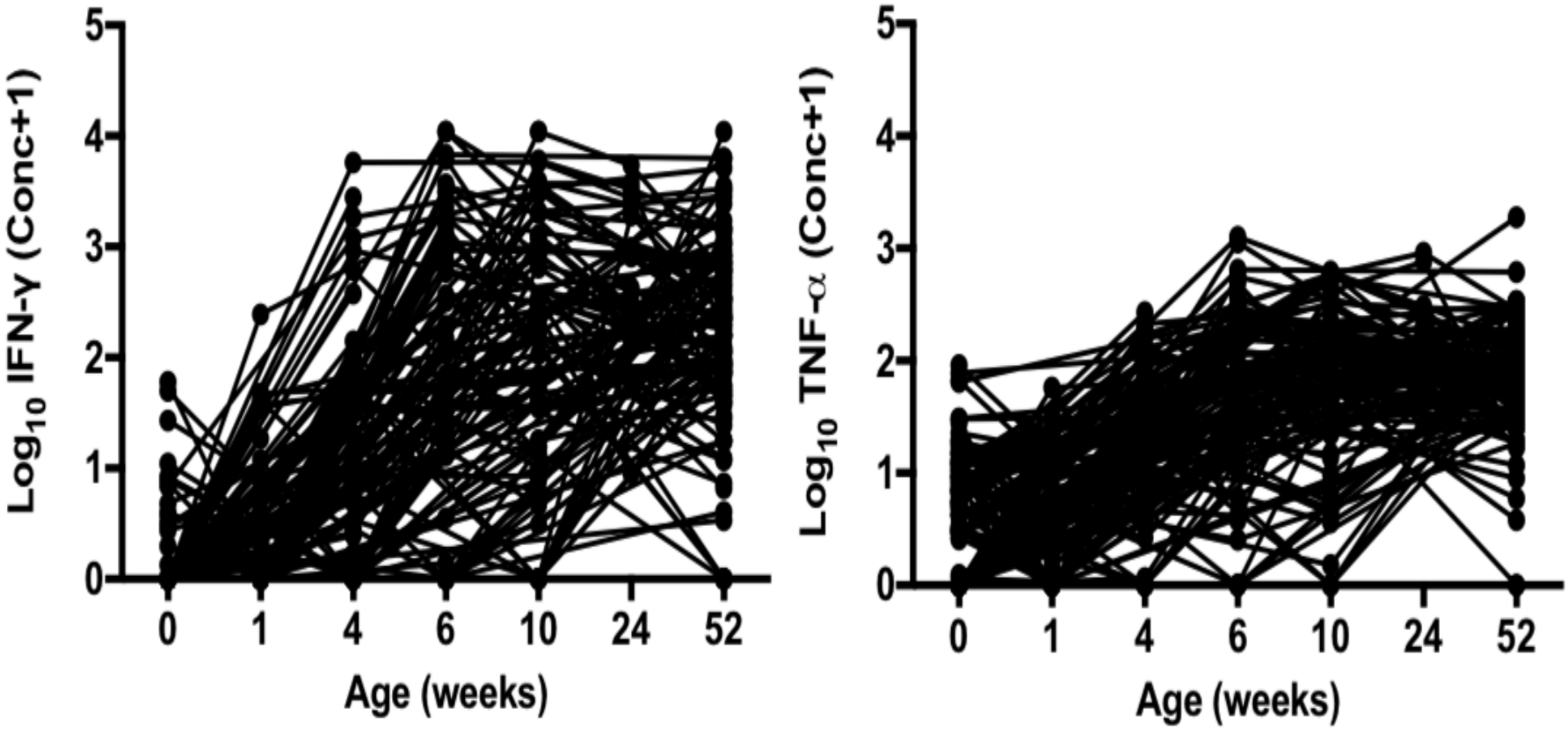

Figure 4.18. Kinetics of individual infant IFN-γ and TNF-α responses. Longitudinal changes in concentrations of IFN-γ and TNF-α in supernatants from infant samples stimulated with PPD and measured by Luminex®assay. Each line represents an individual infant cytokine response. Concentrations of cytokines are shown after subtraction of responses in unstimulated cultures. n=132 for cord blood, 63 for 1-week visit, 65 for 4-week visit, 57 for 6-week visit, 62 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

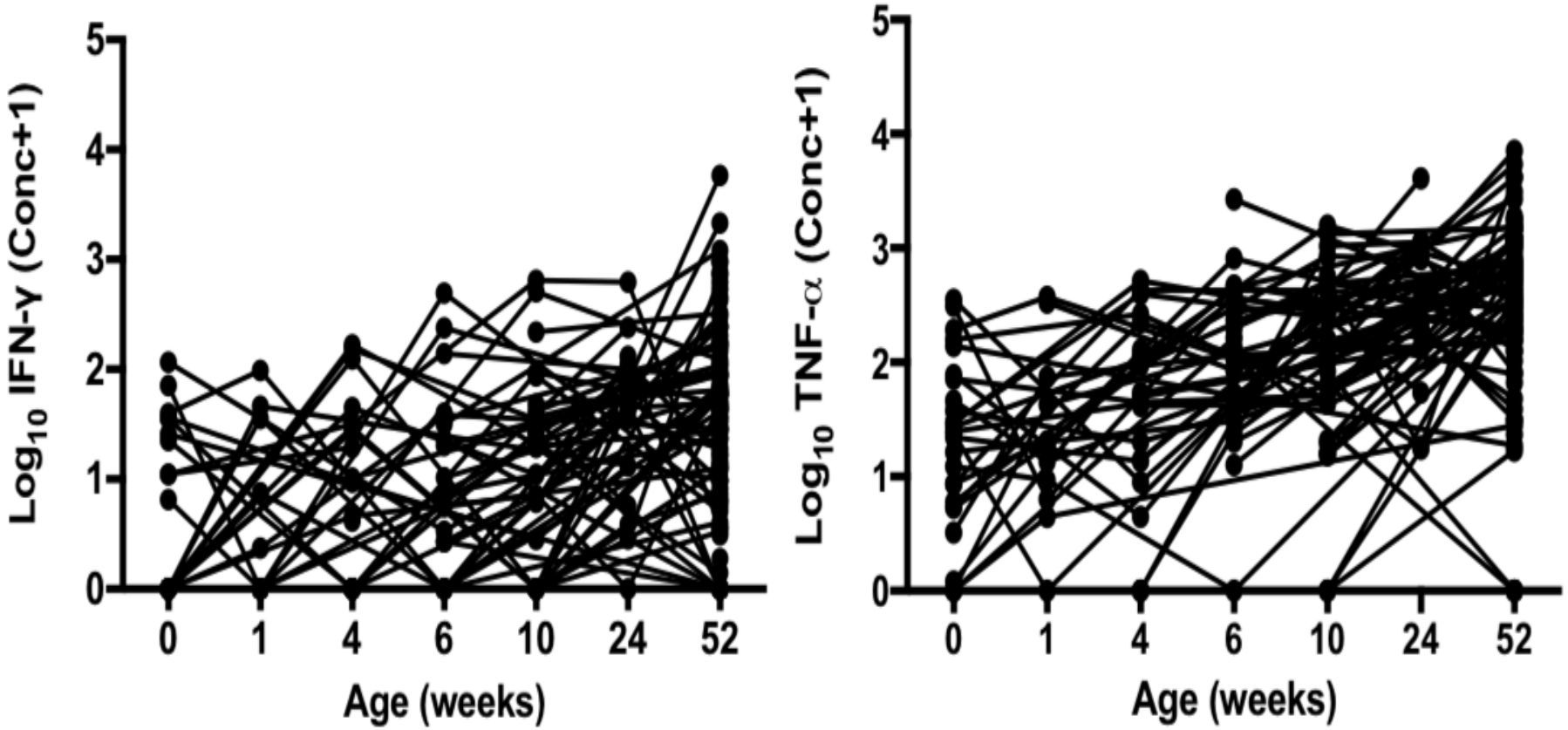

Figure 4.19. Kinetics of individual infant IFN-γ and TNF-α responses. Longitudinal changes in concentrations of IFN-γ and TNF-α in supernatants from infant samples stimulated with ESAT-6/CFP-10 and measured by Luminex®assay. Each line represents an individual infant cytokine response. Concentrations of cytokines are shown after subtraction of responses in unstimulated cultures. n=27 for cord blood, 12 for 1-week visit, 22 for 4-week visit, 24 for 6-week visit, 34 for 10-week visit, 15 for 24-week visit and 103 for 52-week visit.

4.3.4. Comparison of responses at the different time points in the main infant BCG study

Having looked at the time course of the BCG-induced priming of the immune response, and the establishment of the peak in response after BCG immunisation in infancy, the associations between responses at the different time points was assessed. This is important because it can tell, for example, whether the peak in response to BCG immunisation is related to responses at the other time points. Tables 4.8 and 4.9 show the results for responses to PPD and ESAT-6/CFP-10, respectively.

For PPD, responses at the early time points (up to week 4) were significantly different from responses at the later time points, for most cytokines and chemokines assessed, with higher responses at the later time points (Table 4.8).

For ESAT-6/CFP-10, there were few significant differences observed overall. Notable significant differences were for IFN-γ and TNF-α between early and later time points, and between later time points, with higher responses at the later time points (Table 4.9).

Table 4.8. Comparison of responses to PPD at different time points in the main infant BCG study

Cytokine/ Chemokine	Age (weeks)																			
	0 vs 1	**0 vs 4**	**0 vs 6**	**0 vs 10**	**0 vs 24**	**0 vs 52**	**1 vs 4**	**1 vs 6**	**1 vs 10**	**1 vs 24**	**1 vs 52**	**4 vs 6**	**4 vs 10**	**4 vs 24**	**4 vs 52**	**6 vs 10**	**6 vs 24**	**6 vs 52**	**10 vs 24**	**10 vs 52**	**24 vs 52**
IFN-γ	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns
TNF-α	ns	s	s	s	s	s	s	s	s	s	s	ns	s	s	s	ns	ns	ns	ns	ns	ns
IL-2	ns	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	s	ns	ns	s	ns	s	ns
IL-1α	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	s	ns	ns	s	ns	s	ns
IL-1β	ns	ns	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	s	ns	s	ns
IL-8	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-1Ra	ns	ns	ns	ns	ns	s	ns	ns	ns	ns	s	ns	ns	ns	s	ns	ns	s	ns	ns	ns
IL-12p40	ns	ns	s	s	s	s	ns	s	s	s	s	ns	ns	ns	s	ns	ns	ns	ns	ns	ns
IL-10	ns	ns	ns	ns	s	s	ns	ns	ns	s	s	ns	ns	s	s	ns	ns	s	s	s	ns
IL-5	s	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-13	s	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-17A	s	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
GM-CSF	s	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	s	ns
IP-10	s	s	s	s	s	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MCP-1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MIP-1α	ns	s	s	s	s	s	s	s	s	s	s	ns	s	ns	ns	ns	ns	s	ns	s	ns
MIP-1β	ns	s	s	s	s	s	s	s	s	s	s	ns	s	ns	ns	ns	ns	s	ns	s	ns

Significant and non-significant differences are indicated as “s” and “ns”, respectively. The numbers of infants tested at each time point are shown in Table 4.6. A Kruskal-Wallis test was used to compare infants at the different time points, with adjustment for multiple comparisons using Dunn’s multiple comparisons test.

Table 4.9. Comparison of responses to ESAT-6/CFP-10 at different time points in the main infant BCG study

	Age (weeks)																			
Cytokine/ Chemokine	**0 vs 1**	**0 vs 4**	**0 vs 6**	**0 vs 10**	**0 vs 24**	**0 vs 52**	**1 vs 4**	**1 vs 6**	**1 vs 10**	**1 vs 24**	**1 vs 52**	**4 vs 6**	**4 vs 10**	**4 vs 24**	**4 vs 52**	**6 vs 10**	**6 vs 24**	**6 vs 52**	**10 vs 24**	**10 vs 52**	**24 vs 52**
IFN-γ	ns	ns	ns	ns	s	s	ns	ns	ns	ns	s	ns	ns	ns	s	ns	ns	s	ns	s	ns
TNF-α	ns	ns	ns	ns	s	s	ns	ns	ns	s	s	ns	ns	ns	s	ns	ns	s	ns	s	ns
IL-2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	s	ns	ns	ns	ns	ns	ns
IL-1α	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-1β	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-1Ra	ns	s	s	s	s	s	ns	ns	ns	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-12p40	s	ns	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-10	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-13	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-17A	ns	ns	ns	ns	ns	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
GM-CSF	ns	s	ns	ns	s	ns	ns	ns	ns	ns	ns	ns	ns	s	ns	ns	s	ns	ns	ns	s
IP-10	ns	s	s	s	ns	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MCP-1	s	s	s	s	s	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MIP-1α	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MIP-1β	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Significant and non-significant differences are indicated as “s” and “ns”, respectively. The numbers of infants tested at each time point are shown in Table 4.7. A Kruskal-Wallis test was used to compare infants at the different time points, with adjustment for multiple comparisons using Dunn’s multiple comparisons test.

4.3.5. Correlations between production of individual cytokines and chemokines

Having examined differences in responses to mycobacterial antigens between the different time points, the correlations between concentrations of IFN-γ and the cytokines and chemokines measured were examined using Spearman rank correlation to assess the strength and direction of any relationships. This is important as this analysis would determine whether there is a relationship, for example, between pro-and anti-inflammatory cytokines.

The results are shown in Table 4.10 and Appendix T for PPD-specific responses, and in Table 4.11 and Appendix U for responses to ESAT-6/CFP-10 stimulation.
For PPD-specific responses, 9 out of 17 cytokines and chemokines measured at the different time points correlated strongly or very strongly (coefficient of 0.60 and above) with IFN-γ and these included TNF-α, IL-1α, IL-1 β, IL-5, IL-13, IL-17A, GM-CSF, IP-10 and MIP-1β (Table 4.10).

Of the 17 cytokines and chemokines induced by stimulation with ESAT-6/CFP-10, nine showed strong/very strong correlation with IFN-γ and these included TNF-α, IL-1α, IL-1β, IL-12p40, IL-10, GM-CSF, IP-10, MIP-1α and MIP-1β. Strongly positive correlations with TNF-α, IL-10 and MIP-1β with IFN-γ were also observed in cord blood (Table 4.11).

Table 4.10. Correlation between concentrations of IFN-γ and other cytokines and chemokines in PPD-stimulated culture supernatants measured by Luminex® assay.

Cytokine/ Chemokine	Cord blood (n=132)	1 week post-BCG (n=63)	4 weeks post-BCG (n=65)	6 weeks post-BCG (n=57)	10 weeks post-BCG (n=62)	24 weeks post-BCG (n=15)	52 weeks post-BCG (n=103)
TNF-α	0.20	0.33	**0.69**	**0.70**	**0.67**	**0.81**	**0.66**
IL-2	-0.04	0.29	0.31	0.49	0.34	-0.13	0.35
IL-1α	0.11	-0.01	0.24	**0.62**	0.37	**0.85**	**0.61**
IL-1β	0.18	-0.09	0.19	0.50	0.21	**0.83**	0.33
IL-8	0.15	-0.10	0.11	0.00	0.08	-0.30	0.35
IL-1Ra	0.01	-0.18	0.28	0.41	0.11	0.31	0.30
IL-12p40	0.05	0.23	0.35	0.36	0.39	0.45	0.46
IL-10	0.10	-0.11	0.11	0.25	0.21	0.26	0.23
IL-5	0.16	0.13	0.35	0.40	0.25	**0.68**	**0.63**
IL-13	0.27	0.15	**0.62**	**0.66**	0.72	**0.72**	**0.75**
IL-17A	0.38	0.42	0.52	0.25	0.15	**0.61**	0.44
GM-CSF	0.37	0.26	0.45	0.44	0.44	0.54	**0.76**
IP-10	-0.05	0.11	0.19	0.21	0.21	-0.18	**0.62**
MCP-1	0.11	-0.21	0.04	-0.02	-0.06	-0.44	0.04
MIP-1α	0.17	0.11	0.44	0.58	0.29	0.32	0.48
MIP-1β	0.10	0.10	0.33	**0.64**	0.27	0.30	0.45

Values are correlation coefficients obtained using Spearman rho test. Coefficients ≥0.6 are shown in bold.

Table 4.11. Correlation between IFN-γ and other cytokines and chemokines in ESAT-6/CFP-10-stimulated culture supernatants measured by Luminex® assay.

Cytokine/ Chemokine	Cord blood (n=27)	1 week post-BCG (n=12)	4 weeks post-BCG (n=22)	6 weeks post-BCG (n=24)	10 weeks post-BCG (n=34)	24 weeks post-BCG (n=15)	52 weeks post-BCG (n=103)
TNF-α	**0.68**	0.59	**0.63**	**0.70**	0.37	0.55	**0.63**
IL-2	0.34	-0.36	0.45	0.39	-0.22	0.19	0.29
IL-1α	0.40	0.38	0.54	0.27	0.22	**0.79**	0.58
IL-1β	0.41	**0.66**	0.47	0.24	0.12	**0.84**	**0.64**
IL-8	-0.12	0.17	0.33	-0.24	0.26	0.20	0.15
IL-1Ra	0.17	-0.12	0.20	-0.08	-0.12	0.59	0.56
IL-12p40	0.59	**0.60**	0.58	0.25	0.49	0.53	**0.74**
IL-10	**0.68**	0.54	0.23	**0.68**	0.20	0.06	0.31
IL-5	0.18	0.24	-0.03	-0.31	0.23	0.19	0.27
IL-13	0.33	0.34	0.10	0.41	0.59	0.59	0.33
IL-17A	0.44	0.42	0.31	-0.18	0.19	-0.20	0.19
GM-CSF	0.45	**0.66**	0.38	0.19	0.38	**0.62**	0.55
IP-10	0.32	0.58	0.51	0.01	0.27	0.07	**0.61**
MCP-1	-0.25	-0.16	0.24	-0.25	0	0.07	0.19
MIP-1α	0.58	0.58	0.34	0.13	0.21	**0.85**	0.53
MIP-1β	**0.65**	0.43	0.37	-0.06	0.08	**0.86**	0.54

Values are correlation coefficients obtained using Spearman rho test. Coefficients ≥0.6 are shown in bold.

4.3.6. The impact of maternal LTBI on infant responses to mycobacteria.

In the previous section, longitudinal infant responses to mycobacterial antigens over time were shown. Here I now show whether these responses differ according to maternal LTBI status.

Crude associations between maternal and infant factors and the infant responses to mycobacterial antigens were examined using a random effects regression model. Tables 4.12 and 4.13 show the results for responses to PPD and ESAT-6/CFP-10, respectively. For both mycobacterial antigens, infants of mothers with LTBI, compared to those without, had lower IL-1Ra response (crude mean difference, 95% confidence interval (CI) -0.25 (-0.36, -0.06) and -0.22 (-0.40, -0.04) for PPD and ESAT-6/CFP-10, respectively (Tables 4.12 and 4.13). For responses to ESAT-6/CFP-10, maternal BCG scar was associated with increased IL-12p40 responses (0.33 (0.11, 0.55), and infants of younger mothers, compared to those of older mothers, had lower MIP-1β responses (-0.36 (-0.68, -0.03) (Table 4.13).

In multivariate analysis, after adjusting for maternal BCG scar, maternal age, parity and infant gender the association between infant IL-1Ra to PPD and maternal LTBI was lost (adjusted mean difference, 95% CI -0.14 (-0.35, 0.07)). Infants of younger mothers, compared to the older mothers, produced more IL-1α to PPD stimulation (0.35 (0.02, 0.68)) (data not shown).

For responses to ESAT-6/CFP-10 stimulation, the negative association of infant IL-1Ra with maternal LTBI was lost (0.05 (-0.31, 0.42)). The association between maternal BCG scar and infant IL-12p40 responses was also lost (adjusted mean difference, 95% CI 0.07 (-0.30, 0.66)). However, the negative association between maternal age and infant MIP-1β responses was strengthened (-0.70 (-1.27, -0.13)). There were also negative associations between infant IL-1Ra and female infant gender (-0.38 (-0.72, -0.04)), infant IL-10 and maternal BCG scar (-0.44 (-0.85, -0.03)). Parity was positively associated with infant IL-13 responses to ESAT-6/CFP-10, in that the infants of multigravida mothers, compared to primigravida ones, had higher IL-13 concentrations (data not shown).

Table 4.12. Cytokine and chemokine responses to PPD in BCG-vaccinated infants, showing crude associations with maternal and infant factors.

Cytokine/ Chemokine	Maternal LTBI	Maternal BCG scar	Maternal age	Parity	Infant gender
	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)
IFN-γ	-0.06 (-0.33, 0.20)	-0.11 (-0.37, 0.15)	0.09 (-0.19, 0.38)	-0.00 (-0.43, 0.42)	0.03 (-0.22, 0.28)
TNF-α	0.00 (-0.16, 0.17)	-0.06 (-0.24, 0.11)	-0.03 (-0.21, 0.16)	-0.26 (-0.55, 0.03)	-0.02 (-0.19, 0.15)
IL-2	-0.001 (-0.15, 0.15)	-0.03 (-0.17, 0.11)	0.02 (-0.13, 0.17)	0.01 (-0.21, 0.24)	-0.12 (-0.26, 0.01)
IL-1α	-0.07 (-0.26, 0.11)	-0.03 (-0.20, 0.19)	0.08 (-0.12, 0.27)	0.03 (-0.27, 0.34)	-0.02 (-0.20, 0.17)
IL-1β	-0.01 (-0.29, 0.06)	0.02 (-0.14, 0.18)	0.06 (-0.09, 0.22)	0.13 (-0.14, 0.41)	0.01 (-0.13, 0.16)
IL-8	-0.05 (-0.32, 0.22)	0.09 (-0.11, 0.30)	0.05 (-0.19, 0.30)	0.07 (-0.23, 0.38)	0.09 (-0.13, 0.32)
IL-12p40	0.05 (-0.08, 0.18)	-0.04 (-0.16, 0.08)	-0.07 (-0.20, 0.06)	0.07 (-0.12, 0.26)	0.03 (-0.07, 0.14)
IL-1Ra	**-0.25 (-0.36, -0.06)**	0.04 (-0.08, 0.17)	0.01 (-0.05, 0.07)	-0.06 (-0.27, 0.14)	0.09 (-0.03, 0.22)
IL-10	-0.03 (-0.10, 0.04)	-0.00 (-0.07, 0.06)	0.01 (-0.11, 0.13)	-0.05 (-0.16, 0.06)	0.01 (-0.05, 0.07)
IL-5	-0.03 (-0.23, 0.17)	-0.01 (-0.20, 0.18)	-0.03 (-0.24, 0.17)	-0.12 (-0.45, 0.20)	-0.02 (-0.21, 0.16)
IL-13	-0.06 (-0.23, 0.15)	-0.04 (-0.26, 0.18)	0.01 (-0.23, 0.24)	0.03 (-0.34, 0.40)	-0.03 (-0.25, 0.18)
IL-17A	0.01 (-0.14, 0.16)	0.01 (-0.14, 0.15)	-0.05 (-0.21, 0.11)	-0.11 (-0.36, 0.14)	-0.04 (-0.18, 0.11)
GM-CSF	-0.03 (0.26, 0.11)	-0.05 (-0.28, 0.18)	0.04 (-0.20, 0.29)	-0.14 (-0.53, 0.26)	-0.02 (-0.25, 0.21)
IP-10	-0.18 (-0.41, 0.05)	-0.01 (-0.27, 0.24)	0.05 (-0.23, 0.34)	-0.08 (-0.51, 0.35)	-0.11 (-0.38, 0.15)
MCP-1	0.13 (-0.19, 0.43)	0.00 (-0.28, 0.28)	0.13 (-0.19, 0.45)	-0.25 (-0.76, 0.26)	-0.02 (-0.31, 0.26)
MIP-1α	-0.07 (-0.26, 0.12)	-0.12 (-0.30, 0.07)	-0.03 (-0.23, 0.16)	-0.13 (-0.45, 0.18)	-0.05 (-0.23, 0.13)
MIP-1β	-0.05 (-0.23, 0.14)	-0.03 (-0.21, 0.15)	-0.04 (-0.23, 0.15)	-0.10 (-0.40, 0.20)	-0.08 (-0.26, 0.09)

The values are log coefficients and 95% confidence interval analysed using random effects regression model.

Table 4.13. Cytokine and chemokine responses to ESAT-6/CFP-10 in BCG-vaccinated infants, showing crude associations with maternal and infant factors.

Cytokine/ Chemokine	Maternal LTBI	Maternal BCG scar	Maternal age	Parity	Infant gender
	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)	Coefficient (95% CI)
IFN-γ	-0.01 (-0.26, 0.25)	0.21 (-0.06, 0.48)	-0.28 (-0.60, 0.03)	-0.21 (-0.88, 0.46)	-0.05 (-0.35, 0.25)
TNF-α	0.02 (-0.22, 0.25)	0.03 (-0.22, 0.29)	-0.15 (-0.45, 0.14)	-0.39 (-1.00, 0.23)	-0.26 (-0.54, 0.03)
IL-2	0.02 (-0.094, 0.12)	0.02 (-0.10, 0.14)	-0.06 (-0.20, 0.08)	0.23 (-0.08, 0.54)	0.12 (0.00, 0.25)
IL-1α	-0.12 (-0.34, 0.11)	0.17 (-0.07, 0.41)	-0.13 (-0.42, 0.17)	-0.03 (-0.53, 0.47)	-0.17 (-0.45, 0.10)
IL-1β	-0.14 (-0.36, 0.08)	0.10 (-0.13, 0.33)	-0.15 (-0.41, 0.10)	-0.16 (-0.69, 0.38)	-0.17 (-0.45, 0.08)
IL-8	-0.09 (-0.46, 0.27)	0.29 (-0.09, 0.68)	-0.27 (-0.71, 0.17)	-0.46 (-1.30, 0.38)	0.13 (-0.30, 0.56)
IL-12p40	-0.10 (-0.31, 0.11)	**0.33 (0.11, 0.55)**	-0.18 (-0.43, 0.07)	0.07 (-0.43, 0.58)	-0.09 (-0.32, 0.15)
IL-1Ra	**-0.22 (-0.40, -0.04)**	0.10 (-0.10, 0.30)	-0.14 (-0.36, 0.07)	-0.19 (-0.60, 0.23)	0.01 (-0.20, 0.22)
IL-10	-0.09 (-0.25, 0.07)	-0.04 (-0.21, 0.14)	-0.13 (-0.33, 0.08)	-0.07 (-0.48, 0.33)	0.10 (-0.09, 0.29)
IL-5	-0.02 (-0.10, 0.06)	0.00 (-0.08, 0.08)	-0.03 (-0.13, 0.07)	0.07 (-0.100, 0.23)	0.03 (-0.06, 0.13)
IL-13	-0.08 (-0.21, 0.06)	0.01 (-0.13, 0.15)	0.03 (-0.15, 0.21)	0.36 (-0.02, 0.74)	0.14 (-0.03, 0.31)
IL-17A	-0.08 (0.09, 0.24)	0.03 (-0.15, 0.22)	-0.07 (-0.30, 0.15)	-0.12 (-0.59, 0.35)	0.0 (-5.16, 0.25)
GM-CSF	-0.02 (-0.22, 0.17)	0.11 (-0.09, 0.32)	-0.07 (-0.31, 0.17)	0.10 (-0.40, 0.61)	-0.12 (-0.35, 0.10)
IP-10	-0.06 (-0.35, 0.23)	0.09 (-0.21, 0.40)	-0.16 (-0.53, 0.21)	-0.35 (-0.90, 0.19)	-0.03 (-0.39, 0.32)
MCP-1	-0.08 (-0.53, 0.36)	-0.03 (-0.47, 0.42)	-0.14 (-0.65, 0.37)	-0.39 (-1.40, 0.62)	0.22 (-0.28, 0.71)
MIP-1α	-0.27 (-0.57, 0.02)	0.20 (-0.11, 0.52)	-0.24 (-0.61, 0.13)	-0.22 (-0.89, 0.45)	-0.25 (-0.60, 0.10)
MIP-1β	-0.20 (-0.46, 0.06)	0.16 (-0.12, 0.44)	**-0.36 (-0.68, -0.03)**	-0.22 (-0.83, 0.39)	-0.25 (-0.56, 0.05)

The values are log coefficients and 95% confidence interval analysed using random effects regression model.

The lack of association between maternal LTBI and infant cytokine and chemokine responses was confirmed in cord blood, and in infant samples by comparing responses in infants of mothers with and without LTBI using a Kruskal-Wallis and Dunn's multiple comparison tests. The results are illustrated in Figures 4.20A and 4.20B for responses to PPD, and Figures 4.21A and 4.21B for responses to ESAT-6/CFP-10.

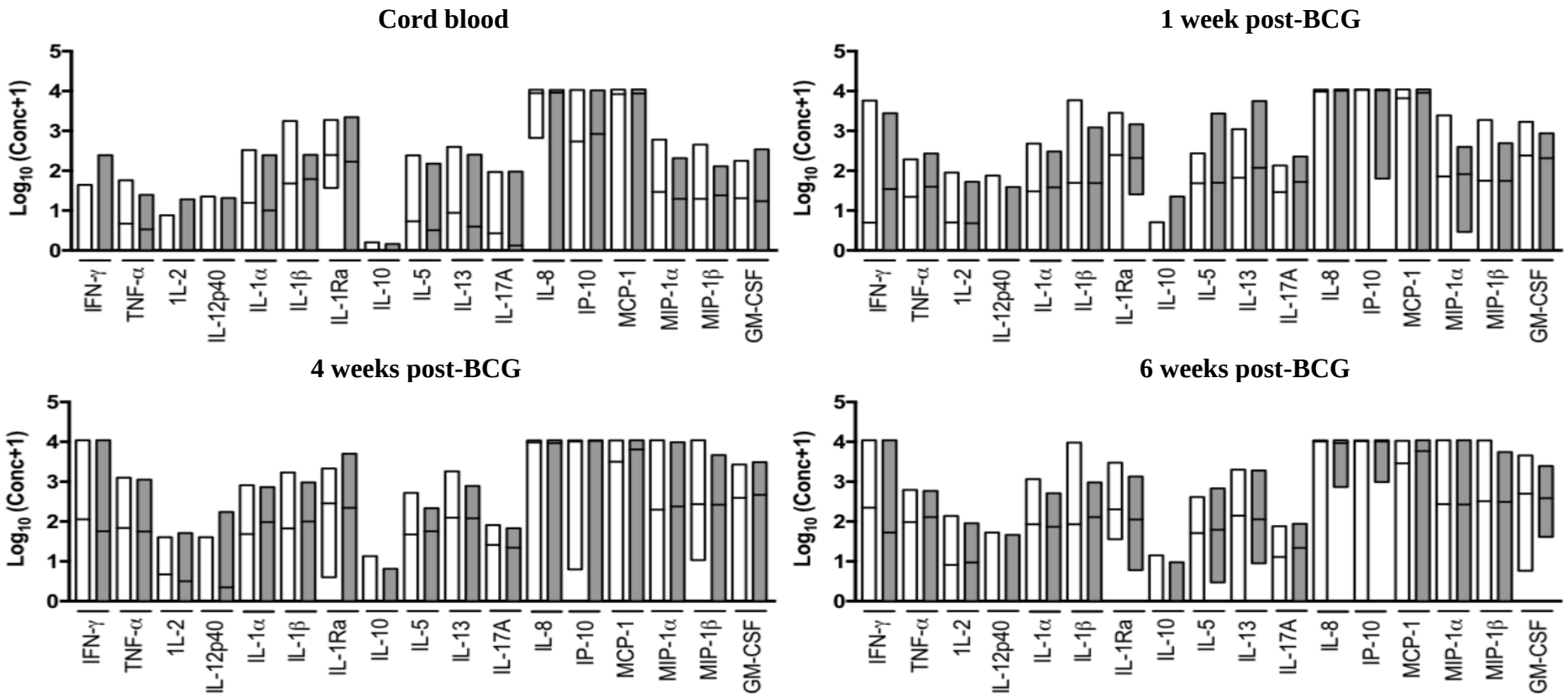

Figure 4.20A. Cytokine and chemokine responses to PPD measured by Luminex® assay. Clear and grey bars represent infants of mothers without (LTBI-) and with (LTBI+) LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by *. A Kruskal-Wallis test was used to compare infants in the two groups, with adjustment for multiple comparisons using Dunn's multiple comparisons test. Numbers of infants for LTBI- and LTBI+ groups: 82 versus 50 for cord blood, 36 versus 27 for 1-week, 42 versus 23 for 4-week, 32 versus 25 for 6-week, 37 versus 25 for 10 week, 9 versus 6 for 24-week, and 65 versus 38 for 52-week visits.

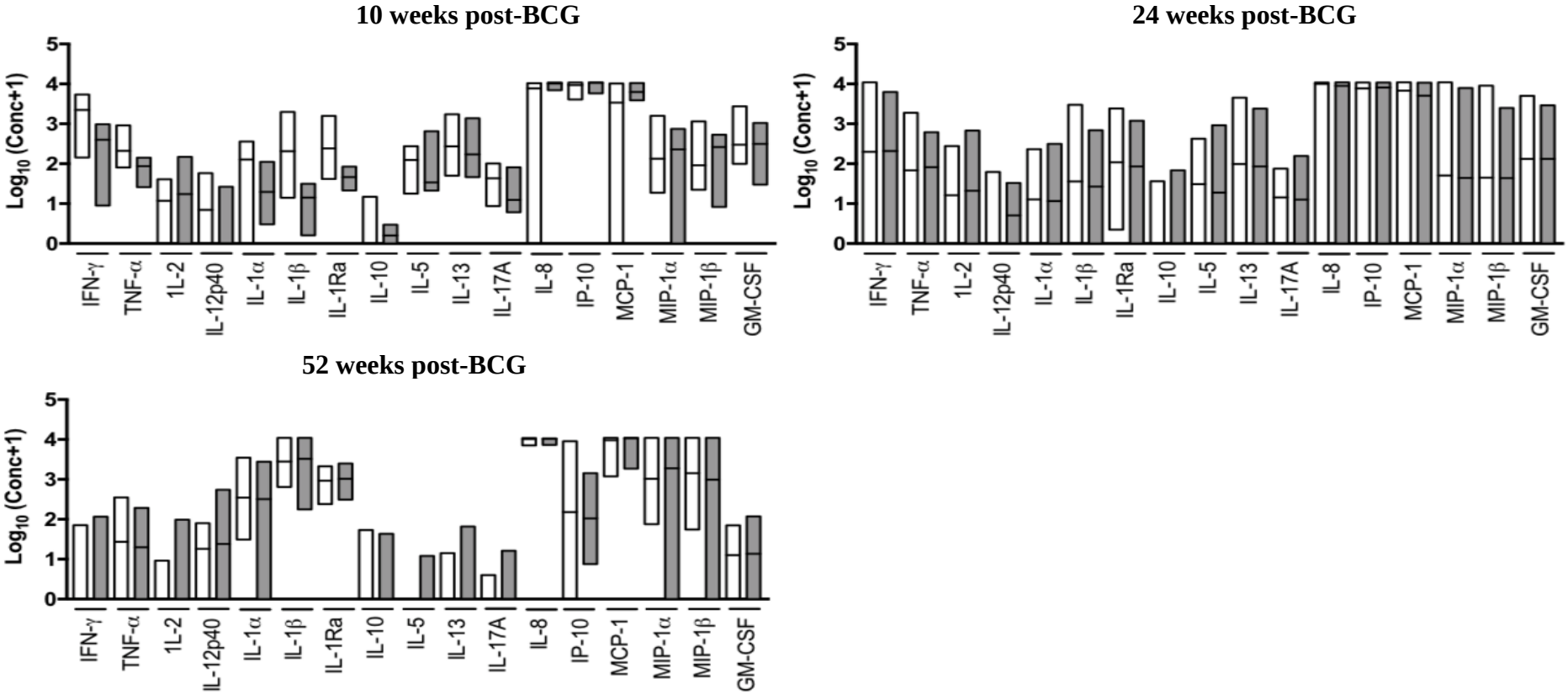

Figure 4.20B. Cytokine and chemokine responses to PPD measured by Luminex® assay. Clear and grey bars represent infants of mothers without and with LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by *. A Kruskal-Wallis test was used to compare infants in the two groups, with adjustment for multiple comparisons using Dunn's multiple comparisons test). Numbers of infants for LTBI- and LTBI+ groups: 82 versus 50 for cord blood, 36 versus 27 for 1-week, 42 versus 23 for 4-week, 32 versus 25 for 6-week, 37 versus 25 for 10 week, 9 versus 6 for 24-week, and 65 versus 38 for 52-week visits.

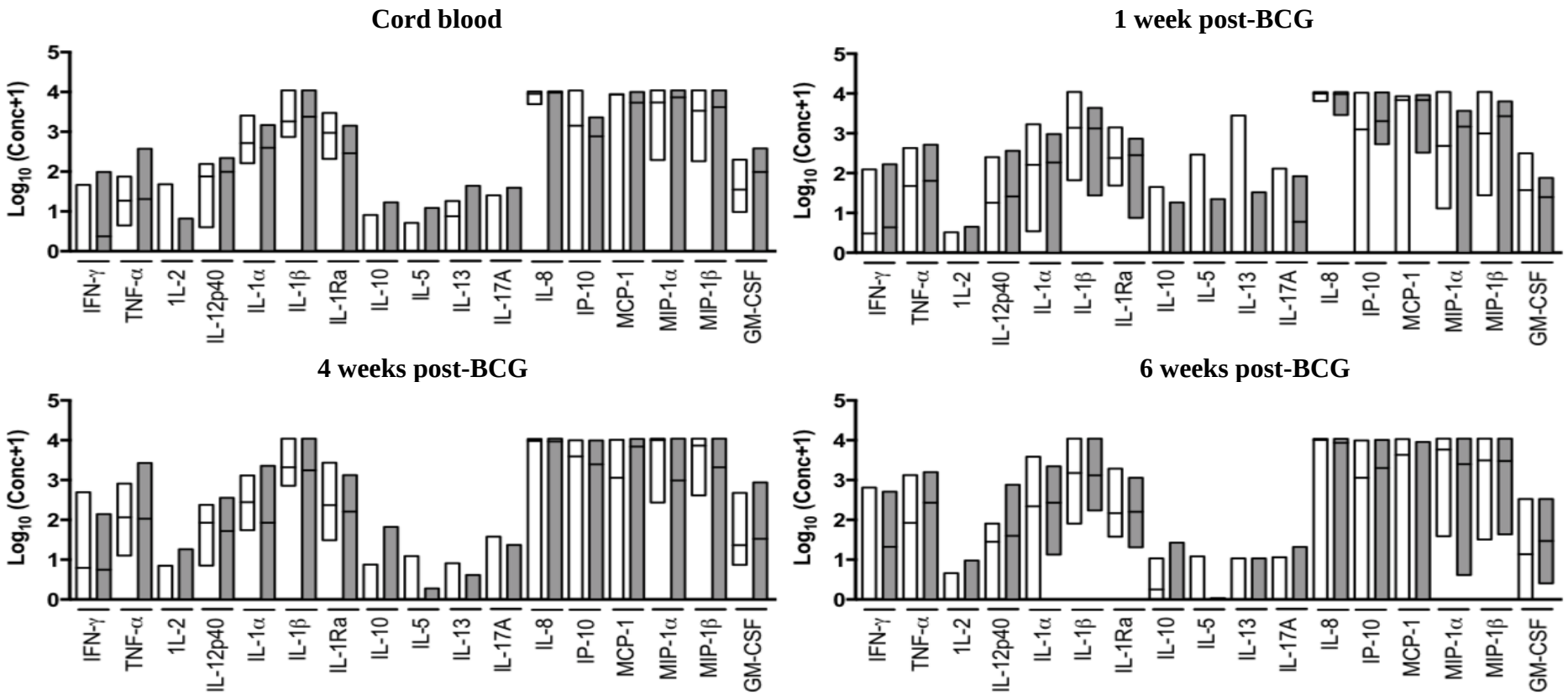

Figure 4.21A. Cytokine and chemokine responses to ESAT-6/CFP-10 measured by Luminex® assay. Clear and grey bars represent infants of mothers without and with LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by *. A Kruskal-Wallis test was used to compare infants in the two groups, with adjustment for multiple comparisons using Dunn's multiple comparisons test). Numbers of infants for LTBI- and LTBI+ groups: 17 versus 10 for cord blood, 7 versus 5 for 1-week, 14 versus 8 for 4-week, 13 versus 11 for 6-week, 21 versus 13 for 10 week, 9 versus 6 for 24-week, and 65 versus 38 for 52-week visits.

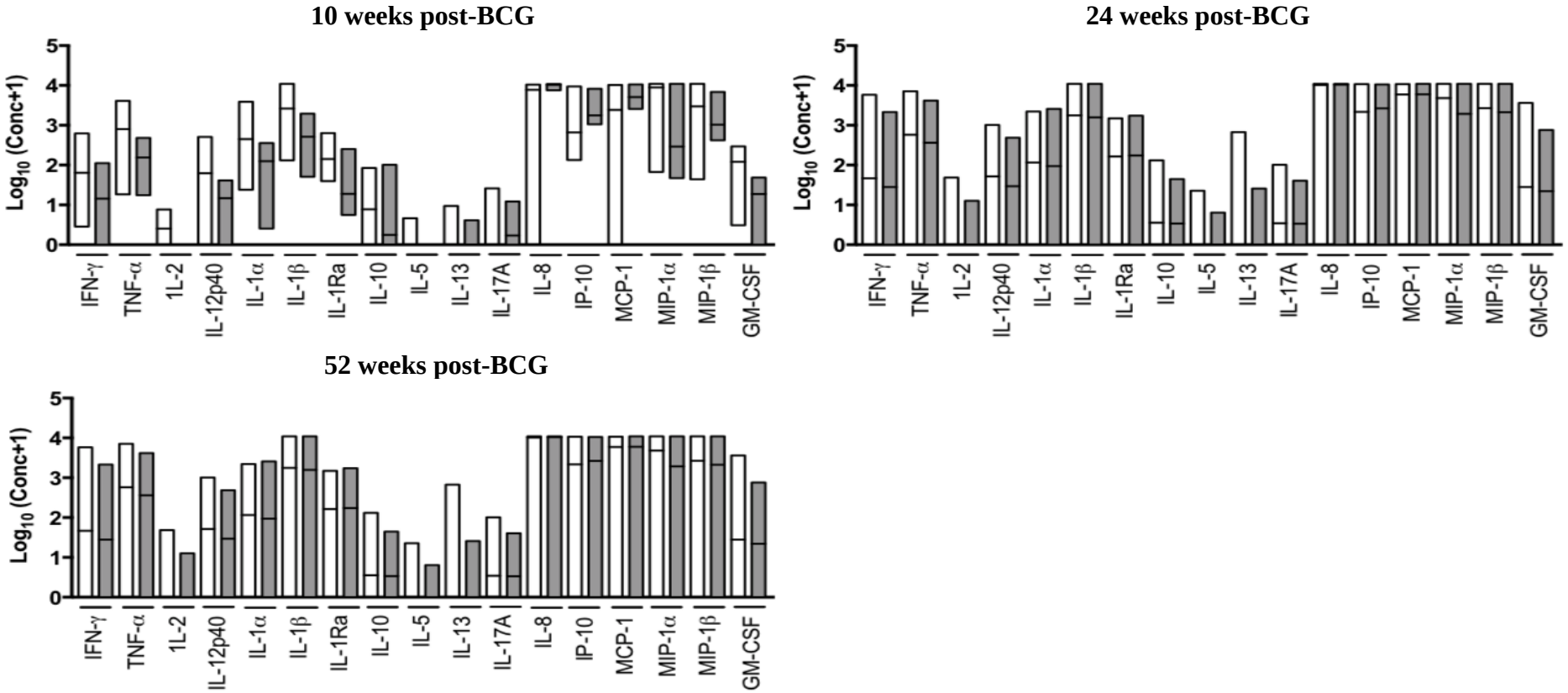

Figure 4.21B. Cytokine and chemokine responses to ESAT-6/CFP-10 measured by Luminex® assay. Clear and grey bars represent infants of mothers without and with LTBI, respectively. The horizontal lines represent the median. Statistically significant differences are shown by *. A Kruskal-Wallis test was used to compare infants in the two groups, with adjustment for multiple comparisons using Dunn's multiple comparisons test). Numbers of infants for LTBI- and LTBI+ groups: 17 versus 10 for cord blood, 7 versus 5 for 1-week, 14 versus 8 for 4-week, 13 versus 11 for 6-week, 21 versus 13 for 10 week, 9 versus 6 for 24-week, and 65 versus 38 for 52-week visit.

4.4. Discussion

As expected, a mycobacteria-specific immune response was detected in BCG immunised infants in both the pilot and the main infant BCG studies. The frequency of IFN-γ-producing CD4+ T cells after PPD stimulation peaked at one week after BCG immunisation in the pilot study and dropped at six weeks, while the proportion of IL-2 and TNF-α-producing CD4+ T cells increased to six weeks. A peak in BCG-induced response was reported at 6-10 weeks post-BCG immunisation by Soares and colleagues (220). The pilot study was limited by a short follow up time of six weeks that did not enable an assessment of peak in response at later time points.

In the pilot infant BCG study, maternal infection with *M. tuberculosis* was associated with lower infant CD4+ T cell responses to PPD at one week after neonatal BCG immunisation, as measured by flow cytometry. It is policy for BCG to be given at birth in most developing countries and LTBI is common in these countries. It is possible that the lower immune responses in early life may result in poor longer-term responses and the subsequent infection with *M.tuberculosis* or progression to active TB disease. It may further call for the treatment of LTBI in women of childbearing age. However, such a short-term effect of maternal LTBI on infant responses does not provide enough immunological evidence to advocate for treatment of LTBI in mothers.
Jones *et al.*, in a recent study showed no difference in BCG-induced responses between infants of mothers with and without *M. tuberculosis* infection at 10 weeks of age (359). The data presented in this thesis (Chapter 4 and published in Transactions of the Royal Society B (326)) differed with that of Jones and colleagues: first, both TST and T-SPOT.TB were used to define maternal LTBI in this study, versus the QuantiFERON-TB Gold test alone in Jones's study. The non-stringent criteria used by Jones and colleagues might have resulted in loss of smaller differences between infants of mothers with and without LTBI. Second, this study used BCG-Russia, versus the Danish strain in Jones' study, and as mentioned above, strain-specific differences in immune responses to BCG immunisation have previously been reported (277, 292). Third, infants in this study were immunised at birth, versus six weeks for Jones *et al*. Early effects of maternal LTBI on infants such as we have observed may have been missed since immune responses were only assessed at 10 weeks in Jones' study. Fourth, a 24-hour cell stimulation assay was used in this study, versus a 6-day WBA by Jones *et al*.

There are also obvious differences in the characteristics of the assays used (intracellular cytokine staining and flow cytometry in this study, versus WBA in the study by Jones and colleagues). Short-term cultures target memory cells, but unfortunately the frequencies of memory T cells are quite low in humans, which makes the responses from short-term cultures quite low. Longer-term cultures likely allow memory T cells to proliferate and expand in frequency, allowing for easier identification. Long term cultures, compared to short-term cultures, do not give an accurate measure of frequency however, but do allow for better detection of antigen-specific responses. The choice of which assays to use should therefore be carefully considered. For the pilot infant BCG study, a short term assay was used for looking at innate responses, and we took the opportunity to examine T cell responses by flow cytometry as an additional measure. In the main infant BCG study, a longer term culture was used to assess adaptive responses. Taken together, it would have been more appropriate to undertake a broader and less biased approach, such as cytometry by time of flight (CyTOF) (360-362), to further explore some of the findings in the pilot infant BCG study.

A limitation of the pilot infant BCG study was the small sample size, and the many outcome measures. Since correction for multiple comparison testing was not done due to small sample size, some of the differences reported as statistically significant could have been by chance. We have therefore taken caution in interpreting the results.

The importance of IFN-γ in immunity to TB has previously been demonstrated (156-158). Although necessary, IFN-γ alone is not sufficient to provide protection against TB (199). It is therefore important to assay other cytokines and chemokines induced by BCG that might show promise as biomarkers of protection against TB. Up to 17 cytokines and chemokines were measured in culture supernatants in response to stimulation of infant blood by mycobacterial antigens in the main infant BCG study.

The median IFN-γ production in the 6-day cultures was higher in PPD-stimulated samples than in ESAT-6/CFP-10-stimulated samples at all time points. PPD is a crude antigen preparation from *M.tuberculosis* which contains mainly degraded protein antigens, most of which are cross-reactive with the antigens in the BCG vaccine, as well as NTM, thus explaining the higher responses. The ESAT-6 and CFP-10 antigens are in

the region of difference- (RD) 1 region that is deleted in the BCG vaccine (363, 364), but expressed in certain NTM species including *M.kansasii*, *M.szulgai*, *M. marinum* and *M. rigadhense* (365, 366). The prevalence of NTM in infants in Uganda is 3.7% (versus 4.6% in adolescents), and *M.szulgai* (one of the ESAT-6/CFP-10-expressing NTM) was among the species recently isolated (367). The lower IFN-γ responses to ESAT-6/CFP-10 than to PPD may indicate reduced exposure to *M. tuberculosis* (or one of the other NTM that express these antigens, such as *M.szulgai*) in this setting. However, the Th1 responses (IFN-γ and TNF-α) to ESAT-6/CFP-10 increased with age, showing the possibility of exposure to *M.tuberculosis* or NTM. It would be important to establish the prevalence of ESAT-6/CFP-10-expressing NTM, such as *M.szulgai*, in this setting as this can interfere with tests that use the RD1 antigens.

The PPD-stimulated cultures also produced more Th2 cytokines (IL-5, IL-13) than ESAT-6/CFP-10-stimulated samples. It is possible that the Th2 responses observed in response to stimulation with PPD may be because of an overall stronger response to the antigen. In a study involving Malawian infants, there was evidence of Th2 responses following BCG immunisation (325). Th2 cytokines were also observed in unvaccinated infants in a study in The Gambia (225). There was good correlation between the Th1 and Th2 cytokines measured.

Some of the chemokines had overall high concentrations above the top standard value. The reagents come ready to use and were all pre-mixed. Ordering the analytes individually and making up the mix would be an alternative option, but this approach may be costly and time consuming. For studies of infant immunology, kits are perhaps not the best to use and testing individual cytokines and diluting where necessary should be advocated for.

For most of the cytokines and chemokines measured, the peak of the response to PPD was around 24 weeks of age, later than the 6-10 week period reported by Soares and colleagues (220). The responses at the early time points were significantly lower than those at later time points, highlighting BCG-induced immune responses and maturation of responses with age. For IFN-γ and TNF-α, the responses to ESAT-6/CFP-10 at the early time points were also different from the responses at the later time points.

It is critical that we know when the peak of the immune response is, as that will inform future prime boost strategies based on BCG prime, as to when the boost should be given. The absence of efficacy against *M.tuberculosis* infection or TB disease after boosting BCG-primed responses with MVA85A vaccine (200) may have resulted from not using the appropriate time point for boosting.
Not all infants peaked at the same time point, highlighting individual differences in infant responses to vaccines. Studies in humans have demonstrated that BCG immunisation of infants induces immunological memory (231, 310). Most studies have shown that BCG immunisation induces CD4+ T cells with effector memory characteristics in both humans (226, 227, 237) and animal models (228, 368). Soares and colleagues previously reported that the memory CD4+ T cells induced by BCG immunisation of infants have a central memory phenotype, but with an effector memory function (220). Data will be available from the main infant BCG study to enable us to analyse the phenotype and function of T cells at the peak of BCG-induced immune responses, but is not part of this body of work.

Concentrations of IFN-γ in the antigen-stimulated cultures correlated with several cytokines and chemokines produced in response to PPD or ESAT-6/CFP-10. This shows that infants in this study are capable of mounting complex mycobacteria-specific immune responses of various strength and direction.

Overall, maternal LTBI was not associated with infant responses. Other factors that remained associated with infant responses after controlling for confounding factors included female infant gender (associated with lower IL-1Ra responses), maternal BCG scar (associated with lower infant IL-10 responses), maternal age (infants of older mothers produced more MIP-1β, but less IL-1α) and parity (infants of multigravid mothers produced more IL-13). These observations demonstrate the importance of looking at other maternal and infant factors when evaluating infant responses to vaccines.

In summary, infant responses peaked around 24 weeks of age and overall, maternal infection with *M.tuberculosis* was not associated with the infant response to BCG immunisation. The poor efficacy of BCG vaccine in the tropics may therefore be as a

result of combination of factors, other than single infections such as *M. tuberculosis* infection.

Chapter 5

Antibody responses in BCG immunised infants, and the influence of maternal LTBI

Having shown the peak of infant response following BCG immunisation and the lack of associations between maternal LTBI and infant T cell responses, the infant IgG response to PPD and the effect of maternal LTBI on these responses were assessed. Although antibody responses are not thought to be central to immunity against intracellular pathogens such as *M.tuberculosis*, there has been recent interest in the B cell and antibody response in TB (164, 165, 369).

5.1. Introduction

Antibodies, produced by plasma cells and plasmablasts, are important in the host defense against infections. Newborns acquire IgG from their mothers through the placenta (370, 371), and these maternally derived antibodies play an important role in protecting the neonates from infections (372, 373). However, these antibodies have been reported to interfere with infant responses to vaccines (374).

Several sero-epidemiological studies aimed at assessing the decay of maternal antibodies in infants have been carried out, but with conflicting results. Maternal antibodies to pertussis (375), respiratory syncytial virus (373) and CMV (376) infections have been shown to decay in less than three months after birth. In a study in Nigeria, the biological half-life of maternal measles virus antibodies was reported to be 33 days (377). Other studies have reported the decay of maternal antibodies to be between 5-12 months (373, 376, 378-386). Differences in the rate of decay of maternal antibodies to dengue virus with the age of the infants has also been reported, where antibodies decayed faster in some infants than in others (379). Antibody decay has also been shown to vary during vaccination and natural infection: maternal antibodies to paramyxovirus infection decayed faster if the mothers were vaccinated (2.4 months) than when the mothers had natural infection (3.8 months) (387).

Maternal antibodies have been shown to impair infant responses to most childhood vaccines (388, 389) and this is thought to result in high mortality and morbidity from vaccine-preventable diseases. However, there are reports of reduced mortality (390) and morbidity (391) when neonates were immunised during the period when maternal antibodies were still high.

The relationship between mycobacteria and the host is thought to be dynamic during LTBI. There may be differences in the concentrations of *M.tuberculosis*-specific antigens and antibodies in people with LTBI than those without. Transplacental transfer of mycobacterial antigens has been demonstrated in animal models (392). Maternal LTBI might therefore lead to *in utero* sensitisation (287), or tolerance (such as reported for maternal helminths) (288, 289) of the fetus. There might also be interference of passively transferred maternal *M.tuberculosis*-specific antibodies with the BCG vaccine.

To my knowledge, the decay in maternally acquired antibodies during *M.tuberculosis* infection, and the influence of maternal LTBI on the antibody responses have not previously been reported. Since there was no effect of maternal LTBI on infant T cell responses observed, it was expected that there would be no effect on antibody responses.

In this chapter, the results of the kinetics of antibody responses in infants and the influence of maternal LTBI on the infant antibody responses are presented.
The specific objectives included:

1. Assessment of the longitudinal changes in infant antibody responses in the pilot infant BCG study and the main infant BCG study.
2. To investigate the effect of maternal LTBI on infant antibody responses.

5.2. Results from the pilot BCG study

5.2.1. Demographic and clinical characteristics of the participants

The characteristics of the mothers and infants included for analysis of humoral responses were the same as for infant T-cell responses in the pilot study described in section 4.2.1. Briefly, compared to those without LTBI, mothers with LTBI were older (mean age 27.5 versus 23 years, p=0.002), less likely to have a BCG scar (47% versus 70%, p =0.001) and less likely to be primigravida (32% versus 48%, p=0.001), and their children were more likely to be of male gender (53% versus 35%, p=0.001) (Table 4.1).

5.2.2. Longitudinal changes in IgG concentrations

Plasma samples obtained from cord blood and from infant samples obtained at one and six weeks after BCG immunisation were assayed for IgG specific for PPD and TT using an ELISA assay. The PPD-specific IgG response was the main outcome of interest. Antibodies to TT were analysed for comparison. At each time point, fifty-four samples were assayed, 38 from infants of mothers without LTBI and 16 from infants of mothers with LTBI. Figure 5.1 illustrates the distribution of concentrations of IgG specific for PPD and TT in the three sample types.

There was a decrease in PPD-specific IgG concentrations at one week after birth (175 ng/ml [0–1100], compared to the concentration in cord blood (median [IQR]: 5600 ng/ml [3300–11050]), p=0.001) and again at six weeks (0.00 ng/ml [0.00–288], p=0.004; Figure 5.1A and Table 5.1).

The concentration of TT-specific IgG, by contrast, was high in cord blood (46750 ng/ml [42000–49950], dropped by half one week after birth (21125 ng/ml [18988–22650] p=0.001) and did not change much between one and six weeks (19550 ng/ml [13750–22038] p=0.252; Figure 5.1B and Table 5.1).

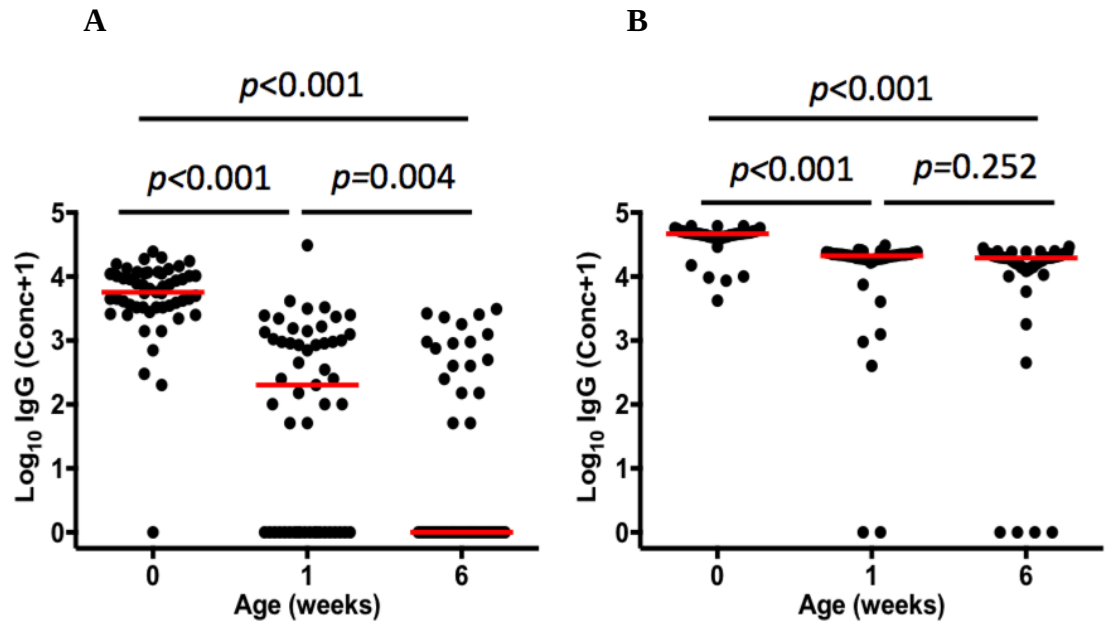

Figure 5.1. Longitudinal changes in PPD- and TT-specific IgG concentrations with age. PPD- (A) and TT-specific (B) responses are shown. Cord blood and infant plasma samples were obtained at one and six weeks after birth. All infants were BCG vaccinated within 24 hours after birth. Tetanus vaccination was given at 6 weeks after birth. Concentrations of IgG in log10 (concentration +1) were measured by ELISA. Each symbol represents an individual infant antibody response. For each plot, the horizontal line represents the median differences in responses between the visits were analysed using the Mann–Whitney test; n=54 for all three time points.

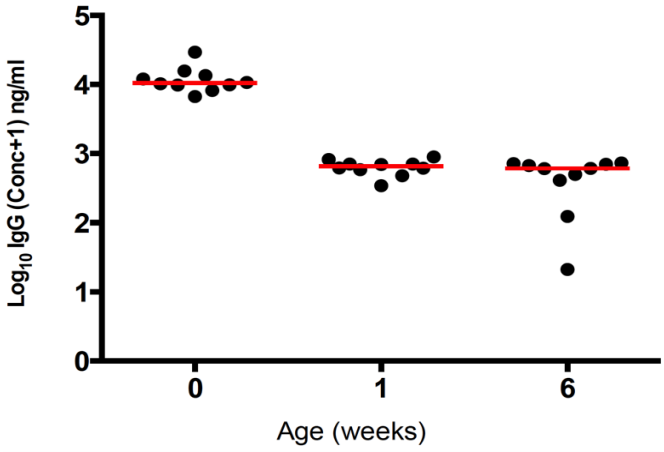

Figure 5.2. Longitudinal changes in PPD-specific IgG concentrations with age. Showing pilot infant BCG study samples re-tested using PPD from the main infant BCG study. n=10 for all three time points.

Table 5.1. IgG responses to PPD and TT in infancy.

Sample/visit	**Number of observations**	**Median responses to PPD**	**IQR**	**Median responses to TT**	**IQR**
Cord blood	54	5600	3300-11050	46750	42000-49950
1 week	54	175	0-1100	21125	18988-22650
6 weeks	54	0	0-288	19550	13750-22038

Antibodies were measured by ELISA. The values are shown as medians in ng/ml with the interquartile range (IQR).

5.2.3. Impact of maternal infection with *M. tuberculosis* on PPD-specific immune responses in infancy.

Having shown the longitudinal changes in infant antibody responses with age, the influence of maternal LTBI on these responses was analysed. This is important because if there was a difference in infant responses by maternal LTBI, then this may contribute to the poor efficacy of BCG in this setting or it may call for the treatment of pregnant women with LTBI.

There were no differences in PPD-specific IgG concentrations between the infants of mothers with and without LTB, at any of the time points (Figure 5.3).

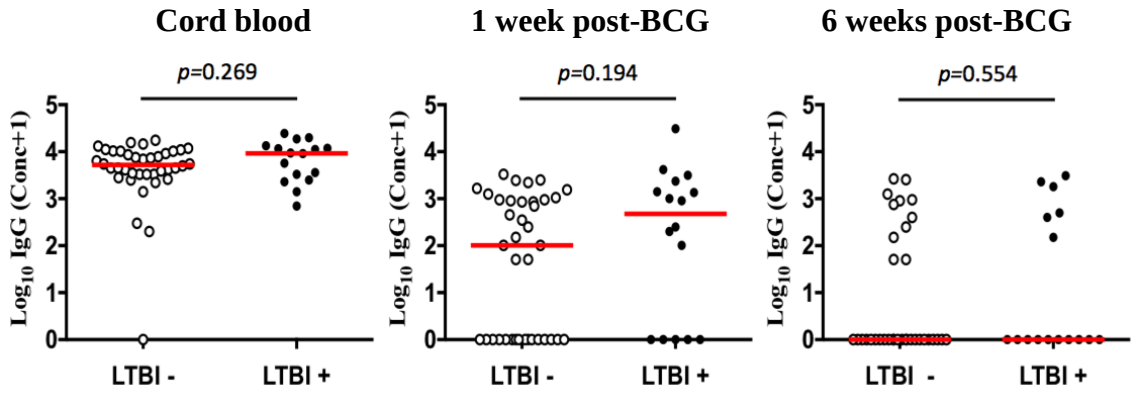

Figure 5.3. The impact of maternal infection with *M. tuberculosis* on the infant antibody responses. Cord blood was sampled and infant plasma samples were obtained at one and six weeks after birth, and compared between infants of mothers without and with LTBI; IgG concentrations were measured by ELISA. Each symbol represents an individual infant antibody response. For each plot, the horizontal line represents the median infant responses. Differences in responses between LTBI exposed and unexposed infants were analysed using the Mann–Whitney test. n=38 for infants of mothers without LTBI (open circles) and n=16 for infants of mothers with LTBI (closed circles).

5.3. Results from the main infant BCG study

5.3.1. Longitudinal changes in IgG concentrations

Having shown the kinetics of IgG responses in the pilot infant BCG study, responses in the main infant BCG study were examined.

Plasma samples obtained from cord blood (n=222) and infant samples collected at 1 (n=92), 4 (n=91), 6 (n=98, 10 (n=103), 14 (n=36), 24 (n=36) and 52 (n=102) weeks after BCG immunisation were analysed for IgG specific for PPD and TT using ELISA, with IgG antibodies to PPD as the main outcome. The distribution of concentrations of IgG specific for PPD and TT by age is illustrated in Figures 5.4A and 5.4B, respectively. Compared to the concentration in cord blood (median [IQR]: 8200 ng/ml [4000–12450]), PPD-specific IgG concentrations decreased at 1 week after birth (389.5 ng/ml [279.25-611.5], $p<0.0001$) and then remained stable up to 52 weeks (291 ng/ml [156–365.25], Figure 5.4A, Table 5.2).

By contrast, the concentration of TT-specific IgG was high in cord blood (618000 ng/ml [417000–808500], dropped less dramatically by 1 week after birth (396000 ng/ml [163000–590000] $p<0.0001$) and showed little change between 1 and 24 weeks (230000 ng/ml [126500-354500], but dropped further at 52 weeks (63000 ng/ml [20000-147000], Figure 5.4B, Table 5.2).

The median PPD-and TT-specific antibody concentrations are shown in Figures 5.4C and 5.4D, respectively. There is a slight increase in IgG responses at 24 weeks of age, probably as a result of the DPT-HepB-Hib vaccine given at 6, 10, and 14 weeks after birth (shown by arrows).

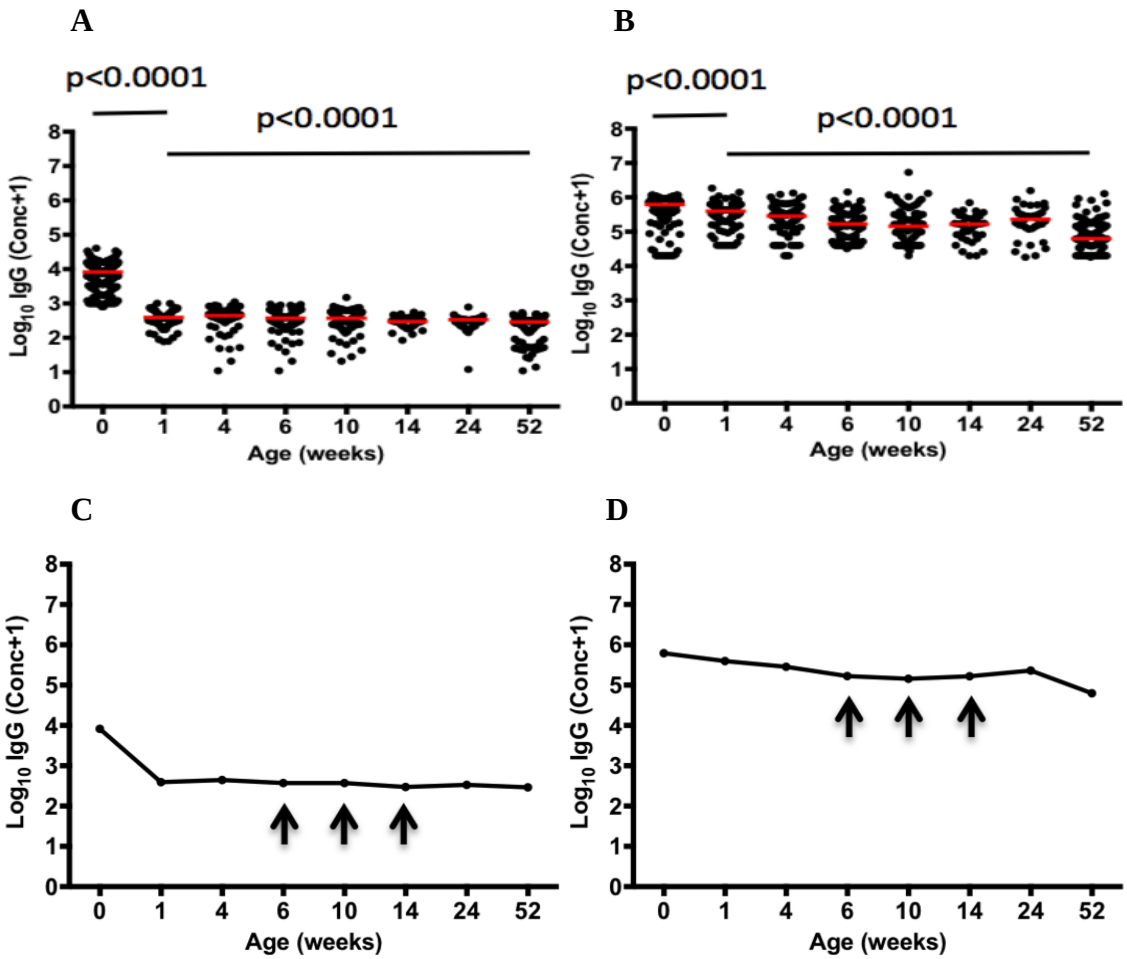

Figure 5.4. Longitudinal changes in PPD-and TT-specific IgG concentrations. Cord blood and infant plasma samples obtained at 1, 4, 6, 10, 14, 24 and 52 weeks after BCG immunisation were tested for IgG to PPD (A) and TT (B) by ELISA. Each symbol represents an individual infant antibody response. For each plot, the horizontal line represents the median (further illustrated for PPD-(C) and TT-specific (D) responses); n=222 for cord blood, n=92 for 1 week, n=91 for 4 weeks, n=98 for 6 weeks, n=103 for 10 weeks, n=36 for 14 weeks, n=36 for 24 weeks and n=102 for 52 weeks time points. Arrows illustrate the timing of DPT vaccination in infants.

Table 5.2. IgG responses to PPD and TT in infancy.

Samples/Visit	**Number of observations**	**Median response to PPD**	**IQR**	**Median response to TT**	**IQR**
Cord blood	222	8,200	4000-12450	618000	417000-808500
1 week	92	3,89.5	279.25-611.5	396000	163000-590000
4 weeks	91	441	328-640	284000	136000-452000
6 weeks	98	372.5	239.25-591	168000	52000-328000
10 weeks	103	370	250-542	144000	40000-318000
14 weeks	36	297	215.25-393	166000	70000-265000
24 weeks	36	335.5	245.25-364.5	230000	126500-354500
52 weeks	102	291	156-365.25	63000	20000-147000

The values are shown as medians in ng/ml with the interquartile range (IQR).

5.3.2. The impact of maternal LTBI on PPD-specific IgG responses in infancy.

Having shown the longitudinal changes in infant antibody responses with age, the influence of maternal LTBI on these responses was examined. This is important because if there was a difference in infant responses by maternal LTBI, then this may contribute to the poor efficacy of BCG in this setting and argue for the treatment of LTBI in pregnant mothers.

The impact of maternal LTBI on the infant IgG responses was assessed in **c**ord blood, and in plasma samples obtained at 1, 4, 6, 10, 14, 24 and 52 weeks after birth, by comparing responses in infants of mothers with and without LTBI. This is illustrated in Figure 5.5. As with the pilot infant BCG study data, and in agreement with the results presented in Chapter 4, there were no differences in PPD-specific IgG concentrations between infants of mothers with and without LTBI at the different time points.

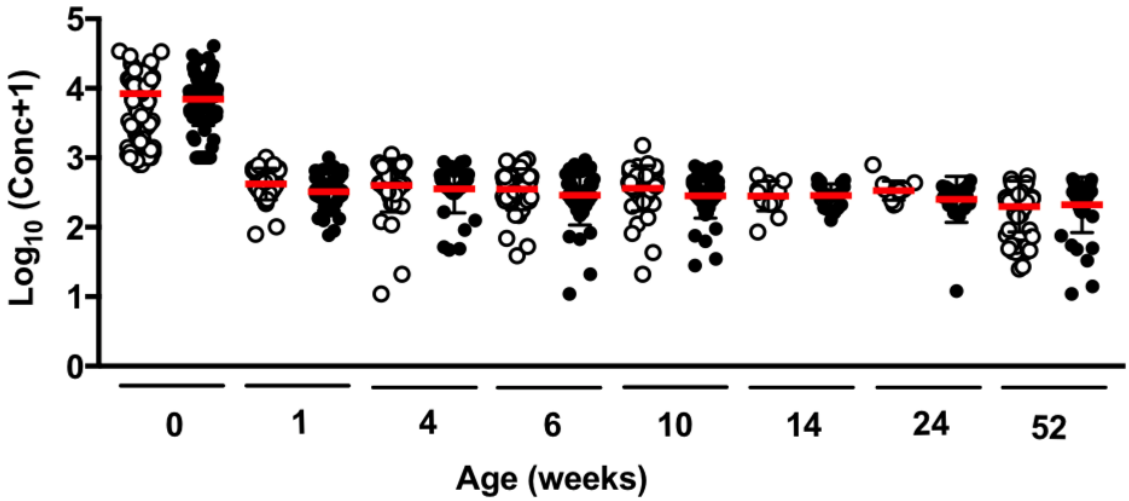

Figure 5.5. The impact of maternal infection with *M. tuberculosis* on the infant antibody responses. IgG concentrations were measured in cord blood, and in infant plasma samples obtained at 1, 4, 6, 10, 14, 24 and 52 weeks post-BCG by ELISA. Each symbol represents an individual antibody response. The horizontal line represents the median; the number of participants for LTBI-negative versus LTBI-positive at each time point were: 122 versus 100 for cord blood, 50 versus 42 for 1 week, 51 versus 40 for 4 weeks, 56 versus 42 for 6 weeks, 55 versus 48 for 10 weeks, 15 versus 21 for 14 weeks, 15 versus 21 for 24 weeks and 60 versus 42 for 52 weeks. Multiple comparisons of infants of mothers without (open circles) and with (closed circles) LTBI were performed using Kruskal-Wallis test.

5.4. Discussion

There have been relatively few studies of antibodies in TB other than attempts to develop antibody-based diagnostic tests. However, maternal IgG antibodies can cross the placenta, and could influence the response of the infant to BCG immunisation. The concentration of maternally derived mycobacteria-specific antibodies dropped rapidly in the first week of life. This is the first time such a finding has been reported. For the pilot infant BCG study, the concentration of antibodies dropped further by six weeks of life, whereas in the main infant BCG study, antibody concentrations remained stable up to age one year.

By contrast, the concentration of TT-specific IgG was high in cord blood, dropped less dramatically by one week after birth and changed little between one week and six weeks (pilot infant BCG study), and between one week and age one year (the main infant BCG study).

In human studies, variations in the decay of maternally derived IgG antibodies have been reported, with some studies showing a shorter (373, 375-377) and others longer (373, 376, 378-380) clearance time. However, this has further been shown to depend on whether the antibodies are elicited as a result of immunisation of the mothers or due to natural infection (387). These two studies differed in important aspects: first, different lots of PPD were used in the two studies. Second, different BCG vaccine strains were used (BCG-Russia for the pilot infant BCG study, versus Danish strain for the main infant BCG study). Strain-specific differences in infant T cell responses have previously been reported (277, 292), but no one has looked at the antibody responses. Third, the pilot infant BCG study was conducted during the months of February to May (considered to be a wet season in Uganda), whereas the recruitment for the main infant BCG study was spread over a long period of time (covering both wet and dry seasons). The season of birth has been reported to influence T cell infant responses (393, 394). It is possible that there was a birth season effect on infant antibody responses to vaccines, though a study in the Gambia did not observe that (395). There is a plan to repeat these assays when samples are collected from the infants at all the time points using a new batch of PPD, as well as looking at responses to other mycobacterial antigens such as ESAT-6/CFP-10 and Ag85A. BCG immunisation has been reported to induce long-

lived humoral responses in infants in this setting (174). It was a missed opportunity that we only measured total IgG responses instead of IgM responses, which would have indicated new antibodies produced by the infants.

The rapid drop in infant antibody concentrations at one week was therefore unexpected. It is possible that antibodies to PPD bound to BCG vaccine or mycobacterial antigens, thus reducing their concentration in circulation. This may have an effect on the efficacy of BCG vaccine. Immune complexes formed between maternally derived antibodies and vaccine antigens (where the live-attenuated vaccines may be neutralized) or antigens from mycobacteria may be taken up by phagocytic cells or deposited in the tissues where they can initiate inflammatory responses or are presented to CD8+ T cells in the context of MHC class I molecules, inducing cell mediated responses (396). Antibodies may also block specific antigen sites (397-400) or change the stability of the antigens (401, 402). This can influence the processing and presentation of antigens and subsequent T cell responses. Other mechanisms by which maternal antibodies interfere with infant responses to vaccines include their effect on B cell responses by binding to FcγRIIB on neonatal B cells, masking of B cell epitopes thus inhibiting responses. Samples collected from a randomized comparison of delayed versus immediate BCG immunisation (352) would present an opportunity to assess the association between infant BCG immunisation and decline of maternally derived antibodies. The rapid drop in antibody responses to PPD at six weeks after BCG immunisation in the pilot infant BCG study, which was not observed in the main infant BCG study, could be a real phenomenon since different strains of BCG vaccine were used in the two studies (BCG Russia in the pilot infant BCG study, versus BCG Danish in the main infant BCG study). It is also possible that this was an assay-related difference since different batches of PPD antigen were used in the two studies. The PPD batches for the two studies were obtained from SSI. Re-testing antibodies in samples from the pilot infant BCG study using PPD from the main infant BCG study did not show the drop in antibodies observed at six week after BCG immunisation (Figure 5.2), indicating that the drop in antibody concentration at six weeks in the pilot infant BCG study is partly related to the batch of PPD used. PPD is a late stage treated culture supernatant from *M.tuberculosis*. It therefore has a lot of secreted antigens. However, it is very degraded and has many small peptides that may not bind well to the ELISA plate.

Maternally derived TT-specific antibodies, by contrast, dropped less dramatically by first week of life and remained stable over time in both the pilot and the main infant BCG study. However, in the main infant BCG study, there was no boost in TT-sspecific responses following priming. This may be as a result of inhibitory effects of maternally derived anti-TT antibodies (403, 404) or maternal antibodies are masking the generation of antibodies in infants. However, other studies have shown that infant responses to DPT are not affected by maternally derived antibodies (405, 406). This agrees well with the data presented here. Low potency and poor vaccine handling have also been reported by as possible cause of lack of boost in TT-specific responses (407). This is unlikely to be the cause in our studies, as the vaccines were handled and stored according to good clinical practice (GCP). Although the responses to TT were high they were within the measurable range for the assay as illustrated for standard curves (A, for pilot infant BCG study and B, for main infant BCG study) and sample IgG concentrations (C, for pilot infant BCG study and D, for main infant BCG study) in Figure 2.10. The same purified IgG standard (from GenScript, NJ, USA) was used in ELISAs for both studies. Also, samples from the same infant collected at different time points were run on the same plate. There could have been a boost in response after vaccination at 6, 10 and 14 weeks, but this could have been masked by the overall high combined maternally derived and infant antibody concentrations as illustrated by the hypothetical Figure 5.6. It was therefore difficult to separate infant antibody responses from maternally derived antibodies. Measurement of IgM, which would have been infant derived, would be recommended for future studies.

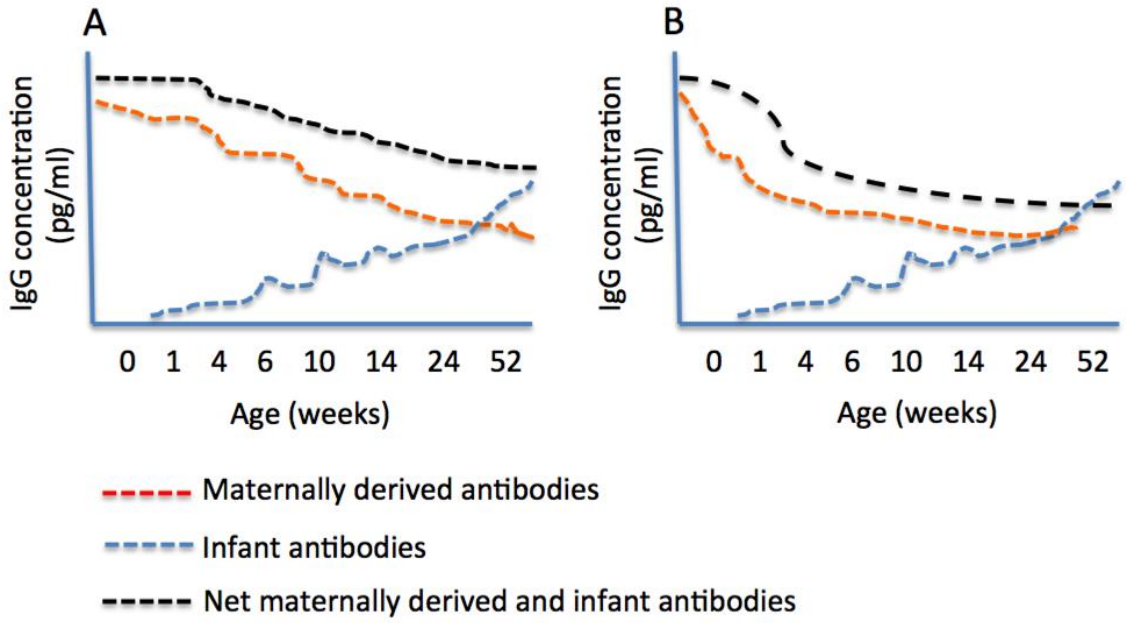

Figure 5.6. Possible dynamics of maternally derived and infant TT antibody concentrations. Maternally derived antibody decay is shown in red, infant antibody production shown in blue and combined maternal and infant antibody concentrations are shown in black. A, shows a situation where maternally derived antibodies decay slowly and B, is where maternally derived antibodies decay rapidly. The overall effect is high antibody responses to TT.

There were some infants who did not generate antibody responses to PPD or TT at some time points in pilot infant BCG study. This could be due to the different strains of BCG vaccine used (BCG Russia in the piot infant BCG study, versus Danish strain in the main infant BCG study).

There were no differences in PPD-specific IgG concentrations between the infants of mothers with and without LTBI at any of the time points.

In conclusion, IgG response to PPD in the infants in these studies waned rapidly in the first week, but there were no associations with maternal latent *M.tuberculosis* infection. The data from the pilot infant BCG study supports waning of maternally derived antibodies in the first week of life. Maternally derived TT antibodies remained stable over time and there was no effect of booster DPT dose on infant responses.

Chapter 6

General discussion

6.1. The hypothesis and major findings

It was hypothesized that maternal latent infection with *M.tuberculosis* would influence the infant response to BCG immunisation. To test this, pregnant women were tested for LTBI and their infants followed up to one year after BCG immunisation. ELISA, Luminex®, intracellular cytokine staining by flow cytometry and gene expression microarray were used to analyse the maternal, cord blood and infant samples collected.

6.1.1. The infant BCG studies

Two studies were conducted: the pilot infant BCG study and the main infant BCG study. These studies differed in important respects: first, the number of study participants and follow up period varied (smaller sample size and short follow up time for the pilot infant BCG study, versus larger sample size and longer follow up of up for the main infant BCG study. In the pilot infant BCG study, cord blood and infant samples were obtained at one and six weeks, whereas in the main infant BCG study, cord blood and infant samples were collected at 1, 4, 6, 10, 14, 24 and 52 weeks after BCG immunisation. Second, different BCG vaccine strains were used (BCG-Russia for the pilot infant BCG study, versus BCG-Danish strain for the main infant BCG study). T cell responses and gene expression profiles have been reported to vary with BCG strain (277, 292, 408). Third, different immunological techniques were used for analysis of T cell responses in the two studies (intracellular cytokine staining by flow cytometry for the pilot infant BCG study, versus WBA and Luminex® for the main infant BCG study). Fourth, mononuclear cells were stimulated for 24 hours in the pilot infant BCG study, versus 6-day stimulation of whole blood in the main infant BCG study.
The objectives of the two studies were also slightly different. The pilot study was aimed at determining immediate cytokine secreting ability of cells (so innate cells such as monocytes and ILCs, effector T cells and perhaps a few effector memory cells), whereas the main infant BCG study was aimed specifically at measuring memory T cell responses. So, the length of the assays measured slightly different cell subsets and this might account for any real differences observed.

6.1.2. Infant innate responses

Innate responses in the pilot infant BCG study were tested by culturing whole maternal and cord blood samples with a number of stimuli that would activate TLR1/2, TLR2/6, TLR4, TLR7/8, TLR9, DC-SIGN and Dectin-1. The concentration of cytokines and chemokines in stimulated samples were moderately high in maternal blood and in cord blood. High concentrations of certain cytokines and chemokines have been reported in neonates and adults (337). *In utero* sensitisation to maternal infections has been shown to prime responses in the infants (287), a possible reason for similar responses observed in maternal and cord blood samples. Common genetic factors between the mothers and their neonates may also be responsible for the similarities in innate responses observed. Increased acute phase responses as a result of the normal delivery process has also been reported to increase responses in the neonates (334). So the birth process may have contributed to the similar responses in maternal and cord blood samples since none of the infants was born by caesarian section.

6.1.3. Impact of maternal factors on innate responses

Maternal LTBI was associated with higher IP-10 responses in the cord blood. IP-10 is a proinflammatory chemokine responsible for the trafficking of immune cells, including leucocytes, and with a role in apoptosis, cell growth and proliferation (409). Human and murine studies have shown increased Th1 responses in an IP-10 environment (181, 410). IP-10 has also been shown to have a potential as a diagnostic marker for TB (411-414). Further assessment of the role of IP-10 in immunity in infants is needed.

There was a positive association between maternal BCG scar and cytokine responses in cord blood. This may be due to common genetic factors between the infants and their mothers that determine scar formation and subsequent infant responses, or that the factors associated with scar formation in the mothers are passed on to the infants. The differences in infant response may also relate either to the mother's BCG immunisation status or to the quality of mothers' BCG-induced responses.

6.1.4. Maternal factors and gene expression profiles in infants

Maternal LTBI was associated with down-regulated interferon and inflammatory response pathways at one week after BCG immunisation. Genes associated with the mitogen-activated protein kinase (MAPK) signaling pathway, such as JUNB (Jun B proto-oncogene), FOS (Fos proto-oncogene) and FOSB (FBJ murine osteosarcoma viral

oncogene homolog B), were also down-regulated one week after BCG immunisation. The MAPK signaling pathway is important in the regulation of innate immune responses as well as in the induction of inflammatory responses (415). Maternal LTBI was associated with up-regulated interferon and inflammatory response pathways at six weeks after BCG immunisation. It was thought that the down-regulated gene expression profile observed at one week would be maintained for some time, so the change in direction after six weeks was unexpected. These observations support the hypothesis that *in utero* exposure to maternal *M.tuberculosis* influences the infant innate response to BCG, but only shortly after birth. A larger study is needed to confirm these findings. BCG immunisation has been reported to influence infant gene expression profiles (416, 417) and different strains of BCG have been shown to induce different gene expression profiles in infants (408). A comparison of gene expression profiles in infants who received BCG-Russia in the pilot BCG study with the infants in the main infant BCG study who were given BCG-Danish would show if there are any differences in responses to different strains of BCG. This is now being done with our collaborators at the Universsity of Oxford.

The expression of genes in the interferon and inflammation response pathways was increased in infants of mothers with a BCG scar at one and six weeks after BCG immunisation. DNA replication and cell cycle progression pathways were also up-regulated in infants of mothers with a BCG scar at one week. These observations highlight the importance of assessing the influence of maternal immunisations on infant responses to vaccines as they show associations with important pathways in infants.

6.1.5. The peak of BCG-induced infant T cell responses

In the pilot infant BCG study, the frequency of CD4+ T cells expressing Th1 cytokines, combined, after stimulation with PPD peaked at one week after BCG immunisation. This might be expected if the focus was on innate responses. For the main infant BCG study, the peak of the response to PPD measured by Luminex® was around 24 weeks of age, later than the 6-10 week period reported by Soares and colleagues (220). Th1 responses to ESAT-6/CFP-10 increased with age, showing the possibility of exposure to *M.tuberculosis* or NTM. These are two new findings.

As discussed in section 6.1.1 above, the differences between the pilot infant BCG study and the main infant BCG study might have resulted in the differences observed.

Together, these two studies add to the knowledge on peak in response to BCG immunisation. The 24-hour stimulation of PBMCs gave a peak response at one week after BCG immunisation, whereas for 6-day stimulation of whole blood, the peak in response was around 24 weeks after BCG immunisation. A study in mice showed that response to PPD in the spleen (examined using *ex vivo* ELISPOT) peaked at 12-32 weeks after BCG immunisation (418).

6.1.6. Maternal factors and infant T cell responses

In the pilot infant BCG study, maternal infection with *M. tuberculosis* was associated with impaired infant CD4+ T cell responses one week after neonatal BCG immunisation when responses were assessed using intracellular cytokine staining and flow cytometry after a 24-hour stimulation period. Jones *et al.* in a recent study showed no difference in BCG-induced responses after 6-day stimulation, measured by intracellular cytokine staining and flow cytometry and Luminex® assay, between infants of mothers with and without *M. tuberculosis* infection (359). As discussed in Chapter 4.4, there were important differences between Jones' study and the pilot infant BCG study. First, the methods used for screening for maternal LTBI were different (both TST and T-SPOT.TB in this study, versus the less stringent QuantiFERON-TB Gold test alone in Jones's study). Second, the strains of BCG vaccine used were different (BCG-Russia in the pilot infant BCG study, versus the Danish strain in Jones' study). Third, the infants in my studies were immunised at birth, versus six weeks for Jones *et al*. Fourth, a 24-hour cell stimulation assay was used in this study, versus a 6-day WBA by Jones *et al*. In the main infant BCG study using 6-day cultures and Luminex® assays, overall, maternal LTBI was not associated with infant responses in agreement with the Jones study**.** These observations show that responses early in life may not reflect what happens later in life, and that it is important to carefully design studies aimed at evaluating infant responses to vaccines as different experiments may give different results.

6.1.7. Maternally derived antibodies and BCG

Maternally derived antibodies dropped rapidly in the first week of life. It is possible that the antibodies to PPD may have bound to the BCG vaccine or mycobacterial antigens derived from it, thus forming an immune complex that has the potential to either reduce or increase responses to childhood vaccines. Neutralization of live-attenuated vaccine

by maternally derived antibodies can reduce the effectiveness of vaccines or protective responses may be elicited when the immune complexes are taken up, processed and presented to T cells, such as reported for CD8+ T cells (396). This may explain why BCG vaccine has good efficacy against disseminated TB in childhood, but poor efficacy in adolescents and adults when maternally derived antibodies have waned. Maternal LTBI was not associated with infant antibody response to PPD.

6.2. Characteristics of assays used

There are important differences in the characteristics of the assays employed in the pilot infant BCG study and the main infant BCG study. These assays and the results obtained using them are illustrated in Table 6.1. As discussed in Chapter 4.4, it is important to carefully select which assays to use in studies like these since these may reflect different types of immune responses and give different results (419).

Table 6.1. Associations between maternal LTBI and maternal BCG scar and results obtained using the different immunological techniques

Assays performed	Longitudinal changes in infant responses	Association between maternal LTBI and infant responses	Association between maternal BCG scar and infant responses	Comments
ELISA	Concentration of antibodies to PPD dropped rapidly in the first week of life	No effect on antibody concentrations	Effect of maternal BCG scar was not assessed	Antibodies measured in plasma
Flow cytometry	Responses peaked at 1 week after BCG	Responses impaired at 1 week after BCG	No associations observed	Short-term (24 hour) PBMC cultures
Luminex® for innate responses	Only measured in cord blood samples	No associations observed	Increased pro-inflammatory responses	Short-term (24 hour) whole blood cultures
Luminex® for adaptive responses	Responses peaked around 24 weeks after BCG	No associations	No association	Long-term (6-day) whole blood cultures
Gene expression microarray	Only measured at 1 and 6 weeks after BCG immunisation	Interferon and inflammatory pathways down-regulated at 1 week, but up-regulated at 6 weeks after BCG	Interferon and inflammatory pathways up-regulated at 1 week and 6 weeks after BCG	Unstimulated whole blood samples were used

6.3. Limitations of the studies

The pilot study was limited in that it was observational and exploratory in nature, and the sample size was small relative to the many outcomes assessed. The small sample size made adjustment for confounding factors difficult. As a result, only major exposures (maternal LTBI and maternal BCG scar) were adjusted for. Also, some important later time points were missed in the pilot infant BCG study. The assessment of innate immune responses was cross-sectional and only performed on cord blood. There were therefore no comparison time points after BCG immunisation, making examination of differences between responses pre-and post-BCG immunisation difficult. For infant gene expression profiles in the pilot infant BCG study, there was no comparison group pre-BCG immunisation. The only data available was for responses at one and six weeks after BCG immunisation. Whole blood was used for assessing the gene expression profile, thus the cellular source of genes or pathways was not known. Further analysis using programmes that look at functional dynamics in cells, based on gene expression profiles, would have shed more light on this (420).

Up to 140 infants who had completed follow up to one year were considered for analysis of immune responses in the main infant BCG study. This number was determined by funds available for the reagents and the time frame within which the laboratory work had to be completed. The selection of infants was therefore not randomly done, highlighting the possibility of bias in the analysis. However, the samples were randomized for analysis of responses by Luminex®.

Follow up of the infants in the main infant BCG study has just been completed. Cleaning of clinical, laboratory and socio-demographic characteristics of the participants is underway. As a result, some of the factors thought to be possible confounders, such as maternal helminth infection, have not been adjusted for in the results presented here but it is hoped they will be available by the time of *viva voce* examination. An analysis of results for all the infants is planned (although not as part of my PhD work).

6.4. Implications of the studies

The increased innate responses in cord blood samples of infants of mothers with a BCG scar, compared to those without, has implications for responses to vaccines administered in childhood. The priming of the innate infant response might result in a better adaptive response, and this has important implications on vaccines and their schedule (267, 421). Although no differences were seen for response to BCG in the long term, the use of other vaccine preparations, such as subunit vaccines might show differences. Perhaps the nature of the live BCG gives enough innate signaling to override the subtle effects which may be seen early on. Examination of such an effect using OPV, versus inactivated polio vaccine (IPV) in infants may shed more light.

The main infant BCG study has provided evidence that the infant response to BCG immunisation peaks around 24 weeks after BCG. This is important since little is actually known about the peak of responses following BCG, yet this knowledge is required for vaccines that use a prime-boost strategy. It was expected that there would be differences in peak of response after 24-hour (at one week) and 6-day stimulations (at 24 weeks) as there are important differences between responses in short and long term cultures, and as the two infant studies used different strains of BCG. Several studies, including those that used viral infection models, have demonstrated that memory populations established after the peak effector phase are suitable to boost (220, 252-255, 422, 423). Based on my data, it is possible that the failure in MVA85A vaccine to provide protection might be because of boosting responses too early (200). Based on the observation in the main infant BCG study, it would therefore be recommended that boosting of responses primed by BCG should be performed after the peak in response because the size of the pool of cells recruited into the primary immune response directly affects the resulting pool of memory T-cells capable of responding to subsequent infection (356). A lower recruitment of cells into the priming phase may affect the BCG-specific immunological memory set point. Since correlates of protective immunity to TB are not known, more understanding of what immune responses should be boosted is required.

Delaying boosting to a later time point, for example at 52 weeks, would be optimal, but this introduces the challenges of exposure to NTM (424) and *M.tuberculosis* (425), with

subsequent establishment of LTBI, as reported in section 4.3.3. These results therefore highlight the need to properly optimize the use of vaccines designed for a prime-boost strategy in areas with a high prevalence of NTM and *M.tuberculosis*.

The studies presented in this thesis have shown associations between maternal factors and infant responses. The positive association between maternal BCG scar and increased infant responses is an important observation. Infant BCG scar has been associated with innate training and subsequent heterologous effects in infants (260-269), although a recent study by Kjaergaard and colleagues did not observe nonspecific beneficial effects of BCG vaccination against childhood infection in a high-income setting (271). A recent systematic review of the available literature did not show sufficient evidence of non-specific immunological effects of BCG immunisation (272). The finding that infants of mothers with a BCG scar, compared to those without, had increased innate responses highlights the importance of further understanding host factors such as genetics (or epigenetics) that may be common between the mothers and their infants, and how maternal immunisations and the resulting immune responses influence responses in the newborns.

The finding in the pilot infant BCG study that maternal LTBI was associated with impaired infant T cell responses at one week of life following BCG immunisation might call for the treatment of women with LTBI in pregnancy or those intending to conceive. Tuberculosis screening of women during pregnancy when attending antenatal clinics would identify those latently infected who could benefit from treatment. The first-line drugs for the treatment of TB (with exception of streptomycin which is ototoxic to the fetus) have been shown to be safe for use during pregnancy (426-428), but little is known about treatment-associated complications during pregnancy, including isoniazid-induced hepatitis (426, 429). Another important factor to consider is the cost of treatment, which is US$40.0 per person for the 6-month course (1). However, the later time points did not show an effect of maternal LTBI on infant responses. Again, preliminary analysis of flow cytometry data (not as part of this PhD work) from the main infant BCG study seems to support the results from the Luminex® assays in that no differences were observed between infants of mothers with and without LTBI. It may therefore be difficult to advocate for treating mothers with LTBI based on the short

term effect at one week against a lack of effect at later time points. The pilot infant study had a small sample size, used a different BCG strain and different technique from the main infant BCG study and the responses may therefore have had a stronger effect. There are reports that after a series of passages, the potency of BCG-SSI has changed over time (430, 431), and high responses to PPD and ESAT-6/CFP-10 were observed in the infants in the main infant BCG study. A much larger follow up study would be needed to examine TB cases in the infants of mothers with or without LTBI and those with and without a BCG scar. Very few infants have developed TB (12 with probable TB disease) in the CiSP cohort of about 1474 infants (323), so such a study would need a considerably larger sample size.

The finding that maternally derived antibodies, particularly to PPD, in the infants decayed rapidly in the first week of life needs further understanding, as this may be one of the mechanisms through which BCG exerts its protective effects in childhood or lack of it in adolescent and adult populations.

The results finally highlight the importance of carefully considering assays for use in studies, as different results were observed with the different assays employed.

6.5. Future perspectives

Studies relating the increased infant innate responses observed in the pilot infant BCG study to adaptive responses would give a complete picture of responses following BCG immunisation. The innate and adaptive responses are closely linked and there may also be innate effects from cytokines produced in the adaptive response. Immunological memory within the innate immune system has been a hot topic for discussion recently. It is therefore important that both arms of the immune response are analysed in future studies.

Further investigations of the decay in antibody responses soon after birth, and the mechanisms involved would be important. If the antibodies bound to BCG vaccine as is suggested, it is also important to examine the type of immune responses elicited by the immune complexes formed. This has implications for vaccine development and for the efficacy of BCG vaccination later in life.

If pregnant mothers with LTBI were to be treated, this would offer opportunities for studies on the effect of maternal LTBI treatment on immune responses in their infants.

The main infant BCG study offers opportunities to relate the peak in response following BCG to the phenotype and function of cells involved. Flow cytometry results are available to be analysed for this objective. Samples are also available to perform gene expression microarray for longitudinal responses, including cord blood that was missed in the pilot infant BCG study.

In conclusion, there was evidence of an influence of maternal LTBI on infant responses in the pilot infant BCG study, but not in the main infant BCG study. There is still a need to understand better what BCG does, in order to design better TB vaccines, but also to carry out more studies on the non-specific effects of giving this vaccine to infants so soon after birth.

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Appendices

The pilot infant BCG study

Appendix A. Ethics approval UVRI REC, Uganda

Appendix B. Ethics approval UNCST, Uganda

Appendix C. Ethics approval LSHTM, UK

Appendix D. Information sheet- English

Appendix E. Consent for procedures and use of samples in future

Appendix F. Eligibility form I

Appendix G. Eligibility form II

Appendix H. Screening form

Appendix I. List of equipment, supplies and reagents

The main infant BCG study

Appendix J. Ethics approval UVRI REC, Uganda

Appendix K. Ethics approval UNCST, Uganda

Appendix L. Ethics approval LSHTM, UK

Appendix M. Information sheet- English

Appendix N. Consent for procedures

Appendix O. Consent to use samples and storage for future studies

Appendix p. Screening II Checklist

Appendix Q. Screening II Eligibility Assessment

Appendix R. Maternal BCG scar is associated with increased infant proinflammatory immune responses**.** Mawa, P.A., *et al.,* Vaccine, 2016.

Appendix S. The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to bacille Calmette-Guérin immunisation". Mawa, P.A**.**, *et al.,* Philos Trans R Soc Lond B Biol Sci. 2015; 370.

Appendix T. Correlations between production of individual cytokines and chemokines following PPD stimulation

Appendix U. Correlations between production of individual cytokines and chemokines following ESAT-6/CFP-10 stimulation.

Appendix A

Tel: (256) 41- 321962 (Direct)
(256) 41- 320385/6 (General)
Fax: (256) 41- 320483
Email:directoruvri@ug.cdc.gov

THE REPUBLIC OF UGANDA

UGANDA VIRUS RESEARCH
INSTITUTE (UVRI)
P.O.Box 49 ENTEBBE (U)

Our Ref : GC/127/11/05/20
Your Ref:

26th May 2011

Mawa Akusa Patrice et al,

RE: UVRI SEC review of protocol titled **"The impact of maternal M. tuberculosis and helminth co-infection on infant immune responses to BCG immunization: A pilot study"**

Thank you for submitting the above study dated 7th April 2011 to UVRI Science and Ethics Committee.

This is to inform you that your protocol was reviewed during the SEC meeting of 19th May 2011 and met the requirements of the UVRI Science and Ethics Committee.

UVRI SEC annual approval has been given for you to conduct your study up to 26th May 2012. Annual progress report and request for extension should be submitted to UVRI SEC prior to the expiry date, to allow timely review.

The reviewed and approved documents include;
1. Project proposal
2. Data collection forms
3. Consent forms and information sheet (English and luganda versions)
4. Curriculum Vitae of applicants

You can now commence with your study after registration with the Uganda National Council for Science and Technology (UNCST).

Note: UVRI SEC requires you to submit a copy of the UNCST approval letter for the above study before commencement.

Yours sincerely,

Mr. Tom Lutalo
Chair, UVRI SEC

C.C Secretary, UVRI SEC

Appendix B

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 1024 **September 2, 2011**

Mr. Patrice Mawa Akusa
MRC/UVRI Uganda Research Unit on AIDS
P.O Box 49
ENTEBBE

Dear Mr. Mawa,

RE: RESEARCH PROJECT, "THE IMPACT OF MATERNAL M. TUBERCULOSIS AND HELMINTH CO-INFECTION ON INFANT IMMUNE RESPONSES TO BCG IMMUNISATION: A PILOT STUDY."

This is to inform you that the Uganda National Council for Science and Technology (UNCST) approved the above research proposal on **July 26, 2011.** The approval will expire on **July 26, 2012.** If it is necessary to continue with the research beyond the expiry date, a request for continuation should be made in writing to the Executive Secretary, UNCST.

Any problems of a serious nature related to the execution of your research project should be brought to the attention of the UNCST, and any changes to the research protocol should not be implemented without UNCST's approval except when necessary to eliminate apparent immediate hazards to the research participant(s).

This letter also serves as proof of UNCST approval and as a reminder for you to submit to UNCST timely progress reports and a final report on completion of the research project.

Yours sincerely,

Jane Nabbuto
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

LOCATION/CORRESPONDENCE

Plot 6 Kimera Road, Ntinda
P. O. Box 6884
KAMPALA, UGANDA

COMMUNICATION

TEL: (256) 414 705500
FAX: (256) 414-234579
EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

Appendix C

OBSERVATIONAL/INTERVENTIONS RESEARCH ETHICS COMMITTEE

08 November
Alison M. Elliott
Dear Alison

Study Title:	**The impact of maternal M. tuberculosis and helminth co-infection on infant immune responses to BCG immunisation: a pilot study.**
LSHTM ethics ref:	**6062**
Department:	**Infectious and Tropical Diseases**

Thank you for your email of 3 November responding to the Committee's request for further information on the above research and submitting revised documentation.
The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*	
LSHTM ethics application	n/a		
Protocol	V2.0	04/11/11	
Information Sheet	V2.0	04/11/11	
Consent form	V2.0	04/11/11	

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

Yours sincerely,

Professor Andrew J Hall
Chair

Appendix D

Uganda Virus Research Institute / Entebbe Hospitals

The impact of maternal *M. tuberculosis* and helminth co-infection on infant immune responses to BCG immunisation: a pilot study.

Consent for procedures to study the impact of maternal *M. tuberculosis* and helminth co-infection on infant immune responses to BCG immunisation.

Dear Mothers,

Tuberculosis (TB) is still a great problem in Uganda and many other countries. Many people are infected with TB, although a much smaller number of people actually suffer from TB disease. BCG immunisation has some benefit for young children in tropical countries like Uganda, but it is not so effective here as it is in Northern countries. The reason for the lower effect of BCG in countries like ours is not yet known.

It is possible that when a mother herself has been infected with TB or worms this may alter the development of the immune response in her unborn child in such a way that the baby's BCG immunisation, given soon after birth, is less effective. The aim of this study is to find out whether this is so. It is a small study conducted in preparation for a planned bigger project.

A clinical test called tuberculin skin test (TST) and a laboratory test known as T-spot are available to tell us whether someone has been infected with the organisms that cause TB disease, even if they do not have the disease. Tests are also available to tell us whether someone is infected with worms. If you agree, we would like to use these tests to find out whether you are infected with TB and worms, and to find out whether this TB or worm infection in you will affect your newly born baby's response to BCG.

We would also like to follow up your child until he/she is six weeks of age. We would like to do this to see the immediate effect of mothers' TB or worm infection or lack of it on infants' responses to BCG at one week, and later effects at six weeks of age. The results will provide useful information as to whether the mother's infection with TB organisms or worms has an influence on the baby's response to BCG immunisation.

We also hope that the results of this study will provide important information that will contribute to finding better ways of immunising against TB in countries like Uganda.

If you agree to take part in this study with your child this is what will happen:

- At delivery, a sample of cord blood will be taken after your baby has been delivered.
- The rest of the following procedures will only take place if the delivery goes well, the cord blood is obtained successfully, and both you and your baby are healthy.
- After delivery you will be asked questions about your health and home environment.
- At one week after delivery, we will ask you for a blood sample (2 teaspoons). We will use this blood for an HIV test, for a T-spot test to test for TB infection, and for tests of your immune responses. You will also be given a tuberculin skin test (TST) and you will be asked to come back (or to be visited at home) for this to be read after two or

three days. This is the usual test for TB infection, and will be done for comparison with the results of

- T-spot test. We will also check your stool and blood to find out whether you have worms.
- We will also ask for a blood sample from your child (half a teaspoon) one week after BCG immunisation. We will also ask for another blood sample from your child after six weeks (half a teaspoon). We will use this blood for tests of immunity.
- If you have a positive blood test for TB infection or a positive tuberculin skin test you will be checked for TB disease by a doctor and sent for a chest X-ray. If you are found to have TB disease you will be treated.
- If you are found to have worms, you will be given anti-worm treatment.
- Transport will be provided to take you home from the hospital and field workers may visit you at home to check on your skin test result and on the baby's progress, and to remind you about your follow up visits. A transport refund will be given for each visit when you are asked to come to the clinic.

  Taking part in this study is not expected to cause any major problems for you or your child. However, there will be some discomfort from having blood samples taken and the smaller discomfort of tuberculin skin testing. In general, blood samples will be used for tests of immunity and some may be stored for other tests in future. All the information collected, and the results of tests, will be completely confidential.

  Your right to refuse or withdraw from the research study

  Your participation in this study is voluntary. You and your child are free to drop out of the study at any time. Dropping out of the study will not affect your entitlement to routine government health care and management or to the provision of treatment for TB or worms if you need it.

  If you have any questions about your participation in this study, please feel free to ask the responsible midwife, doctor or field worker. If you prefer, you may speak to one of the principal investigators for this study: Dr Elliott (telephone: 0417 704000) or Mr Mawa Akusa Patrice (telephone: 0417 704000). If you have any questions about your rights as a research subject, you may also speak with the Ethics Committee Chairman from Uganda Virus Research Institute, Dr. Tom Lutalo on 0414 320631. The programme staff will let you use a phone for the call.

Appendix E

Uganda Virus Research Institute / Entebbe Hospitals

The impact of maternal *M. tuberculosis* and helminth co-infection on infant immune responses to BCG immunisation: a pilot study.

<u>Consent for procedures to investigate the impact of maternal *M. tuberculosis* and helminth co-infection on infant responses to BCG immunisation.</u>

Mother's names ……. Child's names …….

Mother's IDNO |___|___|___|___| **Child's IDNO** |___|___|___|___|/|___|

I have read and/or been fully explained the information sheet concerning I and my child's participation in this study and I understand what will be required if we take part in the study.
Our participation is voluntary.
My questions concerning this study have been answered by
……
I understand that at any time I and my child may withdraw from this study without giving a reason and without affecting our entitlement to routine government health care and management

"My signature / thumb print below indicates that I agree for my child and I to take part in this study, for TST test to be performed on me and for blood to be drawn from us"

……
Signature Or right thumb print

Name of mother ……

Date ……

<u>Witness*:</u>

Name …… Signature ……

**for those using a thumb print, this witness must not be a member of the research staff or a study participant*
Date ……

<u>Investigator:</u>

Name …… Signature
…….

Date ……
Note: form to be completed and signed in duplicate. One copy to be given to the mother; one copy to be retained at the clinic.

Uganda Virus Research Institute / Entebbe Hospitals
The impact of maternal *M. tuberculosis* and helminth co-infection on infant immune responses to BCG immunisation: a pilot study.

Consent to use samples and records for future studies

Mother's names ……Child's names …….

Mother's IDNO |___|___|___|___| **Child's IDNO** |___|___|___|___|/|___|

I have been asked for permission to use my samples and records and that of my child for future studies. I have read the foregoing information or it has been fully explained to me. I had the opportunity to ask questions about it and any questions I have asked have been answered to my satisfaction.

"My signature / thumb print below indicates that I agree for part of my specimen and that of my child to be stored for future studies".

……
Signature Or right thumb print

"My signature / thumb print below indicates that I do not agree for part of my specimen and that of my child to be stored for future studies".

……
Signature Or right thumb print

Name of mother ……

Date ……

Witness*:

Name …… Signature ……

**for those using a thumb print, this witness must not be a member of the research staff or a study participant*

Date ……

Investigator:

Name ……Signature …….

Date ……

Note: form to be completed and signed in duplicate. One copy to be given to the mother; one copy to be retained at the clinic.

Appendix F

11/10/2011 BCG study: ELIGIBILITY Form I (ELIG-I)

NAME: …… |___|___|___|__|___|___|/|___|___|
ANCNO

Screening Number: |___|___|___|___| SCRNO

Date: |___|___|/|___|___|/|___|___|___|___| DATEI

➔ Fill these questions in order. If the mother is not eligible, go to Q7 and indicate it.

ELIGIBILITY FOR BCG STUDY:

To be eligible Q1 to Q5 should be "YES"	1=Yes	2=No	
1. Is the participant resident in Entebbe municipality **EBBMUN** or Katabi Sub-county- Kabale Sabaddu B & Nkumba Parish? ***(Resident=expected to live in entebbe for at-least 2 years)***			

If yes to Q.1, Read ***to her the consent notes:***

2. Is she willing to participate in the BCG study?			**PSTUDY**
3. Does she wish to know her HIV test result?			**TKHIV**
4. Is she HIV negative based on ANC record?			**MHIV**
5. Is the pregnancy normal?			**NPREG**
6. (a) Did she take an anti-worm drug during pregnancy?			**AWDRUG**
(b) *If yes to Q.6a,* which drug? (1=albendazole, 2=praziquantel, 3=other			**WDRUG**
7. Is the participant eligible for the BCG study? (1=Yes, 2=			**ELIG**

Appendix G

04-10-2011 BCG study: ELIGIBILITY Form II (ELIG-II)
(When they bring stool sample)

NAME: ……___|___|___|___|___|___|/|___|___|
ANCNO

Screening Number: |___|___|___|___| **SCRNO**

Date: |___|___|/|___|___|/|___|___|___|___| **DATEII**

Please fill all boxes.
To be eligible all these should be "YES"

	1=Yes	No=2	
Eligibility form "I" filled and eligible?	☐	☐	**FILLED**
2. Is the consent form signed?	☐	☐	**SIGNED**
3. Clinical form (Screening form) filled?	☐	☐	**CLINFIL**
4a. Is this pregnancy normal?			**PRGNORM**
4b. Is severe liver disease **ABSENT**?	☐	☐	**LIVERDIS**
5. Is the pregnancy greater than 14 weeks old ?	☐	☐	**PRG14WKS**

If no to Q.5, give appointment after 14 weeks will be complete.

6. Study/Blood samples taken at last visit?	☐	☐	**BLOOD**
7. Stool sample brought?	☐	☐	**STOOL**
8. Haemoglobin result (Hb): |___|.|___| g/dl			**HB**
9. Is this result ≥ 8 g/dl ?	☐	☐	

GREATER

If yes to all (questions 1-9) above, give study drug.

If no to questions 1-7, wait till "yes" if possible.

11. Study drug number: WS|___|___|___|___| **WSIDNO**

If no to Q.9, refer to clinician to consider treatment for hookworm & anaemia.
(1=Yes, 2=No) |___| **REFER**

Appendix H

20/02/2012 BCG STUDY: SCREENING FORM (BCGSCREEN)

Q1.a. Name of mother …
…….....**NAME**
b. Identity Number:
|___|___|___|___| **IDNO**
Q2. Age |___|___| **AGE** Date of Birth (dd/mm/yyyy)
|___|___|/|___|___|/|___|___|___|___| **DOB**
Q3. **ADDRESS:**
Current Residence/ L.C.1 / Barracks:

…….……..……
Phone
number**.....**
(c) Name of L.C.1
Chairperson**……..…….……**
……..
(d) Description of how to find the home (e.g nearest shop/bar/market/hotel e.t.c):
…….…….……
……...…….……
…….....
(e) Two people whom we could contact if we cannot contact the mother:
Name1 **.....** Phone
number2……..
Name1 **.....** Phone number 2**.....**
…….

PREVIOUS PREGNANCIES:
Q4. Is this your first pregnancy? |___| **FPREG**
(1=Yes, 2=No)

WORM TREATMENT:
Q5. Have you ever had any medicine for worms? |___| **WMED**
(1=Yes, 2=No, 3=Don't know)
Q6. If yes to Q5, When was the most recent? ___| RECENT
1 = During this pregnancy 4 = As a child

2 = Within last year but before the last menstrual period 5 = Never
3 = More than 1 year ago 6 = Don't Know

Q7. If yes to Q5,
1 = Mebendazole Worm drug 1 |___| **WDRG1**
2 = Praziquantel Worm drug 2
|___| WDRG2
3 = Albendazole Worm drug 3 |___| **WDRG3**
4 = Other specify **…….…… Q7SPEC**
5 = Don't know |___| **DKNOW**

TB HISTORY:

Q8. Have you ever received treatment for TB disease? |___| **TTB** Year: |___|___|___|___| **YTB**

(1=Yes, 2=No, 3=Uncertain)

Q9. Was the treatment during this pregnancy?

|___| T**TBP**

(1=Yes, 2=No, 3=Uncertain)

Q10. If yes to Q9,

What is the start date of treatment for TB? (dd/mm/yyyy)

|___|___|/|___|___|/|___|___|___|___| **STBRX**

What is the finish date of treatment for TB?

(dd/mm/yyyy)|___|___|/|___|___|/|___|___|___|___| F**TBRX**

Q11. Is TB treatment still on-going?

|___| T**TBON**

(1=Yes, 2=No)

Q12. Have you ever received treatment to prevent TB? |___| **TBP** Year: |___|___|___|___| **YTBP**

(1=Yes, 2=No, 3=Uncertain)

Q13. Was the treatment during this pregnancy?

|___| T**TBP**

(1=Yes, 2=No, 3=Uncertain)

Q14. If yes to Q13,

What is the start date of treatment for TB? (dd/mm/yyyy)

|___|___|/|___|___|/|___|___|___|___| **STBP**

What is the finish date of treatment for TB? (dd/mm/yyyy)

|___|___|/|___|___|/|___|___|___|___| **FTBP**

Details of TB or preventive treatment history**.....**

Ask if they have their TB treatment card to see at home and bring to next visit.

Q15. Have you ever lived in a house with someone with TB? |___| **HHTB** Year: |___|___|___| **YHHTB**

(1=Yes, 2=No, 3=Uncertain)

Q16. If yes, how close you were to the patient (indicate the closest contact) |___| **HHR**

(1=shared bed, 2=shared bedroom, 3=shared living room, 9=can't tell)

BCG Scar

Q17. Any previous BCG vaccination scar? |___| **BCGSC**

(1=Yes, 2=No, 3=Uncertain)

Q18. If yes, how many BCG scars? |___| **BCGNO**

BCG vaccine data

Manufacturer**……..…… …BCGMAN**

Lot number**…….....BCGLOT**

Polio vaccine data

Manufacturer**…… …POLMAN**

Lot number**…… POLLOT**

Name of Interviewer: …….

Signature of Interviewer:……..

Date: (dd/mm/yyyy) **___|___|/|___|___|/|___|___|___|___| DATE**

THANK YOU.

Appendix I

Major equipment, supplies and reagents

- Biosafety Cabinet Class 2 (Walker Safety Cabinets, United Kingdom)
- Water-bath (Julabo Labortechnik Gmbh, Seelbach, Germany)
- Incubator (Revco Habitat, Thermo Fisher Scientific, Washington, DC, USA)
- Fridge/freezers (+4/-20^0 C) (Lec Medical, Merseyside, United Kingdom)
- Freezer (-80°C) (New Brunswick Scientific, United Kingdom)
- Centrifuge (Rotanta, HAHN Gastedern Gmbh Waldstrasse, Germany)
- Microscope (Olympus corporation, Tokyo, Japan)
- Vortex (Scientific industries, Boloemia, NY, USA)
- Pipette AID (Jencons, San Diego, CA, USA)
- P200-1000µL multi-channel pipetter (Thermo Fisher Scientific, Washington, DC, USA)
- 96 well (round bottomed) tissue culture plates with lids (Beckton Dickinson, NJ, USA)
- 10µL-1000µL pipette tips (Gilson, Luton, Bedfordshire, United Kingdom)
- Microtubes (National Scientific supply, Claremont, California)
- Weighing scale
- Thermometer
- Stethoscope
- Syringes and needles
- Working tray
- Transparent ruler
- Ball pen
- Sharps and disposal container
- Blood collection tubes
- RPMI 1640 medium (Life Technologies Corporation, NY, USA)
- Hepes buffer (Sigma-Aldrich, MO, USA).
- Penicillin/Streptomycin (Sigma-Aldrich, MO, USA)
- L-glutamine 200mM (Sigma-Aldrich, MO, USA).
- Tuberculin PPD (RT 23 (Statens Serum Institut, Copenhagen, Denmark)
- BCG vaccine

Appendix J

UNHRO
Uganda National Health Research Organisation

Uganda Virus Research Institute
Plot 51-59, Nakiwogo Road, Entebbe
P.O. Box 49, Entebbe-Uganda
Tel: +256 414 320 385 / 6
Fax: +256 414 320 483
Email: directoruvri@uvri.go.ug

REPUBLIC OF UGANDA

Our Ref: GC/127/13/08/10

Your Ref: ……

20th August 2013

Profs. Alison Elliott, Pontiano Kaleebu, Dr. Stephen Cose,

RE: UVRI SEC review of protocol titled **"The Impact of maternal infection with Mycobacterium tuberculosis on the infant response to BCG immunisation."**

Thank you for submitting the above study dated 05th August 2013 to UVRI Science and Ethics Committee.

This is to inform you that your study was reviewed during the SEC meeting of 15th August 2013 and members had the following queries for you to address;

1. Women will be defined as positive for *M.TB* infection if the two tests used are positive yet pregnant women do not respond well to the Tuberculin skin Test. The PI is requested to comment on this.
2. The PI is requested to provide the sensitivity of the second test, the TSPOT.TB as the committee was not very familiar with it.
3. It is unclear how positivity for the two tests will be determined and the PI is required to give the scoring criteria.
4. The BCG dose to be used in the study should be approved by NDA as this appears not to be in use by the MOH.
5. The protocol mentions other infections that will be assessed but there is no objective relating to them, Please clarify. (Page 4 0f 6 of the Application form)
6. The sections of the consent form need to be well formatted.
7. The PI is requested to show that the sample size is adequate given that it is different in the two sites, UK and Uganda. What power calculations were done for the comparisons?

Chairman's action will suffice once the above concerns are addressed.

Yours sincerely,

Mr. Tom Lutalo
Chair, UVRI SEC
C.C Secretary, UVRI SEC

Appendex K

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

17/02/2014

Our Ref: HS 1526

Dr. Stephen Cose
MRC/Uganda Virus Research Institute on AIDS
Uganda Virus Research Institute
Entebbe

Re: Research Approval: **The impact of maternal infection with Mycobacterium tuberculosis on the infant response to BCG Immunization**

I am pleased to inform you that **20/01/2014**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **20/01/2014** to **20/01/2017**.

Your research registration number with the UNCST is **HS 1526**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated local Institutional Review Committee (IRC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local IRC for review with copies to the National Drug Authority.
4. Unanticipated problems involving risks to research subjects/participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1	Research proposal	English	1.1	31 July 2013
2	Consent Form	English, Luganda	1.1	6 Jan 2014
3	Consent for Storage	English, Luganda	1.1	6 Jan 2014

Yours sincerely,

Leah Nawegulo Omongo
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

cc Chair, Uganda Virus Research Institute SEC, Entebbe

LOCATION/CORRESPONDENCE

Plot 6 Kimera Road, Ntinda
P. O. Box 6884
KAMPALA, UGANDA

COMMUNICATION

TEL: (256) 414 705500
FAX: (256) 414-234579
EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

Appendix L

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Stephen Cose
Lecturer
CR / ITD
LSHTM

13 January 2014

Dear Dr. Cose,

Submission Title: The impact of maternal infection with Mycobacterium tuberculosis on the infant response to BCG immunisation

LSHTM Ethics Ref: 7104

Thank you for your letter of 8 January 2014, responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval for the amendment having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Information Sheet	Consent Form UG V1.0.doc	7/31/2013	1.0
Information Sheet	Information Sheet V1.1.doc	1/6/2014	1.1
Protocol / Proposal	Protocol V1.1.doc	9/19/2013	1.1

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an Amendment form onthe online application website. The Principal Investigator is reminded that all studies are also required to notify the ethics committee of any serious adverse events which occur during the project via anAdverse Event form on the online application website. At the end of the study, please notify the committee via anEnd of Study form onthe online application website.

Yours sincerely,

Professor John DH Porter
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Appendix M

MRC/UVRI/Entebbe Hospital/Kisubi Hospital

The impact of maternal infection with Mycobacterium tuberculosis on the infant response to BCG immunisation

Dear Mothers,

Tuberculosis (TB) is still a great problem in Uganda and many other countries. Many people are infected with TB, although a much smaller number of people actually suffer from TB disease. BCG immunisation has some benefit for young children in tropical countries like Uganda, but it is not so effective here as it is in Northern countries. The reason for the lower effect of BCG in countries like ours is not yet known.

It is possible that when a mother herself has been infected with TB, that this may alter the development of the immune response in her unborn child in such a way that the baby's BCG immunisation, given soon after birth, is less effective. The aim of this study is to find out whether this is so. Immune responses are the body's way of protecting you against infections.

A clinical test called tuberculin skin test (TST) and a laboratory test known as TSPOT.TB are available to tell us whether someone has been infected with the organisms that cause TB disease, even if they do not have the disease. If you agree, we would like to use these tests to find out whether you are infected with TB or not, and to find out whether this infection in you will affect your newly born baby's response to BCG.

We would also like to follow up your child until he/she is one year of age. We would like to do this to see the immediate and longer term effects of a mother's TB infection, or lack of it, on their infants' responses to BCG at one week, and later effects at four, six, ten and 52 weeks of age. The results will provide useful information as to whether

the mother's infection with TB organisms or worms has an influence on the baby's response to BCG immunisation.

We also hope that the results of this study will provide important information that will contribute to finding better ways of immunising against TB in countries like Uganda.

If you agree to take part in this study with your child this is what will happen:

- At delivery, a sample of cord blood (2 teaspoons) will be taken after your baby has been delivered.
- A sample of the placenta will also be obtained to check for malaria and for studies of immune responses, **as well as gene and gene expression studies**.
- The rest of the following procedures will only take place if the delivery goes well, the cord blood is obtained successfully, and both you and your baby are healthy.
- After delivery you will be asked questions about your health and home environment.
- At one week after delivery, we will ask you for a blood sample (2 teaspoons). We will use this blood for an HIV test, for a test for TB infection, **tests of your immune responses and for tests of gene and gene expression**. If you are found to be HIV positive, you will be counselled and referred to an appropriate care provider. You will not be asked to continue with the study.
- **You will not be able to be identified as a result of agreeing for us to use your DNA or RNA. These studies are for research purposes only, and no personal information will be attached to the results of these studies.**
- You will also be asked to provide a stool sample to test for worm infections
- You will also be given a tuberculin skin test (TST) and you will be asked to come back (or to be visited at home) for this to be read after two or three days. This is the usual test for TB infection. We will also check your stool and blood to find out whether you have worms and malaria.
- When you return to have your TST result read, we may ask you to continue with the study, based on your TST and TB blood test results.
- We will ask you (and your baby) to continue in the study if you are confirmed to have a TB infection.
- Mothers without TB infection (and their babies) will be chosen to continue by a simple lottery method.

- If you are positive on one TB test and negative on the other, you will not be asked to continue with the study. A test result such as this probably means that you do not have TB.
- We will also ask for a blood sample from your child (half a teaspoon) one or four weeks after BCG immunisation. We will also ask for another blood sample (half a teaspoon) from your child after six weeks (if your baby gave blood at one week), or at ten weeks (if your baby gave blood at four weeks). We will use this blood for tests of immunity.
- We will collect blood (one teaspoon) from your baby when they return at age one year, and will also ask you to provide a stool sample from your infant when you arrive.
- If you have a positive blood test for TB infection or a positive tuberculin skin test you will be checked for TB disease by a doctor and sent for a chest X-ray. If you are found to have TB disease you will be treated and your baby will be given treatment to prevent TB.
- If you are found to have worms, you will be given anti-worm treatment.
- Transport will be provided to take you home from the hospital and field workers may visit you at home to check on your skin test result and on the baby's progress, and to remind you about your follow up visits. A transport refund will be given for each visit when you are asked to come to the clinic.
- If you are chosen to continue in the study, we will ask you to continue for approximately one year
- To complete the full study, we expect to recruit 150 mothers with TB infection (and their babies), and 150 mothers without TB infection (and their babies)

  Taking part in this study is not expected to cause any major problems for you or your child. However, there will be some discomfort from having blood samples taken and the smaller discomfort of tuberculin skin testing. In general, blood samples will be used for tests of immunity and some may be stored for other tests in future. All the information collected, and the results of tests, will be completely confidential.

  Your right to refuse or withdraw from the research study

  Your participation in this study is voluntary. You and your child are free to drop out of the study at any time. Dropping out of the study will not affect your entitlement to routine government health care and management or to the provision of treatment for TB if you need it.

If you have any questions about your participation in this study, please feel free to ask the responsible midwife, doctor or field worker. If you prefer, you may speak to one of the principal investigators for this study: Dr Stephen Cose (telephone: 041 7704180) or Prof. Alison M. Elliott (telephone: 041 7704180). If you have any questions about your rights as a research subject, you may also speak with the Ethics Committee Chairman from the Uganda Virus Research Institute, on 0414 321962.

Appendix N

Consent Form - Infant BCG Study

MRC/UVRI/Entebbe Hospital/Kisubi Hospital

Consent for procedures to investigate the impact of maternal infection with Mycobacterium tuberculosis on the infant response to BCG immunisation

Mother's names .....

Mother's IDNO |___|___|___|___|

I have read and/or had the information sheet fully explained concerning my participation, and my child's participation, in this study and I understand what will be required if we take part in the study.

Our participation is voluntary.

My questions concerning this study have been answered by .....

I understand that at any time I and my child may withdraw from this study without giving a reason and without affecting our entitlement to routine government health care and management.

"My signature / thumb print below indicates that I agree for my child and myself to take part in this study"

.....
Name of mother

.....
Signature

Date .....

Or right thumb print

Witness*:

Name ..... Signature .....

Date .....

**for those using a thumb print, this witness must not be a member of the research staff or a study participant*

Person taking consent:

Name ..... Signature .....

Date .....

Note: form to be completed and signed in duplicate. One copy to be given to the mother; one copy to be retained at the clinic.

Comments

UGANDA VIRUS RESEARCH INSTITUTE ENTEBBE SCIENCE & ETHICS COMMITTEE APPROVED VALID UNTIL 20 SEP 2015 P.O BOX 49 ENTEBBE UGANDA

Version 1.1 18/12/2013

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Appendix O

Consent Form for Sample Storage and Future Use - Infant BCG Study

MRC/UVRI/Entebbe Hospital/Kisubi Hospital

Consent for procedures to investigate the impact of maternal infection with Mycobacterium tuberculosis on the infant response to BCG immunisation

Consent to use samples and storage for future studies

Mother's names .....

Mother's IDNO |___|___|___|___|

I have been asked for permission to use my stored samples and that of my child for future studies.

I have read and/or had the information sheet fully explained concerning my participation, and my child's participation, in this study and I understand what will be required if we take part in the study.

Our participation is voluntary.

My questions concerning this study have been answered by .....

I understand that at any time I and my child may withdraw from this study without giving a reason and without affecting our entitlement to routine government health care and management.

"My signature / thumb print below indicates that I agree for part of my specimen and that of my child to be stored for future studies".

.....
Name of mother

.....
Signature

Or right thumb print

Date .....

Witness*:

Name ..... Signature .....

Date .....

**for those using a thumb print, this witness must not be a member of the research staff or a study participant*

Person taking consent:

Name ..... Signature ..... Date .....

Note: form to be completed and signed in duplicate. One copy to be given to the mother; one copy to be retained at the clinic.

Comments

Version 1.0 18/12/2013

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Appendix P

SCREENING II CHECK LIST

Date of Form: (dd/mm/yyyy) |__|__|/|__|__|/|__|__|__|__|

Mothers Name:

……
……

Date of delivery:
(dd/mm/yyyy) |__|__|/|__|__|/|__|__|__|__|

Check that this visit is complete by ticking the boxes corresponding to activities/procedures that have been done. Items not ticked should be reviewed for completeness before the mother is reimbursed. All relevant information worth noting should be documented in the comments column.

Activities/procedures	**Response (tick)**	**Comment, if any:**
Participants reviewed and examined by clinician	☐	
4ml blood sample taken off from mother for repeat HIV test and storage (EDTA vacutainer)	☐	
6ml blood sample taken off from mother for T-Spot assay (Heparin vacutainer)	☐	
TST placed on mother's forearm	☐	
Stool sample received from mother	☐	
Mother's contact details updated	☐	
Mother's transport costs reimbursed	☐	

Date of TST Reading: (dd/mm/yyyy)
|__|__|/|__|__|/|__|__|__|__|

Name of staff……
…….**Initials**|__|__|__|

Appendix Q

SCREENING II ELIGIBILITY ASSESSMENT **ELIG SC-II**

Date of Form:
(dd/mm/yyyy)
|__|__|/|__|__|/|__|__|__|__| **DATE2**

Mothers Name:
…… **MNAME**

Mothers age: **MAGE**
|__|__|years

Mothers weight: **MWT**
|__|__|.|__|**Kg**

Mothers height:
|__|__|__|.|__|cm / |__|__|.|__|m **MHT**

Body Mass Index: **BMI**
|__|__|.|__|__|**Kg/m²**

ELIGIBILITY FOR INFANT BCG STUDY

To be eligible, all responses should be "YES" except Qn 7=No	1=Yes, 2=No	
Eligibility form "I" filled and eligible?	☐	**FILLED**
Is the consent form signed and dated?	☐	**SIGNED**
Address form filled and completed?	☐	**ADDFIL**
Did the mother have a normal vaginal delivery?	☐	**NDEL**
Cord blood collected at delivery?	☐	**CORDBL**
Was birth weight greater or equal to 2.5Kg?	☐	**BWT**
No major congenital abnormalities?	☐	**ABNORM**
Did the baby receive the Danish BCG vaccine from project stocks?	☐	**BCGDAN**
Is the baby well enough to participate in the study?	☐	**CWELL**
. Is the mother happy for her and her baby to continue participating in the study?	☐	**HAPPY**

If eligible, collect mothers stool sample, draw blood for repeat HIV test and T-Spot assay; then place TST antigen on mother's forearm and give appointment for TST reading.

Date of TST Reading+/-Enrolment: |__|__|/|__|__|/|__|__|__|__|

Name of staff……Initials |__|__|__| **INS**

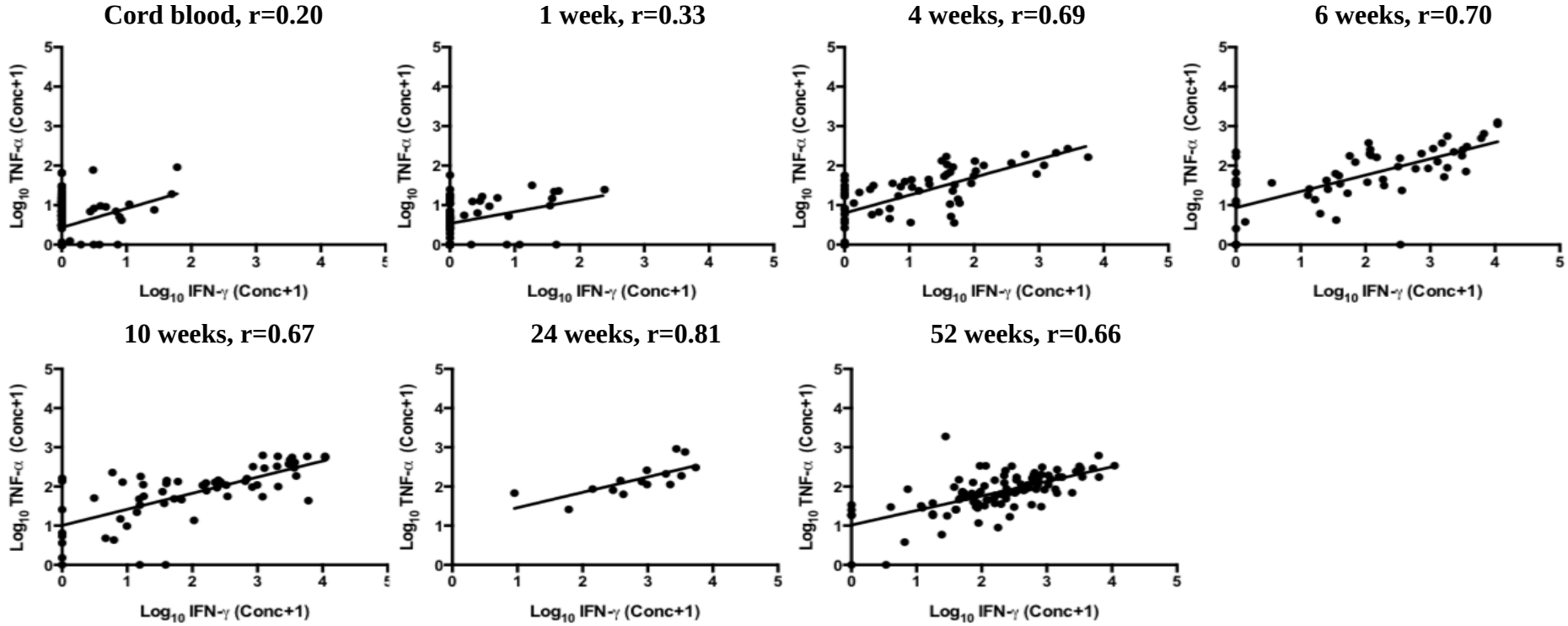
Appendix R.

Maternal BCG scar is associated with increased infant proinflammatory immune responses**. Mawa, P.A,** Webb, E.L., Filali-Mouhim, A., Sekaly, R.P., Nkurunungi, G., Lule, S.A., Prentice, S., Nash, S., Dockrell, H.M., Elliott, A.M., and Cose, C". The original article (in press) is found at the end of the thesis.

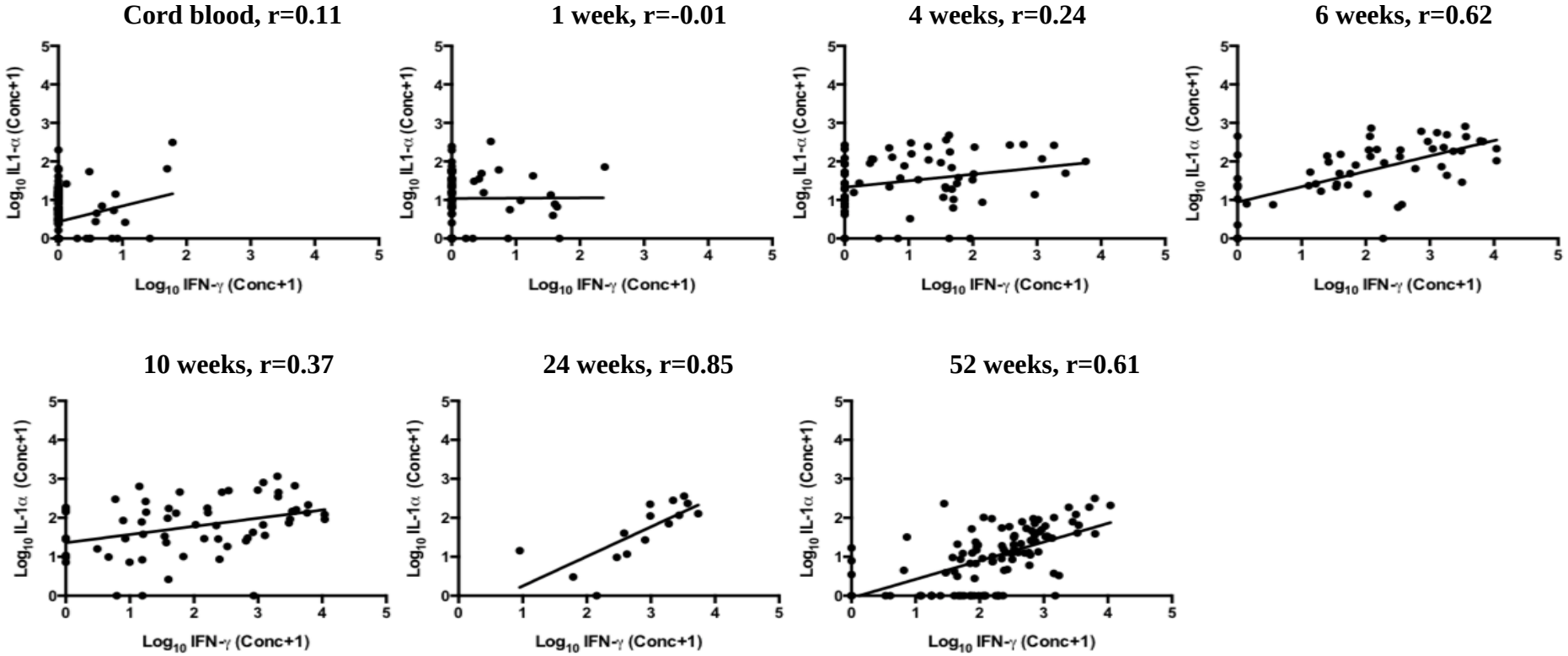
Appendix S.

"The impact of maternal infection with *Mycobacterium tuberculosis* on the infant response to bacille Calmette-Guérin immunisation". **Mawa, P.A.,** Nkurunungi, G., Egesa, M., Webb, E.L., Smith, S.G., Kizindo, R., Akello, M., Lule, S.A., Muwanga, M., Dockrell, H.M., Cose, S., Elliott, A.M. Philos Trans R Soc Lond B Biol Sci. 2015 Jun 19; 370 (1671)". The original article (pages 1-9) is found at the end of the thesis.

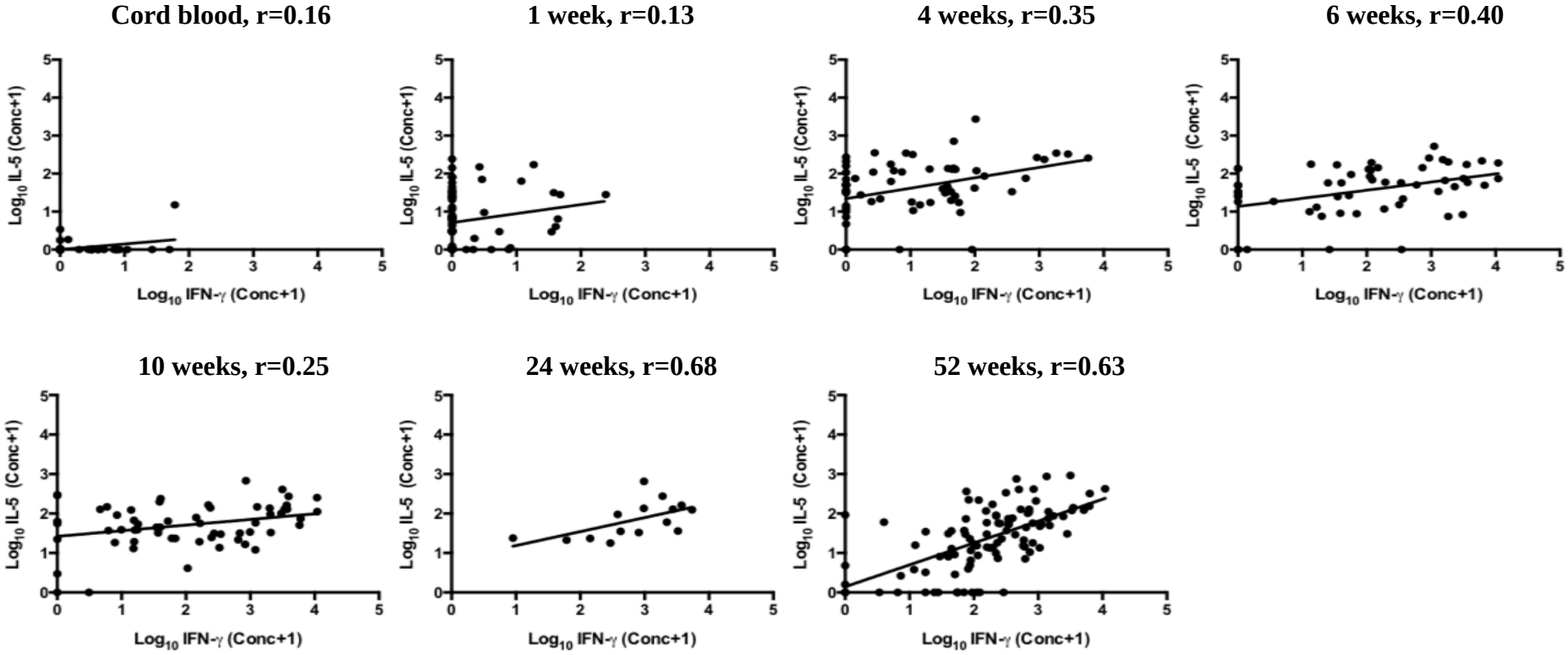
Appendix T.



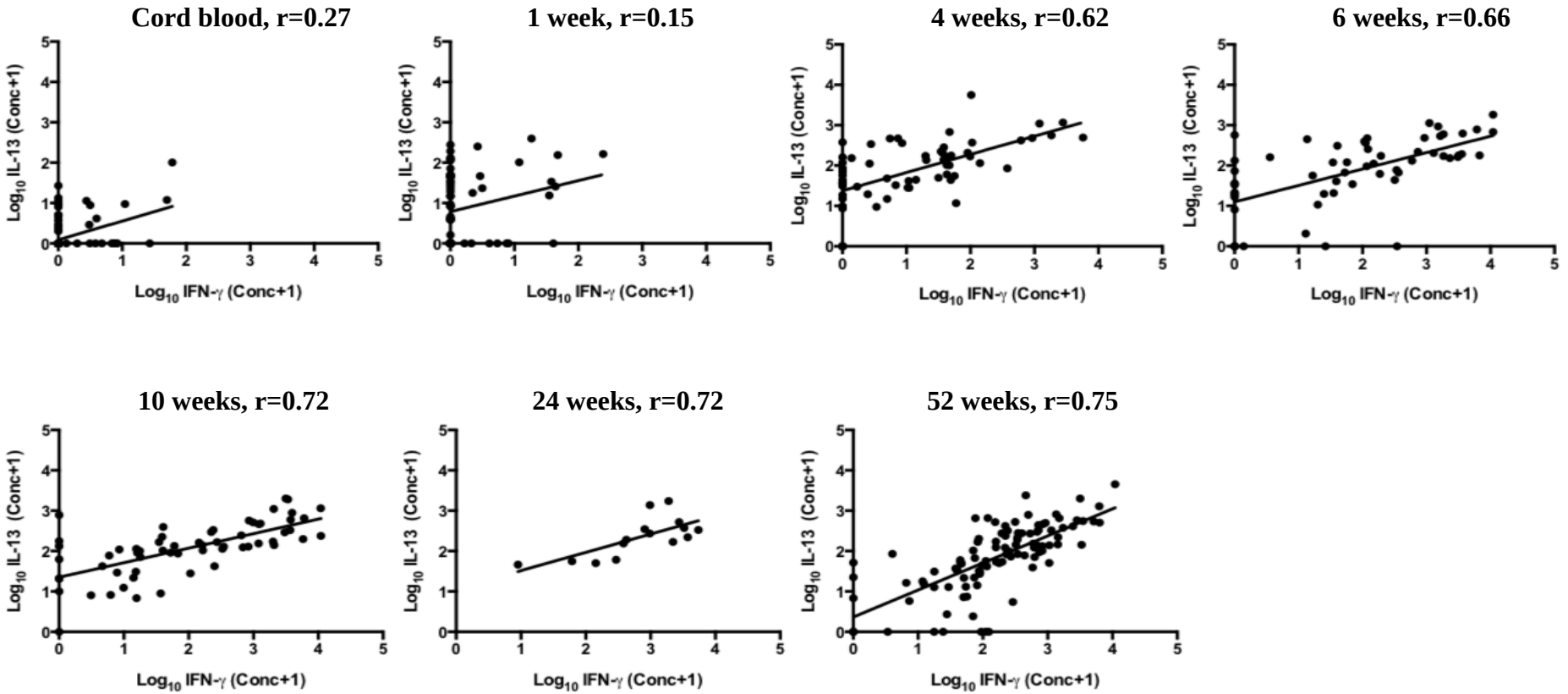
Correlations between concentrations of PPD-induced IFN-γ and TNF-α. Cytokines and chemokines were measured from supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



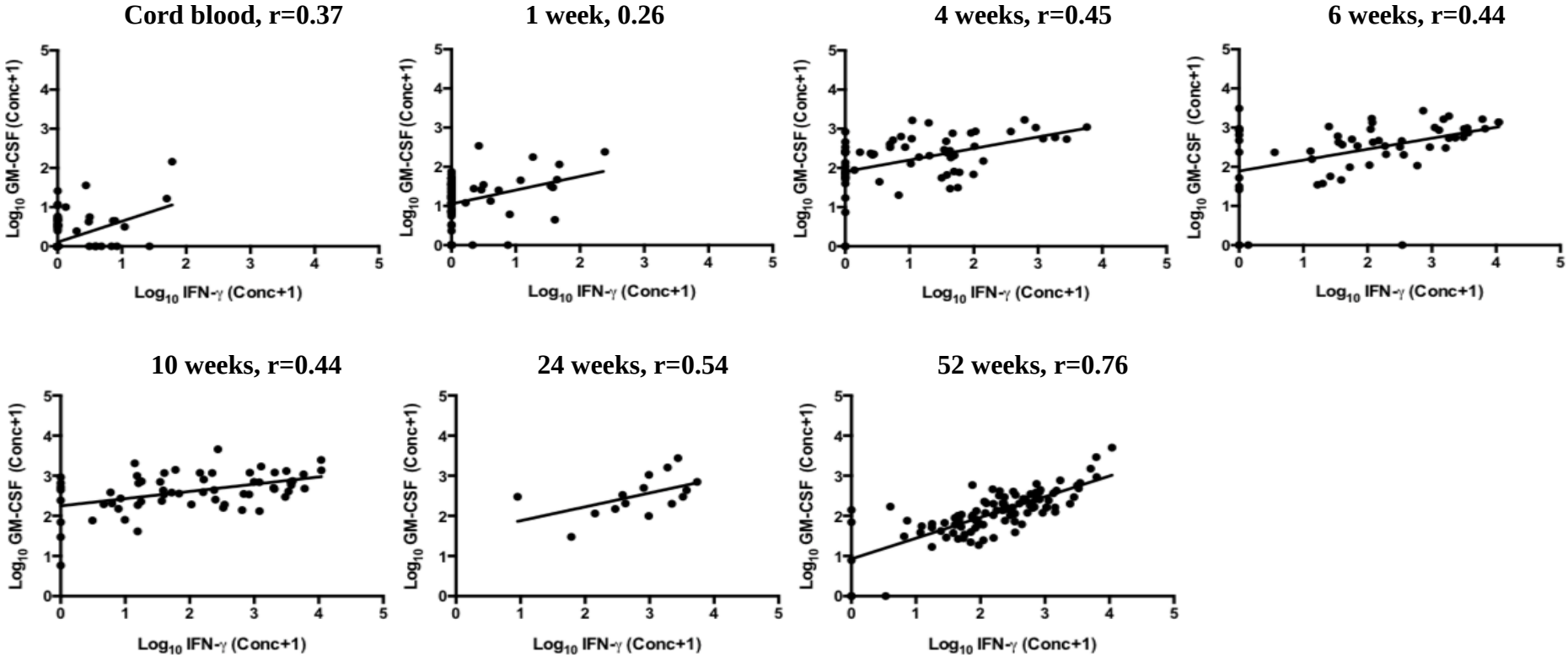
Correlations between PPD-induced IFN-γ and IL-1α. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



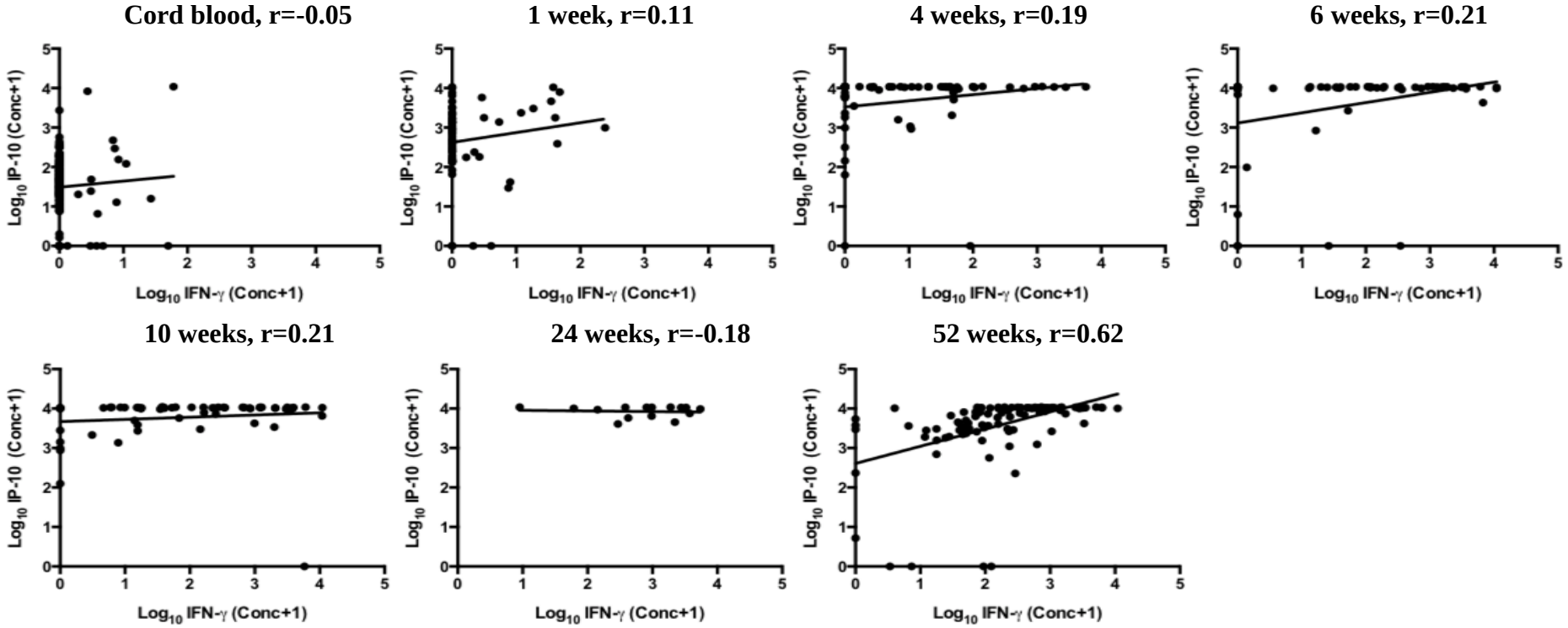
Correlations between PPD-induced IFN-γ and IL-5. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



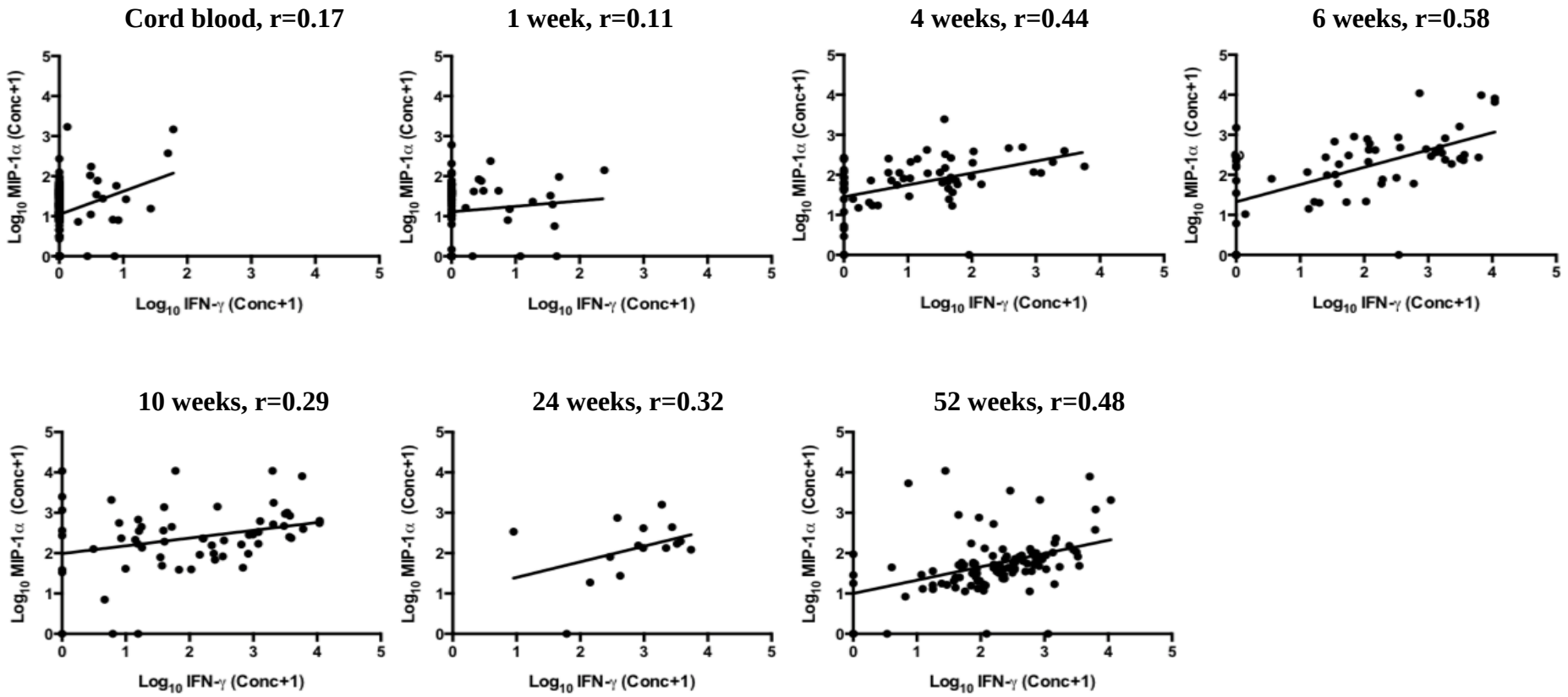
Correlations between PPD-induced IFN-γ and IL-13. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



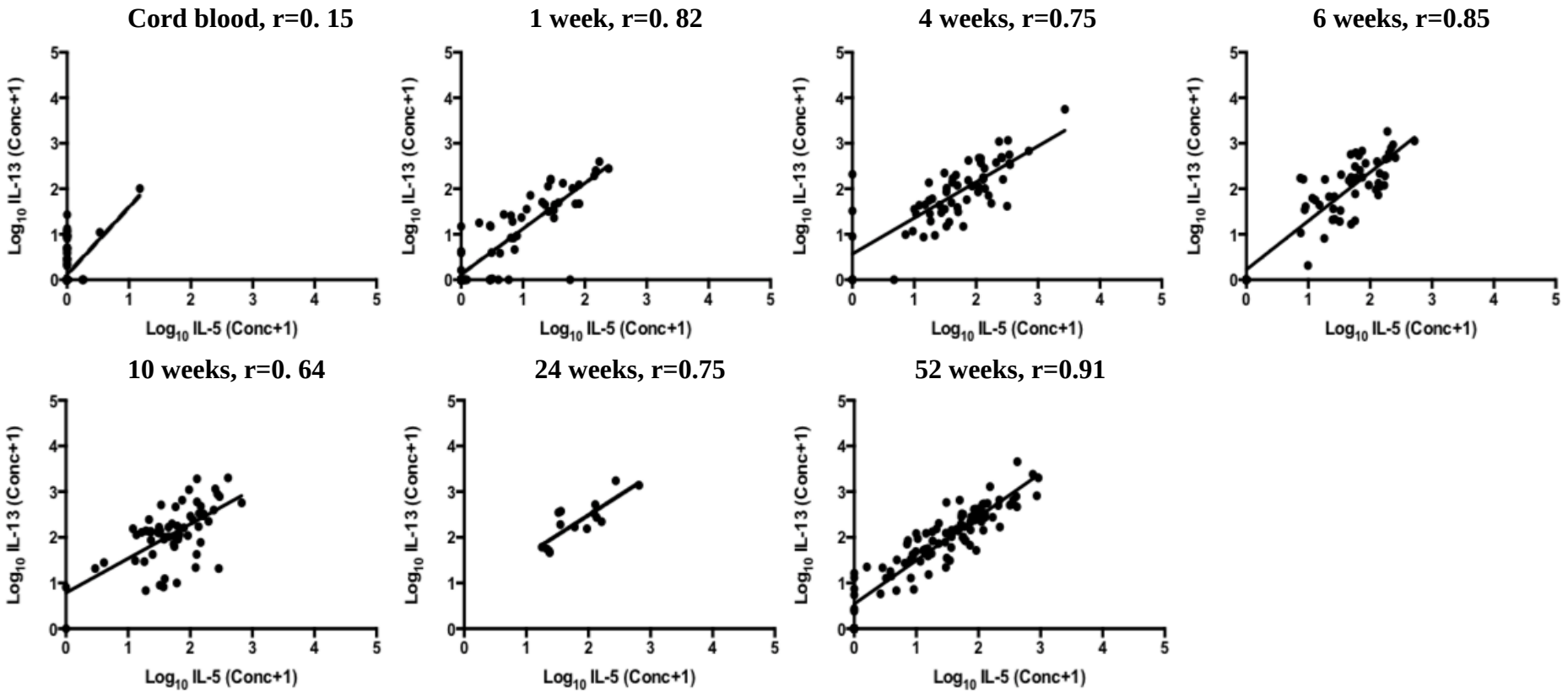
Correlations between PPD-induced IFN-γ and GM-CSF. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



Correlations between PPD-induced IFN-γ and IP-10. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.

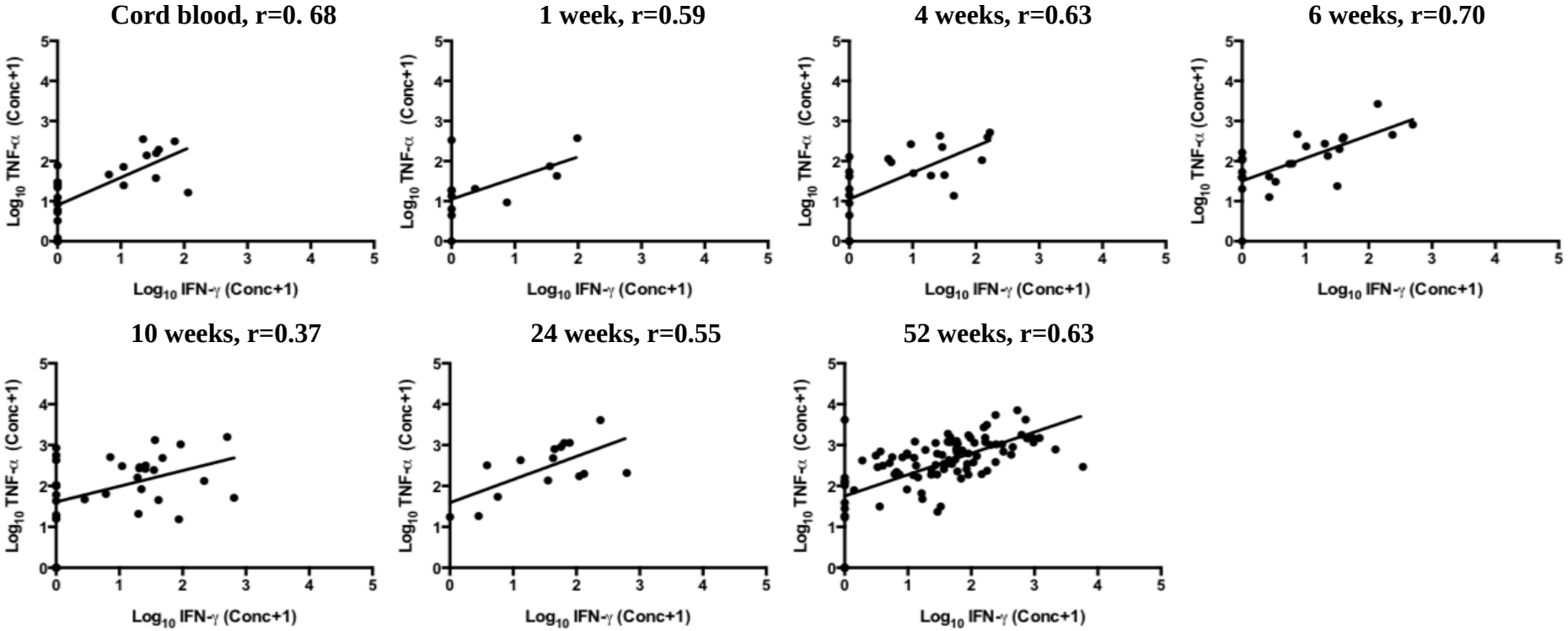


Correlations between PPD-induced IFN-γ and MIP-1α. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.

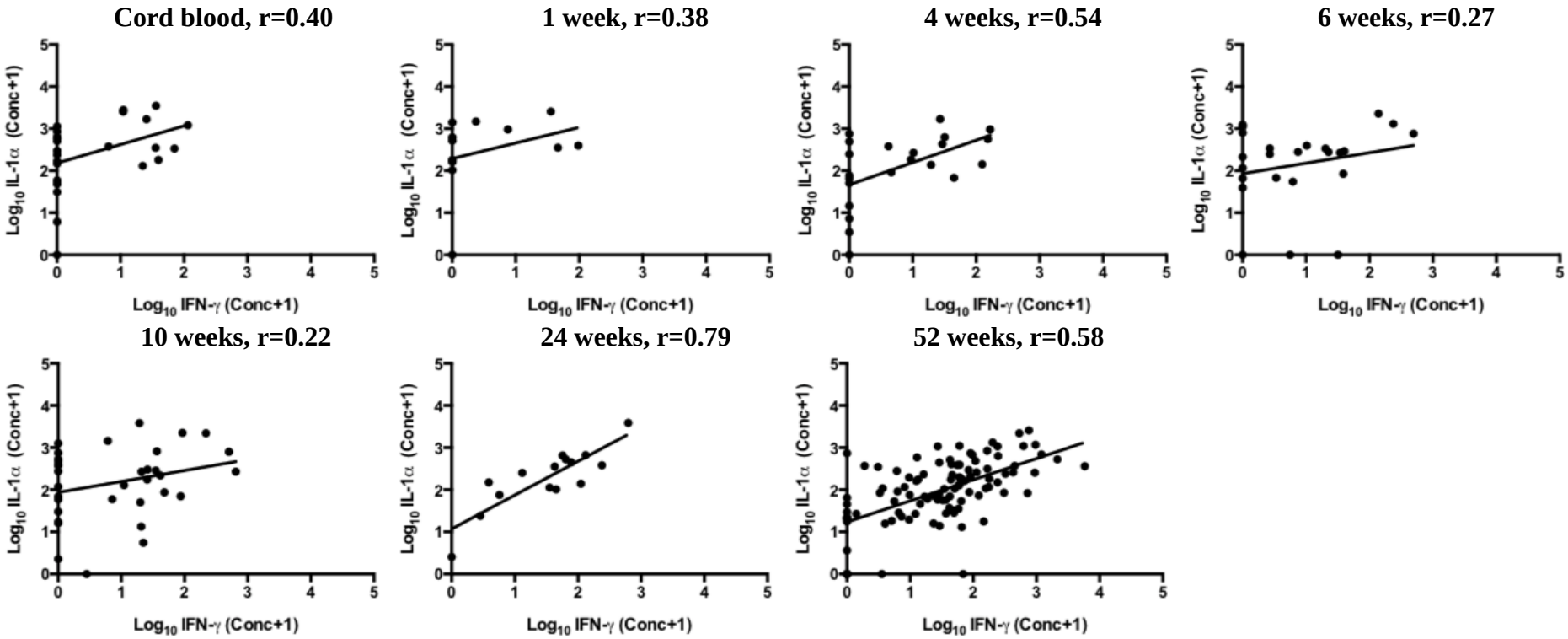


Correlations between PPD-induced IL-5 and IL-13. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.

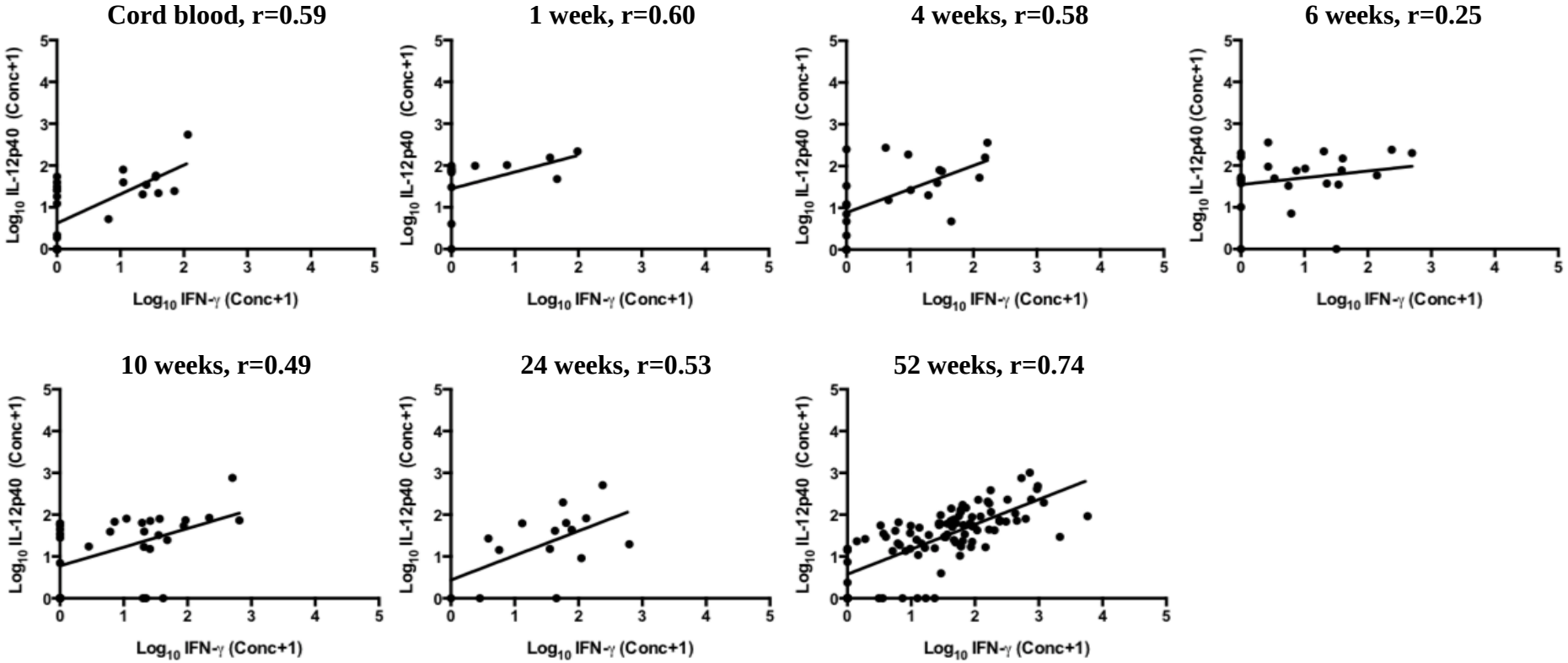
Appendix U.



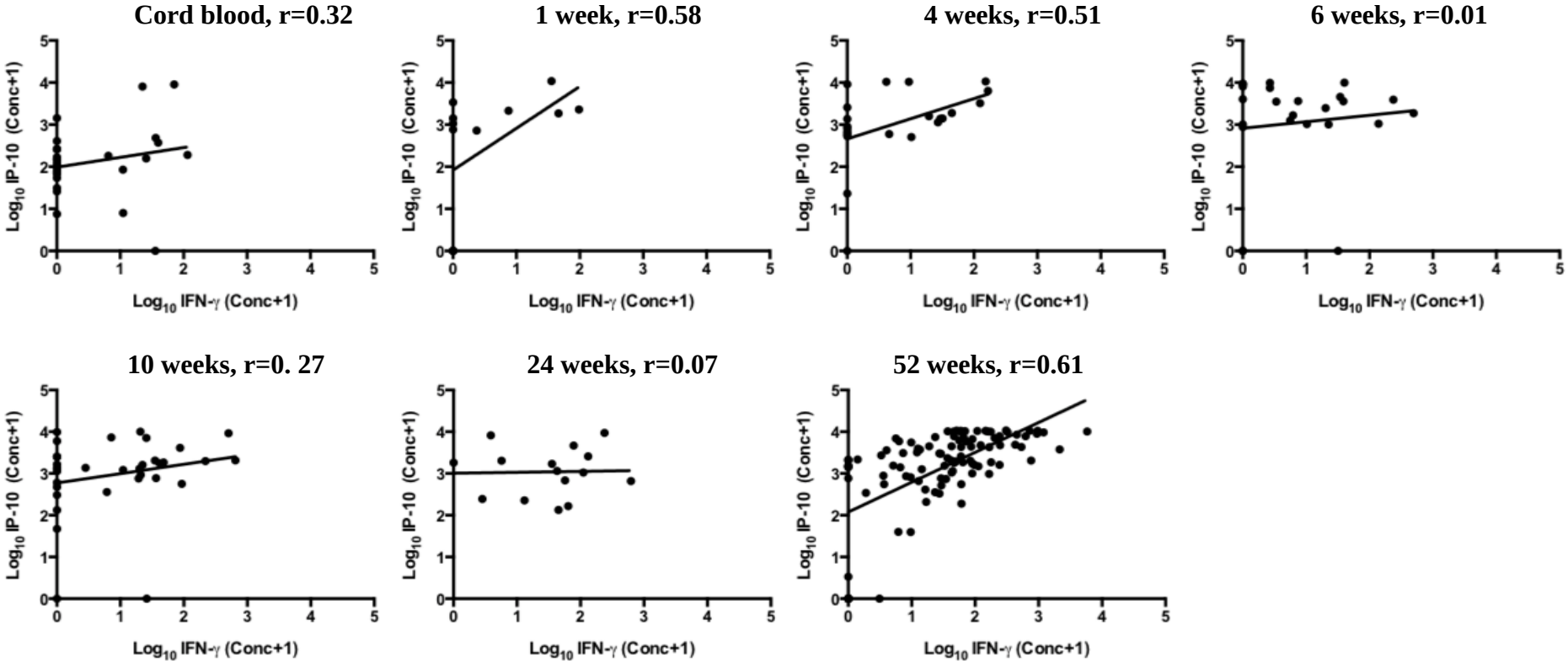
Correlations between ESAT-6/CFP10-specific IFN-γ and TNF-α. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



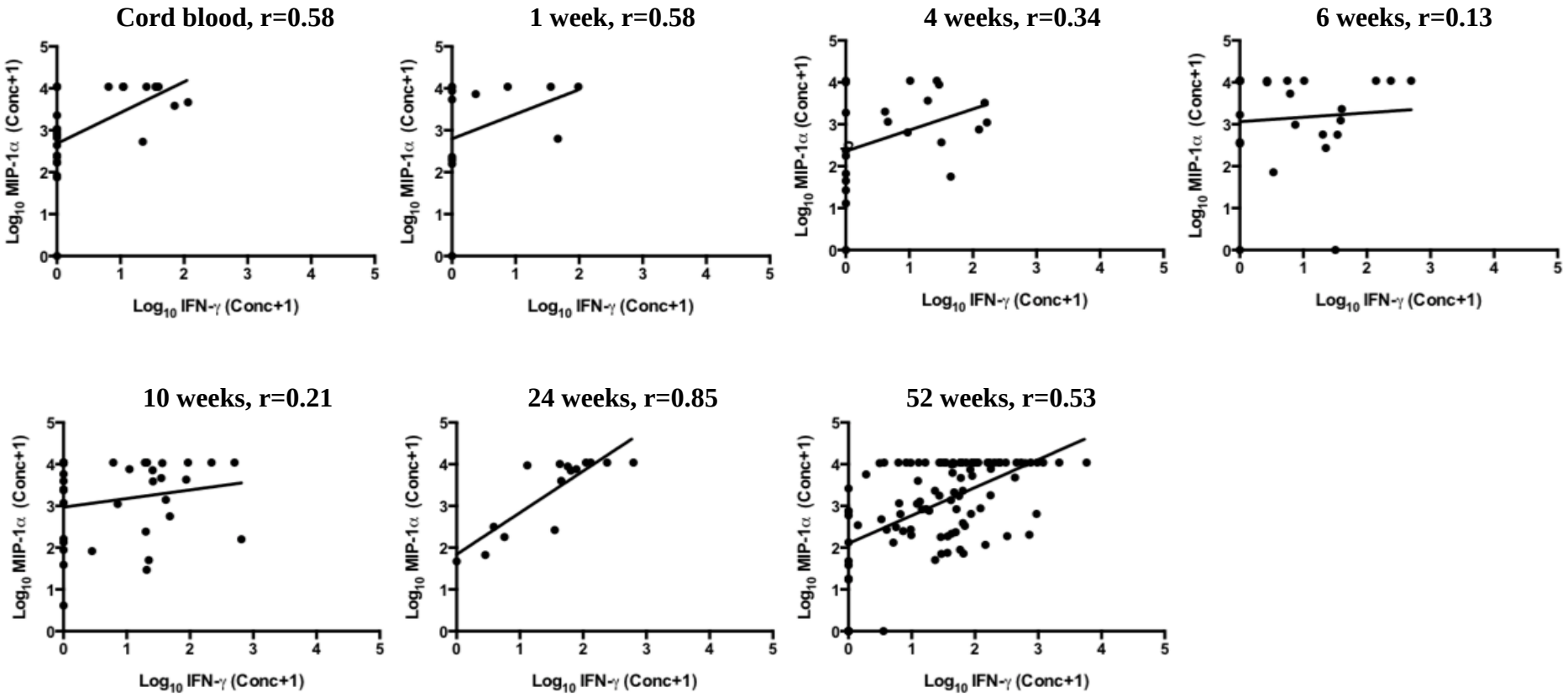
Correlations between ESAT-6/CFP10-specific IFN-γ and IL-1α. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



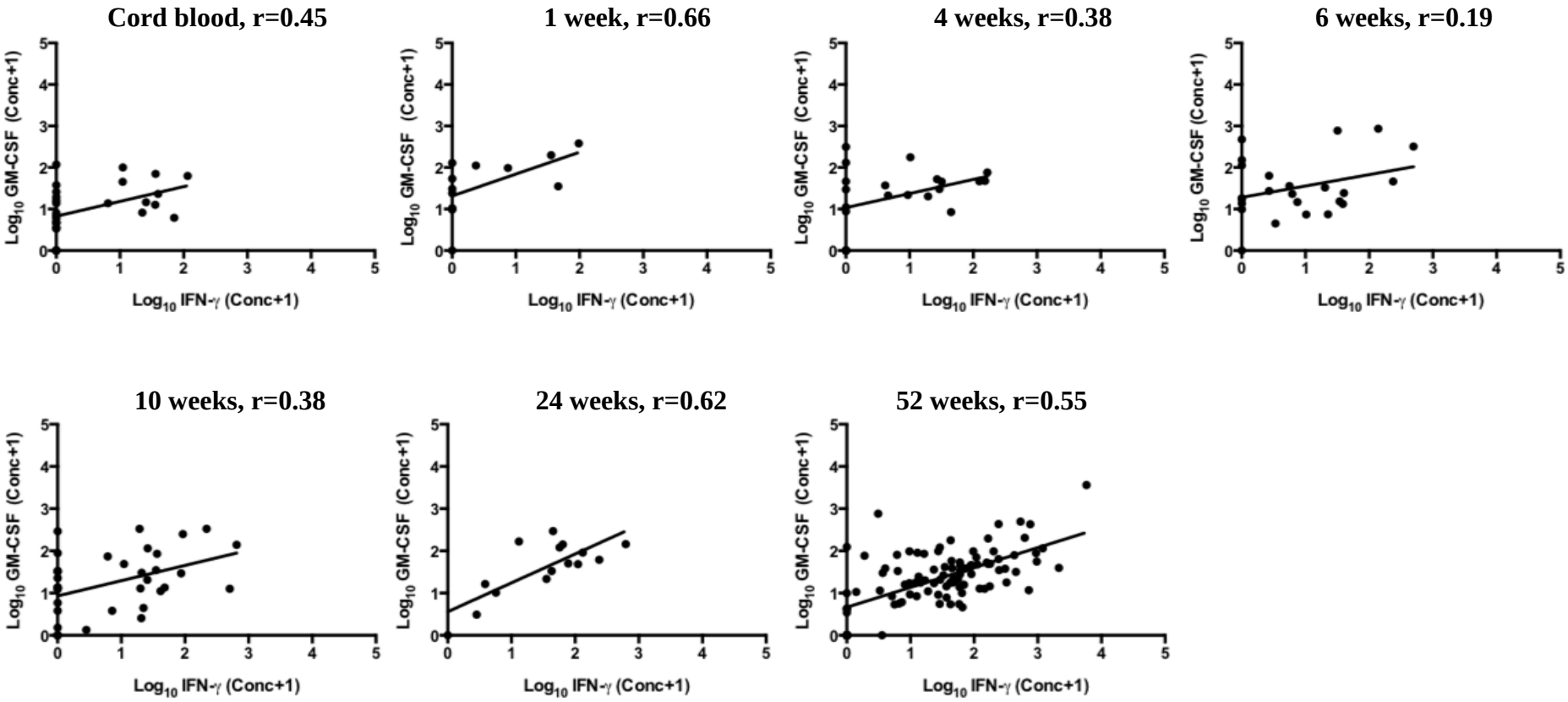
Correlations between ESAT-6/CFP10-specific IFN-γ and IL-12p40. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



Correlations between ESAT-6/CFP10-specific IFN-γ and IP-10. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



Correlations between ESAT-6/CFP10-specific IFN-γ and MIP-1α. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.



Correlations between ESAT-6/CFP10-specific IFN-γ and GM-CSF. Cytokines and chemokines were measured in supernatants after 6-day stimulation of cord blood, and infant blood obtained at 1, 4, 6, 10, 24 and 52 weeks after birth using a 17-plex Luminex® assay.