The influence of maternal HIV and *Mycobacterium tuberculosis* infection on infant immune responses to childhood vaccinations

Dr Christine Elizabeth Jones
Imperial College London, Department of Medicine

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Declaration

I declare that all of the work presented in this thesis is my own, except where I have referenced the work of others.

Dr Christine Jones
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Abstract

Background: Altered immune responses might contribute to the high morbidity and mortality observed amongst uninfected infants exposed to human immunodeficiency virus-1 (HIV) *in utero.* This study examined the influence of maternal HIV and *Mycobacterium tuberculosis* (*Mtb*) infection on infant immune responses to immunisation.

Methods: 109 mother-infant pairs were enrolled from Khayelitsha, Cape Town, South Africa, and were followed for four months. Peripheral blood samples were collected from mother-infant pairs at delivery and from infants at 16 weeks of age, following routine immunisations. Responses to BCG antigens were measured using multi-parameter flow cytometry and multiplex enzyme-linked immunosorbent assays (ELISAs). Specific antibody levels to *Haemophilus influenzae* type B (Hib), pneumococcus, *Bordetella pertussis*, tetanus toxoid and hepatitis B surface antigen were determined by ELISA.

Results: At birth, HIV-exposed, uninfected infants had increased frequencies of proliferating T cells expressing TNF-α and increased levels of TNF-α protein in cell culture supernatants; levels were highest amongst HIV-exposed infants born to *Mtb* sensitised mothers. IFN-γ levels were lower amongst HIV-exposed, uninfected infants compared to unexposed infants. Maternal *Mtb* sensitisation was associated with increased infant IFN-γ levels at birth; infants born to HIV-infected, *Mtb*-sensitised mothers had similar levels IFN-γ compared to unexposed infants.
Following BCG vaccination at 6 weeks of age, the immune response to infant BCG vaccination was unaffected by maternal HIV infection or *Mtb* sensitisation.
Amongst mothers, *Mtb* sensitisation significantly influenced the response to BCG-antigens in HIV-infected but not in HIV-uninfected mothers.
HIV-exposed, uninfected infants had lower specific antibody responses compared with unexposed infants at birth, but had robust responses following immunisation. Deficits in humoral protection against vaccine-preventable diseases were observed amongst HIV infected and HIV-uninfected women.

Conclusions: Antenatal HIV exposure was associated with alterations in immune response to vaccine antigens at birth, however HIV-exposed infants had comparable potential to respond to immunisation.
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Dedicated to Chris, Lucy and Sophie

Contents

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Abbreviations

Ag:	Antigen
AIDS:	Acquired Immunodeficiency Syndrome
BCG:	Bacille Calmette-Guérin
B. pertussis:	*Bordetella pertussis*
CFP:	Culture filtrate protein
CFU:	Colony forming units
CI:	Confidence interval
CMV:	Cytomegalovirus
DRC:	Democratic Republic of Congo
DTP:	Diptheria, Tetanus, Pertussis Vaccine
EGF:	Epidermal Growth Factor
ELISA:	Enzyme linked immunosorbant assay
EPI:	Expanded Programme of Immunisation
GBS:	Group B Streptococcus
G-CSF:	Granulocyte Colony Stimulating Factor
GM-CSF:	Granulocyte-macrophage Colony Stimulating Factor
HAART:	Highly active anti-retroviral therapy
HBsAg:	Hepatitis B surface antigen
HBV:	Hepatitis B
Hib	*Haemophilus influenzae* type B
HIV:	Human Immunodeficiency Virus-1
IFN-γ:	Interferon gamma
IgG:	Immunoglobulin G
IGRA:	Interferon-γ release assay
IL:	Interleukin
IL-1Ra:	Interleukin 1 Receptor Antagonist
IP-10	Inducible Protein 10
IRIS:	Immune reconstitution inflammatory syndrome
IU:	International units
LBW:	Low birth weight
LPA:	Lymphoproliferation assay
LRTI:	Lower respiratory tract infection
M. bovis	*Mycobacterium bovis*
MCP-1:	Monocyte Chemoattractant Protein-1
MDC:	Monocyte Derived Chemokine
MDR:	Multi-drug resistant

MIP-1α	Macrophage Inhibitory Protein-1 α
MIP-1β	Macrophage Inhibitory Protein-1 β
Mtb:	*Mycobacterium tuberculosis*
Ml:	Millilitre
MOU:	Maternal Obstetric Unit
MTCT:	Mother-to-child transmission
NK cells:	Natural Killer cells
NTM:	Non-tuberculous mycobacteria
PBMC:	Peripheral blood mononuclear cells
PMA:	Phorbol 12-myristate 13-acetate
PCP:	Pneumococcal capsular polysaccharide
PCR:	Polymerase chain reaction
PCV:	Pneumococcal conjugate vaccine
PHA:	Phytohaemagglutinin
PMT:	Photomultiplier tubes
PMTCT:	Prevention of Mother to Child Transmission
QFN:	QuantiFERON-TB Gold In-Tube
RR:	Relative risk
SATVI:	South African Tuberculosis Vaccine Initiative
sCD40L:	Soluble CD40 ligand
SEB:	Staphylococcal enterotoxin B
TB:	Tuberculosis
Th1:	Type 1 helper T cell
Th2:	Type 2 helper T cell
TMB:	Tetramethylbenzidine
TNF:	Tumour necrosis factor
TREC:	T-cell receptor excision circles
TST:	Tuberculin skin test
TT:	Tetanus toxoid
Vs:	Versus
VEGR:	Vascular Endothelial Growth Factor
WBC:	White blood cell
WHO:	World Health Organisation

Introduction

Chapter 1: Introduction

1.1 Child survival and immunisation

Despite some progress in recent years, nearly 8.8 million children under the age of 5 years continue to die each year.[1] Infectious diseases account for a large proportion of these deaths, with pneumonia, diarrhoea and malaria dominating the causes of mortality, Figure 1.[1] Immunisation is an important strategy to reduce the burden of preventable deaths. However, in countries where vaccine-coverage is poor, vaccine-preventable diseases continue to ravage young children. In 2008 there were 10,800 pertussis, 1,020 tetanus and 4,000 measles related deaths in the Democratic Republic of Congo (DRC) alone.[2]

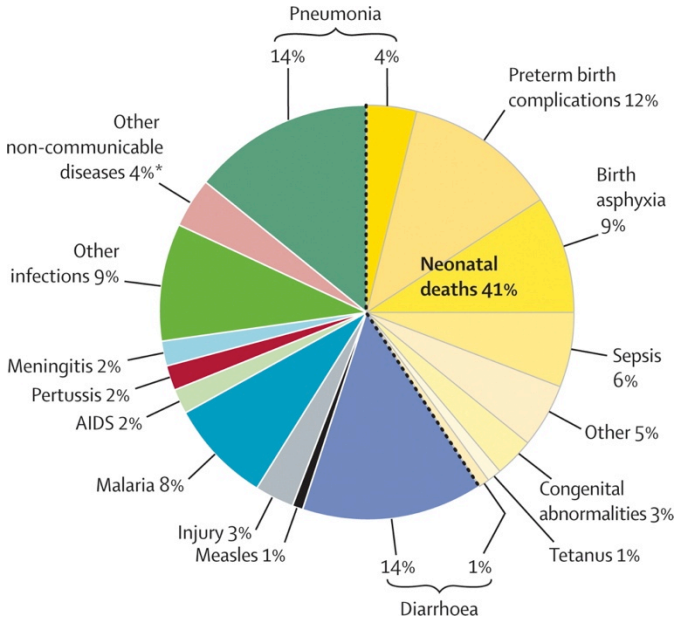


Figure 1. Causes of the 8.8 million child deaths globally.
Adapted from Black, 2010[1]

The availability of vaccines against diseases caused by pneumococcus and rotavirus affords us greater opportunity to impact child survival. It is vital that we continue to improve on the positive trends in vaccine coverage to give each child the opportunity to benefit from these life-saving interventions.[3]

It is important to understand factors that might alter the response to vaccination, such as Human Immunodeficiency virus-1 (HIV). Increasingly, it is recognized that HIV-uninfected

infants born to HIV-infected mothers represent a vulnerable groups of infants with increased rates of morbidity and mortality. However, the impact of HIV-exposure on the response to vaccination is incompletely studied. Bacille Calmette-Guérin (BCG) effectively prevents disseminated tuberculosis (TB) in children, but the protection against pulmonary TB is poor. Understanding immune responses to BCG is important in the development of new anti-TB vaccines. Due to the documented immunological changes in HIV-exposed, uninfected infants the response to vaccination might be altered. Other maternal infections can also prime infant immune response to related and unrelated antigens. There is compelling evidence from mouse models that mycobacterial antigens can be transferred across the placenta and affect cellular immune responses of offspring mice.

The mother-infant study established for this PhD thesis will examine the hypothesis that maternal HIV infection influences infant responses to vaccination, particularly focusing on responses to BCG vaccination. It will also investigate of the role of maternal Mycobacterium tuberculosis (*Mtb*) sensitisation in determining infant responses to BCG vaccination.

1.2 HIV pandemic

There are 33 million people living with HIV worldwide, with Sub-Saharan Africa bearing a disproportionate share of the global burden; two thirds of people with HIV infection live in this region (Data extracted from UNAIDS Report on the Global AIDS epidemic, 2010, unless otherwise indicated[4]).

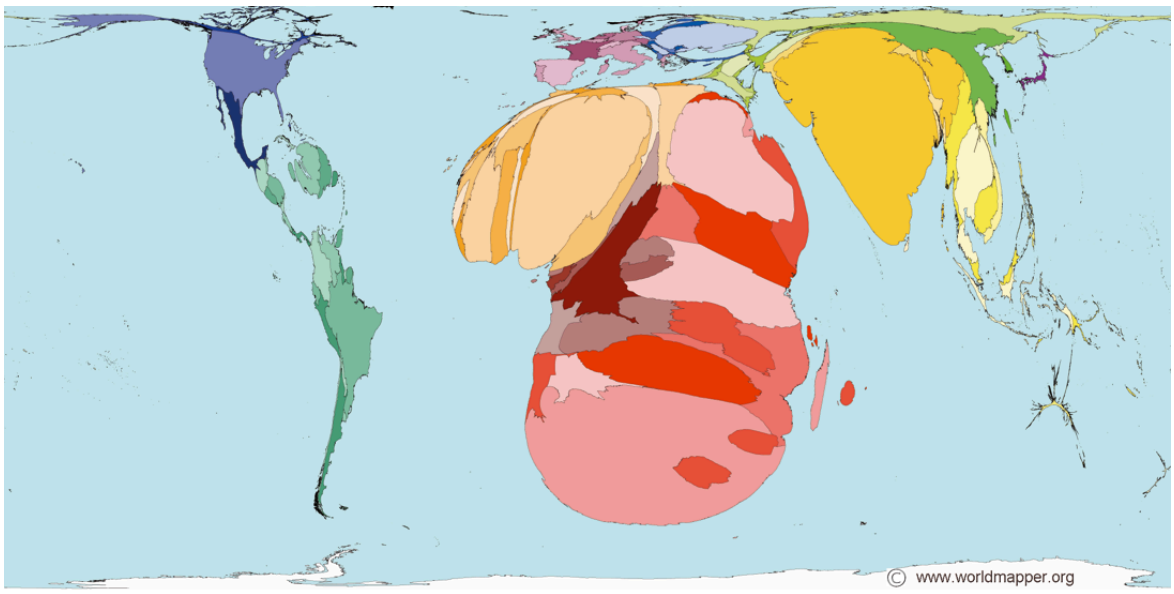


Figure 2: Worldwide HIV prevalence.
Territory size represents the proportion of all people living with HIV aged 15-49 years living there.

Against this bleak backdrop, there are reasons for optimism; the incidence of HIV infection has fallen by more than 25% in 33 high-burden countries over the last 10 years and more than 5 million people are now receiving anti-retroviral treatment. This represents a significant achievement, but the challenge remains to provide access to treatment for the remaining 10 million people eligible for treatment. The challenges these countries face are daunting, not least in South Africa, where 5.6 million people are estimated to be living with HIV.

Women of childbearing age and young children represent a population disproportionately affected by HIV. Women aged 15-24 years are up to 8 times more likely to be HIV-infected compared to age-matched men. As a result, up to one third of women attending public health antenatal clinics in South Africa test HIV positive.[5] The tremendous success of Prevention of Mother to Child Transmission (PMTCT) programmes mean that the vast majority of infants will escape HIV infection themselves. However, even in the absence of infection, children are far from bystanders to the harm inflicted by HIV; globally there are nearly 17 million children who have been orphaned as a result of HIV/AIDS, 90% of whom live in Sub-Saharan Africa.

In 2009, UNAIDS called for the virtual elimination of mother-to-child transmission (MTCT) of HIV infection by 2015. This is potentially realistic in the 10 most severely affected countries; we just need to effectively implement strategies that have been shown to be feasible and effective. Botswana, Namibia, South Africa and Swaziland have achieved more than 80% coverage of antiretroviral medication to prevent MTCT, however in West and Central Africa coverage is only 23%. Effective implementation of programs to prevent MTCT (PMTCT) means that rates of vertical HIV transmission have been lowered to 2.5% in some areas of South Africa.[6] Since 2001, a 25% reduction in the global number of children infected with HIV has been achieved, however, an unacceptable 370 000 children still contracted HIV infection in 2009.

The health and life-prolonging effect of HIV treatment means that there are more women living with HIV who are becoming pregnant. Combined with increased PMTCT coverage, this has led to an increasing number of infants who are exposed *in utero* to HIV infection who do not acquire HIV infection themselves.[7]

1.2.1 Evidence of HIV exposure in uninfected infants

A number of studies have shown that a proportion of healthy, HIV-exposed, uninfected infants have HIV-specific immune responses at birth, showing that exposure to HIV *in utero* has indeed occurred.[8] High frequencies of HIV-specific $CD4^+$ cells are detected in a high proportion of newborns and lower frequencies of $CD8^+$ T cells are detected in a minority, suggesting that transplacental diffusion of HIV-soluble proteins has primed the immune response rather than live virus. These cells have been detected 6 months after birth and become undetectable sometime before the age of 7 years. It is thought that these responses may represent a protective response against primary infection, however, they may simply be a marker of exposure.[9]

1.3 HIV-exposed, uninfected infants and children

A convincing body of evidence shows that HIV-exposed, uninfected infants and children are not unaffected by HIV. They have increased rates of morbidity and mortality, at least in some settings, and multiple immunological changes have been demonstrated.

Evidence of increased mortality rates

HIV-exposed, uninfected infants and children have escaped the high mortality associated with HIV infection, however a number of studies have shown that they are more vulnerable than children not exposed to HIV.

A large prospective study in Zimbabwe enrolling over 3,000 HIV-exposed infants showed that HIV-exposed, uninfected infants were 3.9 times and 2.0 times more likely to die in the first and second year of life respectively compared to unexposed infants. This risk remained significant in sensitivity analyses after the exclusion of infants who tested negative at baseline, but who died before further testing was performed.[10] In line with this, a smaller study in Botswana also found that mortality was more than 4 times higher amongst HIV-exposed infants compared to unexposed infants at 6 months and at 2 years of age.[11] In rural Uganda, the mortality was more than twice as high at 2 years of age.[12] Studies in Uganda and Rwanda found greater than 3 fold increased risk of mortality amongst all HIV-exposed infants (included infected and uninfected infants).[13,14]

Studies examining mortality rates in early infancy have found no difference in mortality up to the age of 6 months in Cameroon and Rwanda,[13,15] whilst others from Rwanda and The

Gambia have shown no difference in mortality rates into later childhood.[16,17] Other studies have confirmed high rates of mortality in HIV-exposed, uninfected infants, however these have not included a comparison group of unexposed infants and children.[18,19]

The causes of death in African HIV-exposed, uninfected infants are similar to those in the population as a whole namely, pneumonia, gastroenteritis, malnutrition and malaria.[1,20]

Advanced maternal immunosuppression places HIV-exposed infants at even higher risk of death in early childhood, independent of maternal death, low birth weight or lack of breast feeding; the mortality rate is more than double once the maternal CD4 count is <350cells/mm^3.[10,18,20-22] Conversely, maternal anti-retroviral treatment significantly reduces the infant mortality rate.[23]

Whilst there is an excess risk of death in HIV-exposed, uninfected infants, this should be seen within the context of the survival rate of HIV-infected infants in the absence of treatment, illustrated in Figure 3. The median survival of vertically infected children in the absence of HIV treatment is only 2-3 years.[10,17,18]

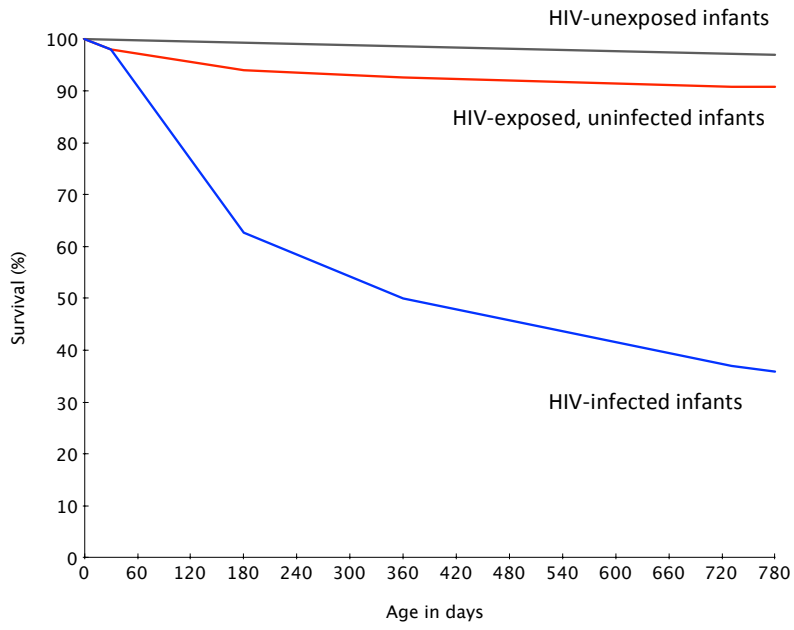


Figure 3. Estimated infant mortality according to HIV status of infant.
Adapted from Marinda et al.[10]

Morbidity

Illness may be more common and more severe in HIV-exposed, uninfected infants. In a large prospective Zimbabwean study, HIV-exposed infants had a 30% increased rate of attendance

at primary healthcare facilities for illness, which was further increased in the context of advanced maternal immunosuppression.[24]

The incidence of specific infections such as lower respiratory tract infections (LRTI) or oral thrush is higher, whereas the frequency of acute diarrhoeal illnesses is similar.[11,24,25] Persistent diarrhoea (greater than 14 days duration) was increased amongst HIV-exposed, uninfected infants in an early study in Zaire (DRC), although this was not statistically significant.[25]

HIV-exposed, uninfected infants not only have an increased rate of illness, but the severity of illness also appears to be higher leading to more frequent hospital admissions. [11,24,26] Amongst children admitted to a large teaching hospital in South Africa with severe pneumonia, HIV-exposed children were more likely to fail to respond to standard treatment after 48 hours of treatment than HIV-unexposed children and were more likely to require admission to intensive care.[28] In children with acute malaria, HIV-exposure is associated with the incidence of acute anaemia, a marker of life-threatening malaria.[34]

A prospective study in a surgical tertiary referral centre in South Africa is the first study to describe a higher rate of surgical complications in HIV-exposed, uninfected infants.[35] These infants more frequently had wound infections and systemic post-operative infections. They had higher in-hospital mortality and those who survived had a longer hospital stay.

The only study to date showing an increased susceptibility to infection in high-income countries was a study in a tertiary referral centre in Belgium.[36] They found that the relative risk (RR) of invasive Group B Streptococcus (GBS, isolated from blood or CSF in infants <90 days) was 19 times higher in HIV-exposed, uninfected infants compared to HIV-unexposed infants (1.55% of HIV-exposed, uninfected infants compared to 0.08% in HIV-unexposed infants). The incidence of late onset disease was significantly higher (1.24% vs. 0.01%, RR 125.2, $p < .0001$) In addition, HIV-exposed, uninfected infants had more severe disease, 5 out of 6 infants presented with sepsis or septic shock, compared to 10 out of 16 controls.

Increased rates of non-infectious morbidity have also been shown. A multi-site study in the United States of America and Puerto Rico showed that hearing loss is more common

amongst HIV-exposed, uninfected children compared to unexposed children, but less common that that seen in HIV-infected children.[37]

Other studies without a HIV-unexposed comparison group of infants confirm high rates of severe infectious morbidity and hospitalization, above what would be expected in the general population. In an Indian study, one third of HIV-exposed, uninfected infants were admitted to hospital in infancy, mainly attributed to infectious morbidity such as gastroenteritis, pneumonia, sepsis or meningitis.[19] In a Latin American and Caribbean study, 60% of infants had one or more infections within the first year, the majority of which were LRTI, skin and mucus membrane infections and sepsis. Nearly 20% of illness led to a hospital admission. Advanced maternal disease was associated with neonatal infections and post-neonatal infections. [33,38,39]

A small descriptive case series of 8 HIV-exposed, uninfected children from a tertiary referral centre in South Africa illustrates that HIV-exposed infants can present with unusual and severe infections more akin to those seen in severely malnourished or immunocompromised children. Even in the absence of HIV infection or other defined immunodeficiency, children presented with severe infections such as *Pneumocystis jiroveci,* the cause of *Pneumocystis* pneumonia, cytomegalovirus (CMV), *Klebsiella pneumoniae*, *Pseudomonas Aeruginosa*, *Acinetobacter baumannii* and varicella.[40] Others have also reported unusual infections such as *Pneumocystis jiroveci* or CMV in these infants[28,41,42]

Studies from Sub-Saharan Africa that have shown little or no difference in morbidity have tended to be studies with smaller numbers of participants, some of which demonstrated similar trends in absence of statistical significance.[16,25,43]

Growth

A large study has revealed differences in the birth weight of HIV-exposed, uninfected infants, however the absolute difference was small (only 6g). The proportion of low birth weight infants (LBW, birth weight <2.5kg) was significantly higher in uninfected infants born to HIV-infected compared to HIV-uninfected mothers, however again, the absolute difference was only small (3%).[10] A study from DRC found that whilst the birth weight of HIV-exposed, uninfected infants was lower, their subsequent growth was similar to the general population.[44]

Other African studies have not shown any effect of HIV-exposure in uninfected infants on either birth weight or growth.[43,45] A large prospective European study also showed that HIV-exposed, uninfected infants have normal patterns of growth throughout childhood, with no differences between children born before or after the availability of anti-retroviral treatment. A possible reason for the lack of difference in growth, that HIV-infection may be associated with less social disadvantage in the European setting.[46]

Although differences in growth have seen observed in some studies, it is unclear whether this has an adverse impact on health outcomes.

Taken together, HIV-exposed infants have specific vulnerabilities, at least in settings where the background rate of infectious morbidity and mortality is high. Advanced maternal disease further accentuates these risks. Even if *in utero* exposure to HIV in the absence of infant infection increases the absolute risk of death or illness by a small amount, the total number of infants affected by this excess risk is substantial, representing a major public health problem that cannot be disregarded.

1.3.1 Reasons for an increased morbidity and mortality in HIV-exposed, uninfected infants

Despite the demonstration of increased vulnerability of HIV-exposed, uninfected infants from the early 1990s, the causes of the disproportionate morbidity and mortality have still not been elucidated. A number of factors may be important, however it is most likely that no one individual reason can explain the whole picture; rather each factor may be just one piece of the 'puzzle', Figure 4.

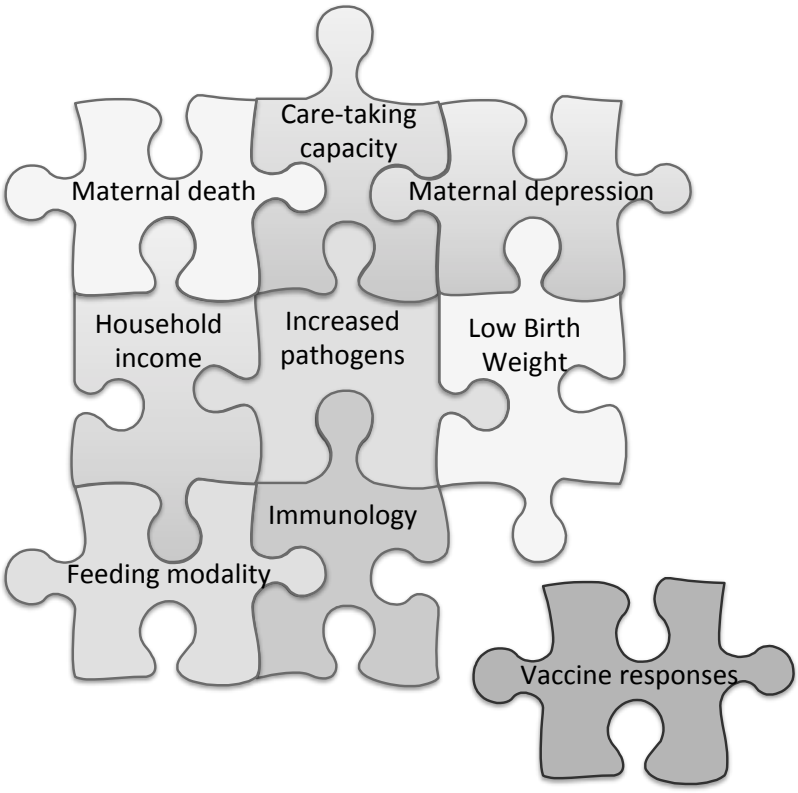


Figure 4. Individual factors which may contribute to the overall picture of vulnerability of uninfected HIV-exposed infants.

Maternal death

HIV-exposed infants are at higher risk of losing their mother or father in childhood. Death of the mother in particular is associated with an increased rate of infant mortality in HIV-exposed, uninfected infants, this also an important prognostic factor irrespective of HIV-exposure.[10,14,18,20,47] Maternal death is also associated with a two fold increased risk of acute diarrhoea and a ten fold increased risk of persistent diarrhoea.[25]

Care-taking capacity

A mother living with HIV may have less ability to care for her child due to her own ill health, particularly when the child is ill too. Whilst there is data that supports this intuitive assumption, a causal link is far from established. Uninfected infants have an increased number of episodes of acute diarrhoea if the mother has advanced HIV infection compared to mothers with lower levels of immunosuppression, this may be related to her capacity to maintain infant hygiene and nutrition in the face of illness.[25] Coverage of childhood vaccines was lower amongst children born to HIV-infected mothers in one study from rural South

Africa, this effect remained after controlling for other significant factors including distance to clinic and level of poverty.[48]

Maternal depression

Maternal depression affects growth and health outcomes of infants and children. High rates of depression are observed in individuals living with HIV, this is likely to be further accentuated for women first diagnosed with HIV in pregnancy.[49,50] Moreover, high levels of maternal depression have been found in this study specific setting.[51] Maternal depression is associated with poor mother-infant interactions, disturbance of cognitive and emotional development, poor infant growth and diarrhoeal illness.[51-55]

Household income

Household income may be lower in families affected by HIV for a myriad of reasons including diversion of funds to pay for medical care, loss of an economically active parent or lost days of paid work due to illness. Lower household wealth was significantly associated with mortality among HIV-exposed, uninfected infants, but was not a risk factor in unexposed infants in a Zimbabwean study.[10] Self-report of food-insecurity is associated with a two-fold increase in mortality before the age of 2 years in uninfected infants born to HIV-infected mothers.[20]

Increased exposure to other pathogens

There may be increased exposure to and transmission of pathogens from HIV-infected family members to infants. Increased rates of symptomatic disease may lead to increased pathogen shedding. HIV-infected adults are more likely to be persistently colonised with *Streptococcus pneumoniae* and these isolates are more likely to be antibiotic-resistant.[56] HIV-infected women have higher carriage rates of GBS, which may be the cause of the high rates of disease in exposed infants.[36] Vaginal candiasis is more prevalent amongst women living with HIV and is more likely to be persistent.[57] The odds of vaginal candidiasis is further increased in women with high viral loads, however combination anti-retroviral treatment does not seem to negate the effect of HIV on vaginal colonisation.[57]

HIV-exposed infants have high rates of exposure to *Mtb*. A study in India observed a high incidence of postpartum TB in HIV-infected women and this was associated with an increased all-cause mortality rate in their exposed, uninfected infants, even after adjustment of multiple other factors.[58] Extremely high rates of TB exposure amongst HIV-exposed (infected and uninfected) infants have been also been observed in a high-TB burden setting

in South Africa. The authors estimated that this would lead to disease rates of 2005/100 000 in this setting.[59]

It has been demonstrated that HIV-exposed, uninfected infants can present with severe respiratory illness due to *Pneumocystis* pneumonia, this pathogen is likely to be acquired from a household member who has HIV-infection. The provision of trimethoprim/sulfamethoxazole prophylaxis to prevent *Pneumocystis* pneumonia in HIV-exposed, uninfected infants was significantly associated with a lower frequency of all-cause illness and or hospital admission, suggesting that the effect of exposure to pathogenic organisms can be mitigated.[60]

There may be increased household transmission of multi-drug resistant (MDR) organisms, including MDR TB, as these are more common in the context of immunosuppression and multiple courses of treatments for infections.

Low birth weight

Low birth weight is associated with an increased risk of death in infants born to HIV-infected and HIV-uninfected women.[10,61] HIV-exposed infants were more likely to be born small-for-gestational age and to be of low birth weight (<2500g) in two South African studies. [45,62] Rates of preterm birth were similar in one of these studies and lower in the other amongst HIV-exposed infants in the absence of maternal combination antiretroviral treatment.

Feeding modality

Replacement feeding may be a factor in populations where the background rate of morbidity and mortality associated with enteric pathogens is high and where provision of safe replacement feeding is limited. Replacement feeding or early cessation of breast-feeding is associated with significantly increased morbidity and mortality rates into the second year of life in HIV-exposed, uninfected African infants, even taking into account the potential for post-natal transmission of HIV and provision of safe replacement feeding. [20,60,63] In one large study in Zambia, mortality amongst HIV-exposed, uninfected children <2 years of age was 17.4% if they were weaned at 5 months compared to 9.7% in children breast–fed beyond 18 months.[20] This was in the context of provision of 'safe' replacement feeding and nutritional interventions. The protective effect of breast-feeding was greatest amongst more immunosuppressed mothers.[20] However, other studies have demonstrated similar mortality rates amongst breast fed and replacement fed HIV-exposed infants.[64]

The omission of breast feeding can not be the only factor at work as the mortality rate remains higher amongst HIV-exposed, uninfected infants compared to breast-fed, unexposed infants even when all in both groups are exclusively breast-fed.[11] Deficiencies in breast milk of HIV-infected mothers are also unlikely be a significant factor; breast milk innate and pathogen-specific immune factors are similar in HIV-infected and uninfected women. [11]

Maternal combination antiretroviral treatment is an important intervention to improve maternal and infant health. The reduction in maternal morbidity and mortality is expected to lead to better outcomes for the infant, but it may not correct all vulnerability factors. Socio-economic factors are likely to be very important, however other factors also exist. Exposed-infants have multiple immunological changes including T-cell subsets, cytokine and chemokine production and maturation factors.

1.3.2 Immunological changes associated with HIV exposure in uninfected infants

Altered T-cell subsets

A number of studies have observed lower $CD4^+$ and higher $CD8^+$ T lymphocytes amongst HIV-exposed, uninfected infants compared to unexposed infants, these changes are lower in magnitude, but of a similar pattern to HIV-infected infants. [27,29-33] $CD4^+$:$CD8^+$ T cell ratio is also reduced, however all these changes appear to be transient and values after 1 year of age are similar to unexposed infants. [27,29-33] Others have found similar frequencies of T cells.[65]

Effects on other cell types

Numbers of B cells and NK cells are similar at birth in most studies, however NK cells may be increased by 12 months of age in HIV-exposed, uninfected infants.[27,30,65] It is postulated that NK cells may have a role in protection against HIV infection in HIV-exposed infants.[65] One study found an increase in $CD19^+CD5^+$ B cells in cord blood of HIV-exposed infants, with differences in the initial maturation of B cells, this may have been influenced by maternal combination antiretroviral treatment and hard drug use.[33]

Quantitative and functional changes in myeloid dendritic cells have been demonstrated in HIV-exposed infants, which could potentially play a role in increased T cell activation.[66]

T cell maturation

T lymphocytes of HIV-exposed, uninfected infants show phenotypic differences compared to HIV-unexposed infants; the immunophenotype is more similar to that observed in HIV-infected infants and children.[27,29,30,65,67] Naïve $CD4^+$ and $CD8^+$ T cells are reduced, whilst memory cells are increased in the neonatal period[27,30,31,65]. Decreased numbers of naïve $CD4^+$ T cells have been recorded even in children aged 7 years, whilst an increase in naïve $CD8^+$ T cells is still evident at 12 months of age.[27,65] Naïve lymphocytes are normally markedly increased whilst memory lymphocytes are significantly reduced in infants compared to adults.[31] The premature shift from naïve to memory cells and immune activation in HIV-exposed, uninfected infants suggests antigenic experience and is observed independently of maternal combination antiretroviral treatment during pregnancy.[27,31,65]

T cell progenitor cells mature and differentiate within the thymus. Naïve $CD4^+$ and $CD8^+$ leave the thymus via the circulation and migrate to peripheral lymphoid tissues. The output of naïve T lymphocytes from the thymus may be reduced in HIV-exposed, uninfected infants, similar to the reduction seen in HIV-infected children. Nielsen et al have demonstrated that the frequency of thymic T-cell receptor excision circles (TREC) is reduced, which suggests reduced thymic output or increased consumption.[30] The reduced thymic function may be caused by an impairment of T cell progenitor function; the total number of progenitor cells is the same, but they display decreased cloning efficacy and reduced ability to differentiate into T cells in thymic organ cultures.[30] This is postulated to account for the decreased $CD4^+$ and $CD8^+$ counts described above.

An increase in interleukin-7 (IL-7) has also been observed around birth in one study, but not subsequent studies.[9,27,33,65] IL-7 is implicated in thymopoiesis and maturation of T cells.

T cell activation

T lymphocytes of HIV-exposed, uninfected infants and children are more activated. An upregulation of CD38 expression has been observed on $CD4^+$ and $CD8^+$ T cells.[27,31,65] $CD8^+$ $CD38^{bright}$ cells were substantially increased in an early study, over 13 times higher in HIV-exposed, uninfected infants compared to unexposed infants and were still increased 3 fold in older HIV-exposed, uninfected children.[27] Soluble markers of immune activation are also higher. Schramm and colleagues observed increased levels of neopterin (reflecting activation of T cells and monocytes) in all HIV-exposed, uninfected infants and increased β_2-m (reflecting activation of $CD8^+$ T cells), SL-selectin (reflecting activation of T cells,

monocytes and neutrophils) only in uninfected infants who lacked HIV-specific T cell responses.[68] GM-CSF was lower only in exposed, uninfected infants who had HIV-specific T cell responses. Expression of early markers of activation, CD25 and CD69, were similar in another study.[30]

Economides and colleagues have shown that cord blood $CD4^+$ and $CD8^+$ T cells from HIV-exposed, uninfected infants behave differently in culture compared to unexposed infants, this is thought to be related to increased cellular activation.[69] Using live/dead stain discriminatory dyes, apoptosis is increased in HIV-exposed, uninfected infants following overnight culture, but is not increased in unexposed infants, leading to a significant difference in the proportion of apoptopic cells between groups of infants after culture, but not directly ex vivo.[69]

Cytokine responses

A number of studies have inconsistently described perturbation of cytokine responses in HIV-exposed, uninfected infants.[9,30,68,70-73] One study observed a lower IL-12p70 response to S*taphylococcus aureus* Cowan in cord blood from HIV-exposed, uninfected infants compared to unexposed infants, this persisted into early infancy, however, this was not confirmed in a subsequent study.[33,70] IL-2 response to phytohaemagglutinin (PHA) was reduced amongst HIV-exposed uninfected infants in one, but not in two other later studies.[30,31,33] Although very low background levels of interferon-gamma (IFN-γ) are detected at birth, one study found a statistically higher levels of IFN-γ in HIV-exposed, uninfected infants.[33,73] Similarly, a proportion of HIV-exposed infants had higher IFN-γ levels in response to PHA and BCG, by 6 weeks this response was similar.[33,73] Others have either observed lower background IFN-γ responses or no difference.[30,74] TNF-α responses seem to be consistently similar.[33,70]

HIV-exposed, uninfected infants may have higher IL-10 levels in cord blood in response to PHA, however this has not been confirmed by other studies.[33,68,70,74] IL-4 was lower in background, PHA and BCG stimulated samples, but similar in another study.[30,33]

Reduced transplacental transfer of maternal antibody

Maternal HIV is associated with reduced transplacental transfer of antibodies.[75] A reduction in passively acquired humoral immunity may play a role in the increased rates of illness seen amongst HIV-exposed infants. This factor will be explored in more detail in section 1.7.2.

Although to date there is insufficient explanation for these immunological observations, these findings suggest that *in utero* exposure to HIV or HIV soluble products has occurred in these infants and that this causes maturation and activation of the infant's immune system without causing infection. However, it is also possible that these maturation abnormalities may be partly explained by exposure to co-infections *in utero*. It is not known whether these findings translate into longer-term immunological disadvantage or how these might influence the infant's ability to respond to vaccination or natural infection.

1.3.3 Vaccine responses

Measurement of vaccine responses represents one approach to test the function of the immune system following controlled antigenic exposure and may allow for a deeper understanding of how immunological perturbations translate into clinical outcomes. However, this has not been well studied in HIV-exposed infants. Analysing responses to BCG vaccination may be particularly important to understand the functional relevance of HIV exposure on $CD4^+$ cells. This is particularly valid in the light of high exposure to *Mtb* HIV-exposed infants.[59] Given BCG is widely administered in countries were HIV prevalence is high, this deserves further study and represents a focus of the work presented in this thesis (see section 1.5.7).

In addition the impact of maternal HIV infection on maternal and infant responses to other vaccines has not been fully studied, section 1.7.2.

1.4 Pre-natal sensitisation of infant T cells

It has been demonstrated that uninfected infants born to HIV-infected mothers have evidence of immunological priming to HIV-specific antigens at birth, section 1.2.1. Other evidence that pre-natal sensitisation does occur and that it can have a long-term impact on immunological memory of the infant comes from maternal infections, diseases and immunisations. Maternal malaria, helminth infection, allergy, influenza vaccination and tetanus vaccination of pregnant women have all been shown to affect the infant.[76-81]

There is also evidence that pre-natal sensitisation to mycobacterial antigens can occur in the mouse model. An elegant study has shown that offspring of mice who have been immunised with mycobacterial antigens in pregnancy have increased post-natal cellular responses to

mycobacterial antigens and are more efficient at controlling infection.[82] In this study, BALB/c mice (albino, laboratory in-bred mice) received antigen (Ag) 85A subcutaneously in the 2nd week of gestation. Ag85A was delivered in the absence of adjuvant, such that a detectable IgG response was not induced. Offspring mice were then immunised intranasally with the same antigens along with adjuvant. Lung-derived lymphocyte antigen-specific IFN-γ recall responses measured a week later were higher in the offspring of the immunised mothers. There was no effect on antibody responses. Offspring of immunised mice had superior protection against challenge with high dose intranasal BCG. They clearly demonstrated that antigen was transported across the placenta to the offspring mice in two ways. Firstly, a group of infant mice were nursed by foster mothers, these mice had similar antigen-specific recall responses to those nursed by the immunised mother, showing that gestational priming was not affected by breast milk, but rather that priming must have occurred *in utero*. Secondly, fluorescent nanocrystals conjugated to Ag85A were delivered to the pregnant mice. Conjugates were demonstrated in placental tissue and in all mice foetuses.

A number of human studies have noted that whilst the majority of infants do not produce IFN-γ in response to mycobacterial antigens at birth, a proportion of infants have significantly higher levels of IFN-γ in response to these antigens.[73,83-87] It is possible that pre-natal sensitisation to mycobacteria might have occurred in these infants and 'primed' their immune system.

Elliott and colleagues observed a possible association between maternal BCG scarring and infant IFN-γ response to mycobacterial antigens following BCG vaccination, however, the confidence interval crossed 1.[88] One small study observed a relationship between the PPD status of the mother and the ability of cord blood lymphocytes to respond to PPD antigens, however, the influence of maternal *Mtb* infection on infant mycobacterial responses at birth and subsequent response to BCG vaccination has not been addressed.[81]

1.5 Bacille Calmette-Guérin Vaccine

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide with an estimated 9.27 million new cases and 1.8 million deaths in 2007.[89] South Africa has one of the highest incidence and prevalence of TB in the world with 948 new cases per 100,000 population per year and 692 cases per 100,000 population in 2006.[89]

Currently, the BCG vaccine is the only licensed vaccine against TB. BCG is a live, attenuated strain of *Mycobacterium bovis (M. bovis)*, which has been in existence since 1921. It has been integrated into the World Health Organization (WHO) Expanded Programme of Immunisation (EPI) since 1974 and is administered at birth or soon after birth in most countries of the world.[90] It is administered to over 100 million children worldwide every year, making it one of the most widely used of all vaccines.[91]

1.5.1 Protective efficacy

BCG is an effective and highly cost-efficient vaccine against disseminated forms of TB in childhood with 73% (95% confidence interval (CI): 67-79%) efficacy against tuberculous meningitis and 77% (95% CI: 58-87%) against miliary tuberculosis.[91] However, BCG has inconsistent efficacy against adult-type pulmonary disease. Estimates of protective efficacy range from 0-80% with an overall effect of 50%.[92,93] In these studies, protection was greater with increasing distance from the equator, thus the efficacy is lowest in countries where protection against TB is most critical. BCG has a limited impact on reducing transmission of adult sputum-positive TB and therefore has had a limited effect on the overall TB epidemic.

It was the previously held view that BCG provided protection up to 10 years after vaccination, however more recent studies have shown that a protective effect may last much longer.[94-96] In Brazil, neonatal BCG vaccination provided protection up to 10-15 years after vaccination and amongst American Indians and Alaskan Natives vaccine efficacy persisted for up to 60 years, however, the exact interval of protective efficacy has not been fully established.[95,96]

1.5.2 Reasons for variation in protective efficacy

Vaccine strain

There are a large number of genetically different vaccine strains of BCG vaccine currently in use throughout the world.[97] Calmette and Guérin originally developed BCG vaccine at the Pasteur Institute in Lille, France by the attenuation of *M. bovis* through repeated sub-culture. Vaccine seed lots were then distributed to laboratories all over the world where further sub-culture to preserve viable organisms led to the emergence of different vaccine strains with phenotypic and genomic diversity.[98,99] Between 1921 and the 1960s, when lyophilised seed lots were created, the vaccines had undergone around 1000 passages.

The considerable evolution of different strains of BCG may be one of the reasons why there is such marked heterogeneity in efficacy of the vaccine across the world. Four suppliers provide three vaccine strains for The United Nations Children's Fund (UNICEF) on behalf of the Global Alliance for Vaccine and Immunisation (GAVI). These three vaccine strains account for a significant proportion of BCG vaccines used.[97] A recent comparison of immune response to these vaccines found that polyfunctional $CD4^+$ T cells, IFN-γ^+TNF^+ CD4 T cells, CD-107^+ cytotoxic CD4 T cells and secreted type-1 cytokines were higher in infants immunised with BCG-Denmark (SSI 1331) or BCG-Japan than BCG-Russia.[100] Highest levels of IL-6, IL-10, MCP-1 and MIP-1β were found with BCG-Japan. In keeping with increased immunogenicity, infants had a larger local reaction to vaccination with BCG-Denmark or BCG-Japan than BCG-Russia. A number of other studies have also found a difference in immune response to different strains of BCG, however, the outcome measures were generally more restricted and there is no consensus as to which vaccine is superior.[101]

A difference in immunogenicity is corroborated by clinical studies that demonstrate variable efficacy afforded by different vaccine strains. These studies are extensively reviewed by Ritz et al.[101] Whilst it is clear that BCG strains are genetically divergent and are associated with variable immunogenicity and efficacy, the lack of clarity regarding superiority means that different strains remain in use throughout the world.

Even if absolute differences in protection are small, an increase of just 1% in protective efficacy could save 18,000 lives and prevent 83,000 cases of TB each year, therefore it is important to establish which of the vaccines strains are associated with the best protection in each country.[101] This vaccine may not be the same for every region of the world, as the same vaccine strain seems to perform differently in different countries. The existing data therefore suggests that vaccine strain alone does not fully explain variation in protection.

Route of administration

Percutaneous administration of BCG-Japan to neonates induces greater specific Th1 immunity than intradermal administration.[102] However, a large randomised controlled trial showed that percutaneous and intradermal administration of BCG-Japan to newborn infants were equivalent in terms of incidence of TB in young children before the age of 2 years.[103] Studies in mice concur that route of administration provides equivalent protection.[104]

Geographical location

BCG provides the best protection at higher latitudes and it is suggested that latitude alone could explain 41% of the variance.[105] For example, vaccine efficacy was found to be 81% in Norway (latitude 65°N of equator), 77-88% in various studies in the UK (53°N), 41% in Australia (20°S) and 22% in Kenya (equatorial), this effect is highly significant, $p < .00001$

Environmental mycobacteria

One of the most compelling reasons postulated for this significant heterogeneity is exposure to environmental mycobacteria, exposure to which increases with proximity to the equator. It is proposed that exposure to mycobacteria blocks (acquired immunity to environmental mycobacteria restricts the growth of BCG) or masks (BCG is unable to provide additional immunity to that induced by environmental mycobacteria) the response to BCG. Fine extensively reviewed this argument first proposed by Palmer and Long in the 1960s.[105] More recent data builds on this hypothesis. Black *et al* found that adolescents from areas where environmental mycobacterial exposure is high have increased baseline IFN-γ responses to *Mtb* purified protein derivative (PPD) compared to adolescents from non-endemic areas and that they fail to increase IFN-γ production after BCG vaccination.[106] It was thought that exposure to environmental mycobacteria accounted for these population differences. However, the same group subsequently found that previous sensitisation to environmental mycobacteria had no effect on the BCG-induced IFN-γ response to *Mtb* PPD in UK school children.[107] When UK- and Malawian-born infants were compared, UK-born infants were significantly more likely to have a strong positive IFN-γ response to BCG vaccination at 3 months and 12 months of age compared to Malawian-born infants and the magnitude of IFN-γ response was higher in the UK-born infants.[108] This is unlikely to be explained by direct exposure to environmental mycobacteria due to the age of the infants, however, it is possible that pre-natal exposures could be important. Genetic and other environmental factors can of course also play a role.

Helminths

Helminth infections are also more common in settings proximal to the equator. Immunological responses to helminths may skew immunity away from a type 1(Th1) response, which is required for protection against *Mtb*, towards a type 2 (Th2) response. BCG vaccination is administered at or around birth in the majority of countries, before acquisition of helminth infection, however *in utero* sensitisation through maternal helminth

infection may affect the postnatal response to BCG vaccination. Studies suggest that the effect varies between species of parasite.

One study in rural Kenya, showed that infants born to mothers with filariasis and schistosomiasis had evidence of helminth-specific T-cell immunity in cord blood in the absence of infection, suggesting that *in utero* sensitisation had occurred. [77] Sensitisation to these parasites was associated with a significantly reduced IFN-γ response to PPD at 1 year of age following BCG vaccination at birth; this effect was still noted in children up to the age of 10 years.

A subsequent randomised, double-blind, placebo-controlled trial of helminth treatment during pregnancy which followed a birth cohort of over 1500 infants in Uganda for one year has shown that maternal helminth infection does not have a significant effect on infant cytokine responses to BCG vaccination.[88] In this study the majority of mothers had at least one helminth infection in pregnancy, the majority of which were hookworm infections. Maternal *M. Perstans* showed a positive association with infant IL-10 production to crude culture filtrate protein (CFP) of *Mtb*, but there was no association with any other cytokine or helminth species. The lack of any significant effect was confirmed by the absence of any response to helminth treatment during pregnancy.

An observational study in Indonesia also found no effect of filarial or helminth infection on infant response to mycobacterial antigens before or after BCG vaccination.[85] They did however note a difference when the mother was infected with intestinal protozoa in terms of TNF-α response to PPD before and after BCG vaccination.

No studies have been conducted which directly examine the protective effect of BCG in infants sensitised to helminths *in utero*.

Other factors

Additional factors that may play at role to a greater or lesser extent include: Genetic differences between BCG vaccinated populations and exposure of the vaccine to sunlight and cold-chain maintenance, although in rigorously conducted studies this is unlikely to be important.

Season of birth may affect response to BCG vaccination and therefore vaccine efficacy. The proportion of Malawian infants who exhibit a positive IFN-γ response to BCG is higher in those born in the hot, dry season compared to those born in the other two seasons[108]. UK-born infants also have higher IFN-γ responses if they are born in the summer months. These results suggest that environmental factors play a role in vaccine-induced immunity, which may include exposure to other infections more common in the winter months or a potential role for Vitamin D.

It should not be neglected to say that socioeconomic factors are also extremely important in the risk of acquisition of infection and progression to TB disease; tuberculosis remains a disease associated with poverty.[109] The factors associated with poverty, such as overcrowding, poor nutritional status and general health status are likely to highly influence risk of disease and therefore measures of protective efficacy. In reality the explanation for the observed variability is likely to be multi-factorial and the reasons may be different in different regions of the world.

1.5.3 Associated benefits of BCG vaccination

All-cause mortality

A reduction in all-cause infant mortality has also been attributed to BCG vaccination (reviewed by Shann[110]). Controlled trials from the 1940s and 1950s show that BCG was associated with an estimated 25% (95% CI: 6 – 41%) reduction in mortality from causes other than TB in the UK and the USA at this time.[110] Observational studies conducted in resource-poor countries with high child mortality show that BCG is associated with a reduction in both morbidity and mortality beyond its effect on TB; this effect seems to be most pronounced in girls.[111] In support of these observations, the birth cohort study in Uganda described above found that female infants had consistently lower responses to mycobacterial and tetanus antigens following routine vaccination compared to boys.[88] Controversy abounds regarding the non-specific effects of vaccination, however supposition that BCG has beneficial effects on all-cause mortality is generally more accepted than the suggestion that the DTP vaccine has a detrimental effect.[112-114]

Leprosy and Buruli Ulcer

BCG provides protection against leprosy, albeit variable. Various studies have demonstrated efficacy of 20-90%, reminiscent of the protection afforded against pulmonary TB.[115] Despite this variability, BCG is credited as an important factor in the decline in leprosy in recent

years. BCG also provides moderate protection for a limited time against *M. ulcerans*, the causative organism of Buruli ulcer and is efficacious against Buruli ulcer related osteomyelitis.[116]

Atopy

BCG vaccination has been associated with a reduced risk of atopy in some studies, but not in others.[117-121] There is marked heterogeneity between the studies in terms of the study design, age and geographic location of the population studied, timing and strain of BCG vaccination, definition of atopy and measurement outcomes, thus the association remains controversial.

Influence on responses to unrelated vaccines

BCG may have an influence on the priming of T and B cell responses to unrelated vaccine antigens, most likely through the enhanced activation of T cells by more mature antigen presenting cells and induction of memory B cells. Ota *et al* found that when BCG was administered at birth at the same time, and in the same arm, as Hepatitis B vaccine, the proliferative, cytokine (IFN-γ, IL-5 sand IL-13) and antibody response to Hepatitis B surface antigen (HBsAg) was increased at 4.5 months of age compared to unvaccinated infants.[122] The effect on type-2 cytokine responses was similar when BCG was delayed until 2 months of age; however, here was no difference in the proliferative, IFN-γ response or antibody response. There was also an increased antibody response to oral polio vaccine with BCG vaccination at 2 months, but not at birth. BCG had a limited effect on responses to tetanus vaccination and no influence on diphtheria vaccine responses (administered as part of DTP vaccine). The timing of BCG vaccination has however not been shown to influence antibody responses in a study carried out in an identical study site as my own where BCG vaccine was administered in the right arm at birth and hepatitis B vaccine administered in the leg at 6, 10 and 14 weeks. (Hesseling, personal communication 2009)

1.5.4 Complications of BCG

The natural history of intradermal BCG vaccination includes shallow ulceration of the injection site of <1cm diameter, which heals over the course of several months, Figure 5. The local reactogenicity of BCG varies with BCG strain and BCG-Denmark (SSI 1331), the vaccine used in South Africa and the UK, is associated with increased reactogenicity. In the minority of cases BCG vaccination is associated with local or distant complications, most commonly in

the context of HIV-infection or children with primary immunodeficiencies. In the pre-HIV era the rate of disseminated BCG disease was <5 cases per million vaccinated infant.[92]

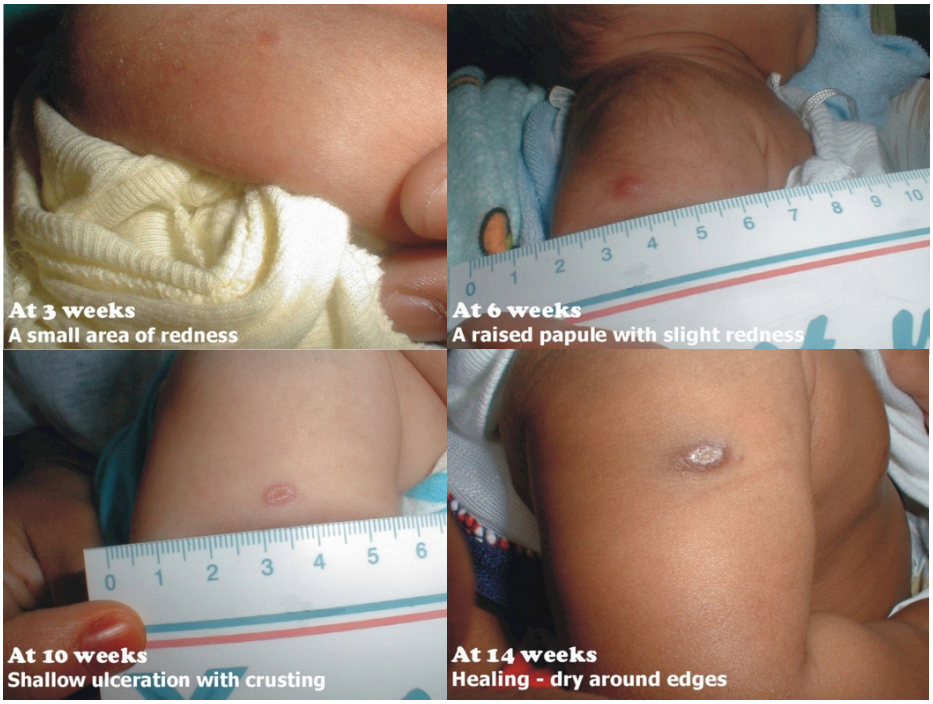


Figure 5. Natural history of BCG vaccination in the infant.
Photos courtesy of G Hussey, SATVI, University of Cape Town.

Local disease includes a BCG injection site abscess of >10mm x 10mm or severe BCG scar ulceration. Regional disease encompasses involvement of regional lymph nodes including ipsilateral axillary, supraclavicular, cervical or upper arm nodes. Involvement of one or more sites beyond a local or regional ipsilateral process, which may include pulmonary secretions, cerebrospinal fluid, urine, bone or skin lesions, constitutes distant disease. Disseminated disease includes any of the manifestations seen in distant disease and / or blood or bone marrow involvement.[123] Disseminated disease is associated with a mortality of >70%.[124] BCG immune reconstitution inflammatory syndrome (IRIS) is BCG disease presenting in an HIV-infected child within 3 months of initiation of highly active antiretroviral treatment (HAART).

Children with suspected BCG disease should be tested for HIV infection, in the absence of HIV infection primary immunodeficiency should be considered including potential defects in the IFN-γ/IL-12 pathway in view of the significantly increased risk in this population. BCG disease may be clinical indistinguishable from TB, therefore microbiological confirmation of

M. bovis by culture or PCR should be sought. The immunocompromised child should be evaluated for distant or disseminated disease. A combination of drugs including Isoniazid, Rifampicin, Pyrazinamide (until *Mtb* infection excluded), Ethambutol and Ofloxacin are used to treat BCG disease.

1.5.5 BCG vaccination of HIV-infected and HIV-exposed uninfected infants

Although BCG has been shown to reduce the incidence of disseminated TB, there is no evidence of BCG-induced protection against TB in HIV-infected infants. In a retrospective study of perinatally infected children there was no difference in incidence of TB in BCG vaccinated infants (44/310, 14%) compared to BCG unvaccinated children (7/44, 11%).[125] Furthermore, in a case-control study in Zambia, BCG had no protective effect in HIV-infected infants.[126]

The suggestion of absence of benefit is supported by more recent BCG immunogenicity data which shows that HIV-infected infants have a severely impaired $CD4^+$ and $CD8^+$ response to BCG vaccination which becomes virtually absent by 9 months of age, in the absence of antiretroviral treatment.[127]

Whilst BCG may not provide any benefit, it is associated with appreciable risk of BCG-related disease in HIV-infected infants. Local or region BCG disease occurs in up to 6% of infants, whilst disseminated disease occurs in an estimated 992/100 000 (95% CI: 567–1495) in the absence of antiretroviral therapy.[123,128] BCG-IRIS occurs in over 15% of infants, however this can be reduced by one third to 5% when combination antiretroviral treatment is initiated before the age of 12 weeks.[129]

In the light of the altered risk-benefit ratio in the context of HIV infection, the WHO revised their recommendations on the use of BCG in 2007.[130] Previously it was recommended that BCG be administered to all infants in high TB burden countries, unless the infant has symptomatic HIV. The revised guidelines now recommend that BCG be withheld from all infants *known* to be infected with HIV. Although this practice has been followed in many TB non-endemic countries for a number of years, there are many practical issues that impact the implementation of this guidance in Sub-Saharan Africa where the brunt of HIV and TB infection is concentrated.[131] BCG is routinely administered at birth before the HIV status of the infant has been established; PCR is not widely available in all countries and even if it is

available, the programmatic capacity is not in place to establish the infant's status before vaccinating infants with BCG. Therefore, these revised WHO guidelines have not resulted in any change in practice in many countries and BCG is still administered at birth in the absence of confirmation of the HIV status of the infant.

Whilst there is no evidence of benefit of BCG in HIV-exposed infants, similarly, there is no strong evidence to the contrary that they would not benefit. One must assume therefore that they do benefit to a similar degree as HIV-unexposed infants. As such, the WHO recommends that infants born to women with HIV and for whom the HIV-status is *unknown*, the BCG should be given, unless the infant is symptomatic.[130]

1.5.6 Immunological responses to BCG

BCG vaccination of newborn infants induces a robust Th1 type immune response of similar magnitude to that of adults.[132-134] Vaccinated infants exhibit strong lymphoproliferative and IFN-γ responses to mycobacterial antigens which are still evident at 1 year of age suggesting that BCG vaccination early in life results in formation of memory T cells.[132] BCG is a potent activator of dendritic cells, the antigen presenting cells required for priming of naïve T cells. The strong Th1 responses elicited by BCG vaccination may be as a result of this powerful activation of dendritic cells or the persistence of BCG during a time of maturation of the immune system. [122,132]

Further dissection of the BCG-induced response shows that it is dominated by multiple $CD4^+$ Th1 cytokine-expressing T cell subsets.[135] Seven distinct populations have been characterised, with similar frequencies of $CD4^+$ T cells producing IFN-γ, TNF-α or IL-2. Polyfunctional cells simultaneously expressing IFN-γ, TNF-α and IL-2 are one of the most frequent of $CD4^+$ T cell subsets. These cells have been thought to be associated with protection against TB, although more recently doubt has been raised.[136] The phenotype of $CD4^+$ T cells expressing IFN-γ or IL-2 after short-term stimulation with BCG antigens is characteristic of effector T cells ($CD45RA^-$ $CCR7^-$ $CD27^+$). Amongst cells expressing IL-2 central memory phenotypes are more common ($CD45RA^-$ $CCR7^+$ $CD27^+$).[135]

BCG was traditionally thought to be a poor inducer of $CD8^+$ T cells, however vaccination of newborn infants does induce a specific and functional $CD8^+$ T cell response, albeit at a lower frequency than that of $CD4^+$ T cells.[133,135,137] These cells are capable of degranulation and

have the potential to up-regulate the expression of cytotoxic molecules such as perforin, granzyme A and granzyme B upon stimulation with BCG antigens. $CD8^+$ T cells have either the capacity to degranulate or produce cytokine such as IFN-γ, but rarely have the capacity to do both simultaneously. IFN-γ-expressing $CD8^+$ T cells are the most frequent subset of type-1 cytokine expressing CD8+ T cells, but there is also a small but distinct population of $CD8^+$ T cells that produce IL-2 in the absence of IFN-γ.[135] Both $CD8^+$ T and $CD4^+$ cells express IL-10 and IL-4, but at very low frequencies in a minority of infants, after short-term stimulation with BCG antigens.[135]

Comparison of secreted cytokines and chemokines in cell culture supernatants from BCG-vaccinated and unvaccinated infants reveals that the immune response induced by BCG vaccination is even more complex and diverse. It involves not only Th1 and Th2 cytokines, but Th17, regulatory, chemokines and growth factors.[86] IFN-γ is the most highly upregulated of these, followed by MIP-1 α, IL-13, IL-1 α and GM-CSF.[86]

Gene expression profiling of 10 week old infants who have been BCG vaccinated at birth shows that a large number of genes are upregulated and down-regulated upon stimulation of PBMCs with BCG antigens.[138] Pro-inflammatory genes are significantly upregulated (IL-6, GM-CSF and IL-1 family member 8). Many of the upregulated genes are associated with the development of macrophages (MΦ1 phenotype), which support a Th1 response. Genes that are significantly downregulated include Fatty acid binding protein 4 (which is downregulated by IFN-γ), transforming growth factor β1 (which is associated with suppression of effector T cell responses).

In view of this complexity, measurement of IFN-γ responses to PPD alone, as has traditionally been used *in vitro* to assess immunogenicity, underestimates the breadth of the immune response to BCG.

Duration of immune response to BCG

Memory responses to BCG vaccination in infancy are long-lived. A longitudinal study in Indonesia showed that IFN-γ, IL-5 and IL-13 responses were increased at 5 months of age, thereafter IFN-γ remained high at the age of 2 years, but IL-5 and IL-13 decreased.[85] Teenagers in the UK still exhibit IFN-γ responses to PPD antigens 14 years after BCG vaccination.[139] When compared to their unvaccinated peers, vaccinated teenagers had significantly higher responses, suggesting that the response was vaccine-attributable.

Mycobacterial-specific responses have been detected even 60 years after BCG vaccination, however, it is highly possible that exposure to cross-reactive mycobacteria plays a role in maintaining the population of memory T cells for such a prolonged period.[140]

1.5.7 BCG-induced immune responses in HIV-exposed uninfected infants

There is limited and inconsistent data regarding BCG-induced immune responses in HIV-exposed infants. One of the first studies to examine the BCG-induced response in HIV-exposed, uninfected infants showed that HIV-exposed infants can have heterogeneous responses mycobacterial antigens.[73] A proportion of infants had similar responses to HIV-unexposed infants, with a significant increase in IFN-γ responses to BCG and PPD compared to pre-vaccination, reflecting an appropriate response to BCG vaccination. However, a proportion of HIV-exposed infants had a significantly higher IFN-γ response to mycobacterial antigens at birth. This was followed by only a moderate increase, no increase, or even a decrease in response at 6 weeks post BCG. A failure to increase IFN-γ responses following BCG in a third of HIV-exposed infants in this study may correspond to a different level of protection afforded by BCG, although there is no clinical data to assess functional responses to BCG at present. Alternatively, these infants may have similar immune capacity at birth compared to non-exposed infants at 6 weeks of age due to *in utero* priming. Despite the different pattern of response, the median response at 6 weeks was the same in HIV-exposed and unexposed. The only parameter that was measured in this study was IFN-γ levels in response to PPD in a whole blood assay, the cells that produced IFN-γ could therefore not be identified. As discussed in section 1.5.6 this is an incomplete assessment of the broad response to BCG.

Following this study, Mansoor *et al* observed no difference in total $CD4^+$ cytokine response, total IFN-γ, IL-2 or TNF-α $CD4^+$ response, or in the IFN-γ^+IL-2^+TNF-α^+ CD4 response between HIV-exposed and unexposed infants.[127] Elliott and colleagues have recently also not found any difference in BCG response.[88]

Recently, both Miles and Mazzola both assessed proliferative responses to mycobacterial antigens using carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution or by visually estimating the proportion of blast lymphocytes.[29,141] Miles found a tendency towards reduced proliferative capacity in response to PPD (no measure of statistical significance was reported), but similar responses to BCG antigens at 10 weeks following vaccination. The ex

vivo IFN-γ response at 2 weeks was similar amongst the two groups.[29] Mazzola found a lower proportion of $CD3^+$ blast cells, but a higher proportion of proliferating $CD4^+$ T cells in HIV-exposed infants aged 6 months to 18 months compared to unexposed infants.[141]

There is a paucity of data regarding BCG-induced immune responses in HIV-exposed infants. In particular there is a lack of data comparing mother and infant responses and little longitudinal data. The studies described here did not comprehensively study the breath of the response to BCG. More research is required to fully understand the influence of maternal HIV on infant responses to BCG vaccination. This thesis aims to bridge this gap in knowledge.

1.5.8 Vaccine-induced correlates of protection

Our understanding of what constitutes a vaccine-induced protective response against *Mtb* is incomplete. Moreover, it is not even known whether infants with higher responses to mycobacterial antigens will in fact have increased protection against TB disease following BCG vaccination. IFN-γ production in response to PPD has been widely used as a measure of vaccine efficacy and was thought to provide the best correlate of protection.[142] However, more recently it has become clear that although this may indicate 'vaccine take', determination of IFN-γ secretion alone does not consistently correlate with protection against TB.[83,104,143]

Most clinical trials of new TB vaccines assess the vaccine-induced immune response in the peripheral blood with a particular focus of the quality of the response as assessed by measurement of polyfunctional cells. These cells simultaneously express IFN-γ, TNF-α and IL-2. It has been thought that such cells are a marker of protective immunity, however new data has cast doubt on this theory. Kagina and colleagues carried out an elegant case-control study that collected blood samples at 10 weeks of age from 5,662 infants vaccinated with BCG within 24 hours of birth and then followed them up over 2 years.[144] They then compared samples from 29 infants who developed TB with a randomly selected group of 55 infants who were exposed to TB in the household, but who did not develop disease. As an additional control group 55 infants who did not develop TB within the follow-up period were randomly selected from the cohort of infants who did not have a household TB contact. These latter two groups were considered as protected against TB. Samples collected at 10 weeks of age from these three groups were then retrospectively analysed for BCG-specific

intracellular cytokine expression in stored $CD4^+$ and $CD8^+$ T cells. They found that there was no difference in the frequency of BCG-specific $CD4^+$, $CD8^+$ or γδ cells expressing IFN-γ, TNF- α or IL-2 individually or in any combination and no correlation with protection against TB.

Ideally, one would measure immune responses to BCG that correlate with BCG-induced protection against TB in order to define clinically relevant differences between populations. However, until such time as we fully understand the correlates of vaccine-induced protection, one should measure components of the immune system that are currently viewed as being important in protection against TB, section 1.6. Unfortunately our understanding of the immunity against *Mtb* is also likely to be incomplete and even what we do know may not necessarily translate into immune correlates of protection against disease. We are therefore left with assessment of vaccine immunogenicity, which is likely to be a measure of vaccine 'take' rather than protective response.

1.5.9 Future prospects for an improved protection against TB

The need for a new vaccine against tuberculosis is evident and urgent. The ideal new vaccine would be superior to BCG, protect against *Mtb* infection, efficacious against all forms of TB, in all age groups and populations, safe in HIV infected individuals, effective even in persons who are infected with *Mtb* and affordable in all countries; a formidable challenge. There are 14 candidate vaccines currently in clinical trials, however the hurdles facing the assessment and eventual implementation of a new vaccine are sizable.[145] A major obstacle to the vaccine pipeline is the lack of defined correlates of vaccine-induced protection against TB as described above. In the absence of defined biomarkers of protection, clinical trials currently are limited to measuring vaccine 'take'. Using clinical disease as an endpoint necessitates large sample sizes and prolonged follow-up. Active case-finding, prophylaxis of household contacts and effective treatment coupled with a paucity of highly sensitive and specific diagnostic tools makes this task very difficult. In addition to all these obstacles, new vaccines may be affected by the same factors that influence the protective efficacy of BCG.

There are 3 main vaccine approaches. Firstly, pre-exposure vaccines that aim to prevent infection and primary disease prior to exposure. Such vaccines would be administered soon after birth and would most benefit infants and young children, where the highest rate of progression from primary infection to disseminated disease is seen. This approach may employ a heterologous "prime-boost" strategy.[146] This strategy is likely to use either BCG or

a modified more immunogenic version of BCG, and then boost with a new sub-unit vaccine. BCG is likely to be retained in some form due to its consistent protection against disseminated mycobacterial infection and reduction in all-cause mortality. A second boost vaccine is then used to expand memory T cells common to the prime and boost vaccines. There are two such vaccines currently in phase IIb clinical trials enrolling large numbers of infants in South Africa: MVA85A/Aeras485 and AERAS-402/Crucell Ad35[147,148]. Another adjuvanted recombinant fusion protein vaccine, M72, is currently in phase II trials.

A second approach is to prevent disease in individuals who are infected with *Mtb*, a post-infection approach. This approach is particularly attractive given the vast numbers of people infected with *Mtb*. A third approach is an immunotherapeutic vaccine, which could be used to shorten duration of TB treatment or reduce the risk of relapse.

Novel TB vaccines would be of greatest benefit in highly TB-endemic countries. HIV is also highly prevalent in these areas; many of these adults with HIV infection are co-infected with *Mtb*. Their children are particularly at risk of TB, since the majority of childhood TB cases are a result of transmission from a close household contact.[149] BCG, and any novel TB vaccines, will be administered to infants who have been exposed *in utero* to HIV and some of their mothers are likely to be co-infected with *Mtb.*

1.6 Protection against TB

The immune response to *Mtb* is multifaceted and complex and involves both the innate and adaptive system. Whilst many cells are involved in the immune response, the interplay of the antigen-presenting cell with CD4 T cells plays a central part.[150]

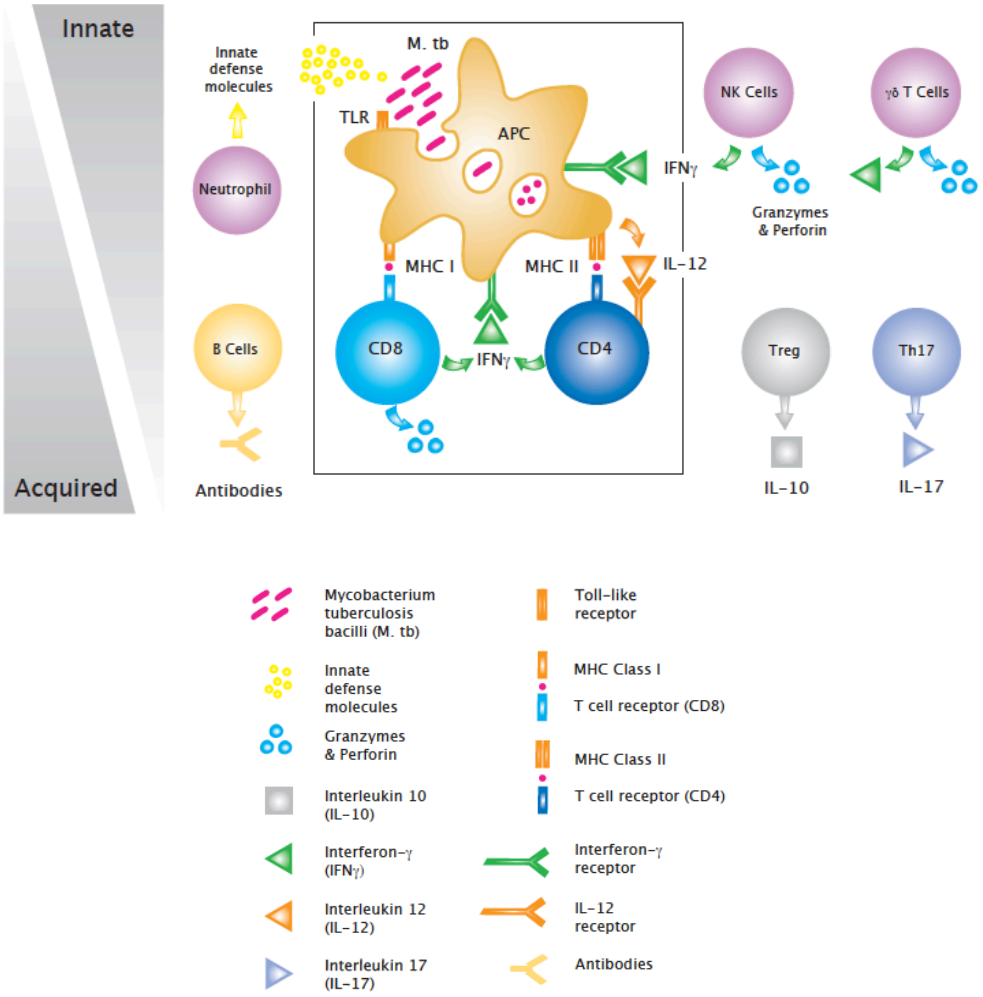


Figure 6. The interplay of the innate and adaptive immune response to *Mtb*.
From Jones *et al*.[150]

Inhaled Mtb is first exposed to antimicrobial peptides and proteins in the airways.[151-153] These elements have bactericidal and immunodulatory properties, in particular enhancement of Toll-Like Receptor (TLR) signalling and enhancement of *Mtb* uptake by macrophages.[154-156] Macrophages resident in the terminal alveoli engulf *Mtb* and the recognition of mycobacterial cell wall products by TLR2 and 4 results in activation of a signalling cascade. This ultimately results in the release of inflammatory cytokines and chemokines, which facilitates the recruitment of other cells such as NK cells and γδ T cells to the area. *Mtb* also infects dendritic cells which become activated and upregulate major histocompatibility complex (MHC) class II molecules, co-stimulatory molecules and inflammatory cytokines are secreted such as IL-1, IL-12 and TNF-α.[157] Dendritic cells migrate to the draining lymph nodes where MHC class II molecules on mature dendritic cells present *Mtb* antigens to $CD4^+$ T cells to initiate the adaptive response.[157] Cytokines such as IL-12p70

cause proliferation and differentiation of naive T cells. The T cells then move back to the primary site of infection in the lung. Chemokines alter blood vessels at this site and provide directional cues to guide the T cells to leave the circulation and enter the lung parenchyma.

Activated T cells, predominately $CD4^+$ T cells, then recognise the *Mtb*-derived antigens on the surface of infected macrophages and activate macrophages through the production of type 1 cytokines such as IFN-γ, IL-2 and TNF-α. Macrophages kill mycobacteria, however the mechanisms by which macrophages kill *Mtb* in humans is not clear.[158] $CD8^+$ T cells are present and are able to kill infected macrophages. Multiple T cells and macrophages organise themselves into granulomas aided by TNF-α, which results in containment of the organism and either killing or progression to mycobacterial latency.

1.6.1 Cell mediated immunity

Cell-mediated immunity is essential in protection against *Mtb* and $CD4^+$ T cells are of primary importance.[159] This is immediately apparent in settings with a high burden of HIV and TB such as South Africa where the annual risk of TB in HIV infected individuals is 5-10 fold higher even amongst HIV-infected individuals on anti-retroviral treatment and highest amongst those with the lowest CD4 counts.[160] The TB incidence of HIV-infected South African infants is at least 20 fold higher than in HIV-uninfected infants.[161]

$CD8^+$ T cells are also likely to play a role in the production of IFN-γ and TNF-α, which contributes to the activation of macrophages.[159] $CD8^+$ T cells produce molecules such as perforin or granzymes, which cause lysis of macrophages infected with *Mtb* and results in apoptotic cell death.[162] In addition, they release granulysin that enters the macrophage through the perforin pore and is directly toxic to *Mtb* within the macrophage.

NK cells may play a number of roles. They have cytotoxic ability, can directly lyse cells and can also release IFN-γ to enhance macrophage killing and prime for subsequent Th1 response.[163]

γδ cells are also able to release IFN-γ and granulysins.[164] They are able to recognise non-proteinaceous antigens and respond rapidly. In addition to IFN-γ, they produce IL-17 in response to *Mtb*. IL-17 is involved in neutrophil recruitment and may be responsible for inflammatory damage associated with *Mtb*. Th-17 T cells are a further source of IL-17. [165]

B cells, although not traditionally thought to be important in protection against *Mtb*, may shape the immune response by influencing cytokine production, such as IL-10, and by bacillary containment. It is hypothesised that immunoglobulin may act on Fcγ receptors and influence antigen presenting cell maturation.[166] This aspect remains incompletely described, but is worthy of further research.

1.6.2 Cytokines

Whilst a large number of cytokines may be involved in the immune response to *Mtb*, several seem to be essential in both the mouse model and in humans[167]

IFN-γ is key in the immune response to TB as demonstrated by mice and humans deficient in IFN-γ being more susceptible to mycobacterial infections.[168,169] It is produced by $CD4^+$, $CD8^+$ and innate cells. It triggers activation of the macrophage, which can then inhibit or kill the infecting organism. TNF-α works synergistically with IFN-γ to enable the macrophage to kill the organism and is also important for granuloma formation.

IL-12 is produced by macrophages and dendritic cells and drives the immune response towards a type 1 response.[170] Its essential role is demonstrated by individuals who have defects in IL-12 production and the IL-12 receptor who have increased susceptibility to mycobacterial infections, so called Mendelian susceptibility to mycobacterial diseases (MSMD)[171]

A number of cytokines with primarily regulatory functions such as IL-17, IL-22 and IL-23, IL-10 and TGF-β have recently been described and are associated with the Th17 lineage and regulatory T cells. IL-17 may have a protective role against TB, since IL-17-expressing memory cells are induced by vaccination against TB.[147] Data defining the role of these cytokines in TB immunity are currently limited and further investigations are required.[159]

1.6.3 Chemokines

Chemoattractant cytokines (chemokines) are small proteins secreted from a variety of cell types during the inflammatory process which act on more than one type of leucocyte.[172] They have a myriad of functions including providing directional cues for movement of leucocytes in development, homeostasis and inflammation and the promotion of

lymphocyte activation and differentiation.[172,173] Chemokines are involved in host defence against mycobacterial infection by mediating neutrophil recruitment and infiltration of monocytes and lymphocytes.[10,174,175]

The role of chemokines in the movement of naïve T lymphocytes through the circulation to the lymph nodes where they encounter antigen and are transformed into memory cells may be particularly relevant as HIV-exposed infants have reduced numbers of naïve cells and increased memory cells.[27,65] Moreover, chemokines are important in the biology of HIV as their receptors are used by HIV as co-receptors to gain entry into cells.[172] The measurement of chemokines as well as cytokines may therefore reveal interesting differences in the immune response to mycobacterial antigens in infants exposed to HIV and or TB *in utero.*

1.7 Humoral protection against vaccine-preventable diseases

Prior to acquisition of immunity from vaccination or natural exposure, infants are protected by passively acquired immunity from the mother. Maternal HIV may influence the efficacy of this process. The level of antibody at birth may affect the infant's own response to vaccination.

1.7.1 Maternal antibodies confer passive protection to the infant

At birth, healthy, full-term neonates produce only 5% of the total adult levels of Immunoglobulin G (IgG), despite this, most of these infants have serum levels of IgG greater than or equal to maternal levels.[75,176] This is explained by the active trans-placental passage of maternal antibodies in the third trimester, which provides passive immunity to the infant prior to acquisition of immunity from natural infection or immunisation. Placental transfer of antibodies is principally restricted to IgG and can be affected by maternal antibody titres, maternal immunisation and infection, and infant gestational age.[177] The quantity of antibody transferred to the infant has implications for the duration and degree of protection against specific pathogens.[75]

1.7.2 Maternal HIV infection affects transplacental transfer of antibodies

Maternal HIV negatively correlates with transplacental transfer of measles specific IgG and this may explain, at least in part, the observed increase in measles seen in the first 9 months of life in HIV-exposed infected and uninfected infants.[176,178,179] However, this has not been

borne out in all studies.[75] It is postulated that HIV interferes with the active transport of IgG across syncytiotrophoblasts by blocking IgG-specific Fc receptors. Published data shows that higher levels of maternal viraemia correlates with reduced placental transfer of measles IgG.[180]

Placental transfer of pneumococcal IgG is also reduced in maternal HIV infection. Conversely, transfer of tetanus IgG does not seem to be affected by maternal HIV infection, despite the fact that it is actively transferred across the placenta in the same way as measles IgG.[75] It is not known what immunological differences underlie this phenomenon.

1.7.3 Influence of maternally derived antibodies on infant vaccine responses

Passively acquired antibodies are important in providing protection against pathogens in newborns early in life, however, maternally derived vaccine-specific antibodies may inhibit the infant's ability to respond to vaccination. Timing of immunisation is therefore critical in order to reduce the period of vulnerability to pathogens and ensure infant seroconversion and levels of antibodies that correlate with protective immunity.

The mechanism by which maternal antibodies may interfere with infant vaccine responses is not known. However, the level of maternal antibody seems to be a critical factor. Very high levels of maternally derived vaccine-specific antibodies are associated with inhibition of the immune response of the infant and low levels seem to have little or no effect, as documented for pneumococcal conjugate (PCV), measles and pertussis vaccines.[176,181,182] It is possible that differences will be seen with different vaccines.

1.7.4 Expanded Programme of Immunisation

The revised South African Expanded Programme on Immunisation (EPI) was implemented at the study site in July 2009. The principal changes to the schedule were the addition of a seven-valent pneumococcal conjugate vaccine (PCV_7) and a rotavirus vaccine; a change from whole cell pertussis to acellular pertussis and a change from oral polio to inactivated polio at 10 and 14 weeks. The schedules are detailed in Table 1.

Age of infant	Vaccine	Route	Previous	2009 Schedule
At birth	BCG	ID	√	√
	OPV	PO	√	√
6 weeks	OPV	PO	√	√
	RV	PO		√
	DTP/Hib	IM	√	
	DTaP-IPV/Hib	IM		√
	HBV	IM	√	√
	PCV_7	IM		√
10 weeks	OPV	PO	√	
	DTP/Hib	IM	√	
	DTaP-IPV/Hib	IM		√
	HBV	IM	√	√
14 weeks	OPV	PO	√	
	RV	PO		√
	DTP/Hib	IM	√	
	DTaP-IPV/Hib	IM		√
	HBV	IM	√	√
	PCV_7	IM		√
9 months	Measles	IM	√	√
	PCV_7	IM		√

Table 1. Infant Immunisations routinely delivered in the South African public sector
BCG (Danish strain 1331, Statens Serum Institute, Denmark); OPV: Oral Polio vaccine (Sabin); RV: Rotavirus vaccine (ROTARIX, GlaxoSmithKline, Belgium); DTP/Hib: Diphtheria, Tetanus, Pertussis and Haemophilus influenzae type b combined vaccine (COMBact-HIB®); DTaP-IPV/Hib: Diphtheria, Tetanus, acellular Pertussis, inactivated Polio and Haemophilus influenzae type b combined vaccine (Pentaxim®); HBV: Hepatitis B vaccine (Heberbiovac, Cuba); PCV7: Pneumococcal 7-valent conjugate vaccine (Prevenar®, Wyeth, USA); Measles (Rouvax). ID: intradermal; PO: oral; IM: Intramuscular. All vaccines supplied by Sanofi Pasteur unless otherwise indicated.

1.8 Conclusions

In summary, TB remains a problem worldwide and the only currently available vaccine against TB has variable efficacy. The HIV pandemic further exacerbates the problem of TB. The success of PMTCT programmes has resulted in fewer children being vertically infected with HIV, however there are a growing number of children who are exposed to HIV but are not themselves infected. There is accumulating evidence that *in utero* exposure to HIV affects the developing immune system and it is possible that these changes might have negative clinical consequences. Whether these immunological changes have an impact on the infant's ability to respond to BCG vaccination is a question of relevance and importance,

particularly since these infants are more likely to be in contact with an adult with TB based on the association between HIV and TB in adults.

Exposure to other maternal infections or disease processes has already been shown to impact on infant immune responses. It is possible that maternal *Mtb* infection could prime the developing immune system in the same way and may affect responses to other mycobacterial antigens such as BCG, potentially resulting in altered protection against *Mtb*.

The immune response to *Mtb* is diverse and complex and involves both the innate and adaptive immune response. Consequently, reliable immune correlates of protection against TB are still not known, but they are likely to require more complex measurements than enumerating the levels of IFN-γ in response to PPD or live mycobacteria. It is therefore indicated and timely to take a broader approach in investigating immune responses to BCG than measuring single cytokines.

This study aims to investigate the impact of maternal HIV infection and *Mtb* sensitisation on immune responses to BCG in the infant. This is of clinical relevance and has implications for the current vaccine schedule as well as for new TB vaccines now progressing into clinical trials. If immune responses to BCG are found to be deficient, boosting vaccination or delaying vaccination until perturbations of the immune system have passed may be required. If maternal TB infection negatively impacts the infant, this would have implications for future studies assessing treatment of *Mtb* infection in pregnancy.

This study also addresses the impact of maternal HIV infection on humoral response to other vaccines routinely administered to infants in the EPI schedule. Whilst there are several studies showing a relationship between antibody levels in mother/infant pairs at birth and other studies examining vaccine-specific antibodies pre- and post-vaccination, there are no published studies relating the effect of HIV-exposure on maternal and infant levels of vaccine-specific antibodies at delivery to post vaccination levels for a broad range of routinely administered EPI vaccines.

1.9 Study hypotheses

Hypotheses

1. *In utero* exposure to maternal HIV infection and / or maternal *Mtb* infection alters infant immune responses to mycobacterial antigens at birth and following BCG vaccination.
2. Maternal HIV infection is associated with altered antibody titres to vaccine-preventable diseases. HIV-infected mothers have reduced placental transfer of vaccine-specific IgG to infants and consequently HIV-exposed, uninfected infants have reduced vaccine-specific antibody titres at birth compared to HIV-unexposed infants.

Specific aims

1. To conduct a prospective cohort study of mother/infant pairs in a setting with a high burden of HIV and TB and to examine the influence of maternal HIV infection on infant responses to mycobacterial antigens at birth and at 16 weeks of age, following BCG vaccination at 6 weeks of age.
2. To examine the influence of maternal *Mtb* sensitisation on infant responses to mycobacterial antigens at birth and at 16 weeks of age.
3. To investigate the influence of both maternal HIV infection and maternal *Mtb* sensitisation on infant responses to mycobacterial antigens at birth and at 16 weeks of age.
4. To compare baseline responses to mycobacterial antigens between HIV infected and uninfected women.
5. To compare baseline responses to mycobacterial antigens between *Mtb* sensitised and *Mtb* unsensitised women.
6. To determine the effect of both HIV infection and *Mtb* sensitisation on maternal responses to mycobacterial antigens.
7. To determine the association of maternal and infant responses to mycobacterial antigens.
8. To measure anti-*Bordetella pertussis*, anti-*Haemophilus influenzae*, anti-tetanus, anti-pneumococcal and anti-Hepatitis B specific IgG in mothers and infants and to determine the effect of maternal HIV infection on these responses. This specific aim is detailed further in section 6.1.

Materials and Methods

Chapter 2: Materials and Methods

2.1 Study setting

The study was conducted in a community health facility in Site B, Khayelitsha, Western Cape Province, South Africa. Khayelitsha is an urban informal settlement located on the outskirts of Cape Town, South Africa.

Figure 7. Study setting: Site B, Khayelitsha, Cape Town, South Africa.

2.2 Ethical considerations

Approval was obtained from the Maternal Obstetric Unit (MOU) at Site B and from the Province of the Western Cape to conduct this study. Ethical permission was granted by the NHS Research Ethics Committee (LREC 07/H0720/178), the Ethics Committee at University of Cape Town (Ref: 382/2008, IRB00001938, FWA: 00001637) and the Committee for Human Research at Stellenbosch University (Ref: N08/10/278, IRB0005239, FWA: 00001372). Mothers were reimbursed for travel expenses but no financial incentive was given for participation in the study.

2.3 Study design

The study was a cohort study design. Mothers and their infants were recruited in the postpartum period from the postnatal ward and a blood sample was drawn from the mother and infant. BCG vaccination was administered to the infant within the study at 6 weeks of age. A further blood sample was collected from the infant at 16 weeks of age to assess immune responses to vaccination. This time point was chosen because BCG-specific $CD4^+$ T cell responses peak at 10 weeks post-vaccination when the vaccine is delayed until after the immediate peripartum period.[184] An overview of the study design is given below.

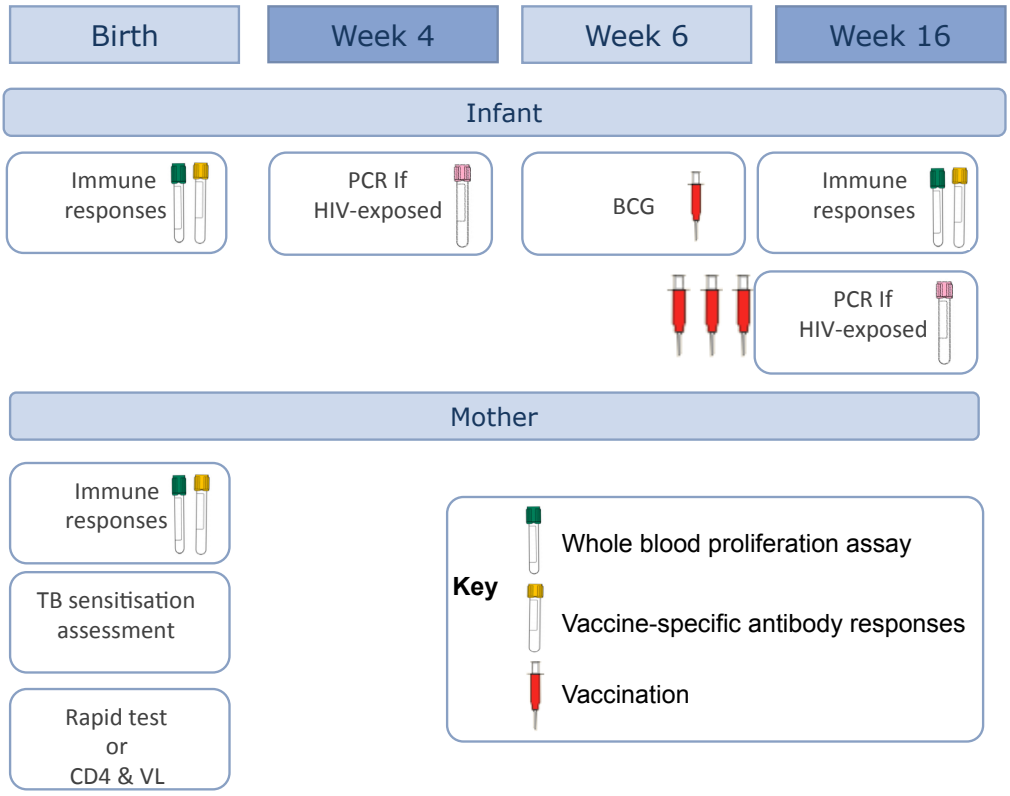


Figure 8. Overview of study design

2.4 Study Participants

Study participants were recruited between March 2009 and January 2010. Women were eligible for the study if they had delivered a live-born, healthy infant at the Site B MOU within the previous 24 hours, were aged 18 or more, were healthy, knew their HIV infection status and were willing and were able to provide written informed consent for herself and for her infant.

Mothers were excluded from the study if they were planning to move outside of the study area during the next 4 months, were on TB treatment or had clinical evidence of active TB, or if there was a current close household contact with active TB.

Infants were excluded from the study if they were born at less than 36 weeks gestational age, had a birth weight of less than 2.5 kg, were unwell at birth, required admission to the Special Care Baby Unit, if there was congenital abnormality or if there was a twin birth.

2.4.1 Recruitment

Information about the study was displayed in the antenatal clinics and the study community counsellor discussed the study with small groups of women. Further information was given to eligible women following delivery. Consecutive eligible women were sequentially enrolled irrespective of their HIV status within 24 hours of delivery. Once sufficient numbers of HIV-uninfected women were enrolled, HIV-infected women were preferentially recruited. A study nurse or study counsellor obtained written informed consent in the participants' home language. Women voluntarily disclosed their HIV status to the study team.

During analysis of results, mothers and infants were retrospectively allocated to one of the following groups based on the maternal HIV rapid test results and the QuantiFERON-TB Gold In-Tube (QFN) results:

HIV - QFN -	HIV - QFN +
HIV+ QFN-	HIV + QFN +

The QFN result was used as a reflection of maternal *Mtb* sensitisation and as a proxy for TB infection.

2.5 Clinical measures and blood sampling

Participants were reviewed at birth, 2, 4, 6 and 16 weeks postpartum, details of each contact is given below. Additionally, infants were seen for unscheduled visits at maternal request.

Birth clinical review

Following recruitment to the study, demographic and clinical data was collected from each participating mother to assess maternal health, which included a symptom based screening tool to screen for active TB. If active TB was suspected, women were referred to TB services and excluded from the study if active TB was confirmed. An example of one of the data collection forms used is attached in Appendix I. A single investigator (Dr C Jones) examined each infant to ensure that they were healthy.

Two to three mls of peripheral blood was collected from the infant within the first 24 hours of life and 10mls of blood was collected from the mother. This included a rapid HIV test (Abbott Determine™ HIV-1/2, Tokyo, Japan) to confirm the HIV status of all mothers who tested negative during pregnancy; all women received pre- and post-test counselling.

A tuberculin skin test (TST) was performed using 2 IU (0.1ml) of PPD RT/23 (SSI, Copenhagen, Denmark, Lot 1540A) on the left volar aspect of the forearm. The TST was dual read at a visit to the study clinic at 48-96 hours. A positive TST was defined as a tuberculin skin test of 10mm or more induration in mothers who were HIV negative and 5mm of induration or more for mothers who were HIV positive. The TST was performed following blood sampling to ensure that boosting of the QFN test did not occur.[185] All women who were HIV-infected and who had a positive skin test, regardless of symptoms, were referred to local TB services.

Clinical review at 2 weeks

A structured questionnaire was completed during a telephone call at two weeks postpartum. This included questions to assess TB exposure and symptoms of TB.

Clinical review at 4 weeks

All infants exposed to HIV *in utero* returned to the study clinic at 4 weeks of age for a HIV PCR test (Amplicor HIV-a DNA kit, Version 1.5, Roche molecular systems Inc., Branchburg, NJ).

Clinical review at 6 weeks

At six weeks infants were reviewed to ensure that they are healthy and were vaccinated with 0.05ml of intradermal BCG Vaccine SSI (Danish strain 1331, SSI, batch 108031A) in the right deltoid region. HIV-infected infants (PCR positive at 4 weeks) did not receive BCG based on current WHO recommendations.[130]

Clinical review at 16 weeks

Clinical information was collected and BCG scarring status (presence of scar, scar size, ulceration or abscess formation) was assessed. Blood sampling was repeated for each infant to assess immune responses to BCG vaccination and antibody responses to other vaccines administered in the EPI schedule. A HIV PCR was performed for any HIV-exposed infant to ensure that there has been no post-partum transmission of HIV, for example through breast milk if the mother had chosen not to adhere to exclusive formula feeding.

At study exit the community counsellor administered a structured questionnaire to assess reasons for participating in the study, satisfaction with the study and to assess the socio-economic status of study participants. Participants were interviewed in a private location separate from the study nurse or doctor to minimise the influence on participant's responses.

2.5.1 Sample handling

Samples were sealed in sample bags and then transported to the laboratory site at the University of Cape Town from the clinical site in Khayelitsha in a secondary container at room temperature. Samples were transported by the investigator (C. Jones) to the laboratory site and processed within 4 hours of sample collection.

2.5.2 Laboratory assessment of maternal *Mtb* sensitisation

The QuantiFERON-TB Gold In-Tube (QFN) test is a commercially available whole blood assay measuring IFN-γ release in response to ESAT6, CFP10 and TB7.7 antigens. QFN (Cellestis, Victoria, Australia) assays were performed in the laboratories of Professor W. Hanekom, South African Tuberculosis Vaccine Initiative (SATVI) by laboratory technical staff according to manufacturers instructions. Manufacturers define a positive test as TB antigen minus Nil as greater or equal to >0.35 IU/ml (where the TB antigen – Nil is also greater than or equal to 25% of the Nil value and the Nil value is less than or equal to 8.0 IU/ml).

2.5.3 Assessment of prevalent tuberculosis in infants.

Infants were screened for prevalent tuberculosis at each study visit using a clinical screening tool. In addition, IFN-γ release in response to ESAT6/CFP10 fusion protein was assessed using a standard ELISA. A QFN test was performed for any infant with a positive response. A positive response was defined as a value greater than twice the detection limit (>39pg/mL).[106] This is a technical cut off rather than a biological cut off. A chest x-ray and gastric aspirate was performed if there was any suspicion of TB disease. The method used for IFN-γ ELISA is described in section 2.6.8

2.5.4 Assessment of maternal helminth sensitisation and infection

Previous sensitisation to the helminth *Ascaris* was quantified using *Ascaris* specific IgE. This routine assay was performed at the University of Cape Town Lung Institute by Bartha Fenemore using a fully automated commercial ELISA system (ImmunoCAP, Phadia AB, Uppsala, Sweden). Evidence of active helminth infection was detected by microscopic examination of a single stool sample per participating mother using the formulin-ether concentration method.[186] This test was performed by the National Health Laboratory Service (NHLS) at Tygerberg Hospital.

2.5.5 Haematological parameters

A Coulter AcT diff Hematology Analyzer (Beckman Coulter) was used to assess haematological parameters such as haemoglobin concentration.

2.5.6 Clinical Data management and statistics

A study-specific Filemaker Pro (Filemaker Inc. version 10.0v3) relational database was used to capture clinical, laboratory and experimental data. All personal information was excluded from the database.

Statistical analysis was performed using SPSS (version 20) and GraphPad Prism (version 5.0a, 2008). Mean values and standard deviation are quoted for normally distributed data sets and the unpaired t test used. Median values and interquartile ranges are quoted for non-normally distributed data and the Mann-Whitney test is used. The Chi-squared or Fisher's exact test was used to compare proportions. Agreement between the TST and QFN test was estimated using Cohen's Kappa coefficient.

2.6 Laboratory Assays and data analysis

2.6.1 Optimisation of antigens

Appropriate concentrations of antigens to stimulate samples in the whole blood assay were selected in a series of optimisation experiments.

Positive control

SEB was tested at a final concentration of 5, 1, 0.5 and 0.05μg/mL. A final concentration of 1μg/mL gave readily detectable responses in both healthy adult donors, infants at birth and at 16 weeks.

Mycobacterial-specific antigens

BCG was tested at a final concentration of $1x10^6$, $5x10^5$, $1x10^5$, $5x10^4$ CFU/mL in healthy adult donors, based on studies in the published literature.[73,102,165,187,188] The concentration that resulted in the highest cytokine response in healthy adult donors was $5x10^5$CFU/mL. No response was found in infants at birth. Subsequent experiments on samples from four 16-week old infants enrolled in the study confirmed that a strong IFN-γ response to this concentration of BCG was found at 10 weeks post BCG vaccination.

Non-mycobacterial, specific antigen

A non-mycobacterial, but specific antigen was selected to investigate whether any differences were specific to BCG or whether they are a result of generalized changes in the immune response. Tetanus was chosen because this is another antigen that all the infants in the study would encounter as part of the primary vaccination schedule.

Tetavax Tetanus vaccine was tested at a final concentrations of 0.016, 0.16, 1.6 and 3.2 International Units (IU)/ml, based on that used previously in a similar flow cytometry assay.[189] A concentration of 1.6IU/mL was found to produce the highest cytokine response by ELISA and a concentration of 0.16IU/mL was optimal using flow cytometry.

2.6.2 Six-day whole blood Ki67 lymphoproliferation assay

Diluted whole blood was stimulated with BCG, tetanus (Tetavax Tetanus vaccine) or SEB in a 6-day whole blood assay. After 24 hours of culture a sample of supernatant was collected for analysis by multiplex ELISA. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were added on day 6 of the assay to induce non-specific activation of T cells and production of cytokines to allow assessment of the functional capacity of antigen-specific T cells. Addition

of Brefeldin A caused intracellular accumulation of these cytokines. Cells were stained with fluorescent-conjugated antibodies, including Ki67, and analysed by multi-parameter flow cytometry to assess antigen specific lymphoproliferation and intracellular cytokine expression.

A whole blood assay was chosen because only very small volumes of blood are required, which is a critical consideration when collecting samples from infants. Immediate antigenic stimulation of whole blood is a more sensitive method of measuring the induced T cell response compared to stimulation of peripheral blood mononuclear cells (PBMCs) that have been isolated, stored and then defrosted.[190] Background responses may also be lower in whole blood assays. A longer-term stimulation was chosen because it may provide a better assessment of memory responses compared to the effector response measured in shorter-term assays.[135] Following sample collection, blood was processed and stimulated with antigens within 4 hours in order to maximise the sensitivity of the assay.[190]

Ki67 is a nuclear protein that is expressed during all active phases of the cell cycle (G_1, S, G_2 and mitosis), but is absent in resting cells (G_0), making it a useful marker of proliferation.[191] It reflects the division of cells and also the capacity of cells to divide. It has most commonly been used in oncology as a prognostic indicator for disease free survival, disease recurrence and progression in breast and prostate cancer. More recently it has been developed as a novel marker in whole blood proliferation assays. Ki67 levels peak after 6 days of culture.[189] The sensitivity of the Ki67 assay to detect proliferating cells is similar to an assay employing dye dilution, but is greater than that of the bromodeoxyuridine (BrdU) BrdU assay. [189]

Whole blood was diluted 1:10 with warm RPMI 1640 tissue culture medium containing L-Glutamine (Walkersville, MD, USA). 1250µl of diluted blood was added in singulate to unstimulated wells and 1225µl was added to all other wells in 24-well flat-bottomed tissue culture plates. Whole blood was incubated with antigens at a final concentration of 1µg/ml SEB (Sigma-Aldrich, St Louis, MO, USA), 5 X 10^5 CFU/ml BCG Vaccine SSI (Statens Serum Institut, Copenhagen S, Denmark, batches 108031A and 108047A), 1.6IU/ml and 0.16IU/ml Tetavax tetanus vaccine (Sanofi Pasteur, Lyon, France, lot A6117-1) at 37°C, 5% CO2, 80% humidity.

After 24 hours 150µl of supernatant was collected and stored at -80°C.

On day 6, a further 500µl of supernatant was removed. 3µl of Brefeldin A (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 0.5mg/ml was added to all conditions. 16µl of PMA (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 1µg/ml and 160 µl of Ionomycin (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 50µg/ml was added to all stimulated wells.

After 4 hours of incubation at 37°C, 5% CO2, 80% humidity, 80µl of 20mM EDTA was added and incubated for 15 minutes at room temperature to aid detachment of cells from the plastic wells. Cells were re-suspended and incubated with alternative lysing solution (150mM NH_4CL, 10mM $KHCO_3$, 1mM Na_4EDTA) for 10 minutes and then centrifuged for 10 minutes at 1600rpm at 25°C. Supernatant was discarded. The process was repeated twice. The cells were washed with PBS (BioWhittaker, MD, USA) and then incubated for 30 minutes with a viability dye (LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen, Eugene, Oregon, USA).

After centrifugation at 1600rpm for 10 minutes, cells were re-suspended and fixed in FACS lysing solution (BD Biosciences, San Jose, CA, USA). Cells were centrifuged at 1600rpm for 10 minutes and re-suspended in 'cryo solution' (10% DMSO, 40% Foetal Calf Serum and RPMI) and stored at -80°C.

2.6.3 Cell surface marker and intracellular cytokine staining

Cells were stained with fluorescent conjugated antibodies to determine surface markers and intracellular cytokine expression profile of antigen-specific proliferating T cells by multi-parameter flow cytometry. All reagents and equipment were supplied by BD Biosciences, San Jose, CA, USA, unless otherwise stated.

White cells were thawed, washed in 2mls of 1 x PBS, pelleted by centrifugation at 1440rpm for 5 minutes and re-suspended in Perm/Wash Solution to permeabilise the cells. After 10 minutes incubation, samples were re-pelleted and the supernatant discarded. Cells were incubated at 4°C for 1 hour in the dark with the following fluorescent-conjugated monoclonal antibodies: anti-CD8 PerCP-Cy5.5 (SK-1), anti-IFN-γ Alexa Fluor 700 (B27), anti-IL-2 FITC, anti-TNF-α PE-Cy7, anti-Ki67 PE, anti-IL-17A Alexa Fluor 647 and anti-CD3 Qdot 605 (clone UCHT1, Invitrogen, Eugene, Oregon, USA). Anti-mouse Ig, κ, or anti-rat Ig, κ for anti-IL-2 FITC, compensation beads were incubated with individual antibodies. Following

incubation, 1ml of PermWash was added to the cells and beads. Samples and controls were pelleted and re-suspended in 150 µl of Perm/Wash. Immediately prior to acquisition samples were filtered using a 100 micron pore nylon monofilament filtering mesh (Technical Fabrics UK Ltd) to remove any debris and reduce cell doublets.

A LIVE/DEAD compensation control was required for correction of spectral overlap, cells proved a more appropriate control than beads. Blood was collected from a single healthy donor, red cells were lysed with alternative lysing solution, cells were then washed and pelleted by centrifugation. Half of the sample was subjected to 90°C in a heat block for 10 minutes to render the cells non-viable. The 'live' cells and 'dead' cells were then mixed together and stained with LIVE/DEAD Violet Dead Cell Stain. Cells were washed, pelleted and then fixed with FACS lysing solution and aliquots were made use in each flow cytometry run.

2.6.4 Multi-parameter flow cytometry and controls

All available sample volume was acquired on a BD LSRFortessa Flow Cytometer (model 649225B7, power 690W, manufactured Sept 2010) configured with 5 lasers and 19 detectors using FACSDiva software (BD Biosciences, San Jose, CA, USA). Optimal photomultiplier tube voltages were established for this study (section 4.1.3). BD Cytometer Setup and Tracking (CS&T) Beads were used to set baseline settings of cytometer and to provide target values for reproducible application settings for each flow cytometry run. The CS&T beads were run daily to track cytometer performance and ensure data consistency over time. Single-stained anti-mouse (or anti-rat) beads Ig, κ, beads were used to calculate compensation in FACSDiva for each run. Cell doublets were excluded by plotting forward scatter-area against forward scatter-height followed by side scatter area against side scatter height. Dead cells were excluded using LIVE/DEAD Fixable Violet Dead Cell Stain and compensation was performed using the control described above. Dead cell discrimination is based on the principle that the fluorescent reactive dye in LIVE/DEAD cell stain binds to cellular proteins (amines). Viable cells have an intact membrane, thus the stain only reacts with amines on the cell surface resulting in dim staining. Dead cells have a damaged membrane therefore allowing the reactive dye to binds to amines within the cells in addition to those on the surface, resulting in more intense staining. Antibody binds non-specifically to dead cells, which can result in an overestimation of the proportion of antigen-specific cells, therefore exclusion of such cells is critical.

2.6.5 Multi-parameter flow cytometry data analysis

Multi-parameter flow cytometry data was analysed using FlowJo v 9.4.11 (TreeStar, Ashland, OR). In order to minimise variability in analysis, gating was performed by a single operator using a pre-determined gating strategy and template, with minor adjustments made to account for individual differences in samples.[192] Combinations of antigen-specific cytokine-producing cells were determined by Boolean Gating in FlowJo. Background subtraction of proliferating cytokine-producing cells was performed using Pestle v 1.7 (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Where background subtraction of the value of the unstimulated samples from the value of the stimulated samples resulted in values less than zero, negative values were set to zero. To eliminate the systematic bias inherent in increasing only negative values, all measurements below a nominal value of 0.01 (for frequencies of cells expressing intracellular cytokines) or 0.5 (for total frequency of proliferating cells) were also set to zero. These values were decided based on the distribution of negative values and positive values close to zero. Display of multiple combination of cells was performed using SPICE v 5.22.[193]

All participants responded to the SEB positive control; the frequency of proliferating $CD8^-$ T cells in the SEB stimulated sample was greater than the median plus 3 times the median absolute deviation of negative control in all cases.

Samples were excluded from analysis if there was no distinct population of live cells separate from dead cells, this typically occurred when the percentage of live cells was less than 5% of total events. Samples that contained less than 1000 live $CD3^+$ cells were also excluded.

2.6.6 Statistical analysis of multi-parameter flow cytometry data

SPSS (version 20) and GraphPad Prism (version 5.0a, 2008) were used for statistical analysis of multiparameter flow cytometry data. The assumption of normality of distribution of data was assessed by visual inspection of Normal Q-Q plots and Shapario Wilk's test ($p < .05$ was considered significant and the null hypothesis of normality was rejected). Square root transformation was applied to non-normally distributed data (data positively skewed). Where transformation resulted in normally distributed data, parametric tests were applied to transformed data, otherwise non-parametric testing was applied to untransformed data.

Where statistical models were sensitive to extreme data points, such as the two-way ANOVA, outliers were identified by calculation of studentized residuals (defined as greater than +/-3 SD). Transformation of data was applied to minimize the effect of these, however where extreme values remained, the infrequent outliers were assigned the next lowest (or highest as appropriate) value in the dataset.

Levene's Test of Equality of Error Variances was used to test for homogeneity of variances ($p > .05$) in models requiring that variances between group combinations of the dependent variable are equal, such as the two-way ANOVA.

A two-way ANOVA was run to test interaction between maternal HIV status and QFN status on frequencies of specific T cells. This model also tested the main effect for each factor, namely HIV and QFN status alone. Where the interaction term was significant, simple main effects were also tested. Simple main effects examine the difference between all combinations of factors, for example the difference between HIV infected mothers with QFN negative tests versus HIV infected mothers with QFN positive tests. Bonferroni adjustment of the significance term is applied to these pairwise comparisons to correct for multiple comparisons; the corrected p value is reported. Only interaction effect and main effects are reported where the interaction term is non-significant ($p > .05$).

Where data remained non-normally distributed, the Kruskal-Wallis test was used to compare the frequencies of specific T cells in the four groups of mothers or infants based the maternal infection status, namely: HIV-uninfected, QFN negative; HIV-uninfected, QFN positive; HIV-infected, QFN negative and HIV-infected, QFN positive. Where the Kruskal-Wallis test was statistically significant, pairwise comparisons were performed using Dunn's (1964) procedure with Bonferroni correction for multiple comparisons; adjusted p values are reported.

The distribution of multiple combinations of cytokine-producing T cells across groups of mothers or infants was compared using a global distribution comparison statistic (a non-parametric permutation test).[193]

A mixed ANOVA was run to determine if there was a difference between paired infant responses to BCG vaccine antigens at birth (pre-vaccination) and at 16 weeks (post BCG

vaccination) and whether maternal infection status influenced the difference in this response. The assumptions required for the mixed ANOVA model were fulfilled, unless otherwise indicted, namely: no outliers were detected; square root transformed data was normally distributed; there was homogeneity of variances; Box's test of equality of covariance matrices ($p > .05$) revealed homogeneity of covariances and finally the assumption of sphericity was fulfilled, Mauchly's Test for Sphericity ($p > .05$).

The relationship between maternal and infant responses was assessed using Spearman's Rank Order correlation, since data did not conform to the assumption of linearity and normality.

2.6.7 Multiplex ELISA

Supernatants were collected from the whole blood assay after 24 hours of incubation with BCG, tetanus toxoid or SEB and were analysed by multiplex ELISA to determine levels of secreted chemokines and cytokines. This technique is particularly suited to studies involving infants as large numbers of analytes can be measured in small volumes of blood.

The principal of multiplex ELISA is based on the use of fluorescent-coded beads, so-called microspheres, coated with antibodies to measure a large number of analytes simultaneously. Two lasers detect the individual fluorescent signal from each microsphere along with the tagged detection antibody.

Initial exploratory work was undertaken using a 27-plex and 13-plex plates (MILLIPLEX MAP, Millipore, Billerica, MA, USA) that were part of a collaborator's ongoing study and a number of wells were kindly made available to me by Gerhard Walzl, University of Stellenbosch, South Africa. These plates contained the following: 27-plex cytokine / chemokine panel - Epidermal Growth Factor (EGF), Eotaxin (CCL11), Fibroblast Growth Factor (FGF-2), FMS-related tyrosine kinase 3 Ligand (FLT-3-L), Fractalkine, Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte-macrophage Colony Stimulating Factor (GM-CSF), Growth-related Oncogene (GRO or CXCL1), IFN-α, Interleukin (IL) 1 Receptor Antagonist (IL-1Ra), IL-1α, IL-3, IL-9, IL-12p40, IL-15, IL-17, Interferon-γ (IFN-γ) Inducible Protein 10 (IP-10 or CXCL10), Monocyte Chemoattractant Protein-1 (MCP-1 or CCL2), MCP-3 , Monocyte Derived Chemokine (MDC), Macrophage Inhibitory Protein-1 α (MIP-1α, CCL3), MIP-1β (CCL4), Soluble CD40 ligand (sCD40L), Tumour Necrosis Factor-α (TNF-α), TNF-β, Vascular

Endothelial Growth Factor (VEGF). 13-plex high sensitivity panel: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12p70, IL-13, IFN-γ, GM-CSF, TNF-α.

The main study employed customised 20-plex plates (MILLIPLEX MAP) containing the following cytokines and chemokines: Epidermal Growth Factor (EGF), Fractalkine, Granulocyte-macrophage Colony Stimulating Factor (GM-CSF), Interleukin(IL)-1 Receptor Antagonist (IL-1RA), IL-12p40, IL-17, Interferon-γ (IFN-γ), Inducible Protein 10 (IP-10 or CXCL10), Monocyte Chemoattractant Protein-1 (MCP-1 or CCL2), Monocyte Derived Chemokine (MDC), Macrophage Inhibitory Protein-1 β (MIP-1β, CCL4), Soluble CD40 ligand (sCD40L), Tumour Necrosis Factor-α (TNF-α), IL-1β, IL-2, IL-6, IL-7, IL-8 (CXCL8), IL-10 and IL-13. The cytokines and chemokines were selected based on a thorough review of the literature and data from pilot experiments from the collaborating laboratories as detailed below.

Cytokine / Chemokine	Reason for inclusion	Reference
Proinflammatory cytokine		
IFN-γ	Known key role in immune response to TB Upregulated by BCG	86, 138, 168, 169
TNF-α	Upregulated by BCG	86, 138
IL-2	Induced by BCG vaccination Decreased in HIV-exposed uninfected infants	31, 86
IL-12p40	Decreased in HIV-exposed uninfected infants	70
IL-1β	Upregulated by BCG	
IL-6	Upregulated by BCG	86, 138
Th17 Cytokine		
IL-17	Upregulated by BCG	86
Anti-inflammatory cytokine		
IL-13	Upregulated by BCG Higher in infants protected by BCG	86 Unpublished observations, Hanekom 2010
IL-1RA	Antagonist of IL-1β	
Regulatory Cytokine		
IL-10	Upregulated by BCG Increased in HIV-exposed uninfected infants	74, 86
Growth Factor		
GM-CSF	Upregulated by BCG Predictor in model of infants protected by BCG	86 Unpublished observations, Hanekom 2010
IL-7	Increased in HIV-exposed uninfected infants	27
Chemokines		
IL-8	Upregulated by BCG	86
IP-10	Upregulated by BCG	86, 138
MIP-1β	Upregulated by BCG	86, 138
MCP-1	Involved in immune response to TB	Hasan 2005
EGF	Lower in infants protected by BCG	Unpublished observations, Hanekom 2010
Fractalkine	Lower in infants protected by BCG	Unpublished observations, Hanekom 2010
MDC	Upregulated by BCG	138
sCD40L	Increased in HIV-exposed uninfected infants	Romano 2006

Samples were run in singulate by a single researcher (Dr C Jones) without reference to the patient grouping. Manufacturers instructions were followed. SEB-stimulated samples were not run as a positive control in this assay due to high cost, however these samples were run on a separate IFN-γ ELISA to confirm that a positive response was detected for each individual.

Following preparation of the filter plate by the addition of assay buffer to pre-wet the filter, undiluted samples and standards were added. Blocking buffer was added and then samples and standards were mixed with fluorescent beads coated with capture antibody and

incubated for 1 hour. Following washing steps, biotinylated detection antibodies were incubated with samples and standards for 30 minutes and then reacted with Streptavidin-Phycoerythrin for a further 30 minutes. Beads were washed and re-suspended in sheath fluid and analysed immediately on a Bio-Plex array reader (Bio-Rad, Hercules, CA, USA). The standard curve for all analytes ranged from 3.2 – 10,000pg/ml. All analyte levels in the quality controls supplied with the kit were within the expected ranges. A single supernatant was run of each on the plates as an inter-plate control.

Multiplex data handling and statistical analysis

The unstimulated sample value was subtracted from the stimulated sample value to determine the response attributable to the antigenic stimulation. Any value of less than 3.2 pg/ml (the lower limit of detection of the assay), or values deemed "Out of Range-Low" by the Bio-Plex Manager software were assigned a value of 1.6 pg/ml. Values of greater than 10,000 pg/ml (the upper limit of detection of the assay), or a values described as "Out of Range-High" were assigned a value of 10,000 pg/ml.

Data was transformed using the natural logarithm (the logarithm to the base e, where e is a constant approximately equal to 2.71). Presented data is back transformed using the inverse function.

A 3-way repeated measures ANOVA with mixed models was run to determine the effect of maternal HIV and TB infection on the level of secreted cytokines and chemokines in mothers and infants. In infants, the effect of time point (pre or post- BCG vaccination) was also considered in the model. A number of analytes had a bimodal distribution (expressed or not expressed) and these were analysed using a generalized estimating equations (GEE) model. Statistical analysis of the multiplex ELISA data was completed using Statistica (v10) and were run in conjunction with Professor Martin Kidd, University of Stellenbosch, South Africa.

2.6.8 Laboratory screening for prevalent TB: Whole blood assay and IFN-γ ELISA

196 μl of 1:10 diluted whole blood was stimulated with SEB 1μg/ml, Early Secreted Antigenic target 6kDa Protein/ Culture Filtrate Protein 10 (ESAT6/CFP10) fusion protein (produced and supplied by T. Ottenhoff, University of Leiden Medical Centre, Netherlands) at a final concentration of 5μg/ml or left unstimulated. Cultures were incubated at 37^0C, 5% CO_2, 80%

humidity. 150 μl of supernatant was harvested after 6 days and stored at -80^{0}C until required.

Ninety-six well plates were coated with mouse anti-human IFN-γ capture antibody (all antibodies and standards supplied by Pharmingen, BD, Oxford, United Kingdom) and incubated overnight at 4 °C. Following washing and blocking steps, samples (diluted 1:4) and recombinant human IFN-γ standards were added and incubated overnight at 4 °C. Bound cytokine was detected with biotinylated mouse anti-human IFN-γ antibody and reacted with avidin-peroxidase conjugate (Sigma-Aldrich, St Louis, MO, USA). O-phenylenediamine di-hydrochloride (OPD) enzyme substrate solution (Sigma-Aldrich) was added for 10 min and the reaction was stopped 2M H_2SO_4 (Sigma-Aldrich). The absorbance was read at 490nm using an ELISA plate reader.

2.6.9 Antibody ELISAs

Serum samples were processed in collaboration with the National Health Laboratory Service (NHLS) Immunology Laboratory, Tygerberg Division. Dr C De Beer and Ms S Naidoo processed the samples under laboratory director Dr M. Esser, using standard international commercially available ELISAs in a semi-automated method.

Bordetella pertussis

Specific IgG to *Bordetella pertussis (B. pertussis)* was measured using SERION ELISA *classic* kits (ESR 120G, Serion Immundiagnostica GmbH, Würzburg, Germany). All reagents were supplied with the commercial kit. The manufacturer's instructions were followed. In summary, 100μl of samples diluted 1:100, standards and controls were incubated in microwells pre-coated with *B. pertussis* IgG antigens (pertussis toxin and filamentous hemagglutinin) for 60 minutes at 37°C in a moist chamber. Unbound material was removed by washing and conjugate (polyclonal goat anti-human-IgG conjugated to alkaline phosphatase) was added to all the wells, except substrate blank. Samples were incubated with conjugate for 30 minutes at 37°C. Unbound conjugate was removed by washing and para-nitrophenylphosphate substrate was added. Following a further 30 minute incubation at 37°C, the resulting colour reaction was stopped with sodium hydroxide stop solution and the colour intensity was measured at 405nm.

Pertussis titers of >30FDA U/ml were regarded as positive (defined by the manufacturer). [194]

Tetanus Toxoid

Levels of specific IgG antibodies against Tetanus toxoid were determined using SERION ELISA *classic* kits (ESR 108G, Serion Immundiagnostica GmbH, Würzburg, Germany). The method used was identical to that used to measure anti-*B. Pertussis* antibodies, with the exception that microwells were coated with Tetanus toxoid. Tetanus antibody levels were classified as providing sufficient protection if >0.1 IU/ml.[195]

Haemophilus influenzae type b (Hib)

Hib capsular polysaccharide specific IgG present in the serum of mother and infant was measured using the VaccZyme™ Human Anti- Hib Enzyme Immunoassay kit (MK016, The Binding Site Ltd, Birmingham, England).

The assay was carried out according to manufacturer's instructions. In brief, 100µl samples diluted 1:100, assay standards and controls were added to microwells pre-coated with Hib capsular polysaccharide antigen. Following 30 minutes incubation at room temperature and washing, samples were incubated with conjugate (peroxidase rabbit anti-human IgG) for 30 minutes. Excess conjugate was removed by a further wash step and then TMB substrate was added to allow visualisation of the bound conjugated antibody. Colour development was stopped after 30 minutes with phosphoric acid. The optical density (OD) of each sample and standard was read at 450nm within 30 minutes using the Bio-Rad PhD™ System. The assay detection limits were 0.11 – 9.0 mg/L. Anti-Hib antibody titers of >1.0mg/l were regarded as protective.[196]

Pneumococcal capsular polysaccharide (PCP)

PCP-specific IgG was measured using VaccZyme™ Anti-PCP Enzyme Immunoassay kits (MK012, The Binding Site Ltd, Birmingham, England). Microwells in the pneumococcal assay were supplied pre-coated with pneumococcal capsular polysaccharide antigens 1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F and incorporated C-polysaccharide antibody absorption, which confers limited protection against pneumococcal infection.[197] The antigens are the same as those in the 23-valent pneumococcal vaccine and are responsible for the majority of commonly encountered virulent serotypes. The assay method was identical to that described for Hib capsular polysaccharide specific IgG. The limits of detection of the assay were 3.3 – 270mg/l. No level of protective immunity has been established for a collective response to multiple pneumococcal serotypes.

Hepatitis B surface antigen (HBsAg)

HBsAb was measured using an Abbott AxSYM® HBsAg kit, v2 (7A40-22, Abbott, Wiesbaden, Germany). All reagents, including controls, were supplied with the commercial kits; manufacturer's instructions were followed. Samples were placed in the Sample Carousel and reagent packs into the Reagent Pack Carousel. Further steps were fully automated. The sample, anti-HBs coated microparticles and biotinylated anti-HBs were combined in the reaction vessel. The reaction mixture was transferred to the matrix cell where microparticles bind to the glass fibre matrix. Anti-biotin Alkaline Phosphatase conjugate was added. Following washing to remove unbound microparticles, substrate (4-Methylumbelliferyl Phosphate) was added. The fluorescent product was measured by the optical assembly. HBsAb values of >10mIU/ml were regarded as seropositive and protective against Hepatitis B infection.

Data management and statistical analysis

Antibody levels of zero or below the assay cut off were assigned an arbitrary value of half the detection limit.[75,198]

The magnitude of specific antibody response between groups was compared using the unpaired t test where data were normally distributed. Where the distribution was non-normal, data were log transformed; the unpaired t test was used if resulting distributions were normal; the Mann-Whitney test was used for non-normal data.

The proportion of mothers or infants with responses considered protective was compared using the Fisher's exact test. HBsAb data had a bimodal distribution and therefore only the proportion of participants with seroprotective levels of Hepatitis B surface antibody results were analysed.

Simple correlations were assessed using Pearson or Spearman correlation in the case of normal or non-normal distribution respectively. A multiple linear regression model was used to assess the relationship between the magnitude of maternal and infant Hib, pertussis, pneumococcal and tetanus responses at delivery in relation to maternal HIV status, treating maternal age, gravidity and household type (informal structure or brick house), a proxy for socioeconomic status in this community, as covariates. Infant gender and birth weight were used as additional covariates in analyses of infant responses at birth. All independent variables were entered into the model simultaneously (forced entry method).

Placental transfer was defined as the ratio of infant to mother specific IgG concentration at birth.[180]

To determine if there was a significant difference in the antibody level between birth (pre-vaccination) and post vaccination between HIV-exposed and unexposed infants, the vaccine-induced change in response (fold change) was compared.

Missing data were excluded from analysis. No correction was made for multiple comparisons as group comparison were pre-planned and the same essential question was asked for each subgroup of mothers and infants. Statistical calculations were completed using SPSS (v20) and GraphPad Prism (v5.0a, 2008). Two-sided P values were calculated and a p value $< .05$ was considered significant. All comparisons were pre-specified except for the comparison of infants who had not received all vaccinations, which was post-hoc.

Results

Chapter 3: Characteristics of the mother-infant cohort

3.1 Establishment of the field site

A field site was established within a community health facility in Khayelitsha, an urban informal settlement. The name of the township, "*our new home',* characterises the origin of the settlement and explains many of the area's difficulties. It is a rapidly expanding area largely populated by migrants from the Eastern Cape in search of employment or education. The majority of the population live in shacks constructed of tin or wood and there are high levels of unemployment, crime and poverty.

These socio-economic problems are compounded by the high prevalence of TB and HIV. The Western Cape province has the highest incidence of TB in South Africa, with an adult tuberculosis incidence of over 1,000 per 100,000 reported in 2009[183] and Khayelitsha itself has the highest incidence in the Cape Town Metropole with rates of 1,614 per 100,000 (City of Cape Town, 2008). Confounding the problem of tuberculosis is a high prevalence of HIV; 51% of TB cases tested for HIV were also HIV-infected (92% uptake of HIV testing) in 2007 (City of Cape Town, 2008). Similarly, the prevalence of HIV amongst pregnant women registering for antenatal care is high in the Western Cape, with 12.3% of attendees testing HIV positive in 2009.[183] At the study specific site, the prevalence exceeds this with 33% of women attending antenatal clinics infected with HIV in 2009 (City of Cape Town, 2009).

However, Khayelitsha district has a well-established Prevention of Mother to Child Transmission (PMTCT) programme and has achieved a reduction in the vertical HIV transmission rate from 12.5% in 2002 to 2.5% in 2010.[6] All women are offered HIV testing at their first antenatal visit with pre-test and post-test counselling. Rapid HIV testing is performed using First Response® HIV 1-2.0, Premier Medical Corporation, an immunochromatographic test performed with whole blood from a finger prick sample.

The uptake of HIV testing at the study-specific antenatal clinic was 98% in 2007 (Western Cape Department of Health, 2007) and 96.9% of HIV-infected women were enrolled in the PMTCT program (A. Hesseling, personal communication). The programme consists of dual therapy for both mothers and infants, starting Zidovudine at ≥ 28 weeks gestation, then Zidovudine for one month to the infant and a single dose of Nevirapine to both mother and infant. Exclusive feeding options are encouraged and mothers are provided with free formula for 6 months, if they chose exclusive formula feeding. The programme follows

infants in the community-based primary health clinic until 6 months of age. Mothers are routinely offered a single infant HIV DNA PCR test (Roche, Amplicor®) at 6 weeks of age.

These characteristics were key considerations when selecting the study site and also explain many of the challenges to setting up the study. It was an ideal site due to the high rates of maternal HIV and *Mtb* infection coupled with a well-established PMTCT program such that the rate of vertical transmission of HIV was low. The PMTCT program continued to follow HIV-exposed infants after birth and ensured ongoing care for these infants.

There were many challenges that were overcome in the establishment of the study site. Permission to conduct the study was sought and gained from two local ethics committees, the health facility and the local community elders. There were a number of safety issues that had to be observed, not least travelling to the health facility through the local community. Access was impossible due to rioting on a small number of occasions.

3.2 Recruitment

3.2.1 Recruitment pilot study

A pilot study was carried out to investigate the optimal timing of counselling and recruitment to the study. Women were counselled and screened for eligibility in the antenatal clinic and sequential eligible women were enrolled (n=15). Consent was re-confirmed following delivery.

In this pilot study, cohort retention was poor; only one quarter of women that were recruited to the study in the antenatal clinic were actually enrolled following delivery. The most common reason was women delivering their infant and being discharged before the study team was available, since most women leave the maternity unit within 8 hours of giving birth. In addition a number of study participants were given the BCG at birth in error and therefore no longer qualified for enrolment.

As a result of this pilot study, a community counsellor administered a structured interview to assess maternal perceptions of recruitment in the postnatal rather than antenatal period. This ensured that women did not feel pressured by the presence of a medical doctor or nurse. Ten women were asked about their perceptions of discussing a research study in the immediate post-partum period and their views on blood sampling from their newborn

infant. The counsellor made an assessment of whether information about the study had been understood by testing recall and understanding. All 10 women thought that it was acceptable to discuss a research study in the immediate postnatal period. All mothers were able to recall the details of the study and all would be willing to have a blood sample taken from their newborn infant, some women would prefer not to be present when this was done. The results of the survey are tabulated and shown in Appendix II. Ethics committees and local senior midwifery staff were consulted and agreed to the change in recruitment strategy.

3.2.2 Recruitment of study population

A study lay community counsellor approached women on the postnatal ward to give women information about the study. Further information was given to interested women, a study nurse or Dr C Jones took informed consent from eligible women. Dr C Jones examined all infants and took blood from each infant. Blood samples were transported by Dr C Jones to the laboratory at the University of Cape town within 4 hours of sample receipt. She then prepared the whole blood culture for each of the 218 blood samples collected from mothers and infants at birth. The same procedure was observed for the 95 samples collected from 16 week old infants.

3.3 Study cohort

Between March 2009 and January 2011, 123 eligible mother-infant pairs were identified. Of these women, 11 declined to participate; 109 maternal-infant pairs were therefore enrolled (91% participation rate). Of these, 47 mothers (43%) were HIV-infected and 62 (57%) HIV-uninfected. All women testing negative for HIV at their antenatal care registration had a further repeat negative HIV test at delivery. One infant (1%) was determined to be HIV-infected at 4 weeks of age and was referred for rapid initiation of anti-retroviral treatment (mother-infant pair subsequently excluded from analysis). Of the original cohort, 95 infants were followed up to 16 weeks of age (87% of infants). Of these, 39 infants were HIV-exposed, uninfected (41%) and 56 were HIV-unexposed (59%). The number of samples for each assay available for these infants is given in the relevant results section. The selection of the study cohort from the population of women giving birth at the study site and screened for eligibility is shown in Figure 9.

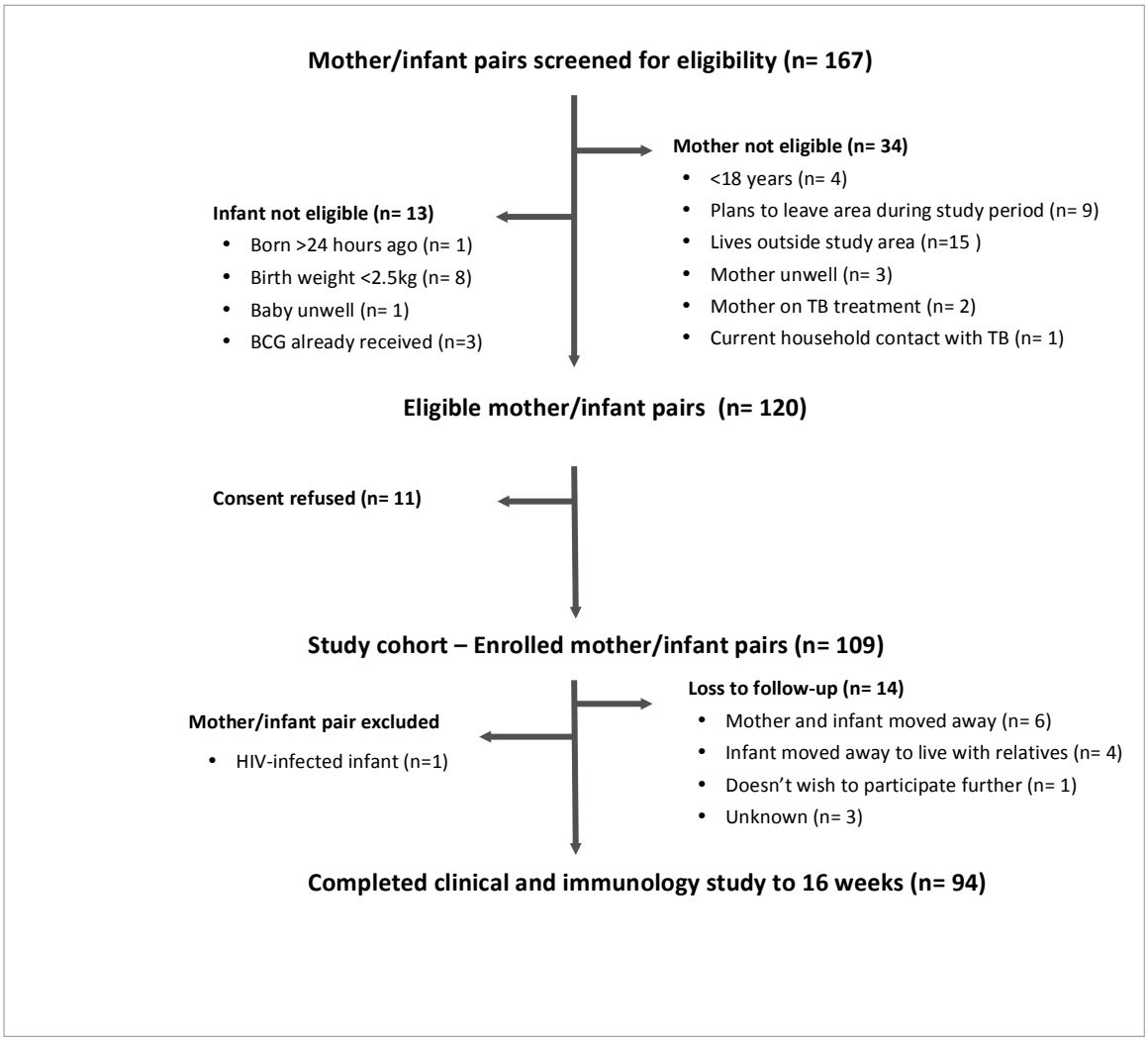


Figure 9. Study cohort flow chart

The study cohort was subsequently divided according to maternal TB infection status based on evidence of *Mtb* sensitisation, as defined by a positive QFN test. The four groups of mother/infant pairs were defined by maternal infection status namely: HIV- QFN- (n=27), HIV- QFN+ (n= 35), HIV+ QFN- (n=25), HIV+ QFN + (n=20). One HIV-infected mother had an indeterminate QFN result (participant data was used when considering HIV status alone, but not when considering the four groups). Cohort clinical data was analysed only by maternal HIV infection status as this has most biological relevance.

3.3.1 Maternal Characteristics

Characteristics of participating mothers are given in Table 2.

	HIV-infected mothers (n=46)	HIV-uninfected mothers (n=62)	p value
Median age in years (IQR)	27.5 (24 – 31.25)	24 (20-27.25)	.001
Normal vaginal delivery	46 (100%)	62 (100%)	1.00
Primigravidity	12 (26%)	30 (48%)	.02
BMI at delivery	28.10 (25.55 - 31.69)	27.28 (24.10 - 33.97)	.95
BMI at 16 weeks	26.17 (23.47 - 28.85)	25.60 (22.96 - 30.51)	.97

Table 2. Maternal characteristics.
'Primigravidity' refers to mothers delivering their first child; BMI: Body Mass Index.

The mean CD4 count among HIV-infected women was 474 cells/μl (SD 252) and the median viral load 730 copies/ml (IQR 357 - 3925). Seven women had CD4 counts <200 cells/μl; 3 of these were already on HAART at enrolment and a further 4 were referred by the study team to commence HAART following delivery. A large proportion of women were only diagnosed with HIV as a result of antenatal screening in this pregnancy (n= 27, 59%).

Most women received antiretroviral medication as part of the PMTCT programme; antenatal AZT or HAART was received by 89% of women (n= 41), intrapartum NVP or ongoing treatment with HAART was administered to 65% of women (n=30). Six women were commenced on HAART prior to conception and a further three women were commenced on HAART in pregnancy. The majority of infants received NVP at birth (n = 44, 96%) and postnatal AZT (n=45, 98%) for varying amounts of time (5-28 days).

3.3.2 Infant characteristics

All infants were full-term and of good weight at birth. There was no difference in growth between HIV-exposed and unexposed infants. All HIV-infected mothers chose exclusive replacement feeding and continued with this practice throughout the study.

	HIV-exposed, uninfected infants	HIV- unexposed infants	p value
Gestation, weeks	38^{+3} (37^{+1} - 39^{+6})	38^{+4} (35^{+1} - 40^{+1})	.36
Female	26 (57%)	34 (55%)	1.00
Birth weight, kg	3.13 (2.90 - 3.45)	3.12 (2.98 - 3.47)	.59
Weight at 6 weeks, kg	4.60 (4.20- 5.19)	4.77 (4.49 - 5.18)	.15
Weight at 16 weeks, kg	6.39 (6.02 - 6.98)	6.73 (6.25 - 7.40)	.16
HC at birth, cm	34 (33 - 35.6)	34 (33-35)	.35
HC at 6 weeks, cm	38 (37-40)	38.3 (37.1 - 39.3)	.56
HC at 16 weeks, cm	42 (41 - 43)	42 (41 - 43)	.98
Length at 6 weeks, cm	54 (51.9 - 56.1)	54.3(53 - 55.9)	.51
Length at 16 weeks, cm	60.5 (58.9 - 63)	61.5(60 - 63)	.18
Breast fed at birth	0 (0%)	62 (100%)	<. 001
Breast fed at 6 weeks	0 (0%)	51 (90%)	< .001
Breast fed at 16 weeks	0 (0%)	37 (66%)	<.001

Table 3. Characteristics of participating infants.
'Breast fed' refers to infants who received breast milk either exclusively or in combination with formula milk. Infants below 2.5kg at birth were excluded from the study. Median values (IQR) are stated for continuous data and the Mann-Whitney U test used to compare groups. Numbers (%) are otherwise stated and groups compared using Fisher's Exact test.

Infants were screened for prevalent *Mtb* infection using a clinical screening tool and in addition IFN-γ release in response to ESAT6/CFP10 fusion protein was assessed using a standard ELISA. One infant was admitted to hospital for a lower respiratory tract infection at 6 weeks of age and was treated for presumed TB. None of the other infants were clinically suspected of having TB. Thirteeen infants had a positive IFN-γ response to ESAT6/CFP10 fusion protein, however all that returned for repeat testing with a standard QFN test and TST had negative results and were clinically well.

3.4 Analysis of clinical data

3.4.1 Assessment of maternal *Mtb* sensitisation

Tuberculin skin testing (TST) was undertaken to guide clinical management as national TB guidance is based on TST rather than QFN testing. This afforded the opportunity to compare results of TST and QFN testing.

TST results were available for 97 women; 12 women did not return to have the TST read. Of these, 81 women returned within 96 hours to have the TST read (median 48 hours, range 48 hours – 8 days). Out of the 16 women who returned later than 96 hours, the majority had large areas of induration, with median responses of 13.5mm (range 0 – 25mm), therefore the TST was not repeated for these women. There is some evidence of good agreement between TST readings on day 2 and day 7.[199]

Positive TST responses, as defined by induration of 5mm in HIV-infected women and 10mm in HIV uninfected women, were found in 59 (61%) of mothers. Negative responses were recorded for 38 mothers (39%). There was no difference in the proportion of positive and negative results amongst mothers between women who returned on time or late for TST readings.

Of the women with available TST results, 53 (55%) had positive QFN results, 43 (44%) had negative results and 1 (1%) had an indeterminate result. Discordant results were common between the TST and QFN tests, Table 4. Only moderate agreement between the tests was found, (Kappa) $\kappa = 0.49$ (95% CI: 0.31 - 0.66, $p < .001$).

		QFN		Total
		Negative	Positive	
TST	Negative	28	9	37
	Positive	15	44	59
Total		43	53	96

Table 4. Agreement between TST and QFN test results.
Numbers indicate number of women in each test category.

The relationship between maternal HIV infection and distribution of TST results (induration in mm) is reflected in Figure 10.

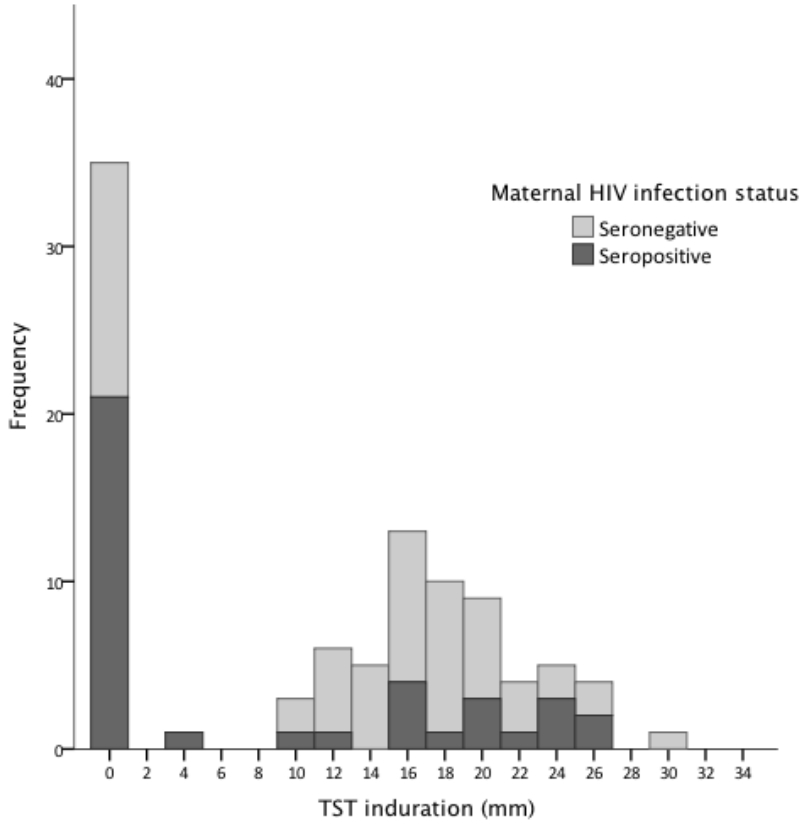


Figure 10. Relationship of maternal HIV infection and distribution of TST response.
Data available for 97 women (39 HIV infected, 58 HIV uninfected).

Positive TST responses were recorded for 16 HIV-infected women (41%) and negative responses for 23 (59%). The rate of positivity was substantially higher in HIV-uninfected women, with 43 women (74%) having a positive result and 15 (26%) having a negative result. The agreement between the TST and QFN test was similar for HIV-infected and uninfected women, Table 5.

HIV-infected mothers		QFN		Total	HIV-uninfected mothers		QFN		Total
		Negative	Positive				Negative	Positive	
TST	Negative	15	7	22	TST	Negative	13	2	15
	Positive	3	13	16		Positive	12	31	43
	Total	18	20	38		Total	25	33	58
	Kappa	0.48 (95% CI 0.21 - 0.75) p= .003				Kappa	0.48 (95% CI: 0.27 - 0.70) p< .001		

Table 5. Agreement between TST and QFN tests was similar for HIV-infected and uninfected women.

3.4.2 Clinical correlates of BCG vaccination in infants

There is no evidence that BCG scar size is associated with protection against TB, however it provides a reliable indicator of BCG vaccination and has been correlated with TST responses.[200]

All infants had a visible BCG scar at 16 weeks. Although there was a trend towards a smaller scar amongst HIV-exposed infants (5.9mm, SD 2.3mm) compared to HIV-unexposed infants (7.0mm, SD 2.8), the actual difference was very small (1.1mm) and this did not reach statistical significance (p= .05). Only one infant had abscess formation associated with BCG vaccination and no infant had right axillary adenitis. Of note, the single infant who was HIV-infected did not receive the BCG vaccine, in line with WHO guidance.[130] The single infant treated for TB disease had the BCG deferred until after treatment was complete.

3.4.3 Infant Morbidity

Between birth and 16 weeks of age, a higher proportion of HIV-exposed infants were taken to local health facilities due to ill health than HIV unexposed infants (24% vs 4%, RR 2.34, 95% CI 1.5 – 3.5, p= .006). However, a similar proportion attended the research clinic for unscheduled visits (29% vs 41%, p= .28). When considering attendance at both clinics the trend persisted (58% vs 45%, RR 1.3, 95%CI: 0.8-2.3), however it no longer reached statistical significance. There were similar numbers of hospital admission amongst HIV-exposed and unexposed infants. HIV-exposed infants had more hospital admissions for infections compared to HIV-unexposed infants (13% of HIV-exposed infants compared to 7% of unexposed infants), however this did not reach statistical significance, p > .05. HIV-exposed infants were admitted to hospital with the following diagnoses: pneumonia (4), meninigitis (1) and pyloric stenosis (1). The 8 HIV-unexposed infants who has a hospital admission had skin sepsis (1), pneumonia (3), jaundice (3), removal of accessory digits (1).

3.4.4 Maternal helminth infection and sensitisation

Stool samples were available from 69 women; 9 of these samples were positive for Helminths (13%) and all but one were samples from HIV-uninfected women. *Ascaris* was detected in 7 samples, Trichuris in 5 samples, and Entamoeba in 1. Following confirmation of a positive test, women received treatment.

Ascaris-specific IgE results were available for 105 women. This test revealed that 21 women were sensitised to *Ascaris (*specific IgE >0.35). Of these women 5 women had evidence of current infection on stool sample; 2 women with active *Ascaris* infection had *Ascaris*-specific IgE levels of <0.35.

3.4.5 Haematological parameters

HIV-infection has been associated with changes in haematological parameters both in the mother and in their uninfected offspring, especially in the context of PMTCT treatment with zidovudine. Estimation of haematological indices was performed on a sub-set of the cohort. Data was available for 48 mothers (35 HIV infected and 14 uninfected), 46 infants at birth (33 HIV-exposed and 13-unexposed infants) and 64 infants at 16 weeks of age (30 HIV-exposed and 34 HIV-unexposed).

There was no statistically significant difference in haemoglobin levels amongst HIV-infected and uninfected mothers (11.1 vs 12.7g/dl, p= .14), or their infants either at birth (18.08 vs 18.80g/dl, p= .30) or at 16 weeks (11.0 vs 11.1g/dl, p= .31). There was however a significant difference in the red blood cell (RBC) count in HIV-infected mothers compared to HIV-uninfected mothers (3.6 vs 4.2 $x10^6$/µl, p= .002). HIV-exposed, uninfected infants might have lower RBC counts compared to HIV-unexposed infants (4.9 vs 5.3 $x10^6$/µl, p= .07)

As is well established, infants at birth had significantly higher haemoglobin levels compared to mothers (p< .0001) or compared to at 16 weeks of age (p< .001). By 16 weeks of age levels between mothers and infants were comparable (p= .63).

HIV-infected mothers had significantly lower levels of white blood cells (WBC) compared to HIV-uninfected women (10.6 vs 13.5 x 10^9/l, p= .01). However there was no difference in WBC between exposed-uninfected infants compared to unexposed infants at birth (16.6 vs 15.8 x 10^9/l, p= .69, or at 16 weeks of age (8.6 vs 9.4 x 10^9/l, p= .30). Similarly, there was no significant difference in lymphocyte count at birth (4.90 vs 4.95 x 10^9/l, p= .86) or at 16 weeks (5.35 vs 5.95 x 10^9/l, p= .20) between HIV exposed and unexposed infants. Granulocyte and monocytes count was similar amongst all infants at birth and at 16 weeks.

3.4.6 Maternal socioeconomic status

Mothers infected with HIV were significantly more likely to live in an informal housing structure ('shack') than a brick house compared to HIV-uninfected mothers and less likely to have access to running water or a flush toilet in the house. These measures provide a good reflection of socio-economic status in the study community. Despite the high levels of poverty prevalent in the community, a large proportion of households owned commodities such as a mobile phone (91%), television (80%), DVD player (62%) or fridge (67%). The majority of women had completed at least secondary education (93%) and most households had someone in regular employment (75%). There was no difference in these indices between HIV-infected and uninfected women, Table 6.

	HIV-infected women (%)	HIV-uninfected women (%)	p
Informal housing structure	79	55	.01
Running water in house	32	55	.02
Flush toilet in house	29	52	.03
Cell phone	95	89	.47
Landline	0	2	1
Electricity	87	89	.75
Fridge	73	63	.29
DVD player	70	57	.2
Television	87	75	.18
Radio	65	59	.67
Car	14	11	.75
Bicycle	5	5	1.00
Completed secondary school	92	93	1.00
Mother in regular employment	21	23	1.00
Anyone in household employed	71	77	.63

Table 6. Indices of socioeconomic status in HIV-infected and uninfected women.
Ownership of commodities is per household.

3.5 Qualitative analysis of study participation

A questionnaire was administered at study exit in order to assess perceptions surrounding study participation. Women felt that they had a free choice about whether or not to participate in the study; only 5 (5%) said that they felt compelled to participate (all of these subsequently reported being satisfied with the study). There were a number of reasons

given for study participation; the principal motivation was improved access to healthcare, Figure 11.

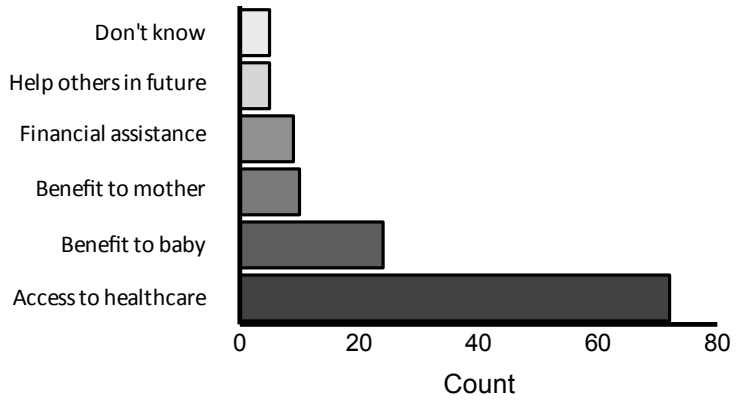


Figure 11. Reasons for study participation.
"Financial assistance" refers to the reimbursement of travel expenses to attend the health clinic, R20 (approximately £1.60).

All women reported being 'very satisfied' or 'satisfied' with study participation. They reported a number of advantages of taking part in the study (Figure 12) and the majority did not cite any problems with the study; one woman said it was difficult to travel to the clinic and one found it too time consuming. No one felt that taking part in the study caused her to be stigmatised, which was a potential concern as study participation can be synonymous with being HIV positive in this setting.

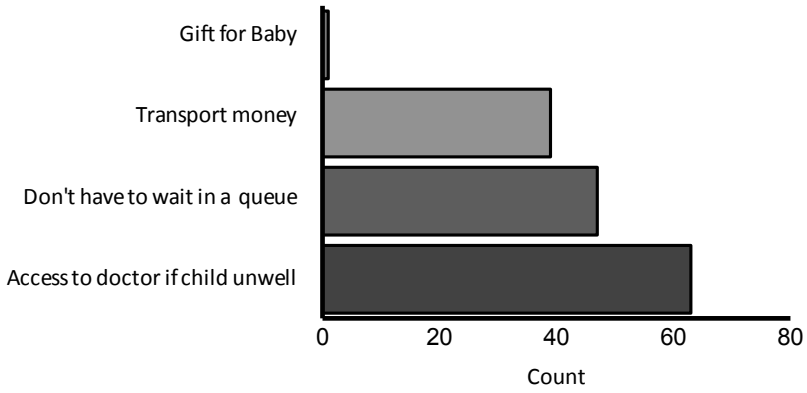


Figure 12. Advantages of study participation.
Women were reimbursed for travel expenses to attend the health clinic, R20 (approximately £1.60) and they received a baby's rattle of nominal value on completion of the study.

The majority of mothers did not mind the blood test for themselves (n=77, 83%), although a minority did find it 'a little upsetting' (n=11, 12%) or 'very upsetting' (n=5, 5%). Most mothers also did not mind the blood test for the infant (n=62, 67%), however it was upsetting to 17 women (18%) and very upsetting to 14 (15%).

3.6 Summary of results

Women living with HIV were older and more likely to have had more than one child than mothers who were not infected with HIV. All women had comparable nutritional status, as measured by BMI.

Mothers with HIV infection had relatively well preserved CD4 counts and moderately low viral loads. More than half of women were only diagnosed with HIV in the current pregnancy. Three women were currently on HAART and most of the remaining women had received anti-retrovirals as part of PMTCT. The majority of eligible infants also received NVP and AZT as part of PMTCT.

All infants were born by normal vaginal delivery at a similar gestational age. There was no difference in birth weight or subsequent growth of infants. All HIV-exposed infants received exclusive replacement feeding, whereas all HIV-unexposed infants were breast fed at birth. Of these infants, the majority was still receiving breast milk at 6 weeks, but only two-thirds by 4 months of age were breast-fed.

Discordant results were common between TST and QFN tests, however, agreement between tests was similar amongst HIV-infected and uninfected women.

All infants had evidence of a BCG scar at 4 months of age, however HIV-exposed infants tended to have smaller scars. The actual difference was small and did not reach statistical significance, $p = .05$.

HIV-exposed infants may have had increased morbidity. They had significantly more attendances to primary health clinics (this excluded routine visits to PMTCT clinics) and a higher proportion were admitted to hospital in the first 4 months of life for serious infections, however this did not reach statistical significance at the $p < .05$ level.

Active helminth infections were uncommon amongst women in this setting, however over 20% showed evidence of previous sensitisation to *Ascaris*.

HIV-infected mothers tended to have lower haemoglobin levels and had significantly reduced RBC counts compared to uninfected mothers. HIV-exposed infants had similar haemoglobin levels at birth and 16 weeks compared to unexposed infants. HIV-infected women had lower WBC than uninfected mothers, however their infants also similar WBC counts at birth compared to unexposed infants.

HIV-infected women were more likely to live in an informal housing structure and have no household running water or toilet, however ownership of commodities, education level and employment was similar amongst groups of women.

Women participated freely in the study and they cited improved access to healthcare as the main motivation for taking part. All women were generally satisfied with the study and they perceived the main benefits to be access to doctor, not having to wait in long queues for appointments or medication and the reimbursed transport costs. Most women expressed that they did not mind the blood sampling of themselves or their infants, however a number were upset by the blood sampling of their infant.

Chapter 4: Results of proliferative and intracellular cytokine responses to BCG

This aspect of the study aimed to assess the frequency of proliferating T cells in response to BCG antigens in uninfected HIV-exposed and unexposed infants at birth and 10 weeks following BCG vaccination. The frequency of these cells was also compared in infants born to mothers with and without evidence of *Mtb* sensitisation. The interaction effect of maternal HIV and *Mtb* sensitisation on the frequency of proliferating T cells was also examined. Multiparameter flow cytometry was also used to explore the functional profile of BCG-induced $CD8^-$ and $CD8^+$ $Ki67^+$T cells amongst infants at birth and at 16 weeks of age. Baseline BCG-induced proliferative and functional profiles in mothers were analysed, this allowed for associations with infant responses to be examined.

4.1 Optimisation of flow cytometry panel

4.1.1 Selection of antibodies

It was intended to include an anti-CD4 antibody in the flow cytometry panel, however optimization studies showed that recognition of $CD4^+$ T cells by surface staining was compromised when cells had been stimulated with PMA and ionomycin, Figure 13. PMA and ionomycin strongly downregulate the surface expression of $CD4^+$ on T cells, therefore the $CD3^+$ $CD8^-$ T cell subset was assumed to represent $CD4^+$ T cells.[135,201]

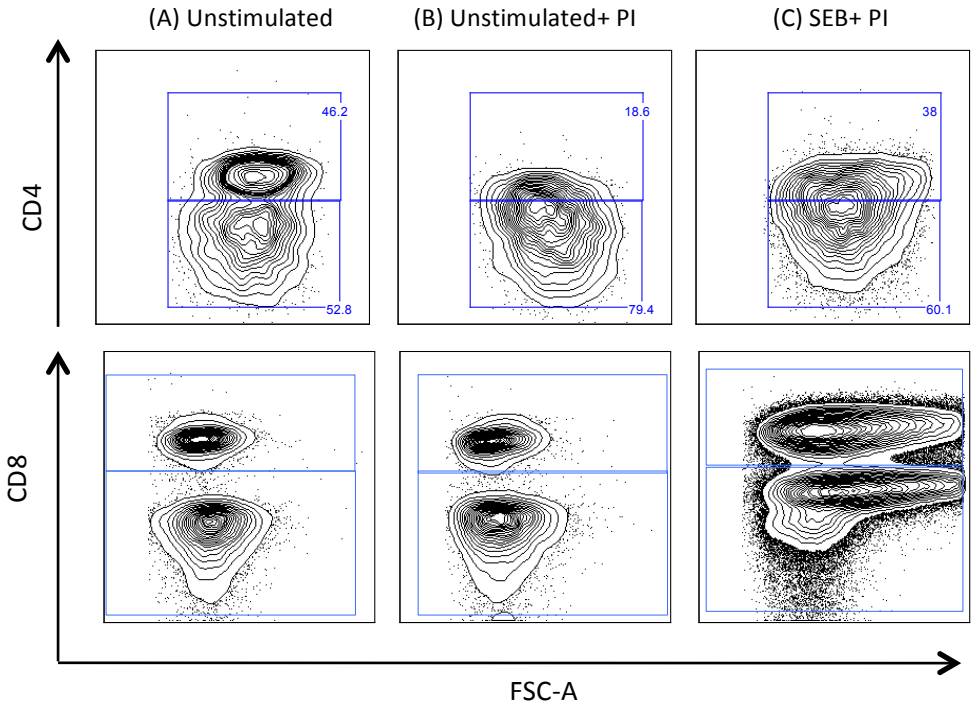


Figure 13. Surface expression of CD4, but not CD8, is downregulated on $CD3^+$ T cells stimulated with PMA and ionomycin.
The plots shown are gated on live CD3+ cells: (A) Cells incubated with medium only for 6 days; (B) Cells incubated with medium for 6 days, PMA and ionomycin (PI) added on day 6; (C) Cells incubated with SEB for 6 days, PMA and ionomycin added on day 6.

The panel of fluorescent-conjugated antibodies used to detect surface and intracellular markers was designed to minimise spectral overlap. The emission spectra were sufficiently different to allow individual signals to be distinguished. The excitation and emission spectra, the lasers and bandpass filters used are represented in Figure 14.

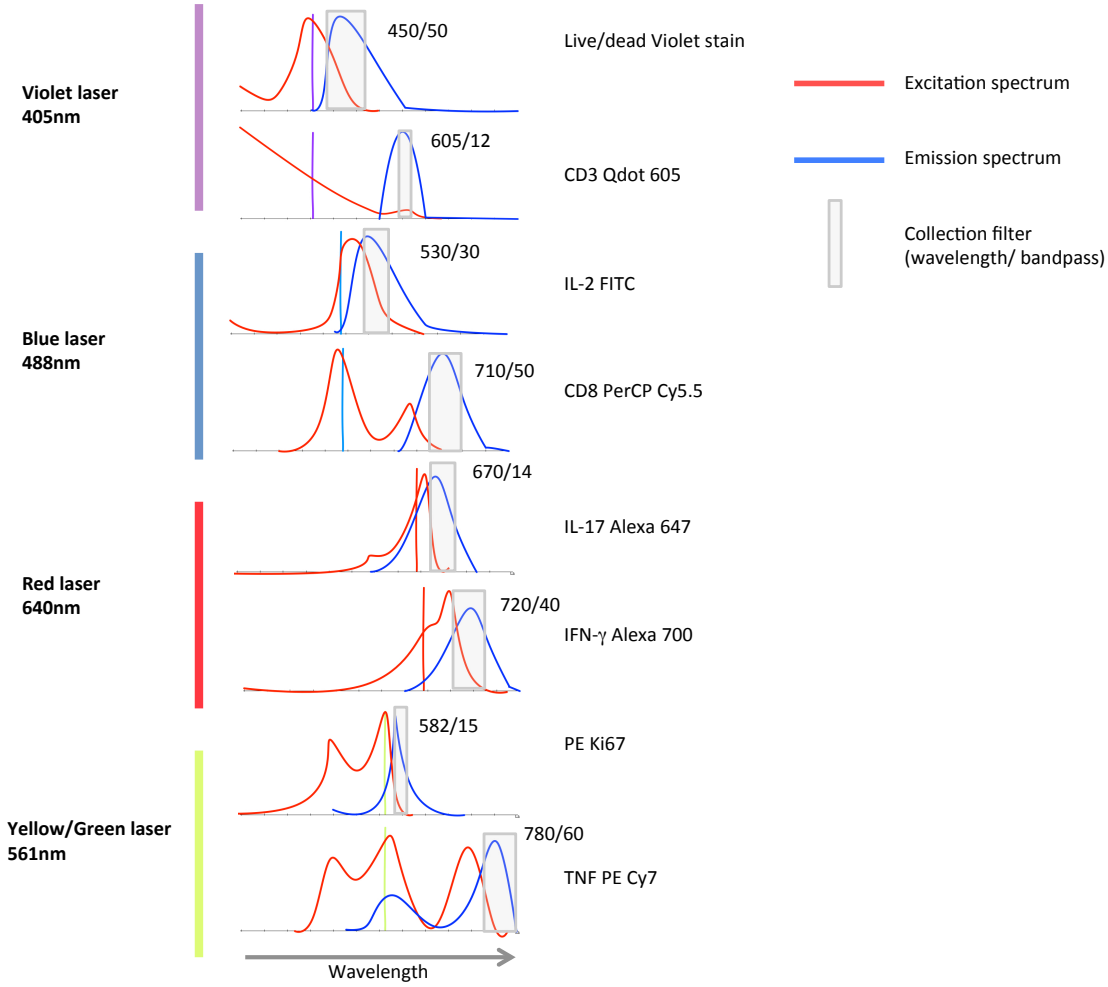


Figure 14. Spectra of fluorescent-conjugated antibodies used in flow cytometry panel.

Table 7 illustrates that this panel of antibodies resulted in minimal spectral overlap. Spill-over of light between detectors was mathematically eliminated using compensation.

	IL-2	CD8	IL-17	IFN-γ	Live/ Dead	CD3	Ki67	TNF
IL-2		1.61	0	0	0	1.32	0	0
CD8	0		8.00	9.01	0	0	0.07	7.57
IL-17	0	0.33		17.79	0	0	0	0.97
IFN-γ	0.07	4.64	2.26		0.09	0	0.05	7.62
Live/Dead	0.12	0.12	0	0		1.68	0.10	0
CD3	0	0	0	0	0		0.61	0
Ki67	0.41	5.84	0	0	0	12.87		0.82
TNF	0.15	1.14	0	0.15	0	0.28	1.61	

Table 7. Spectral overlap (%) between fluorescent-conjugated antibodies in flow cytometry panel.

4.1.2 Titration of antibodies

Cells were stained with a series of two-fold dilutions of antibody to determine the concentration that achieves optimal staining, with low level background staining and good resolution between the positive and negative populations. The stain index was used in conjunction with the visual assessment of populations. This is calculated using the formula:

$$\frac{1.645*(\text{median of positive population} - \text{median of negative})}{(95\% \text{ of negative} - 5\% \text{ of negative})}$$

The stain index aims to normalise the spread of the positive signal to the spread of the negative signal, where the negative signal may be autofluorescence, unstained cells or compensated cells from another dye dimension. The point at which the stain index starts to plateau is the optimal concentration of antibody

A sequential process was used whereby the optimum concentration of surface markers (CD3 and CD8) was initially determined. These antibodies were then used together to determine the optimum concentration of Ki67. These three antibodies were then used together to determine the optimum concentration of the intracellular antibodies. Finally all the antibodies were added together in the 'mastermix' to ensure the panel worked together. The plots below illustrate titration of Ki67 PE where a decreasing concentration of antibody is associated with loss of resolution between Ki67 positive and negative cell populations, Figure 15. The stain index was highest at 0.8µl.

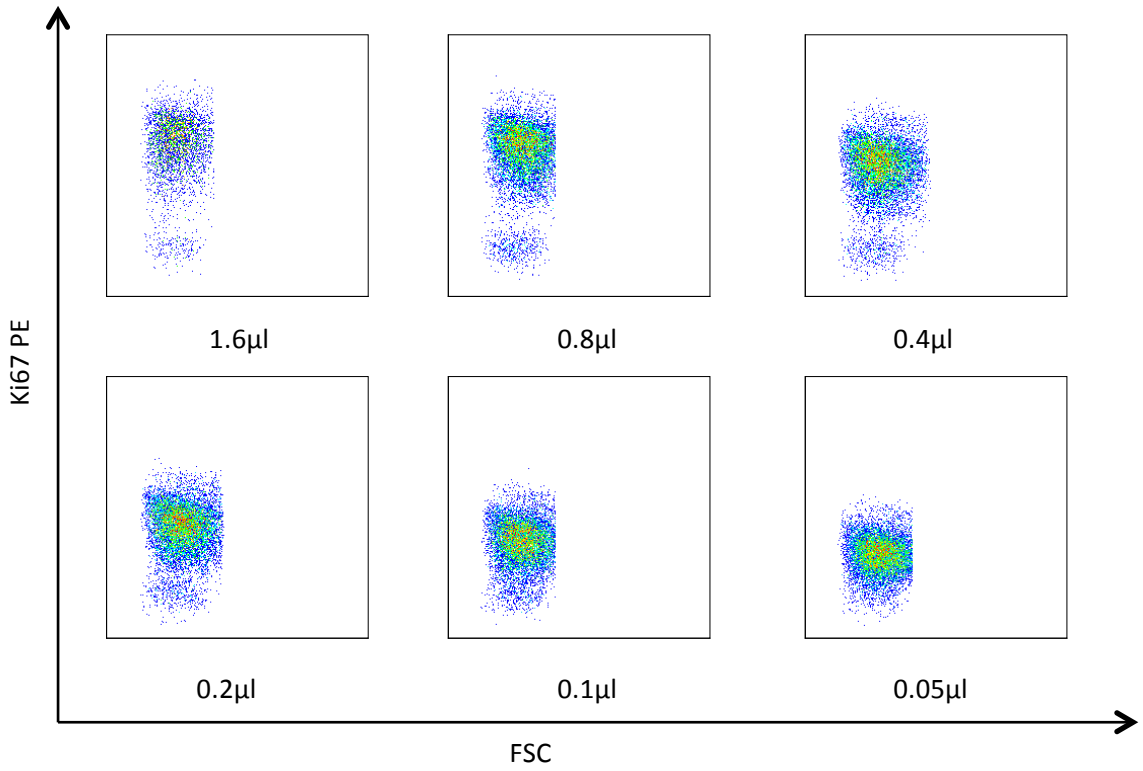


Figure 15. Titration series of Ki67 PE.
Loss of resolution of positive and negative cells populations occurs at decreasing antibody titres. Quantities represent number of µl of antibody added to final volume of antibody mastermix of 50 µl.

Titration series of other antibodies showed poor resolution of populations at higher concentrations of antibody, as antibody tends to bind non-specifically to cellular elements causing high background staining, Figure 16.

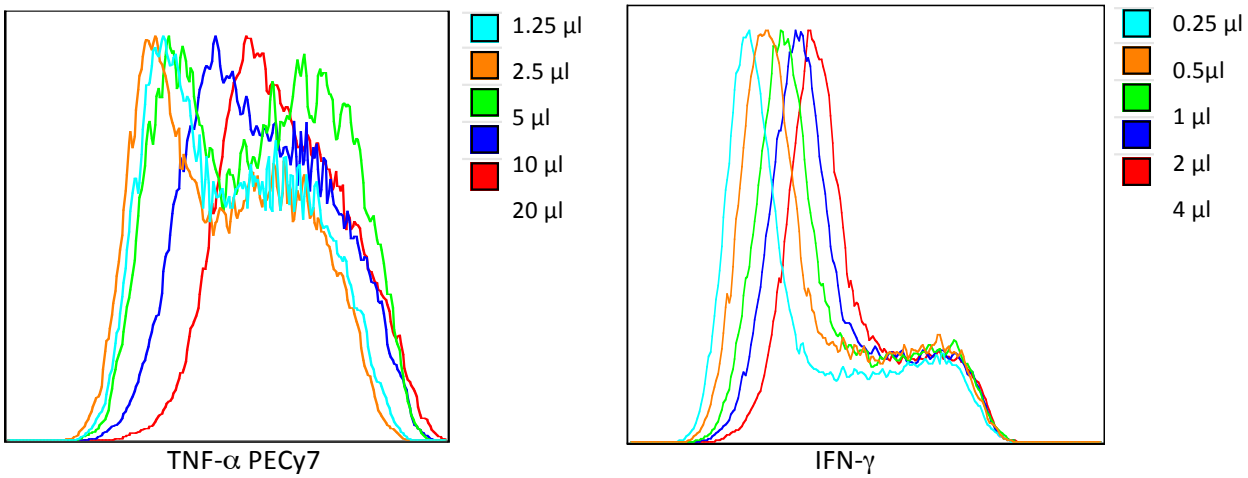


Figure 16. Loss of resolution between negative and positive populations occurs at higher antibody titres of PECy7 and AlexaFluor700.
Quantities represent number of µl of antibody added to a final volume of antibody mastermix of 50 µl.

Table 8 shows the optimum concentration of antibody determined by the titration experiments.

Fluorescent-conjugated antibody	Antibody titre
CD3 Qdot 605	0.5 µl
CD8 PerCP Cy5.5	2.5 µl
Ki67 PE	0.8 µl
IFN-γAlexaFluor700	0.5 µl
IL-2 FITC	1 µl
IL-17 AlexaFluor 647	3 µl
TNF PE Cy7	3 µl

Table 8. Optimised antibody titres.

4.1.3 Optimisation of Photomultiplier tubes (PMT) voltages

Voltages were optimised so that populations of cells were distinguishable and that all were visible on scale using an unstained sample and a fully stained sample. Voltages required minimal alteration for specific samples (<10%).

Laser and filter	Parameter	Voltage
	FSC	381
	SSC	281
488-530/30	IL-2 FITC	482
488-710/50	CD8 PerCP Cy5.5	485
640-670/14	IL-17 AlexaFluor 647	604
640-720/40	IFN-γ AlexaFluor700	555
405-450/50	Live Dead Marker	429
405-605/12	CD3 Qdot 605	636
561-582/15	Ki67 PE	519
561-780/60	TNF PE Cy7	566

Table 9. Optimised voltages for multi-parameter flow cytometry assay

4.2 Gating strategy

A hierarchical gating strategy was used to select proliferating single-cell $CD8^-$ and $CD8^+$ T cell populations, Figure 17. Gates for proliferating $CD8^-$ and $CD8^+$ T cells expressing cytokines were set using the unstimulated control (Nil well). However, where no population was visible in the unstimulated control, the preceding gate of CD8 versus Ki67 was widened to visualise the unproliferating as well as proliferating cells, this then allowed the gate to be set using the unstimulated control. A Boolean combination was used to determine T cells producing multiple combinations of cytokines.

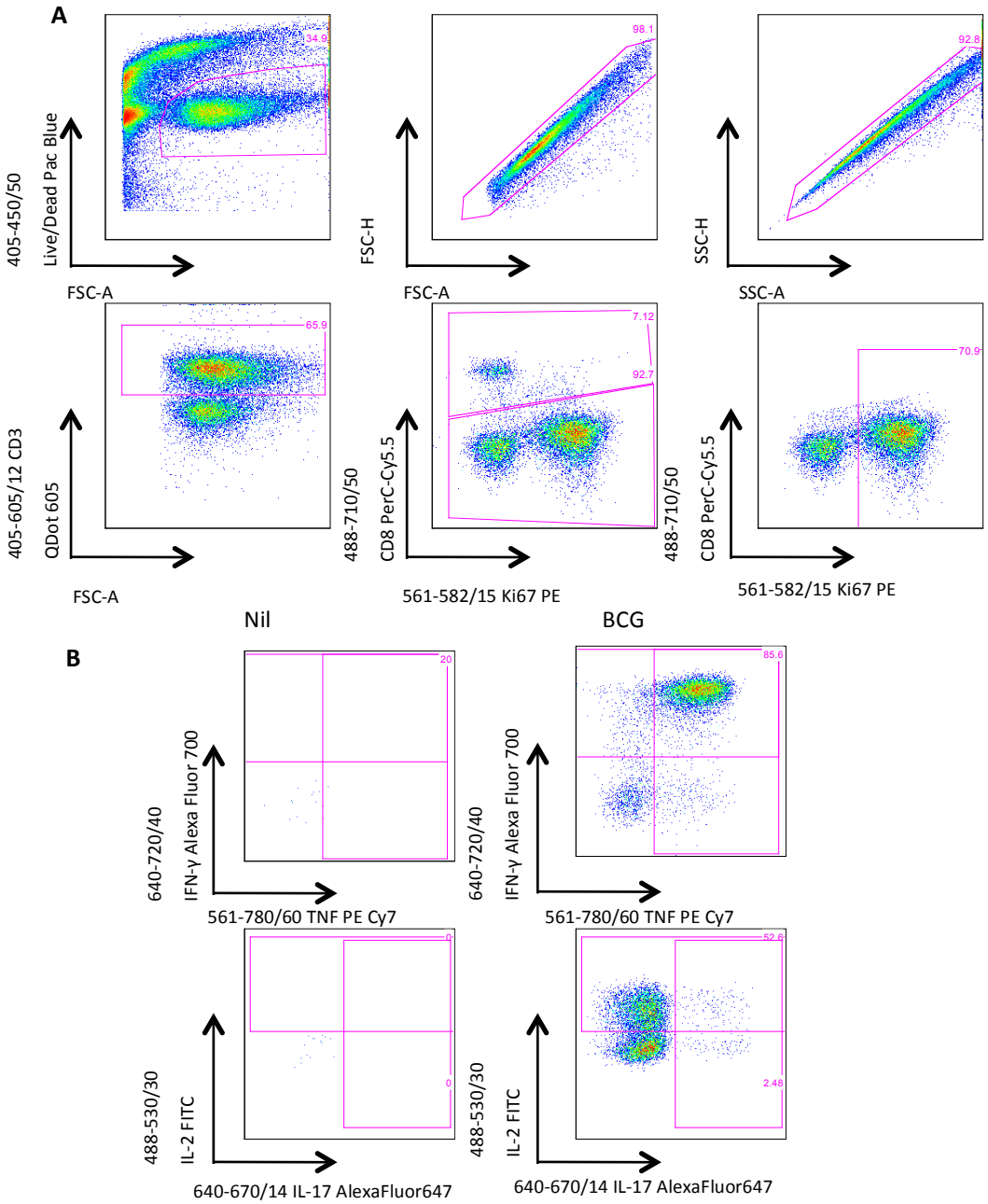


Figure 17. Hierarchical gating strategy for selection of $CD8^-$ proliferating T cells expressing intracellular cytokines in response to BCG antigens.
Representative dot plots from a single 16 week old infant are shown. (A) Gating strategy used to identify $CD8^-$ proliferating cells T cells. From left to right, live cells were identified from dead cells using a viability dye and cell doublets excluded with forward scatter area versus forward scatter height parameters, followed by side scatter area versus side scatter height. T cells were identified by the expression of cell surface staining with CD3 marker and subsequently differentiated into $CD8^-$ and $CD8^+$ T cells. Proliferating $CD8^-$ T cells were then identified by selecting $CD8^-Ki67^+$ T cells.

(B) Representative dot plots of cytokine expression in $CD8^-Ki67^+$ T cells from unstimulated and BCG stimulated conditions in the same 16 week old infant. Gates for proliferating $CD8^-$ T cells expressing cytokines were set using the unstimulated control. The filters used are specified in association with the laser, eg 488-530/30 indicates the blue laser and the 530/30 filter.

4.3 Analysis of study samples

Following culture of whole blood with BCG antigens for 6 days, the number of viable cells was markedly reduced in a number of samples. A total of 286 mother and infant samples were collected, of these 67 samples were excluded (23%) based on our pre-set exclusion criteria (section 2.6.5). Samples collected from infants at birth were most affected by prolonged incubation, as a result, 52 newborn infant samples (50%) were excluded from analysis. As the infant matured, the percentage of viable cells available for analysis increased; 7 (9%) samples were excluded from infants at 16 weeks. The majority of maternal samples were included, with only 8 (8%) of samples excluded.

Similarly, a number of samples incubated with tetanus toxoid in the whole blood assay were excluded: infants at birth: 34 (32%); infants at 16 weeks: 8 (10%); mothers: 7 (7%). Samples incubated with SEB were least affected by prolonged incubation and fewer samples were excluded: 26 (25%); infants at 16 weeks: 6 (6%); mothers: 4 (4%).

One mother had an indeterminate QFN result, the mother-infant pair was excluded from analysis.

4.4 BCG-induced T cell proliferation

Proliferation of antigen-specific T cells was measured as an assessment of immunological memory and vaccine immunogenicity. BCG-specific $Ki67^+$ T cells were enumerated to assess proliferation potential in response to BCG antigens in infants pre- and post-vaccination (at birth and at 16 weeks of age). Infant proliferative responses were compared based on the infant's exposure to maternal HIV and TB infection *in utero* (maternal TB infection was defined as a positive QFN test). BCG-induced proliferation was also evaluated in maternal

samples to assess the effect of HIV and TB infection on the mother's immunological memory to mycobacterial-specific antigens.

4.4.1 Infants at birth

Prior to BCG vaccination, infants at birth displayed undetectable or low BCG-specific T cell proliferation. BCG-specific $Ki67^{+}CD8^{-}$ T cells represented 2.54% (SD 1.41) of total $CD8^{-}$ T cells and BCG-specific $CD8^{+}$ T cells represented 1.32% (SD 1.11) of total $CD8^{+}$ T cells.

There were no differences in the frequency of BCG-specific $Ki67^{+}CD8^{-}$ T cells between infants based on the maternal HIV (p= .98) or QFN status (p = .36) or when considering the interaction effect of maternal HIV and QFN status (p= .25) on infant responses. Moreover, there was no effect of maternal HIV status (p= .87) or QFN status (p = .42) and no interaction of maternal HIV and QFN status (p= .73) on infant BCG-specific $Ki67^{+}CD8^{+}$ T cells responses at birth.

4.4.2 Infants at 16 weeks

BCG-specific $CD8^{-}Ki67^{+}$ T cells

All infants received BCG vaccination at 6 weeks of age and proliferative responses to the BCG vaccine were measured 10 weeks following later, at 16 weeks of age. All infants had comparable frequencies of BCG-specific CD8-$Ki67^{+}$ T cells, with maternal HIV or QFN status having no effect on the response (Figure 18).

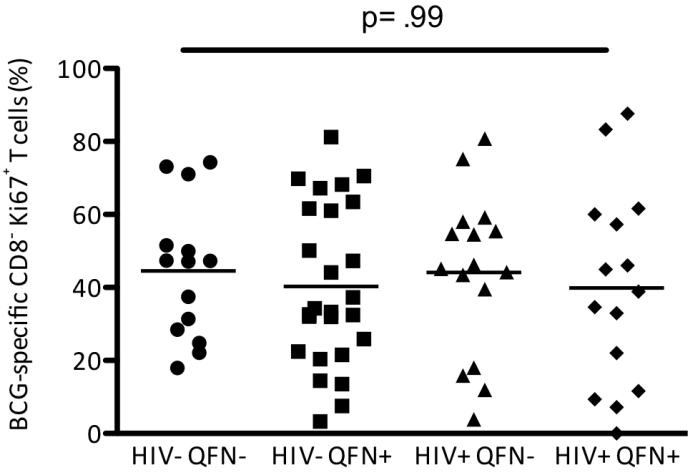


Figure 18. Comparable frequency of BCG-specific $CD8^{-}Ki67^{+}$ T cells in infants following BCG vaccination.

Groups of infants are based on maternal HIV and QFN status: HIV- QFN- (n= 14); HIV- QFN+ (n=26); HIV+ QFN- (n= 16); HIV+ QFN+ (n=15). In a two-way ANOVA model there was no interaction effect of maternal HIV and QFN status (p = .99) or effect of maternal HIV (p = .94) or QFN status (p = .44) on infant $CD8^{-}$ proliferative responses to BCG antigens. The horizontal line represents the mean response.

BCG-specific $CD8^+Ki67^+$ T cells

There were also no differences in the frequency of BCG-specific $CD8^+Ki67^+$ T cells between infants who were exposed to HIV and their peers (p = .10), or between infants born to QFN positive or QFN negative mothers (p = .71). There was no interaction effect of maternal HIV and QFN status on infant BCG-specific $CD8^+$ T cell proliferation (p= .71).

4.4.3 Mothers

BCG-specific $CD8^-Ki67^+$ T cells

There was a trend towards a statistically significant interaction effect of HIV infection and QFN status on BCG-specific $CD8^-$ T cell proliferation in women at delivery, p= .05. When QFN status was considered alone, BCG-induced $CD8^-$ T cell proliferative responses were higher in women who tested QFN positive than in women who tested QFN negative, p< .0005. However, no difference was found when considering maternal HIV status alone, p= .48. Amongst HIV-infected women, those who tested QFN positive had higher frequencies of BCG-induced $CD8^-Ki67^+$ T cells compared to those who tested QFN negative. However, amongst HIV-uninfected women, QFN status did not have a statistically significant effect on BCG-specific $CD8^-$ proliferation (Figure 19A).

Exploratory analysis was done to determine whether re-defining the groups based on both the QFN status and also the TST result altered the results. Mothers who tested negative on both the QFN **and** TST were defined as *Mtb* unsensitised (*Mtb* -). Mothers who tested positive on either the QFN or TST or both were defined as Mtb sensitised (*Mtb*+). A significant difference remained amongst HIV-infected women and no difference was observed amongst HIV-uninfected women, (Figure 19B).

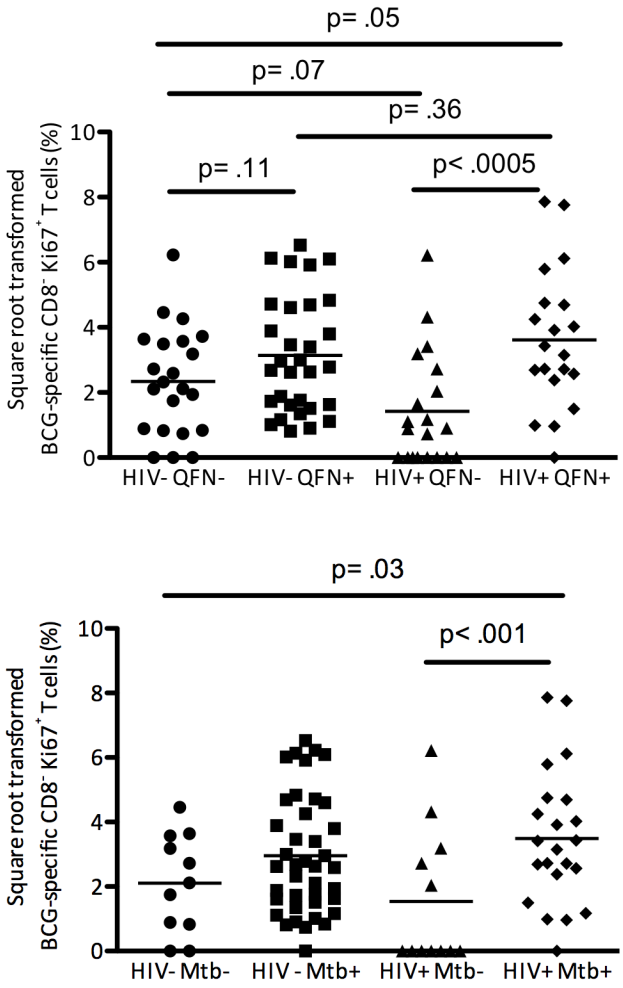


Figure 19. QFN status has a significant effect on BCG-specific CD8$^-$ T cell proliferation in HIV-infected women, but not in HIV-uninfected women.

(A) Groups of women were based on HIV and QFN status: HIV- QFN- (n= 22); HIV- QFN+ (n=31); HIV+ QFN- (n= 20); HIV+ QFN+ (n=20).

(B) Groups of women were based on HIV, QFN and TST status: HIV- *Mtb*- (QFN and TST -, n= 11); HIV- *Mtb* + (QFN or TST or both +, n= 40); HIV+ *Mtb*- (QFN and TST -, n= 12); HIV+ *Mtb*+ ((QFN or TST or both +, n= 22).

Square root transformed data is presented; horizontal line represents the mean response.

BCG-specific CD8$^+$Ki67$^+$ T cells

There was a statistically significant interaction between HIV status and QFN status on BCG-specific CD8$^+$ T cell proliferative responses, p= .04; indicating that the effect of HIV status on BCG-specific CD8$^+$ T cell proliferative responses depended on the TB status of the mother and vice versa (Figure 20). When the QFN status was not considered, HIV-infected women had lower BCG-specific CD8$^+$ proliferative responses compared to HIV uninfected women, p= .04. When considering only QFN status, mothers testing QFN positive and those testing negative had similar responses, p= .13.

HIV infection had a significant effect on BCG-specific $CD8^+$ T cell proliferation in women who were QFN negative, but not in women who tested QFN positive (Figure 20). Amongst HIV-infected women, those who tested QFN positive had higher BCG-specific CD8+ T cell proliferative responses compared to women who tested QFN negative. QFN status had no effect on BCG-specific CD8+ T cell proliferative responses in HIV-uninfected women.

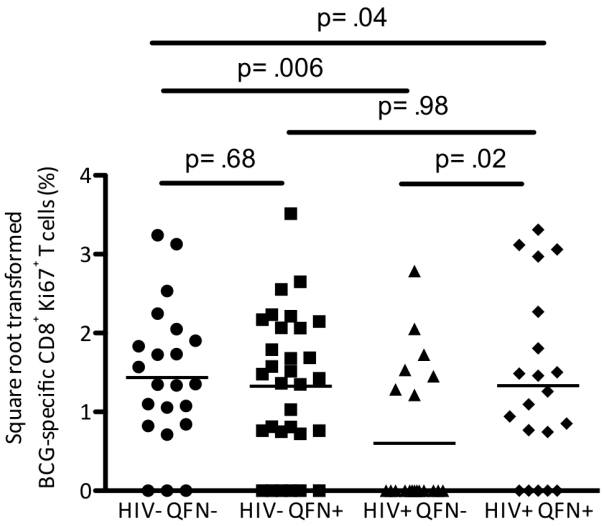


Figure 20. HIV infection has a significant effect on BCG-specific $CD8^+$ T cell proliferation in women testing QFN negative, but not in women who test QFN positive.
Samples available for each group of women: HIV- QFN- (n=22); HIV- QFN+ (n=31); HIV+ QFN- (n= 20); HIV+ QFN+ (n=20). Square root transformed data is presented; horizontal line represents the mean response.

4.5 Tetanus-specific proliferative responses

Tetanus-specific proliferative responses were also measured in mothers and infants to assess whether any effect of maternal HIV and TB infection on BCG-driven proliferation was an antigen- specific or general phenomenon.

Tetanus-specific $CD8^-Ki67^+$ T cells

Tetanus-specific $CD8^-$ proliferative responses were undetectable in infants at birth and in the majority of mothers at delivery (median response 0%, IQR 0.00 - 0.25%) in this assay system. This is in direct contrast to readily demonstrable anti-tetanus antibody responses (Chapter 6:). Following vaccination at 6, 10 and 14 weeks of age, infants at 16 weeks of age had detectable responses (median = 1.23%, 0.00 – 4.85), however there was no statistically significant difference in the response between the four groups of infants, p= .72.

Tetanus-specific $CD8^+Ki67^+$ T cells

Tetanus-specific CD8+ proliferative responses were undetectable in mothers and infants at birth. The response was undetectable in majority of infants at 16 weeks of age (median 0%, IQR 0-1.32%), with no statistically significant difference between the four groups of infants, p= .10.

4.6 BCG-Specific $Ki67^+$ $cytokine^+$ T cell responses

The frequency of BCG-specific T cells expressing cytokines was measured to assess the functional capacity of antigen-specific T cells. Cells that had proliferated during the 6-day incubation period were re-stimulated using PMA and ionomycin to induce intracellular cytokine production, giving information about the functional potential of antigen-specific cells.

4.6.1 Infants at birth

BCG-specific $CD8^-Ki67^+$ $cytokine^+$ T cells

The frequency of proliferating $CD8^-$ T cells expressing IFN-γ (p = .33), TNF-α (p= .18), IL-2 (p= .39) or IL-17 (p= .30) was similar between groups of infants at birth, Table 10.

Group (maternal infection status)	n	$CD8^-Ki67^+$ $IFN\text{-}\gamma^+$	$CD8^-Ki67^+$ $TNF\text{-}\alpha^+$	$CD8^-Ki67^+$ $IL\text{-}2^+$	$CD8^-Ki67^+$ $IL\text{-}17^+$
HIV- QFN -	15	0.07% (0.00 – 0.37)	0.23% (0.09 – 0.87)	0.22% (0.08 – 1.08)	0.07% (0.00 – 0.22)
HIV- QFN $^+$	20	0.11% (0.00 – 0.97)	0.30% (0.02 – 1.31)	0.18% (0.00 – 1.15)	0.00% (0.00 – 0.08)
HIV^+ QFN -	9	0.12% (0.00 – 0.65)	0.38% (0.01 – 1.59)	0.14% (0.02 – 1.00)	0.00% (0.00 – 0.21)
HIV^+ QFN $^+$	8	0.44% (0.13 – 1.84)	1.96% (0.30 – 5.29)	0.50% (0.21 – 1.49)	0.07% (0.00 – 0.36)
Total	52	0.14% (0.00 – 0.56)	0.39% (0.05 – 1.62)	0.21% (0.08 - 1.06)	0.02% (0.00-0.20%)

Table 10. Median (IQR) frequencies of BCG-specific $CD8^-Ki67^+$ T cells expressing cytokines in infants at birth.
A reduced number of samples were available for analysis of $IL\text{-}17^+$ $CD8^-$ T cells (HIV- QFN - 11; HIV- QFN+ 17; HIV+ QFN – 7; HIV+ QFN + 7). Differences between groups were not statistically significant as calculated by the Kruskal-Wallis test.

BCG-specific $CD8^+Ki67^+$ $cytokine^+$ T cells

There were also comparable frequencies of proliferating $CD8^+$ T cells expressing IFN-γ (p = .92), TNF-α (p= .52), IL-2 (p= .38) or IL-17 (p= .47) between groups of infants at birth, Table 11.

Group (maternal infection status)	n	$CD8^+Ki67^+$ $IFN\text{-}\gamma^+$	$CD8^+Ki67^+$ $TNF\text{-}\alpha^+$	$CD8^+Ki67^+$ $IL\text{-}2^+$	$CD8^+Ki67^+$ $IL\text{-}17^+$
HIV- QFN -	15	0.11% (0.00 – 0.44)	0.16% (0.00 – 1.09)	0.13% (0.00 – 0.80)	0.06% (0.00 – 0.58)
HIV- QFN $^+$	20	0.13% (0.00 – 0.81)	0.24% (0.00 – 0.62)	0.24% (0.03 – 1.41)	0.00% (0.00 – 0.10)
HIV^+ QFN -	9	0.15% (0.06 – 0.69)	0.34% (0.00 – 0.53)	0.11% (0.00 – 0.34)	0.07% (0.00 – 0.29)
HIV^+ QFN $^+$	8	0.01% (0.00 – 3.07)	0.49% (0.05 - 4.01)	0.02% (0.00 - 0.20)	0.37% (0.00 – 0.48)
Total	52	0.14% (0.00 – 0.66)	0.24% (0.00 – 0.65)	0.10% (0.00 – 0.79)	0.06% (0.00 – 0.37)

Table 11. Median (IQR) frequencies of BCG-specific $CD8^+$ $Ki67^+$ T cells expressing cytokines in infants at birth.
A reduced number of samples were available for analysis of $IL\text{-}17^+$ $CD8^-$T cells (HIV- QFN - 11; HIV- QFN+ 17; HIV+ QFN – 7; HIV+ QFN + 7). Differences between groups not statistically significant as calculated by the Kruskal-Wallis test.

4.6.2 Infants at 16 weeks

BCG-specific $CD8^-Ki67^+$ T cells

There was no statistically significant interaction effect of maternal HIV and QFN status on the frequency of BCG-Specific $CD8^-$ $Ki67^+$ T cells expressing IFN-γ (p= .63), TNF-α (p= .61), IL-2 (p= .50) or IL-17 (p= .88), Figure 21. Moreover, there was no statistically significant difference between HIV-exposed and unexposed infants in BCG-Specific $CD8^-$ $Ki67^+$ T cells expressing IFN-γ (p= .53), TNF-α (p= .53), IL-2 (p= .90) or IL-17 (p= .40). Similarly there was no difference between infants born to QFN positive or negative mothers in BCG-Specific $CD8^-$ $Ki67^+$ T cells expressing IFN-γ (p= .59), TNF-α (p= .77), IL-2 (p= .93) or IL-17 (p= .79).

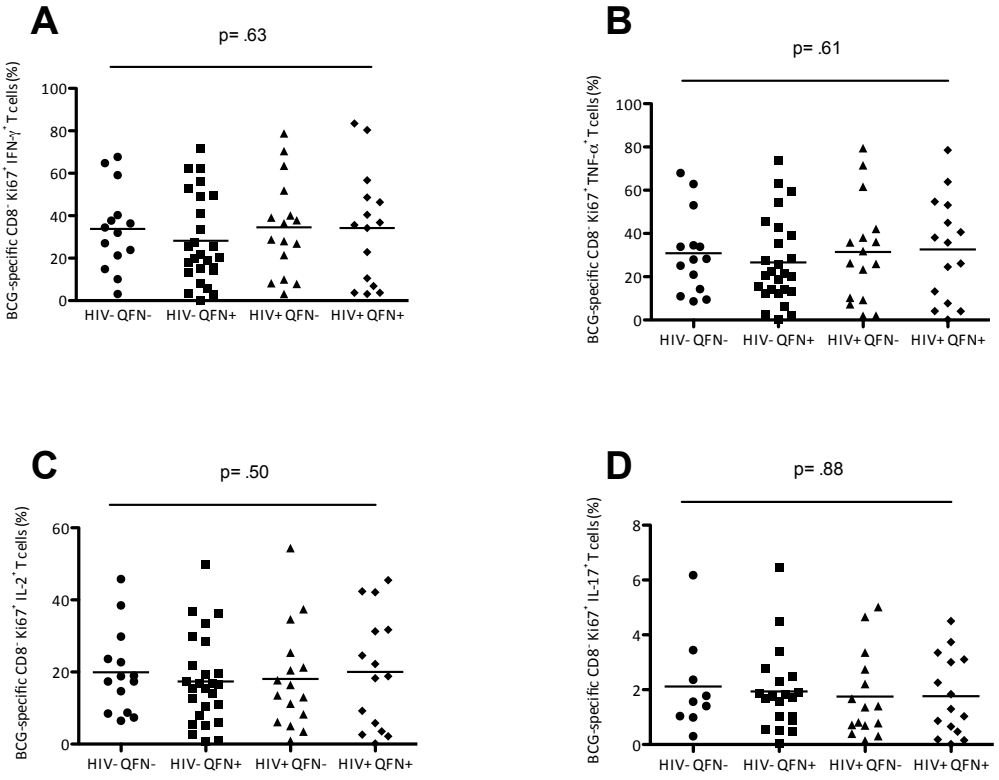


Figure 21. Frequencies of BCG-specific CD8$^-$ Ki67$^+$ T cells expressing IFN-γ, TNF-α, IL-2 or IL-17 in infants at 10 weeks post BCG vaccination.

Groups of infants were defined by maternal infection status, namely HIV- QFN- (n= 14, n=9 for IL-17); HIV- QFN + (n= 26, n=20 for IL-17); HIV+, QFN- (n=16, n=15 for IL-17) and HIV- QFN + (n=15, n=15 for IL-17). Each symbol represents an individual infant and the horizontal line in each plot represents the mean. (A) Frequencies of BCG-specific CD8$^-$ Ki67$^+$ T cells expressing IFN-γ $^+$. (B) Frequencies of BCG-specific CD8$^-$ Ki67$^+$ T cells expressing TNF-α. (C) Frequencies of BCG-specific CD8$^-$ Ki67$^+$ T cells expressing IL-2 (D) Frequencies of BCG-specific CD8$^-$ Ki67$^+$ T cells expressing IL-17.

BCG-specific CD8$^+$Ki67$^+$ T cells

Kruskal-Wallis testing showed that there was no difference between the four groups of infants in the frequency of BCG-specific proliferating CD8$^+$ T cells expressing IFN-γ (p= .77), TNF-α (p= .83), IL-2 (p= .37), or IL-17 (p= .20), Figure 22.

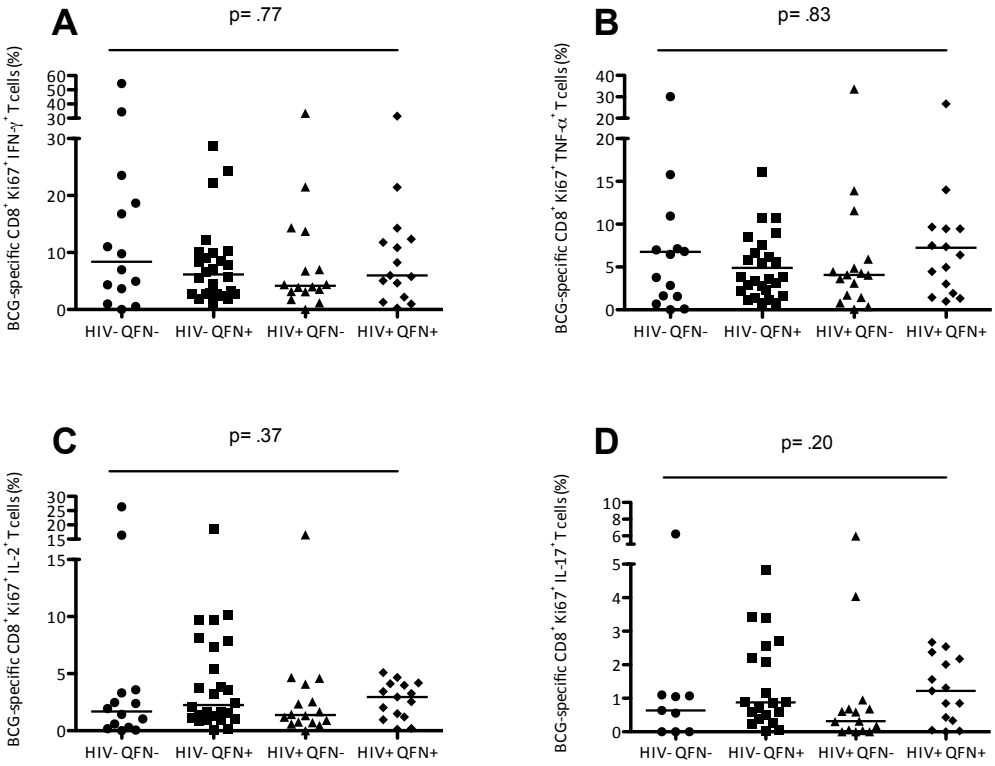


Figure 22. Frequencies of BCG-specific $CD8^+$ $Ki67^+$ T cells expressing IFN-γ, TNF-α, IL-2 or IL-17 in infants at 10 weeks post BCG vaccination.
Groups of infants were defined by maternal infection status, namely HIV-uninfected, QFN negative (n= 14, n=9 for IL-17); HIV-uninfected, QFN positive (n= 26, n=20 for IL-17); HIV-infected, QFN negative (n=16, n=15 for IL-17) and HIV-infected, QFN positive (n=15, n=15 for IL-17). Each symbol represents an individual infant and the horizontal line in each plot represents the median (A) Frequencies of BCG-specific $CD8^+$ $Ki67^+$ T cells expressing IFN-γ^+. (B) Frequencies of BCG-specific $CD8^+$ $Ki67^+$ T cells expressing TNF-α. (C) Frequencies of BCG-specific $CD8^+$ $Ki67^+$ T cells expressing IL-2 (D) Frequencies of BCG-specific $CD8^+$ $Ki67^+$ T cells expressing IL-17.

4.6.3 Mothers

BCG-specific $CD8^-Ki67^+$ T cells

There was a statistically significant difference in the frequency of BCG-specific $CD8^-Ki67^+$ T cells expressing IFN-γ (p= .001), TNF-α (p= .001), IL-2 (p= .003) or IL-17 (p= .04) between the four groups of mothers, as defined by their HIV and QFN status, Figure 23.

Post-hoc analysis revealed differences amongst HIV-infected women who tested QFN negative compared to those who tested QFN positive in the frequency of proliferating $CD8^-$ T cells expressing IFN-γ (median = 0.35% vs 8.72%, p= .001), TNF-α (median 0.45% vs 8.57%, p= .002) or IL-2 (median = 0.35% vs 4.24%, p= .006), but not IL-17 (median = 0.09% vs 0.78%,

p= .61), Figure 23. However, differences in BCG-specific cytokine expressing $CD8^-$ $Ki67^+$ T cells were not present amongst HIV-uninfected women who tested QFN negative compared to those who tested positive (IFN-γ: median= 3.42% vs 5.93%, p=1.00; TNF-α: median = 3.18% vs 6.38%, p= .51; IL-2: median= 1.15% vs 3.43%, p= .64; IL-17: median = 0.64% vs 1.23%, p= .50), Figure 23.

There was a tendency towards lower $CD8^-$ $Ki67^+$ IFN-γ^+ responses in HIV-infected, QFN negative women compared to HIV-uninfected, QFN negative women (p= 0.05), this did not reach statistical significance following correction for multiple comparisons, adjusted p= .29.

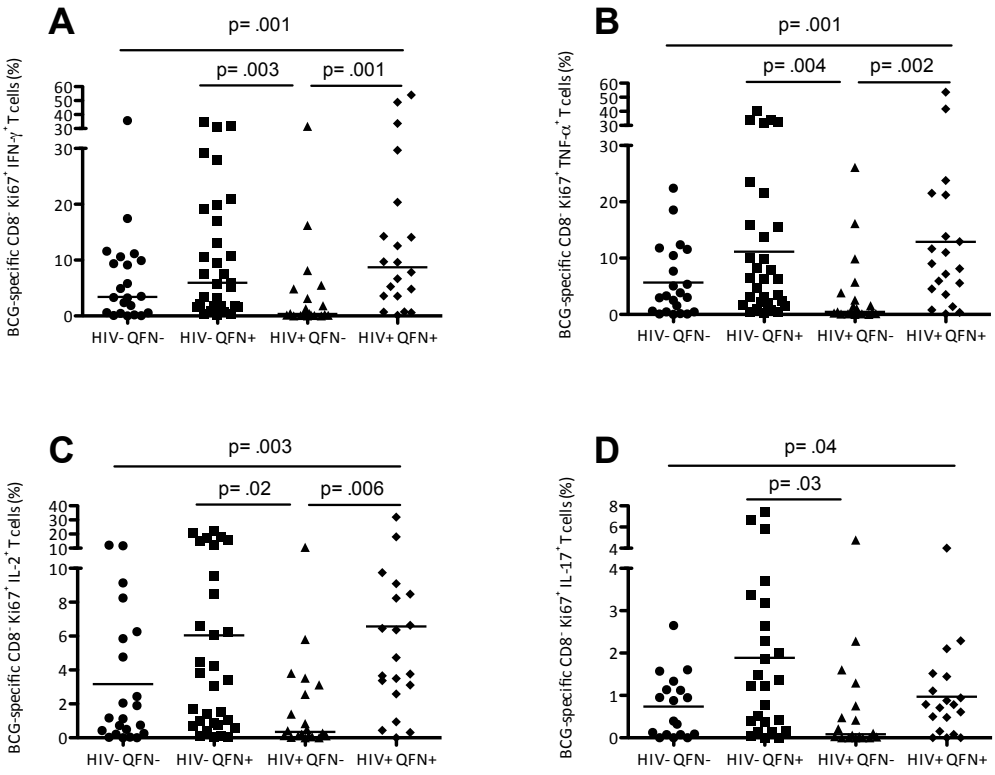


Figure 23. Frequencies of BCG-specific $CD8^-$ $Ki67^+$ T cells expressing IFN-γ, TNF-α, IL-2 or IL-17 in mothers at delivery.

Groups of mothers were defined by infection status, namely, HIV- QFN - (n= 22, n=18 for IL-17); HIV- QFN + (n= 31, n=25 for IL-17); HIV+ QFN- (n=20, n=18 for IL-17) and HIV+ QFN+ (n=20, n=19 for IL-17). Each symbol represents an individual and the horizontal line in each plot represents the median. Only comparisons with adjusted p values <0.05 are given. (A) Frequencies of BCG-specific $CD8^-$ $Ki67^+$ T cells expressing IFN-γ. (B) Frequencies of BCG-specific $CD8^-$ $Ki67^+$ T cells expressing TNF-α. (C) Frequencies of BCG-specific $CD8^-$ $Ki67^+$ T cells expressing IL-2 (D) Frequencies of BCG-specific $CD8^-$ $Ki67^+$ T cells expressing IL-17.

BCG-specific $CD8^+Ki67^+$ T cells

The frequency of BCG-specific Ki67+ $CD8^+$ T cells expressing any of the cytokines measured was much lower than the frequencies measured in the $CD8^-$ T cells. Whilst the frequency of BCG-specific proliferating $CD8^+$ T cells expressing IFN-γ was similar between groups of women (p = .05), there was a statistically significant difference in the frequency $CD8^+$cells expressing TNF-α (p= .006), IL-2 (p= .03) or IL-17 (p= .01),Figure 24.

Consistent with $CD8^-$ responses, HIV-infected women who tested QFN negative had lower frequencies of BCG-specific $CD8^+$ $Ki67^+$ TNF-α^+ T cells compared to those who tested QFN positive (median 0.13% vs 0.96%, p= .04). There was also a trend towards lower IL-2 responses in these women (median 0.04% vs 0.24%, p= .04), however after correction for multiple testing this effect was not statistically significant (p= .21). There was no difference in frequency of $CD8^+Ki67^+IL\text{-}17^+$ cells (median = 0.00% vs 0.09%, p= .68) amongst HIV-infected women who tested either QFN negative or positive.

No differences in the frequency of $CD8^+Ki67^+$ T cells expressing TNF-α (median 0.90% vs 1.44%, p= 1.00), IL-2 (median 0.35% vs 0.49%, p= 1.00) or IL-17 (median = 0.23% vs 0.33%, p= 1.00) were seen in QFN negative compared to QFN positive HIV-uninfected women.

There was a tendency towards lower $CD8^+$ $Ki67^+$ TNF-α^+ responses in HIV-infected, QFN negative women compared to HIV-uninfected, QFN negative women (p= .01), however, this did not reach statistical significance following correction for multiple comparisons, p= 0.06.

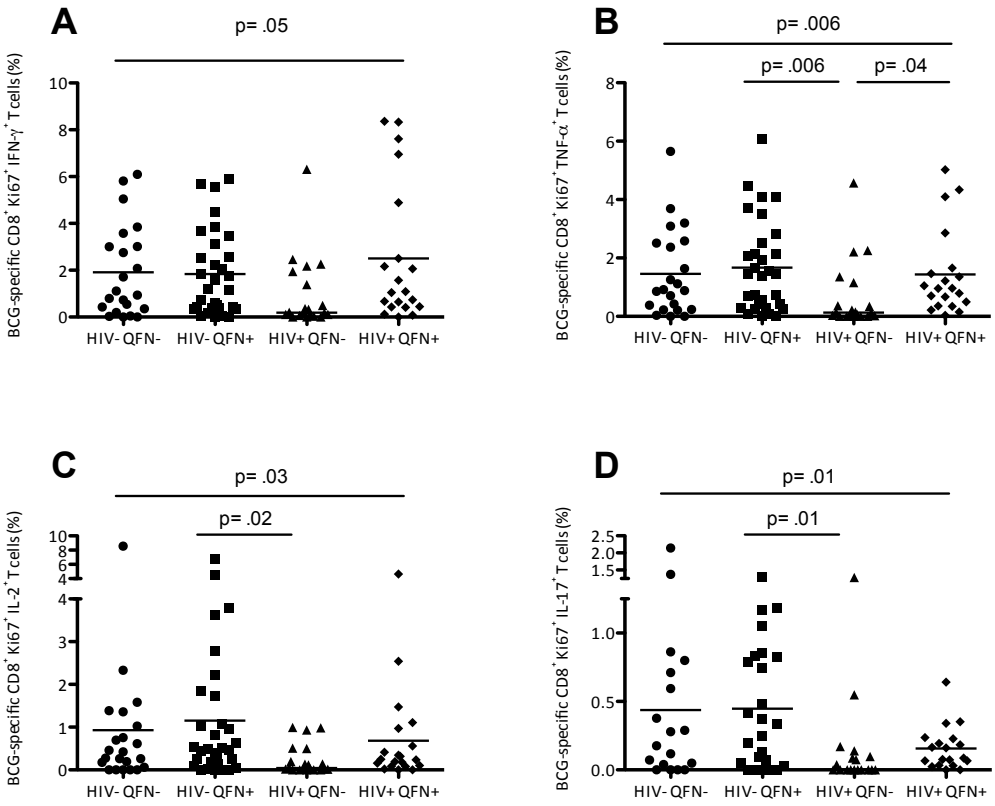


Figure 24. Frequencies of BCG-specific CD8$^+$ Ki67$^+$ T cells expressing IFN-γ, TNF-α, IL-2 or IL-17 in mothers at delivery.

Groups of mothers were defined by infection status, namely HIV- QFN- (n= 22, n=18 for IL-17); HIV- QFN+ (n= 31, n=25 for IL-17); HIV+ QFN- (n=20, n=18 for IL-17) and HIV+ QFN+ (n=20, n=19 for IL-17). Each symbol represents an individual mother and the horizontal line in each plot represents the median. Only comparisons with adjusted p values <0.05 are stated. (A) Frequencies of BCG-specific CD8+ Ki67+ T cells expressing IFN-γ. (B) Frequencies of BCG-specific CD8$^+$ Ki67$^+$ T cells expressing TNF-α. (C) Frequencies of BCG-specific CD8$^+$ Ki67$^+$ T cells expressing IL-2 (D) Frequencies of BCG-specific CD8$^+$ Ki67$^+$ T cells expressing IL-17.

4.7 Tetanus-Specific Ki67$^+$ cytokine$^+$ T cell responses

Tetanus-specific CD8$^-$ and CD8$^+$ cytokine$^+$ T cell responses were barely detectable in mothers and undetectable in the majority of infants at birth with no differences between groups of mothers or infants (Appendix IV). Following vaccination at 6, 10 and 14 weeks of age, 16 week old infants displayed predominately CD8$^-$ Ki67$^+$ cytokine$^+$ T cell responses rather than CD8$^+$ Ki67$^+$ cytokine$^+$ T cell responses. There were no differences in response between groups of infants (Appendix IV).

4.8 BCG-Specific Ki67$^+$ polyfunctional T cell responses

T cells expressing cytokines in multiple combinations were assessed in infants and mothers to assess the pattern of activation of antigen-specific proliferating T cells, giving a sense of the quality of the response. The capability of cells to produce multiple cytokines simultaneously is thought to be associated with superior functional capacity[202].

4.8.1 Infants at birth

BCG-specific CD8$^-$Ki67$^+$ T cells

At birth, HIV-exposed, uninfected infants displayed an increased proportion of BCG-specific TNF-α single-positive CD8$^-$ T cells (median = 0.34%, IQR 0.17- 1.89), compared to infants born to HIV-uninfected mothers (median = 0.12%, IQR 0.00 – 0.49), p= .04 (Figure 25). Exposed infants also showed an increased frequency of specific IFN-γ$^+$TNF-α$^+$ T cells (median 0.2%, IQR 0.04 – 0.99 vs 0.00%, IQR 0.00 – 0.08), p= .007. The frequency of specific CD8$^-$ T cells producing any other combination of cytokines was not different between HIV-exposed and unexposed infants at birth.

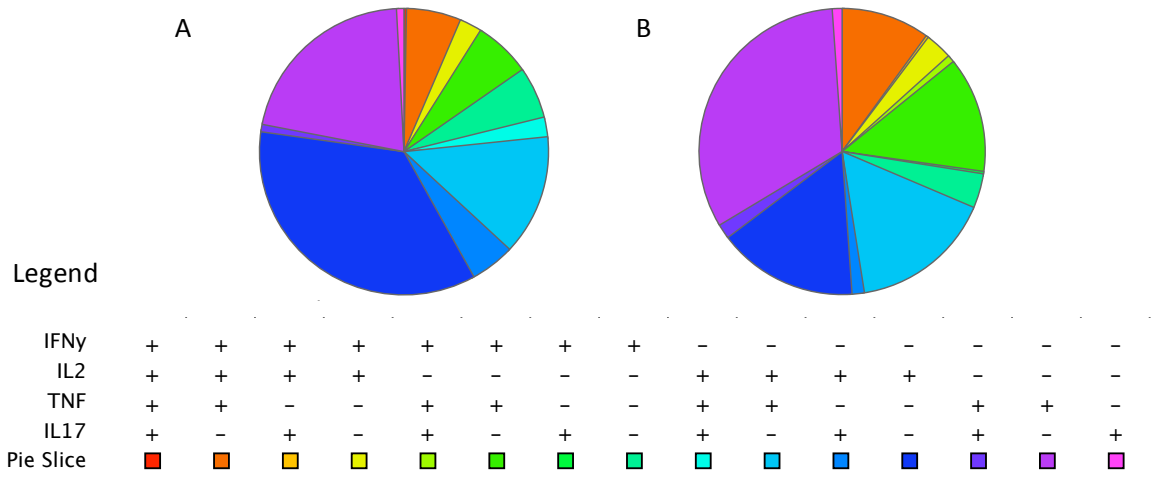


Figure 25. Relative proportions of BCG-specific Ki67$^+$ CD8$^-$ T cells expressing cytokines in different combinations in infants at birth.
A) HIV-unexposed infants, B) HIV-exposed, uninfected infants.

When considering only the QFN status of the mother, the proportion of BCG-specific CD8$^-$ T cells producing cytokines in any combination was similar between groups of infants.

There were no differences between specific $CD8^-$ T cell subsets between the four groups of infants.

BCG-specific $CD8^+Ki67^+$ T cells

The BCG-specific $CD8^+$ T cell response was less varied than that of the $CD8^-$ response; 5 discrete subsets were discernable compared to 15 $CD8^-$ T cell subsets. The majority of infants did not express any BCG-specific $CD8^+$ T cell response at birth.

The pattern of the $CD8^+$ BCG-specific response in infants exposed to HIV was distinct from that observed in HIV-unexposed infants ($p = 0.006$), with specific differences in the frequency of TNF-α single-positive $CD8^+$ T cells ($p= .007$) and IL-2 single-positive $CD8^+$ T cells ($p= .007$). The majority of HIV-exposed infants had detectable TNF- α single positive $CD8^+$ T cells (median = 0.17, IQR 0.00 – 0.99) compared to an undetectable population in most HIV-unexposed infants. They also had higher frequencies of BCG-specific $CD8^+$ IL-2-single positive T cells at birth (median = 0.25%, IQR 0.09 – 0.96) compared to HIV-unexposed infants (median = 0.11%, IQR 0.00 – 0.89), p .007, although this formed a smaller proportion of the overall response.

When considering both the HIV status and the QFN status of the mother, there remained statistically significant differences in the frequency of BCG-specific $CD8^+$ TNF-α-single positive T cells between the four groups of infants, $p= .01$. Infants born to HIV-infected, QFN-positive mothers expressed the highest frequencies (median = 0.47%, IQR 0.13 – 1.28) and the majority of other infants did not express this subset (median= 0.00% for other groups). Following Bonferroni adjustment, statistical significance was only reached for comparison of infants born to HIV-infected, QFN positive mothers compared to infants born to HIV negative, QFN negative mothers ($p= .006$).

4.8.2 Infants at 16 weeks

BCG-specific $CD8^-Ki67^+$ T cells

The predominant response to BCG vaccination was observed in the $CD8^-$ compartment with 12 discrete subsets being expressed; IFN-γ^+TNF-α^+ and IFN-γ^+IL-2^+TNF-α^+ T cells formed the majority of the response, Figure 26. There were no statistically significant differences in BCG-specific $CD8^-$ T cells producing cytokine in any combination in infants at 16 weeks of age.

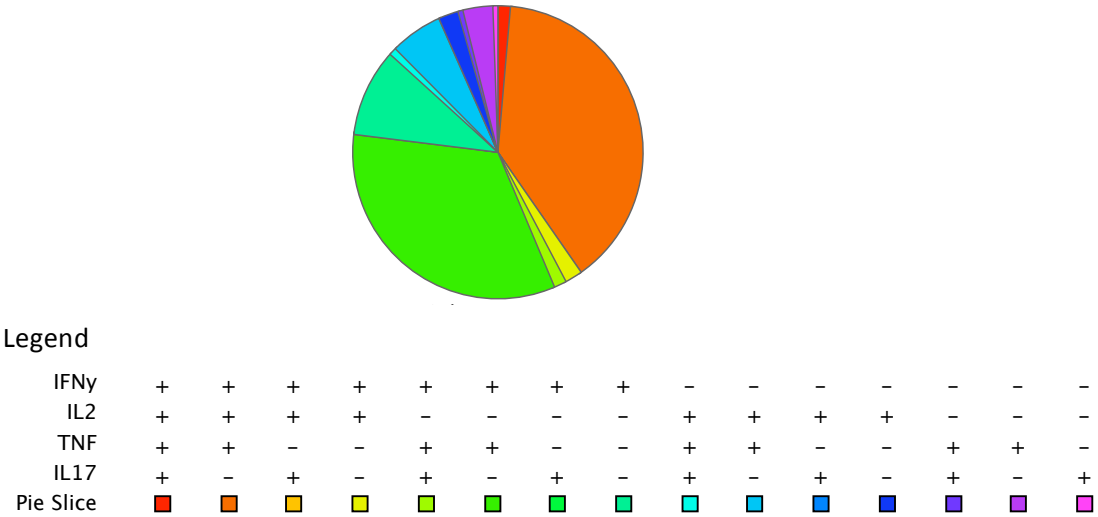


Figure 26. Relative frequencies of BCG-specific CD8- T cell subsets in 16 week old infants (N= 59)

BCG-specific $CD8^+Ki67^+$ T cells

Within the $CD8^+$ compartment 8 discrete subsets were discernable (Figure 27). In keeping with the response to BCG seen in the $CD8^-$ compartment, polyfunctional IFN-γ^+IL-2^+TNF-α^+ T cells were a pre-dominant subset, however the frequency of these cells was substantially lower (median = 1.05, IQR 0.46 - 2.06) compared to frequency in the $CD8^-$ compartment (median of 11.37, IQR 3.56 – 21.70). In contrast, $CD8^+$ cells expressing IL-17 alone or in combination with TNF-α were more frequent and formed a larger proportion of the overall response, Figure 27.

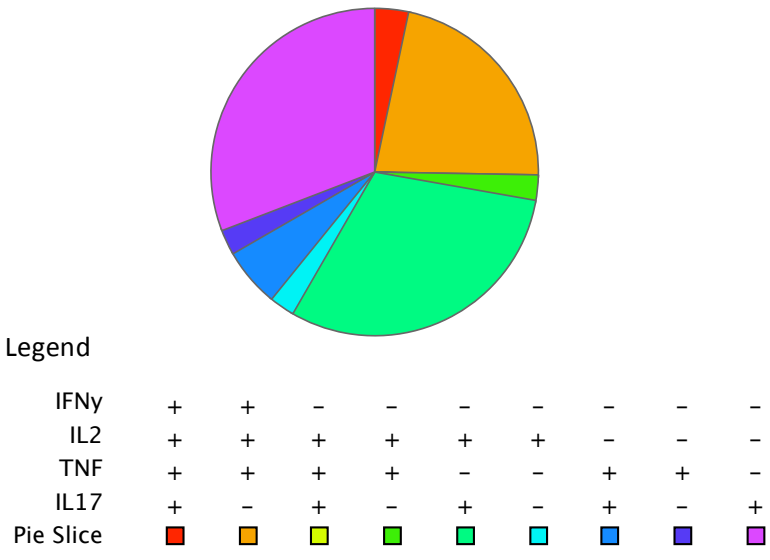


Figure 27. Relative frequencies of BCG-specific $CD8^+$ T cell subsets in 16 week old infants (n=59).

BCG-specific IL-2^+ $CD8^+$ T cells formed a very small proportion of the overall response (Figure 27), however differences in the frequency of these cells were observed between groups of infants. HIV-unexposed infants (n= 30) had higher frequencies of these cells (median = 0.18%, IQR 0.09 – 0.73) compared to HIV-exposed infants (n= 29; median= 0.04%, IQR 0.00 – 0.19), p= .002. Infants born to QFN-positive mothers (n= 35; median= 0.18%, IQR 0.03 – 0.70) also had an increased frequency of these cells compared to infants born to QFN-negative mothers (n= 24; median =0.05%, IQR 0.00 – 0.16), p= .02. The frequency of BCG-specific IL-2^+ $CD8^+$ T cells was also statistically significantly different between the four groups of infants, p= .005. Pairwise comparisons showed that infants born to HIV-negative, QFN-positive mothers had the highest frequency of such cells (n= 20; median= 0.23%, IQR 0.10 – 0.89) and this was statistically significant when compared to infants born to HIV-positive, QFN-negative mothers (n= 14; median= 0.00%, IQR 0.00 – 0.07), p= .003.

4.8.3 Mothers

BCG-specific $CD8^-Ki67^+$ T cells

The frequency of BCG-specific $CD8^-$ T cells producing any combination of cytokine was similar between HIV-infected and uninfected mothers, with the exception of IFN-γ^+IL-2^+TNF-α^+IL-17^+ and IFN-γ^+TNF-α^+IL-17^+ $CD8^-$ T cells, which were lower in HIV-infected mothers (p= .009 and p= .02). These subsets formed a very small proportion of the total response.

A positive QFN response was associated with an increased frequency of BCG-specific IFN-γ^+IL-2^+TNF-α^+IL-17^+ (p= .001), IFN-γ^+ IL-2^+TNF-α^+ (p< .005), IFN-γ^+ TNF-α^+IL-17^+ (p= .008), IFN-γ^+TNF-α^+ (p= .001), IL-2^+TNF-α^+IL-17^+ (p= .005), IL-2^+TNF-α^+ (p= .001), TNF-α^+IL-17^+ (p= .03), IL-2^+ (p= .007) and TNF-α^+ (p= .001) $CD8^-$ $Ki67^+$ T cells.

When both the HIV status and TB were considered, a number of differences in frequencies of BCG-specific $CD8^-$ T cells expressing two, three or four cytokines simultaneously were seen, Figure 28.

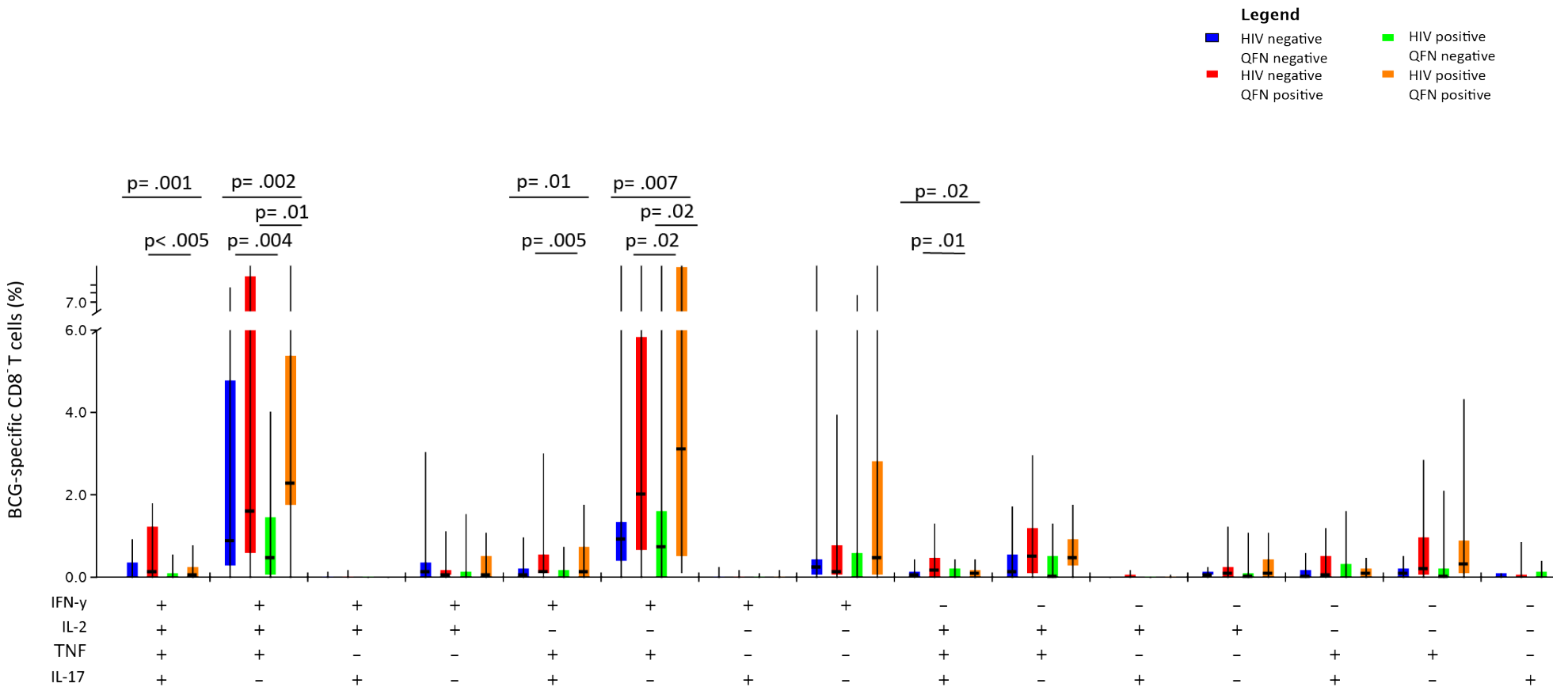


Figure 28. BCG-specific CD8⁻ T cells producing combinations of cytokines in mothers at delivery.
Groups of mothers were defined by infection status, namely: HIV-uninfected, QFN negative (n=18); HIV-uninfected, QFN positive (n= 25); HIV-infected, QFN negative (n=18) and HIV-infected, QFN positive (n=19). Kruskal- Wallis testing was used to compare the four groups of mothers; pairwise comparisons were performed with Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Only statistically significant comparisons are shown. Bars represent the interquartile range, horizontal line represents the median and error bars represent the range.

BCG-specific $CD8^+Ki67^+$ T cells

Compared to HIV-uninfected mothers, HIV-infected mothers had significantly lower frequencies of BCG-specific IFN-γ^+IL-2^+TNF-α^+ (p= .002), IFN-γ^+IL-2^+TNF-α^+IL-17^+ (p= .009), IL-2^+TNF-α^+ (p= .02) and IL-2-single positive (p= .002) $CD8^+$ T cells.

Women with positive QFN tests had increased frequencies of BCG-specific $CD8^+$ IL-2^+TNF-α^+ and TNF-α^- single positive T cells compared to women with negative QFN tests, (p= .01 and p = .03) but similar proportions of other subsets.

When both the HIV status and TB were considered, differences in frequencies of BCG-specific $CD8^+$ IFN-γ^+IL-2^+TNF-α^+, IFN-γ^+IL-2^+TNF-α^+IL-17^+ and IL-2^+TNF-α^+ $CD8^+$ T cells remained and were predominately observed between HIV-infected, QFN-negative women and HIV-uninfected, QFN-positive women, Figure 29.

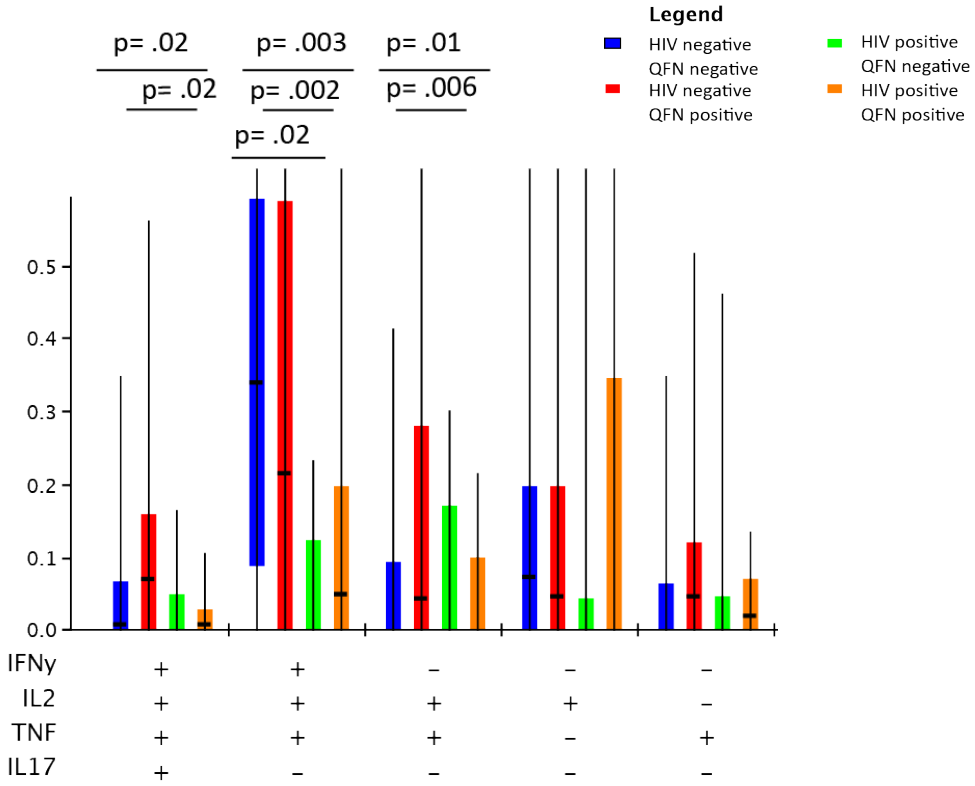


Figure 29. BCG-specific $CD8^+$ T cells producing combinations of cytokines in mothers at delivery. Groups of mothers were defined by infection status, namely: HIV-QFN- (n=18); HIV-QFN+ (n= 25); HIV+ QFN- (n=18) and HIV+ QFN+ (n=19). Only statistically significant comparisons are shown. Bars represent the interquartile range, horizontal line represents the median and error bars represent the range.

4.9 Tetanus-Specific Ki67^{+} polyfunctional T cell responses

Responses to tetanus were barely detectable in infants at birth and in mothers, however responses were detected in infants at 16 weeks of age. There were no differences in frequency of tetanus-specific CD8- or CD8+ T cells expressing cytokine in any combination between any group of infants at 16 weeks (data not shown).

4.10 Paired infant BCG-specific T cell proliferative responses pre- and post-BCG vaccination

BCG-specific responses were measured in infants before and after BCG vaccine. These paired responses were analysed to assess the change in the magnitude of proliferation and also the nature of the functional response to vaccination.

Paired data was available for analysis for a total of 32 infants at birth (pre-vaccination) and at 16 weeks of age (10 weeks post-BCG vaccination). When infants were divided into groups based on the maternal infection status there was paired data available for 7 infants born to HIV negative, QFN negative mothers, 16 infants born to HIV negative, QFN positive mothers, 4 infants born to HIV positive, QFN negative mothers and 5 infants born to HIV positive, QFN positive mothers.

4.10.1 Magnitude of response: frequency of BCG-specific proliferating T cells

Paired BCG-specific CD8-Ki67+ T cell responses

There was a statistically significant difference in the frequency of CD8^{-}Ki67^{+} T cells following vaccination (mean = 36.84, SD 3.50) compared to pre-vaccination (mean 3.35%, SD 1.5) in infants measured at birth and at 10 weeks post vaccination, $p < .0005$.

Maternal infection status did not have an effect on this response; all four groups of infants had similar increases in CD8^{-}Ki67^{+} T cell responses between the two time points, $p = .54$, Figure 30.

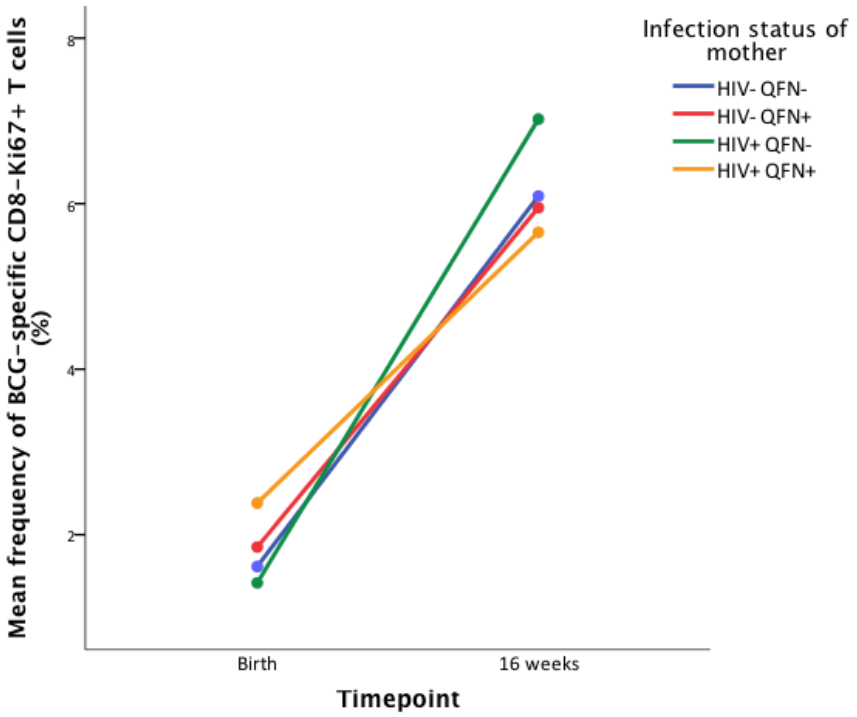


Figure 30. The frequency of BCG-specific $CD8^{-}Ki67^{+}$ T cells significantly increases following BCG vaccination in infants, irrespective of maternal HIV and QFN infection status, p< .0005.
Groups of infants were defined by maternal infection status, namely: HIV-uninfected, QFN negative (n=7); HIV-uninfected, QFN positive (n= 16); HIV-infected, QFN negative (n=4) and HIV-infected, QFN positive (n=5). Data was square root transformed to fulfill the assumptions required for comparison of groups using a mixed ANOVA model. Mean frequencies of square root transformed data is presented.

Paired BCG-specific $CD8^{+}Ki67^{+}$ T cell responses

Although the predominant T cell response to BCG vaccination was in the $CD8^{-}$ T cell compartment, the frequency of $CD8^{+}Ki67^{+}$ T cells were also significantly increased following vaccination, p< .0005 (mean pre-vaccination =2.19%, SD 1.18, mean post-vaccination = 10.18% SD 1.80).

Infant exposure to maternal HIV or TB infection (as defined by a positive QFN test) had no statistically significant effect on the change in frequency of BCG-specific $CD8^{+}Ki67^{+}$ T cells between pre- and post- vaccination, p= .24.

4.10.2 Quality of the response: BCG-specific $CD8^-$ and $CD8^+Ki67^+$ $cytokine^+$ T cell responses

Following BCG vaccination, there was a highly significant increase in BCG-specific $CD8^-$ and $CD8^+$ $Ki67^+$ IFN-γ^+, TNF-α^+, IL-2^+ and IL-17^+ T cells, p< .0005 (p= .003 for $CD8^+$ IL-17^+ T cells), Table 12 and 13. This upregulation was similar for all infants with no statistically differences between the four groups of infants.

			CD8-Ki67+			
Group	Time point	n	IFN-γ^+	TNF-α^+	IL-2^+	IL-17^+
HIV- QFN -	Birth	7 (4)	0.08 (0.06)	0.18 (0.21)	0.49 (0.86)	0.82 (2.68)
HIV- QFN -	16 weeks		24.1 (3.93)	22.75 (2.94)	14.78 (1.65)	8.02 (6.67)
HIV- QFN +	Birth	16(13)	0.29 (0.27)	0.66 (0.57)	0.48 (0.48)	0.09 (0.84)
HIV- QFN +	16 weeks		21.12 (4.76)	20.93 (4.75)	13.27 (2.56)	3.47 (1.31)
HIV+ QFN -	Birth	4 (3)	0.16 (0.16)	0.5 (0.36)	0.39 (0.5)	0.05 (0.15)
HIV+ QFN -	16 weeks		30.54 (0.24)	33.71 (0.35)	13.7 (1.1)	8.46 (4.03)
HIV+ QFN +	Birth	5 (5)	1.36 (0.48)	3.03 (0.88)	1.24 (0.88)	0.03 (0.05)
HIV+ QFN +	16 weeks		22.88 (9.72)	22.75 (7.5)	14.69 (6.59)	2.73 (2.59)
Total (Birth)		32(25)	0.32 (0.3)	0.74 (0.64)	0.57 (0.58)	0.13 (0.84)
Total (16 weeks)			23.14 (4.43)	23.06 (4.03)	13.87 (2.52)	4.42 (2.5)
	Effect of time	p	< .0005	< .0005	< .0005	< .0005
	Effect of time and maternal infection status	p	0.76	0.55	0.98	0.76

Table 12. The frequency of BCG-specific $CD8^-Ki67^+$ T cells expressing IFN-γ, TNF-a, IL-2 or IL-17 were significantly increased in infants following BCG vaccination, irrespective of maternal infection status.
A reduced number of samples were available for analysis of IL-17+ CD8-T cells, indicated in parentheses. A mixed ANOVA model was used to assess differences between groups.

			CD8+Ki67+			
Group	Time point	n	IFN-γ^+	TNF-α^+	IL-2^+	IL-17^+
HIV- QFN -	Birth	7 (4)	0.09 (0.06)	0.1 (0.17)	0.22 (0.11)	0.1 (0.28)
HIV- QFN -	16 weeks		5.24 (3.32)	2.42 (1.62)	0.79 (0.57)	0.32 (0.19)
HIV- QFN +	Birth	16(13)	0.32 (0.47)	0.39 (0.45)	0.43 (0.4)	0.12 (0.81)
HIV- QFN +	16 weeks		4.66 (0.86)	3.74 (0.82)	2.34 (0.67)	2 (2.28)
HIV+ QFN -	Birth	4 (3)	0.29 (0.04)	0.22 (0.1)	0.14 (0.08)	0.02 (0.02)
HIV+ QFN -	16 weeks		10.17 (1.56)	8.14 (0.7)	2.69 (0.37)	4.94 (10.71)
HIV+ QFN +	Birth	5 (5)	1.31 (1.33)	1.72 (1.01)	0.17 (0.41)	0.1 (0.07)
HIV+ QFN +	16 weeks		5.3 (1.14)	4.88 (0.93)	2.17 (0.64)	2.09 (1.44)
Total (birth)		32(25)	0.35 (0.49)	0.41 (0.49)	0.29 (0.29)	0.1 (0.46)
Total (16 weeks)			5.47 (1.46)	4.04 (1.05)	1.94 (0.63)	1.91 (2.5)
	Effect of time	p	< .0005	< .0005	< .0005	0.003
	Effect of time and maternal infection status	p	0.45	0.33	0.29	0.47

Table 13. The frequency of BCG-specific $CD8^+Ki67^+$ T cells expressing IFN-γ, TNF-α, IL-2 or IL-17 were significantly increased in infants following BCG vaccination, irrespective of maternal infection status.
Paired infant samples were collected at birth (pre-BCG vaccination) and at 16 weeks of age (10 weeks post BCG vaccination). A reduced number of samples were available for analysis of IL-17+ CD8-T cells, indicated in parentheses. A mixed ANOVA model was used to assess differences between groups.

In order to determine whether a difference might have been missed due to small numbers of paired samples being available for analysis, groups were combined and re-analysed based only on maternal HIV status or maternal QFN status (ie 2 groups rather than 4). The $CD8^-Ki67^+$ T cell response was similar between HIV-unexposed infants (n=23) and HIV-exposed (n=9), p= .92. The $CD8^+Ki67^+$ T cell response was also similar, p= .65. Similarly there was no difference when groups of infants were analysed based only on maternal TB status (11 infants born to mothers with QFN negative tests, 21 infants born to mothers with positive QFN tests): $CD8^-Ki67^+$, p= .28, $CD8^+Ki67^+$,p= .11.

4.11 Paired infant tetanus-specific T cell responses pre- and post-BCG vaccination

The assumptions required for the mixed ANOVA model were violated more frequently when assessing tetanus-specific T cell responses, making the model more susceptible to type I errors. Therefore, the Wilcoxon-Signed Rank test was run, however this test cannot compare the difference in responses pre- and post-vaccination as well as the effect of maternal infection status.

At 16 weeks of age, following tetanus vaccination at 6, 10 and 14 weeks, there was a highly significant increase in the frequency of tetanus-specific $CD8^-$ ($p< .005$) and in $CD8^+$ ($p= .02$) $Ki67^+$ T cell response compared to responses at birth. In keeping with the overall proliferative response, there was a highly significant increase in tetanus-specific $CD8^-$ $Ki67^+$ IFN-γ^+ ($p< .005$), TNF-a^+($p< .005$), IL-2^+ ($p< .005$), and IL-17^+ ($p= .004$) T cells. There was also highly significant increase in the $CD8^+$ $Ki67^+$ IFN-γ^+ ($p< .005$), TNF-a^+($p= .001$) and IL-2^+ ($p= .001$) T cells, but not IL-17^+ T cells ($p = .93$).

4.12 Association between maternal and infant responses

BCG-specific $CD8^-Ki67^+$ T cell responses in mothers and infants at birth

Spearman's Rank Order correlation was used to assess the association of maternal and infant BCG-specific $CD8^-Ki67^+$ T cell responses.

There was a moderate positive correlation between maternal and newborn infant BCG-specific $CD8^-Ki67^+$ T cell response in the group of QFN positive mothers and their newborn infants (n=27 paired mother and infant samples at birth), $r_s= .43$, $p= .03$, Figure 31. However, no association was seen in QFN negative mothers and their infants (n=22), $r_s= .02$, $p= .92$. No relationship was found between maternal and newborn infant BCG-specific $CD8^-Ki67^+$ T cell responses in HIV infected (n=16, $r_s= .34$, $p= .21$) or uninfected mothers and their infants (n=33, $r_s= .22$, $p= .22$), or indeed in the overall mother-infant cohort (n=49), $r_s= .25$, $p= .09$.

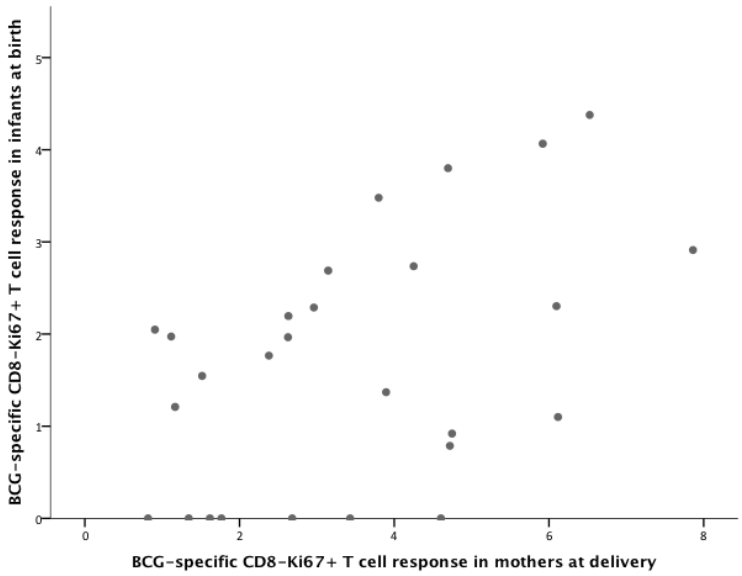


Figure 31. Relationship between maternal and infant BCG-specific $CD8^{-}Ki67^{+}$ T cell responses in QFN positive mothers and their infants.
N=27, r_s= .43, p= .03. Data is square root transformed in order to fulfill the assumption of a monotonic relationship required for the Spearman's Rank Order correlation. Each marker represents a mother-infant pair.

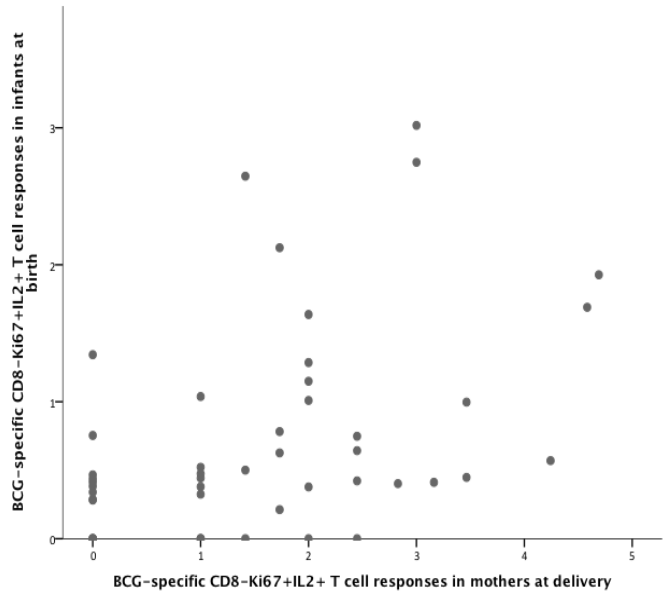
BCG-specific $CD8^{+}Ki67^{+}$ T cell responses in mothers and infants at birth

There was no correlation between maternal and infant BCG-specific $CD8^{+}Ki67^{+}$ T cell response in the overall cohort, r_s= .20, p= .18 or between any of the groups.

4.13 BCG-specific $CD8^{-}$ and $CD8^{+}$ $Ki67^{+}$ $cytokine^{+}$ T cell responses in mothers and infants at birth

There was a moderate positive correlation between maternal BCG-specific $CD8^{-}Ki67^{+}$ $cytokine^{+}$ T cell response and infant BCG-specific $CD8^{-}Ki67^{+}$ $cytokine^{+}$ T cell response for IL-2 (r_s= .49, p=< .0005) and TNF-α (r_s= .39, p= .0006), Figure 32.

A



B

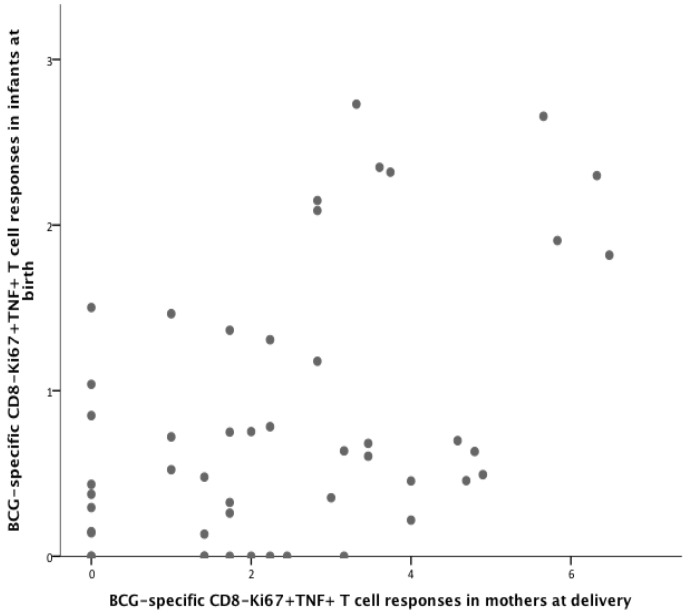


Figure 32. The association between maternal and infant BCG-specific $CD8^{-}Ki67^{+}$ $IL\text{-}2^{+}$ and TNF- α^{+} T cell.

N=49. Data is square root transformed in order to fulfill the assumption of a monotonic relationship required for the Spearman's Rank Order correlation. Each marker represents a mother-infant pair. (A) BCG-specific $CD8^{-}Ki67^{+}$ $IL\text{-}2^{+}$ T cell responses, r_s= .49, p< .0005. (B) BCG-specific $CD8^{-}Ki67^{+}$ TNF- α^{+} T cell responses, r_s= .43, p= .0006.

This association was only observed between HIV-uninfected women and their infants and was not seen in HIV-infected mothers and their infants, Table 14. Maternal and infant $CD8^{-}$ $Ki67^{+}$ $IL\text{-}2^{+}$, TNF-α^{+} and $IL\text{-}17^{+}$ T cell frequencies were positively correlated where the mother had a positive QFN response, Table 14.

An association between maternal and infant responses was also observed in the $CD8^+$ T cell compartment, with a positive correlation between maternal BCG-specific $CD8^+Ki67^+$ IL-2^+ T cell frequencies and infant BCG-specific $CD8^-Ki67^+$ IL-2^+ T cell frequencies, r_s= .45, p = .001. Similarly to the $CD8^-$ responses, there was a relationship between maternal and infant responses in HIV-uninfected mothers and their infants, but not between HIV infected mothers and infants, Table 14. This maternal-infant correlation was also present irrespective of maternal *Mtb* sensitisation.

Group		$CD8^+Ki67^+$				$CD8^-Ki67^+$			
		IFN-γ^+	IL-2^+	TNF-α^+	IL-17^+	IFN-γ^+	IL-2^+	TNF-α^+	IL-17^+
Overall cohort (n=49)	r_s	0.14	0.45	0.22	-0.17	0.27	0.49	0.39	0.23
	p	0.36	**0.001**	0.13	0.29	0.07	**< .0005**	**0.006**	0.15
HIV- (n=32)	r_s	0.37	0.50	0.12	-0.03	0.29	0.53	0.43	0.28
	p	0.04	**0.004**	0.66	0.88	0.11	**0.002**	**0.02**	0.16
HIV+ (n=17)	r_s	-0.18	0.29	0.30	-0.42	0.25	0.37	0.30	0.06
	p	0.49	0.26	0.09	0.16	0.33	0.14	0.24	0.85
QFN - (n=22)	r_s	-0.24	0.44	-0.24	-0.32	-0.05	0.45	0.02	-0.14
	p	0.28	**0.04**	0.28	0.23	0.84	**0.03**	0.93	0.60
QFN+ (n=26)	r_s	0.36	0.50	0.47	-0.10	0.32	0.49	0.52	0.48
	p	0.07	**0.01**	**0.02**	0.65	0.11	**0.01**	**0.007**	**0.02**
HIV- QFN- (n=14)	r_s	0.02	0.58	-0.12	-0.22	-0.05	0.56	0.20	-0.08
	p	0.94	**0.03**	0.70	0.54	0.88	**0.04**	0.49	0.82
HIV- QFN+ (n=18)	r_s	0.59	0.44	0.55	0.09	0.44	0.49	0.56	0.52
	p	**0.01**	0.06	**0.02**	0.74	0.07	**0.04**	**0.02**	0.31
HIV+ QFN- (n=8)	r_s	-0.40	0.00	-0.51	-0.48	-0.10	0.17	-0.12	-0.22
	p	0.33	1.00	0.20	0.33	0.81	0.70	0.78	0.68
HIV+ QFN+ (n=8)	r_s	0.19	0.63	0.04	-0.70	-0.23	-0.17	-0.10	0.25
	p	0.65	0.09	0.93	0.08	0.59	0.69	0.82	0.59

Table 14. Association between maternal and infant BCG-specific $CD8^-$ and $CD8^+$ $Ki67^+$ $cytokine^+$ T cell frequencies at birth.
Data was square root transformed and analysed using Spearman's Rank Order correlation, p < .05 were considered significant.

4.13.1 BCG-specific $CD8^-$ and $CD8^+$ $Ki67^+$ T cell responses in mothers at delivery and infants at 16 weeks

There was no correlation of BCG-specific $CD8^-$ or $CD8^+$ proliferative response between mothers and infants, r_s= .02, p= .88 and r_s= -.07, p= .59 respectively.

4.13.2 BCG-specific $CD8^-$ and $CD8^+$ $Ki67^+$ $cytokine^+$ T cell responses in mothers at delivery and infants at 16 weeks

There was a moderate association between maternal BCG-specific $CD8^+$ $Ki67^+$ $IL\text{-}2^+$ T cell frequencies and infant BCG-specific $CD8^+$ $Ki67^+IL\text{-}2^+$ T cell frequencies at 16 weeks, r_s= 0.32, p= .01. This association was seen in QFN positive mothers and their infants, r_s= 0.35, p= .04, but not in QFN negative mothers and their infants, r_s= 0.18, p= .39. When the cohort was split into the four groups, BCG-specific $CD8^+$ $Ki67^+IL\text{-}2^+$ T cell frequencies of infants born to HIV-infected, QFN positive mothers were positively correlated with the maternal frequency of these cells, R_s= .65, p = .01. There were no other associations between maternal and infant $CD8^-$ or $CD8^+$ $Ki67^+$ $cytokine^+$ T cell frequencies, Table 15.

Group		$CD8^+Ki67^+$				$CD8^-Ki67^+$			
		IFN-γ^+	IL-2^+	TNF-α^+	IL-17^+	IFN-γ^+	IL-2^+	TNF-α^+	IL-17^+
Overall cohort (n=63)	r_s	0.02	0.32	0.10	0.23	-0.11	0.03	-0.01	-0.05
	p	0.88	**0.01**	0.46	0.09	0.39	0.83	0.93	0.74
HIV- (n=32)	r_s	-0.1	0.27	0.06	0.27	-0.18	-0.02	-0.11	0.09
	p	0.57	0.12	0.73	0.17	0.29	0.92	0.55	0.64
HIV+ (n=17)	r_s	0.15	0.36	0.18	0.17	0.00	0.008	0.13	-0.26
	p	0.46	0.06	0.37	0.39	1.00	0.97	0.53	0.19
QFN - (n=22)	r_s	0.16	0.18	0.06	0.12	-0.16	0.02	-0.01	-0.21
	p	0.44	0.39	0.79	0.61	0.44	0.94	0.95	0.36
QFN+ (n=26)	r_s	-0.08	0.35	0.13	0.31	0.01	0.03	0.003	-0.01
	p	0.65	**0.04**	0.45	0.09	0.95	0.89	0.99	0.97
HIV- QFN- (n=12)	r_s	-0.14	0.22	-0.31	0.21	-0.47	-0.17	-0.43	-0.30
	p	0.66	0.48	0.33	0.58	0.12	0.6	0.17	0.44
HIV- QFN+ (n=23)	r_s	-0.12	0.22	0.26	0.33	0.09	0.18	0.11	0.23
	p	0.60	0.31	0.23	0.18	0.67	0.40	0.62	0.37
HIV+ QFN- (n=13)	r_s	0.37	-0.24	0.22	-0.18	-0.11	-0.06	0.19	-0.32
	p	0.22	0.43	0.48	0.57	0.72	0.86	0.53	0.30
HIV+ QFN+ (n=14)	r_s	0.002	0.65	-0.13	0.45	0.04	-0.08	0.007	-0.29
	p	0.99	**0.01**	0.67	0.10	0.89	0.79	0.98	0.30

Table 15. Association between maternal and infant BCG-specific $CD8^-$ and $CD8^+$ $Ki67^+$ $cytokine^+$ T cell frequencies in mothers at delivery and infants at 16 weeks.

4.13.3 Tetanus-specific T cell responses in mothers at delivery and infants at 16 weeks

There was no correlation between maternal and 16 week old infant T cell frequencies (data not shown).

4.14 Summary of key findings

4.14.1 BCG-specific T cell proliferation

There was no effect of maternal HIV or TB infection (defined by a positive QFN test) on BCG-specific T cell proliferation in infants at birth or at 16 weeks of age, following BCG vaccination at 6 weeks of age.

HIV infection was associated with lower maternal frequencies of BCG-specific $CD8^+$, but not $CD8^-$ proliferating T cells. Conversely, higher maternal frequencies of BCG-specific $CD8^-$, but not $CD8^+$, $Ki67^+$ T cells were observed in the presence of TB infection.

Co-infection with TB and HIV had a significant effect on maternal BCG-specific T cell proliferation, both in the $CD8^-$ compartment and the $CD8^+$ compartment; co-infected women had higher frequencies of $Ki67^+$ T cells than HIV-infected mothers without evidence TB infection. In HIV-uninfected women however, TB infection did not have a significant effect on BCG-specific T cell proliferation.

There were no differences in tetanus-specific T cell proliferation in mothers or infants.

4.14.2 BCG-specific intracellular expression of cytokines in proliferating T cells

The total expression of IFN-γ, TNF-α, IL-2 or IL-17 in proliferating T cells was similar amongst groups of infants when responses were measured before BCG vaccination (at birth) and after BCG vaccination (at 16 weeks), with no effect of maternal HIV or TB infection at either timepoint.

Despite similarities observed in their offspring, mothers had significantly different intracellular expression of these cytokines dependent upon both the presence of HIV infection and TB infection. Amongst HIV infected women co-infected with TB, lower frequencies of BCG-specific $CD8^-Ki67^+IFN\text{-}\gamma^+$, TNF-$\alpha^+$ and IL-2^+ T cells were observed and lower TNF-α^+ were observed in the proliferating $CD8^+$ compartment. In the absence of HIV infection, TB infection was not associated with any differences in intracellular cytokine expression. HIV-infected mothers without evidence of TB infection had the lowest frequencies of IFN-γ, TNF-α, IL-2 or IL-17 proliferating $CD8^-$ and $CD8^+$ T cells.

There were no differences in tetanus-specific $CD8^-$ or $CD8^+$ $cytokine^+$ T cell proliferation in mothers or infants.

4.14.3 BCG-specific polyfunctional proliferating T cells

At birth, HIV-exposed, uninfected infants had higher frequencies of IFN-γ^+TNF-α^+ $CD8^-$ T cells, TNF-α single-positive $CD8^-$ and $CD8^+$ T cells and IL-2 single-positive $CD8^+$ T cells compared to their unexposed peers. Infants born to mothers co-infected with HIV and TB had the highest frequencies of BCG-specific $CD8^+$ TNF-α-single positive T cells.

These populations of cells are distinct from the population of cells described above, in which no significant differences were observed, in that they exclusively express these cytokines and no other cytokine in combination. The population above represents the total population of T cells expressing cytokines, which may express other cytokines in combination.

Following vaccination, the predominant response to BCG was $CD8^-$ IFN-γ^+TNF-$\alpha^+$and IFN-γ^+IL-2^+TNF-α^+ T cells. Although BCG-specific IL-2^+ $CD8^+$ T cells formed a small proportion of the overall T cell response, differences in this subset were observed depending on infant exposure to maternal HIV and TB infection. Infants born to HIV-uninfected mothers with evidence of TB infection had the highest frequencies of these cells.

In mothers, HIV infection and TB infection alone as well co-infection with HIV and TB had a significant effect on multiple T cell subsets.

HIV infected women had lower frequencies of BCG-specific IFN-γ^+IL-2^+TNF-α^+IL-17^+ and IFN-γ^+TNF-α^+IL-17^+ $CD8^-$ T cells; these cells formed a small proportion of the overall response.

Women with evidence of TB infection had an increased frequency of BCG-specific IFN-γ^+IL-2^+TNF-α^+IL-17^+, IFN-γ^+ IL-2^+TNF-α^+, IFN-γ^+ TNF-α^+IL-17^+, IFN-γ^+TNF-α^+, IL-2^+TNF-α^+IL-17^+, IL-2^+TNF-α^+, TNF-α^+IL-17^+, IL-2^+ and TNF-α^+ $CD8^-$ $Ki67^+$ T cells.

The interaction of maternal HIV and TB infection had a significant effect on the frequency of BCG-specific IFN-γ^+IL-2^+TNF-α^+IL-17^+, IFN-γ^+IL-2^+TNF-α^+, IFN-γ^+TNF-α^+IL-17^+, IFN-γ^+TNF-α^+ and IL-2^+TNF-α^+IL-17^+ $CD8^-$ $Ki67^+$ T cells in these mothers

Within the $CD8^+$ compartment differences in IFN-γ^+IL-2^+TNF-α^+, IFN-γ^+IL-2^+TNF-α^+IL-17^+, IL-2^+TNF-α^+, IL-2-single positive and TNF-α- single positive $CD8^+$ T cells were observed.

There were no differences in tetanus-specific $CD8^-$ or $CD8^+$ cytokine$^+$ T cell proliferation in mothers or infants.

4.14.4 Paired infant T cell response pre- and post-vaccination

BCG vaccination was associated with a substantial increase in BCG-specific T cell proliferation. Proliferating $CD8^-$ and $CD8^+$ T cells expressing IFN-y, TNF-α, IL-2 and IL-17 were significantly upregulated in infants at 16 weeks compared to at birth. Maternal HIV and TB infection had no effect on the expansion of specific T cells in response to BCG vaccination. At 16 weeks of age the overall frequency of tetanus-specific proliferating T cells as well as cytokine expressing T cells was significantly increased compared to frequencies at birth.

4.14.5 Association between maternal and infant responses

In the presence of maternal TB infection, frequencies of maternal and newborn infant BCG-specific Ki67^+ $CD8^-$ T cells were positively correlated, however, no association was observed in the absence of TB infection. No association was observed in the $CD8^+$ compartment.
At birth, the frequency of BCG-specific Ki67^+CD8^- and $CD8^+$ IL-2^+ T cells were associated with the frequency of these cells in the mother. This correlation was not observed in the presence of maternal HIV infection. At 16 weeks, this correlation persisted in the $CD8^+$, but not the $CD8^-$ compartment.

The frequency of BCG-specific Ki67^+CD8^- TNF-α^+ T cells observed in infants at birth were correlated with the frequency of these cells in the mother, this effect did not persist in infants at 16 weeks of age. The association was seen specifically in infants born to HIV-uninfected mothers with evidence of TB infection.

Chapter 5: Analysis of secreted cytokines and chemokines

Multiplex ELISA was used to determine the levels of secreted cytokines and chemokines in cell culture supernatants collected after 24 hours of incubation with BCG or tetanus antigens in mother-infant pairs. The following analytes were evaluated using a Luminex multiplex ELISA: EGF, Fractalkine, GM-CSF, IL-1Ra, IL-12p40, IL-17, IFN-γ, IP-10, MCP-1, MDC, MIP-1β, sCD40L, TNF-α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10 and IL-13. The coefficient of variation (CV) was so high between sample runs for Fractalkine (79.92%) that evaluation of this analyte had to be disregarded. The CV was less than 20% for most other analytes with a mean CV 13% (range 6-23%).

A 3 way repeated measures ANOVA with mixed models was run to determine the effect of maternal HIV and TB infection on the level of secreted cytokines and chemokines in mothers and infants. Where the distribution of data was bimodal, a generalized estimating equations (GEE) model was used instead. Spearman's Rank Order correlation was used to determine the association between groups.

5.1.1 Exploratory multiplex analysis

Samples collected from four mothers, four infants at birth and four infants at 16 weeks were stimulated with BCG and supernatants were collected after 1 day and 6 days of incubation at 37°C, 5% CO2, 80% humidity. Supernatants were analysed on two MILLIPLEX plates (27-plex and 13-plex) to compare levels of secreted cytokines and chemokines on day 1 and day 6. Some analytes such as TNF-α were higher in the day 1 samples, whilst others such as IFN-γ were higher in day 6 samples. The time of harvest did not seem to affect the levels of others such as MCP-1. Overall, one time point favoured some analytes, but not others, Appendix V. It was decided to use supernatants harvested on day 1 because of the concern that haemolysis seen in a substantial proportion of the newborn infant samples after 6 days of culture could potentially affect the results. This exploratory analysis also informed the selection of cytokines and chemokines to measure in the main study cohort.

5.2 Study cohort

A total of 108 maternal and 106 infant samples at birth and 88 infant samples at 16 weeks were available for analysis, the numbers available for each sub-group are given in Table 16.

Group (maternal infection status)	Mother	Infant at birth	Infant at 16 weeks
HIV-	62	60	54
HIV+	46	46	34
QFN -	52	51	39
QFN +	56	55	49
HIV- QFN-	27	26	22
HIV- QFN +	35	34	32
HIV+ QFN -	25	25	17
HIV+ QFN +	21	21	17
Total	108	106	88

Table 16. Numbers of samples available for analysis for mothers and infants at birth and infants at 16 weeks.

5.3 Influence of Maternal HIV and Mtb sensitisation on cytokine and chemokine responses in infants and mothers

5.3.1 Infants at birth

Responses to BCG antigens

At birth, HIV-exposed, uninfected infants had higher levels of TNF-α protein in response to BCG antigens compared to unexposed infants, p= .03. Comparison of the four groups of infants revealed that maternal HIV infection only had an effect on infant TNF-α responses in the presence of maternal *Mtb* sensitisation, Figure 33.

Infants born to *Mtb* sensitised mothers had higher sCD40L responses to BCG, p= .01. This effect was seen in HIV-exposed and unexposed infants, however the effect was only statistically significant amongst HIV-unexposed infants, p= .02. Infants born to co-infected mothers had significantly higher responses than infants born to HIV-uninfected, QFN negative mothers (p= .01), Figure 33.

Infants born to HIV-infected, QFN positive mothers also had the highest GM-CSF responses to BCG antigens, Figure 33. A difference in IFN-γ response to BCG antigens was seen

between HIV-unexposed infants born to QFN positive mothers and HIV-exposed infants born to QFN negative mothers, Figure 33.

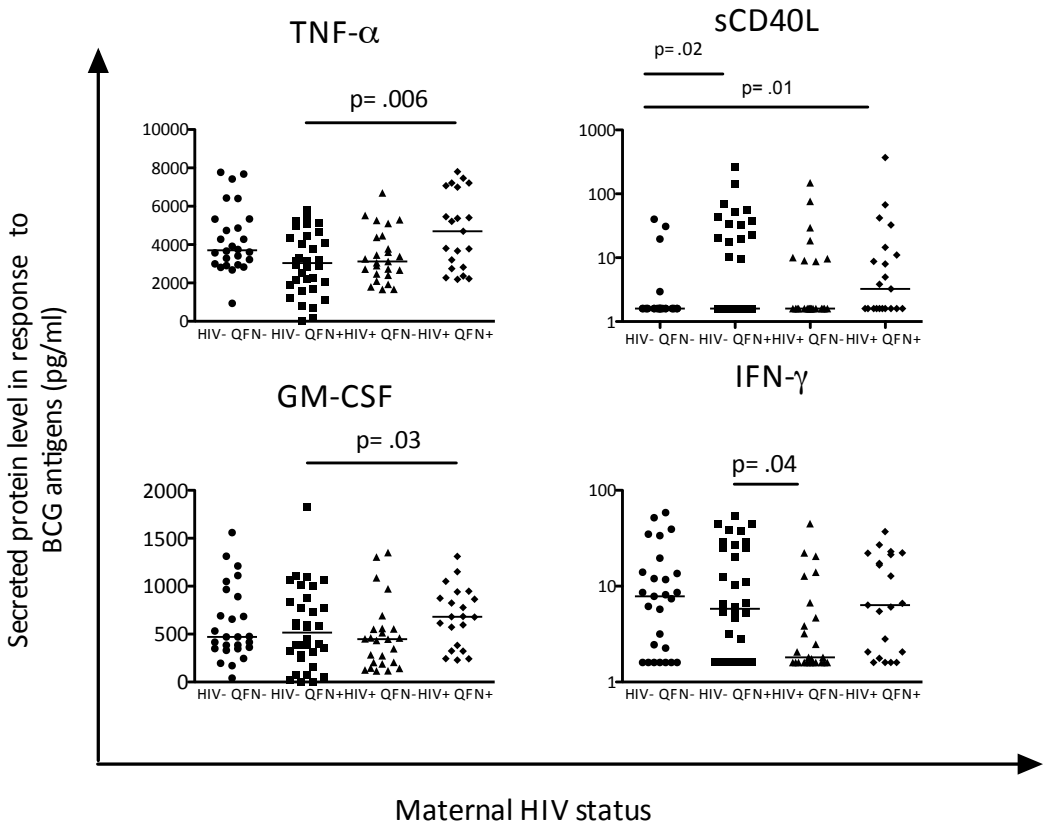


Figure 33. HIV-exposed infants born to mothers with positive QFN tests have increased TNF-α, GM-CSF and sCD40L responses to BCG antigens at birth.
Median values are graphically displayed and only responses that showed a statistically significant difference are shown. IFN-γ and sCD40L data is presented using a log scale.

Responses to tetanus antigens

HIV-exposed infants had statistically significantly lower IL-8 responses to tetanus toxoid compared to HIV-unexposed infants, p= .002. Responses were lower in HIV-exposed infants regardless of maternal QFN status, Figure 34. Infants born to HIV/TB co-infected mothers had the lowest responses.

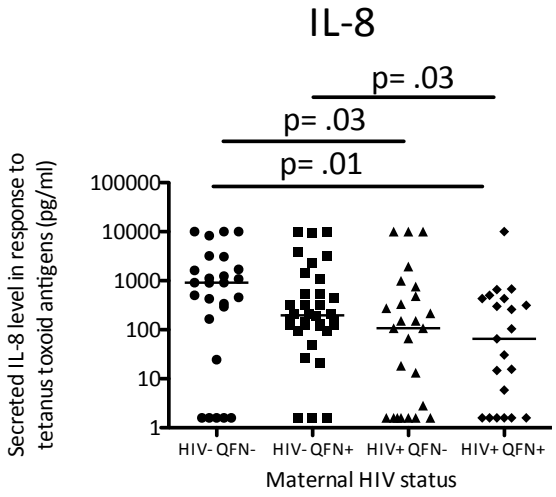


Figure 34. HIV-exposed infants have lower levels of IL-8 released in response to tetanus toxoid.
The horizontal line represents the median and data is graphically displayed on a log scale.

Infants born to HIV-uninfected women with positive QFN tests had the highest levels of MCP-1 released in response to tetanus toxoid. This was statistically significant when compared to HIV-exposed infants born to QFN negative mothers, Figure 35.

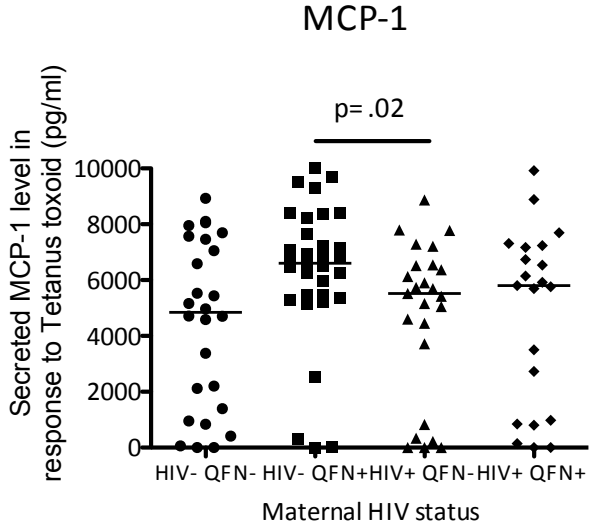


Figure 35. HIV-unexposed infants born to QFN positive mothers have the highest levels of MCP-1 released in response to tetanus toxoid.
The horizontal line represents the median.

Background expression in unstimulated samples

Differences in the background expression levels of IP-10, IL-8, MDC, MCP-1 were observed between the four groups of infants at birth, Figure 36.

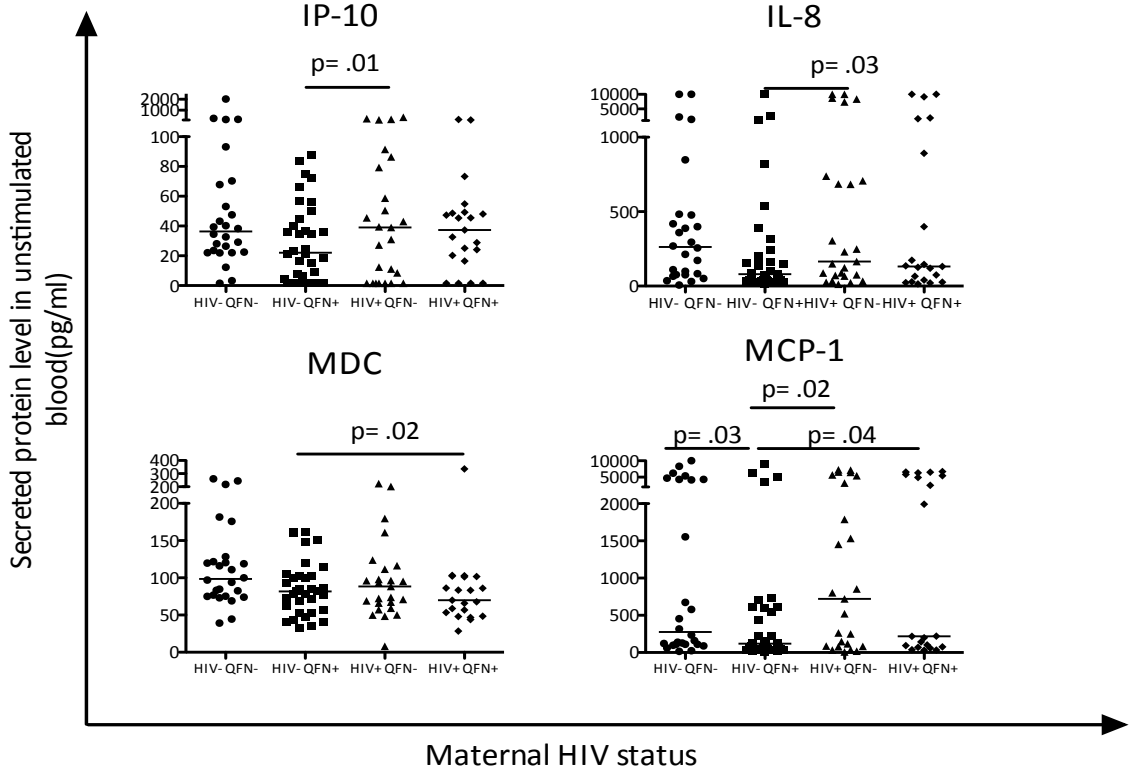


Figure 36. Background expression levels of IP-10, IL-8, MDC and MCP-1 in infants at birth.
The horizontal line represents the median.

5.3.2 Infants at 16 weeks

Following BCG vaccination, there were no differences in the levels of cytokines or chemokines released in response to BCG or tetanus antigens amongst infants; there was no statistically significant effect of maternal HIV or TB alone or in combination.

5.3.3 Mothers at delivery

Responses to BCG antigens

Effect of HIV infection

Mothers infected with HIV had significantly lower IL-1ra, MDC, IFN-γ and IL-10 responses to BCG antigens, Figure 37.

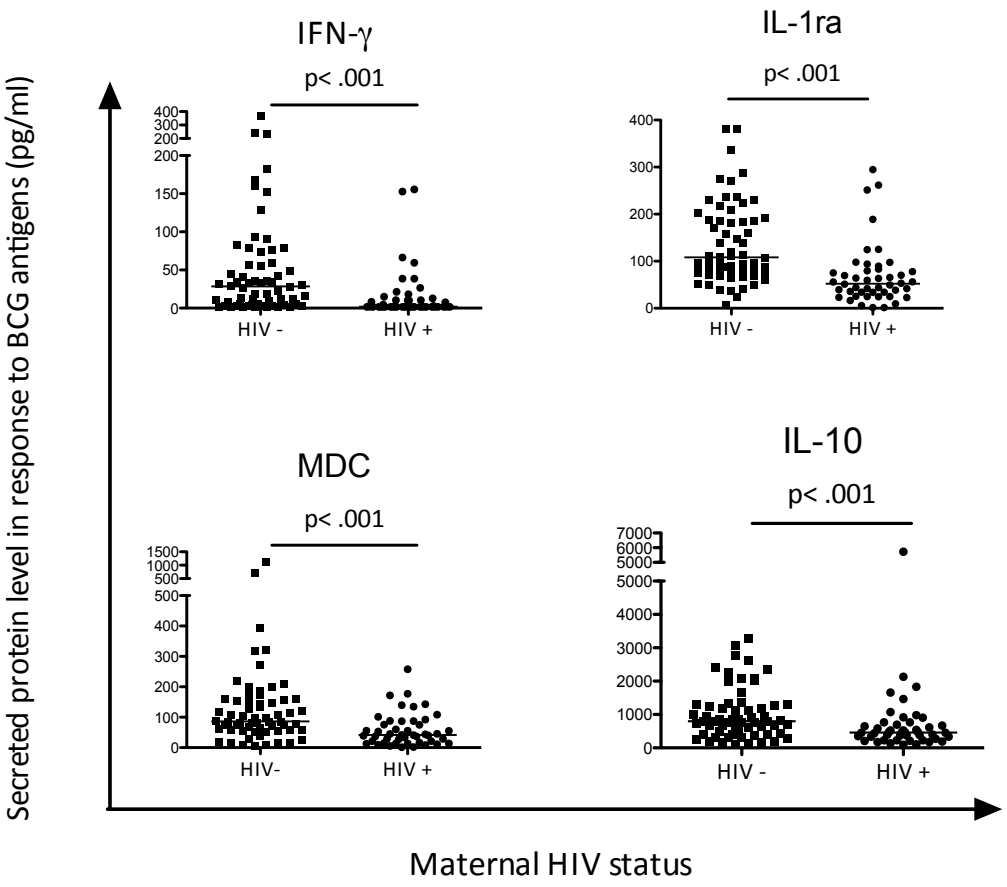


Figure 37. HIV-infected mothers have significantly lower responses to BCG antigens than uninfected mothers.
The horizontal line represents the median value, only responses that showed a statistically significant difference are shown.

Effect of *Mtb* sensitisation

Mothers with a positive QFN tests had higher IFN-γ responses to BCG antigen than those without evidence of TB sensitisation on the QFN test, however there were no differences in any other response, Figure 38.

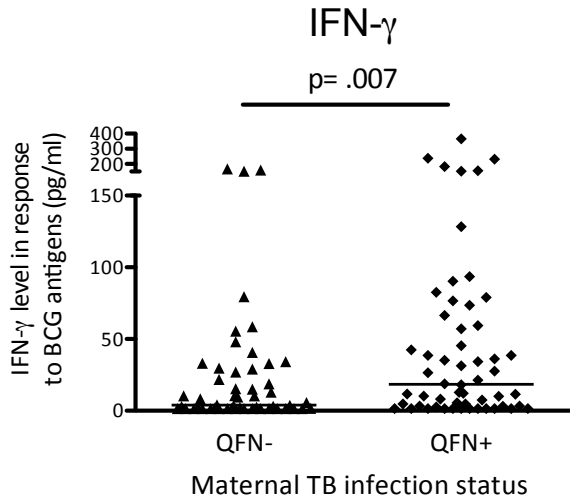


Figure 38. Mothers with TB infection (defined by a positive QFN test) have significantly higher IFN-γ responses to BCG antigens than mothers not infected with TB (QFN test negative).
The horizontal line represents the median value.

Effect of HIV and TB infection status

When considered alone, HIV and TB infection status both had a statistically significant effect on the maternal IFN-γ response to BCG antigens. It follows therefore that there was an interaction effect of HIV and TB infection status on this response. In the presence and absence of evidence of TB infection, HIV-infected women had lower IFN-γ responses to BCG antigens compared to HIV uninfected women. Amongst HIV-infected women, IFN-γ responses were significantly higher in those that were sensitised to *Mtb* compared to those who showed no response the QFN test. However, amongst HIV-uninfected women, there was no difference in IFN-γ response between women who tested QFN negative or positive (p= .25), Figure 39.

Differences in IL-1ra and MDC responses to BCG antigens were observed between HIV infected and uninfected women with and without evidence of TB infection. No significant differences were seen between women who tested QFN positive and those that tested QFN negative, irrespective of HIV infection, Figure 39.

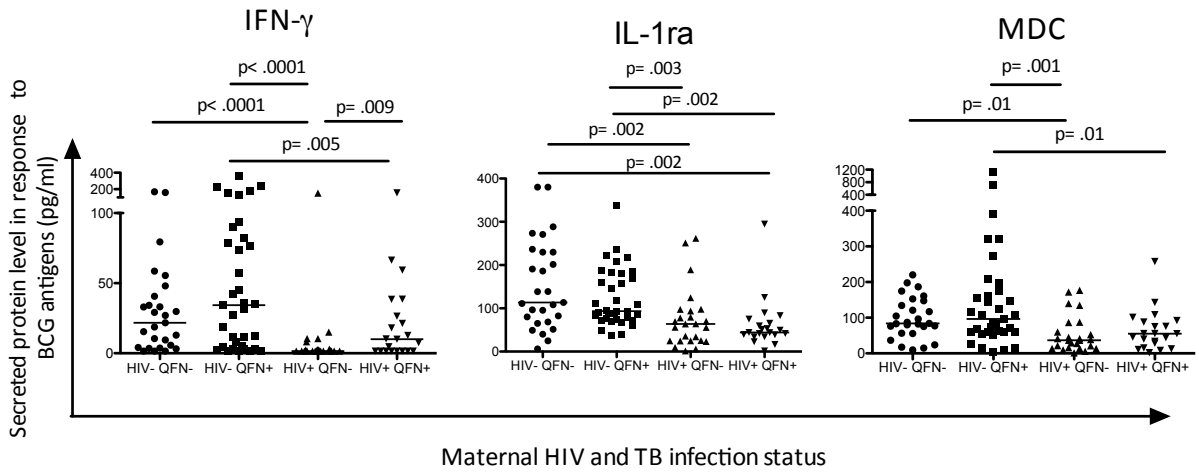


Figure 39. Maternal IFN-γ, IL-1ra and MDC responses to BCG antigens.
The horizontal line represents the median value.

Tetanus

Mothers infected with HIV had significantly lower IL-1Ra and MIP-1β responses to tetanus toxoid compared to uninfected mothers (p= .01 and p= .02 respectively). No differences were seen in any response amongst QFN positive compared to QFN negative women.

5.3.4 Changes in infant cytokine and chemokine levels pre- and post-vaccination

There were a large number of chemokines and cytokines that changed between birth and 16 weeks in age following vaccination with BCG at birth and tetanus at 6, 10 and 14 weeks of age (Table 17).

Cytokine/ Chemokine	Antigen	Response at birth (pg/ml)	Response at 16 weeks (pg/ml)	p
IL-1Ra	Nil	10.91 (8.89 - 13.39)	7.78 (6.19 -7.78)	0.03
IP-10	Nil	25.46 (20.44 - 31.71)	167.50 (131.06 - 167.53)	<0.001
MCP-1	Nil	371.85 (268.47 - 515.04)	1774.01 (1233.05 - 774.79)	<0.001
MDC	Nil	84.80 (77.55 - 92.73)	128.38 (116.13 - 128.32)	<0.001
MIP-1β	Nil	125.22 (96.26 - 162.90)	222.07 (165.54 - 222.14)	0.005
sCD40L	Nil	105.80 (93.09 - 120.23)	87.27 (75.67 -87.28)	0.039
IL-8	Nil	202.03 (144.02 - 283.40)	406.67 (278.57 - 406.69)	0.008
EGF*	Nil	6.28 (1.6 – 12.40)	13.39 (5.9 – 24.25)	<0.001
IL-6*	Nil	4.29 (1.60 – 15.38)	10.19 (4.35 – 47.33)	<0.001
IL-10*	Nil	1.60 (1.60 – 1.60)	1.60 (1.60 – 3.84)	<0.001
TNF-α*	Nil	1.60 (1.60 – 4.88)	4.81 (2.43 – 10.78)	<0.001
IL-1Ra	BCG	161.66 (131.39 - 198.89)	15.96 (12.65 - 15.95)	<0.001
IL-12p40	BCG	34.59 (27.54 - 43.45)	17.78 (13.79 - 17.78)	<0.001
MCP-1	BCG	2386.26 (1402.43 - 4060.28)	646.78 (355.88 - 646.50)	0.002
MDC	BCG	216.16 (168.16)	33.08 (24.94 - 33.07)	<0.001
TNF-α	BCG	3024.21 (2512.99 - 639.43)	1859.38 (1511.79 - 859.99)	<0.001
IFN-γ	BCG	7.13 (5.53 – 9.20)	71.95 (54.02 - 71.92)	<0.001
IL-10	BCG	783.68 (633.80 – 969.55)	177.68 (140.01 - 177.63)	<0.001
sCD40L*	BCG	1.60 (1.60 - 10.82)	1.60 (1.60 - 1.60)	0.02
IP-10*	BCG	1.60 (1.60 – 6.14)	80.60 (1.60 – 284.80)	<0.001
IL-1Ra	TT	10.55 (8.33 - 13.37)	7.40 (5.68 -7.40)	0.026
MCP-1	TT	1769.78 (1071.16 - 924.03)	453.96 (257.61 -453.99)	0.001
MIP-1β	TT	234.77 (156.79 - 351.52)	60.82 (38.57 -60.83)	<0.001
sCD40L	TT	11.93 (8.78 - 16.21)	28.53 (20.19 - 40.33)	<0.001

Table 17: Cytokine and chemokine responses in infants at birth (pre-vaccination) and at 16 weeks (post-vaccination).
Only responses that showed a statistically significant difference are shown. Red bars indicate where a response is upregulated at one timepoint compared to the other; blue bars indicate a downregulated response. Where data was normally distributed, the mean and 95% confidence interval is stated. Where data was non-normally distributed (*), the median and interquartile range is stated.

5.4 Correlation between maternal and infant cytokine and chemokine levels

Maternal and infant cytokine and chemokine responses were significantly correlated for a large number of analytes at day 1 post-partum (Table 18). At 16 weeks of age, infant responses remained correlated with maternal levels for only a few analytes (Table 19).

Cytokine / chemokine	Antigen	Rs	p
EGF	Nil	0.26	< .01
IL-1Ra	Nil	0.54	< .01
IP-10	Nil	0.37	< .01
MDC	Nil	0.31	< .01
MIP-1β	Nil	0.25	< .01
sCD40L	Nil	0.31	< .01
TNF-α	Nil	0.46	< .01
IL-13	Nil	0.20	.04
IL-1β	Nil	0.24	.01
IL-6	Nil	0.22	.02
GM-CSF	BCG	0.38	< .01
IL-1Ra	BCG	0.41	< .01
IL-12p40	BCG	0.35	< .01
IP-10	BCG	0.29	< .01
MCP-1	BCG	0.23	.02
MDC	BCG	0.29	< .01
TNF-α	BCG	0.51	< .01
IFN-γ	BCG	0.27	< .01
IL-10	BCG	0.42	< .01
IL-1β	BCG	0.58	< .01
IL-6	BCG	0.45	< .01
GM-CSF	TT	0.33	< .01
IL-1Ra	TT	0.47	< .01
IP-10	TT	0.45	< .01
MCP-1	TT	0.25	.01
MIP-1β	TT	0.50	< .01
TNF-α	TT	0.44	< .01
IL-1β	TT	0.30	< .01
IL-8	TT	0.40	< .01

Table 18: Correlation between maternal and infant cytokine and chemokine responses at birth.
Only significant correlations are shown.

Cytokine / chemokine	Antigen	Spearman	Spearman p-val
sCD40L	Nil	0.28	< .01
IL-12p40	BCG	0.36	< .01
IL-8	BCG	0.38	< .01
IL-12p40	TT	0.5	< .01

Table 19: Correlation between maternal cytokine and chemokine responses on day 1 postpartum and infant responses at 16 weeks.
Only significant correlations are shown.

5.5 Differences in cytokine and chemokine responses between mothers and infants

The level of secreted cytokines and chemokines in maternal and newborn infant whole blood was measured after 24 hours of incubation with BCG or tetanus antigens or medium alone (unstimulated). There was a significant difference in the pattern of cytokine and chemokine expression in mothers and infants, Table 20. The background expression of analytes was generally higher in infants compared to mothers.

Cytokine/ Chemokine	Antigen	Maternal response (pg/ml)	Infant response (pg/ml)	P
IL-1Ra	Nil	6.93 (5.67 - 8.48)	10.89 (8.89 -13.33)	< .001
IP-10	Nil	57.37 (44.34 - 74.23)	25.42 (19.62 - 32.95)	< .001
MDC	Nil	20.58 (18.66 - 22.7)	84.93 (76.96 - 93.73)	< .001
MIP-1β	Nil	52.01 (40.18 - 67.33)	124.83 (96.29 - 161.83)	< .001
sCD40L	Nil	68.79 (60.27 - 78.52)	105.50 (92.36 - 120.51)	< .001
IL-8	Nil	42.52 (30.65 - 59.01)	201.67 (145 - 280.48)	< .001
IL-1Ra	BCG	73.07 (60.22 - 88.65)	161.3 0(132.89 - 195.79)	< .001
IL-12p40	BCG	12.33 (9.73 - 15.62)	34.69 (27.37 - 43.97)	< .001
MCP-1	BCG	4110.48 (2837.4 - 5954.75)	2386.70 (1645.83 - 3461.06)	.04
MDC	BCG	58.01 (46.66 - 72.11)	216.04 (173.69 - 268.72)	< .001
TNF-α	BCG	1858.11 (1559.18 - 2214.37)	3026.01 (2538.05 - 3607.78)	< .001
IFN-y	BCG	11.90 (9.5 - 14.91)	7.10 (5.66 - 8.9)	< .001
IL-10	BCG	602.47 (492.57 - 736.89)	781.88 (638.92 - 956.81)	.04
sCD40L*	BCG	3395 (2485 – 5055)	26.71 (4.55 – 96.15)	< .001
IL-1β	BCG	1847.47 (1484.18 - 2299.67)	3573.54	< .001
IL-1Ra	TT	7.46 (5.93 - 9.38)	10.57 (8.4 - 13.3)	.005
MCP-1	TT	2925.65 (2011 - 4256.31)	1774.65 (1217.93 - 2585.86)	< .001
MIP-1β	TT	103.25 (72.81 - 146.42)	234.26 (164.97 - 332.65)	< .001
IL-8	TT	45.72 (28.52 - 73.3)	135.13 (84.13 - 217.02)	< .001

Table 20. The pattern of cytokine and chemokine expression is different in mothers and infants at birth.

A 3-way ANOVA model with mixed models using log transformed data was run to determine the effect of maternal HIV status on infant cytokine and chemokines responses. Backtransformed mean responses are stated with 95% confidence intervals and only responses that showed a statistically significant difference are shown. Where cytokines and chemokines were either expressed or not (bimodial distribution), data was analysed using Generalised Estimating Equations(*). The median response is stated for this data. Red bars indicate where a response is upregulated in one member of the mother-infant pair compared to the other; blue bars indicate a downregulated response.

5.6 Summary of key findings

5.6.1 Infants at birth

HIV-exposed, uninfected infants displayed increased levels of TNF-α in cell cultures stimulated with BCG antigens compared to HIV-unexposed infants at birth. The difference in TNF-α levels between HIV-exposed and unexposed infants was most significant in those born to *Mtb* sensitised mothers, suggesting that both the HIV and QFN status of the mother were influential. Infants born to HIV-infected, *Mtb* sensitised mothers had the highest levels of TNF-α.

IFN-γ secretion in response to BCG antigens was lowest amongst HIV-exposed infants born to mothers not sensitised to *Mtb*, whilst HIV-exposed infants born to *Mtb* sensitised mothers had levels similar to HIV-unexposed infants.

In the context of maternal, HIV-exposed infants had higher GM-CSF responses to BCG antigens compared to HIV-unexposed infants.

5.6.2 Infants at 16 weeks

We found no differences at all in secreted cytokine or cytokine response to BCG antigens amongst infants; maternal HIV infection and *Mtb* sensitisation did not significantly influence the response.

5.6.3 Mothers

Analysis of secreted cytokine and chemokine levels in response to BCG revealed that HIV-infected mothers had lower IFN-γ, IL-1ra, MDC and IL-10 levels compared to HIV-uninfected mothers.

Mtb sensitisation significantly influenced maternal IFN-γ responses to BCG antigens amongst HIV-infected but not HIV-uninfected mothers.

5.6.4 Change in cytokine and chemokine response between pre and post BCG vaccination

There was a significant increase in IFN-γ and IP-10 response between birth and 16 weeks, many other responses to BCG stimulation were higher at birth than at 16 weeks.

5.6.5 Association between maternal and infant cytokine and chemokine levels

There was a strong association between maternal and infant TNF-α levels, IL-1β, IL-1Ra, IL-6 and IL-10. There were moderate associations between mothers and newborn infants for a large number of cytokines and chemokines. An association between maternal and 16 week old infant response remained only for IL-12p40 and IL-8.

5.6.6 Differences in maternal and infant cytokine and chemokine levels

There were a number of differences in the immune response to BCG antigens in unvaccinated newborn infants compared to adults. Pro-inflammatory cytokines (TNF-α, IL-12p40 and IL-1β) were elevated in infants compared to adults and immunomodulatory molecules such as IL-1Ra and IL-10 were also increased. IFN-γ responses were lower in infants than in mothers in response to BCG antigens. Some chemokines (IL-8, MDC and MIP-1β) were increased.

Chapter 6: Antibody responses to routine vaccines in HIV-exposed and unexposed infants

6.1 Specific hypotheses

Hypotheses

1. Maternal HIV infection is associated with altered vaccine-induced antibody titres. HIV-infected mothers have reduced placental transfer of vaccine-specific IgG to infants and consequently HIV-exposed, uninfected infants have reduced vaccine-specific antibody titres at birth compared to HIV-unexposed infants.
2. HIV-exposed uninfected infants have increased vaccine-induced antibody titres compared to HIV-unexposed infants post-vaccination.

Specific aims

1. To measure maternal anti-*Bordetella pertussis*, anti-*Haemophilus influenzae*, anti-tetanus, anti-pneumococcal and anti-Hepatitis B specific IgG at delivery in HIV-infected and uninfected mothers.
2. To calculate transfer of maternally derived vaccine-specific IgG to the infant at birth.
3. To measure infant vaccine-specific antibody titres at birth in HIV-exposed and unexposed infants.
4. To determine the association of maternal CD4 count and viral load on placental transfer of vaccine-specific IgG from the mother to the infant at birth.
5. To measure the vaccine-induced antibody titres to routine vaccination with pertussis, *Haemophilus influenzae* type B, tetanus, Pneumococcal conjugate and Hepatitis B in HIV-exposed and unexposed infants at 16 weeks of age.
6. To compare infant and maternal vaccine-induced antibody titres in HIV-infected and uninfected mothers and HIV-exposed and unexposed infants at delivery.

6.2 Study cohort

There were 109 maternal-infant pairs enrolled in the study. Samples were available from 105 mothers (96% of the maternal sample, 47 HIV-infected and 58 HIV-uninfected) at delivery, and from 101 infants (93% of the infant sample; 47 HIV-exposed and 54 HIV-unexposed) at birth. Sample volumes were insufficient for 4 mothers and 8 infants. One infant (1%) was determined to be HIV-infected at 4 weeks of age and was referred for rapid initiation of anti-retroviral treatment (mother-infant pair subsequently excluded from analysis).

Of the 95 infants completing follow-up of the immunological study, samples were available from 94 infants (87% of infants; 38 HIV-exposed and 55 HIV-unexposed) at a mean postnatal age of 16.4 weeks (standard deviation, SD, 1.7). One late follow-up sample was excluded from analysis (collected at 28 weeks after birth). Thus, the final analysis was based on 104 maternal and 100 infant samples collected at birth and 93 infant samples collected at 16 weeks of age.

6.3 Infant specific antibody responses at birth

At birth, HIV-exposed infants had significantly lower specific antibody levels compared to unexposed infants to Hib (0.37mg/l, IQR 0.22 – 0.67, vs. 1.02mg/l, IQR 0.34 – 3.79; P< .001), pertussis (16.07FDA U/ml, IQR 8.87 – 30.43, vs. 36.11FDA U/ml, IQR 20.41 – 76.28; P< .001), pneumococcus (17.24mg/l, IQR 11.33 – 40.25, vs. 31.97mg/l, IQR 18.58 – 61.80; P= .015) and tetanus (0.08IU/ml, IQR 0.03 – 0.39, vs. 0.24 IU/ml, IQR 0.08 – 0.92; P= .006) Figure 40.

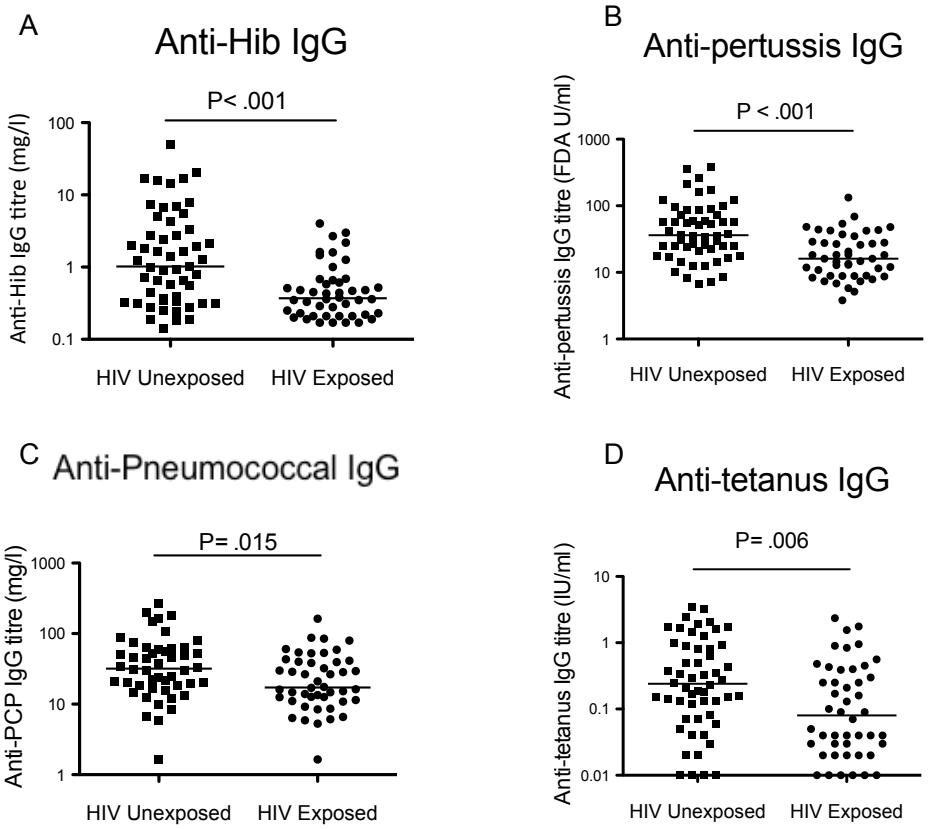


Figure 40. Specific antibody titres in HIV-exposed, uninfected and HIV-unexposed infants at birth. Specific antibodies to (A) Hib, (B) pertussis, (C) pneumococcal capsular polysaccharide (PCP) and (D) tetanus from serum samples collected within 24 hours of birth were non-preferentially analysed on available sample volume by commercially available ELISAs. Horizontal lines indicate median response. There were 53 samples available for HIV-unexposed infants for each assay and 46 samples from HIV-exposed infants for Pertussis and tetanus and 52 and 46 for Hib and PCP respectively.

These lower levels observed in HIV-exposed infants at birth corresponded with a lower proportion of HIV-exposed infants with levels considered to be protective against Hib (17% vs. 52%; P< .001), pertussis (24% vs. 57%; P= .001), tetanus (43% vs. 74%; P= .002) and hepatitis B (21% vs. 54%; P= .012).

In a multiple linear regression model for factors associated with magnitude of specific antibody response at birth, HIV exposure remained associated with reduced Hib titres (unstandardized regression coefficient [b] 0.49, Standard error [SE] b 0.12; P< .001), pertussis (b 0.38, SE b 0.12; P< .001), pneumococcus (b 0.24, SE b 0.10, P= .01) and tetanus (b 0.52, SE b .16; P= .002) levels (Appendix VI). There was no association with maternal age, gravidity, housing structure, infant gender or birth weight for Hib, pneumococcus and

tetanus levels but increased maternal age was associated with higher pertussis specific antibody titers (b 0.02, SE b 0.01; P= .03) [Appendix VI].

6.4 Maternal specific antibody responses

To investigate the mechanisms associated with infant response, specific maternal antibody levels were measured in parallel. HIV-infected mothers had lower specific antibody levels than uninfected mothers to Hib (0.67mg/l, IQR 0.16 – 1.54, vs. 1.34mg/l, IQR 0.15 – 4.82; p= .01) and pneumococcus (33.47mg/l, IQR 4.03 – 69.43, vs. 50.84mg/l, IQR 7.40 – 118.00; p= .03). No differences were observed for pertussis (22.07FDA U/ml, IQR 12.48 – 29.67, vs. 23.64FDAU/ml, IQR 12.87 -54.68; p= .26) or tetanus (0.09IU/ml, IQR 0.03 – 0.33, vs. 0.15IU/ml, IQR 0.06 – 0.67; p= .12) between HIV-infected and uninfected women, Figure 41.

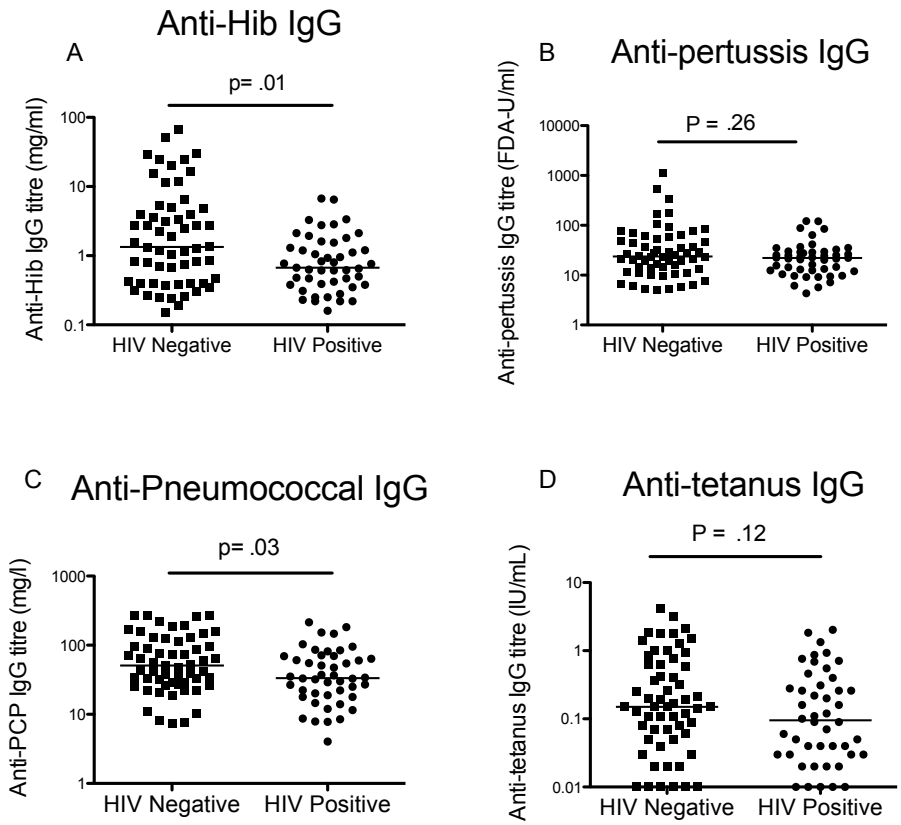


Figure 41. Specific antibody titres in HIV-infected and HIV-uninfected mothers at delivery.
Specific antibodies to (A) Hib, (B) pertussis, (C) pneumococcal capsular polysaccharide (PCP) and (D) tetanus from serum samples collected within 24 hours of delivery were analysed by commercially available ELISAs. Horizontal lines indicate median response. There were 58 samples from HIV-uninfected mothers for each assay and 47 samples available for HIV-infected mothers for pertussis and Hib assays and 46 samples for PCP and tetanus assays.

In a multiple regression model for factors associated with level of maternal specific antibody response, maternal HIV infection remained associated with low Hib and pneumococcal antibody levels; however, there was no significant association with maternal age, gravidity or type of dwelling for any of the specific antibody responses (Appendix VI).

HIV-infected mothers were less likely to have anti-Hib antibody levels considered to be protective (35% vs. 59%; P= .02). The proportion of mothers with protective antibody levels against pertussis (24% vs. 38%; P = .14), tetanus (47% vs. 64%; P = .11) or Hepatitis B (26% vs. 33%; P = .52) was similar in HIV-infected and uninfected mothers. The overall proportion of all mothers with protective antibody levels was low for pertussis (32%), tetanus (41%) and hepatitis B (30%).

In HIV-infected women, CD4 count was positively correlated with the level of antibody to pertussis (r=0.31; P= .04) pneumococcus (r=0.33; P= .03) and tetanus (r=0.37; P= .01), but not with Hib (r=-0.07; P= .63) [Appendix VI]. There was no correlation between maternal HIV-viral load and any specific antibody level (Appendix VI).

In HIV-infected women and their infants, maternal and infant specific antibody responses were correlated for Hib (r= 0.91; P< .001), pertussis (r=0.78; P< .001), pneumococcus (r= 0.86; P< .001) and tetanus (r=0.95; P< .001). In HIV-negative mothers, maternal and infant responses were also correlated for Hib (r=0.95; P< .001), pertussis (r=0.89; P< .001), pneumococcus (r=0.80; P< .001) and tetanus (r=0.93; P< .001).

Association of maternal HIV with placental transfer of specific antibody

The proportion of maternal specific antibody transferred across the placenta to infants was significantly reduced among HIV-infected women and their infants. Using infant:maternal antibody ratios as a proxy for placental transfer, HIV-infected mothers had significant reductions in placental transfer of 23% for Hib, 40% for pertussis, and 27% for tetanus specific antibodies compared to HIV-uninfected mothers; there was a trend toward a reduction in placental transfer of pneumococcal specific antibodies Table 21. Among HIV-infected women, there was no association between maternal CD4 or viral load and placental transfer in HIV-infected mothers (Appendix VI).

	Placental transfer [a]		Percent reduction [b]	P [c]
	HIV-infected mother – exposed uninfected infant pairs (medians and IQR)	HIV-uninfected mother – unexposed infant pairs (medians and IQR)		
Hib	0.57 (0.45 – 0.79)	0.74 (0.61 – 1.00)	23%	.002
Pertussis	0.91 (0.61-1.20)	1.51 (1.15 – 2.06)	40%	< .001
Pneumococcus	0.62 (0.41 – 0.77)	0.73 (0.53 – 0.94)	15%	.05
Tetanus	0.95 (0.60 – 1.12)	1.30 (1.03 – 1.86)	27%	< .001

Table 21. The influence of maternal HIV infection on placental antibody transfer.
[a] Placental transfer of antibody from mother to infant is expressed as a ratio of infant/maternal specific IgG concentration at birth. [b] Percent reduction in placental transfer between HIV-infected and HIV-uninfected mothers; calculated as ratio of the placental transfer from HIV-uninfected mothers to the placental transfer for HIV-uninfected mothers, subtracted from 100. [c] Mann-Whitney test.

6.5 Specific vaccine-induced antibody responses in infants at 16 weeks

In a stratified analysis of infants who had received 1, 2 or 3 doses of DTP-Hib vaccine (n=6, 22 and 65 respectively), there was no difference in antibody levels between infants who had received 1 or 2 doses (Appendix VI); these groups were therefore combined for further analysis. Similarly, data were combined for infants who had received 1 or 2 doses of pneumococcal capsular polysaccharide (n= 15 and 34 respectively). There was no statistical difference in the proportion of HIV-exposed and HIV-unexposed infants who received fewer than 3 doses of DTP-Hib vaccine (25% vs. 16%; P= .31) or fewer than 2 doses of pneumococcal capsular polysaccharide (20% vs. 49%; P=. 06) prior to the 16-week sampling.

Despite initially lower titres at birth, HIV-exposed uninfected infants mounted robust responses following vaccination. In the group that received all 3 scheduled doses of DTP-Hib vaccine, HIV-exposed infants had significantly higher responses to pertussis (270.1FDA U/ml, IQR 84.4 – 355.0, vs. 91.7FDA U/ml, IQR 27.9 – 168.4; P= .006) than unexposed infants, but had similar responses to Hib and tetanus. HIV-exposed infants also had higher levels of pneumococcal-specific antibody than HIV-unexposed infants (47.32 mg/l, IQR 32.56 – 77.80, vs. 14.77 mg/l, IQR 11.06 – 41.08; P= .001). Among infants who had received only 1 or 2 doses of DTP-Hib vaccine, responses were higher in HIV-exposed infants than unexposed infants to Hib (6.46mg/l, IQR 1.74 – 9.29, vs. 0.54mg/l, IQR 0.24 – 4.1; P = .02), pertussis (81.16 FDA U/ml, IQR 38.64 – 195.4, vs. 11.60 FDA U/ml, IQR 5.3 – 39.42; P< .001) and tetanus (1.86 IU/ml, IQR 0.51 – 2.21, vs. 0.50IU/ml, IQR 0.10 – 0.93; P= 0.01) (Figure 42).

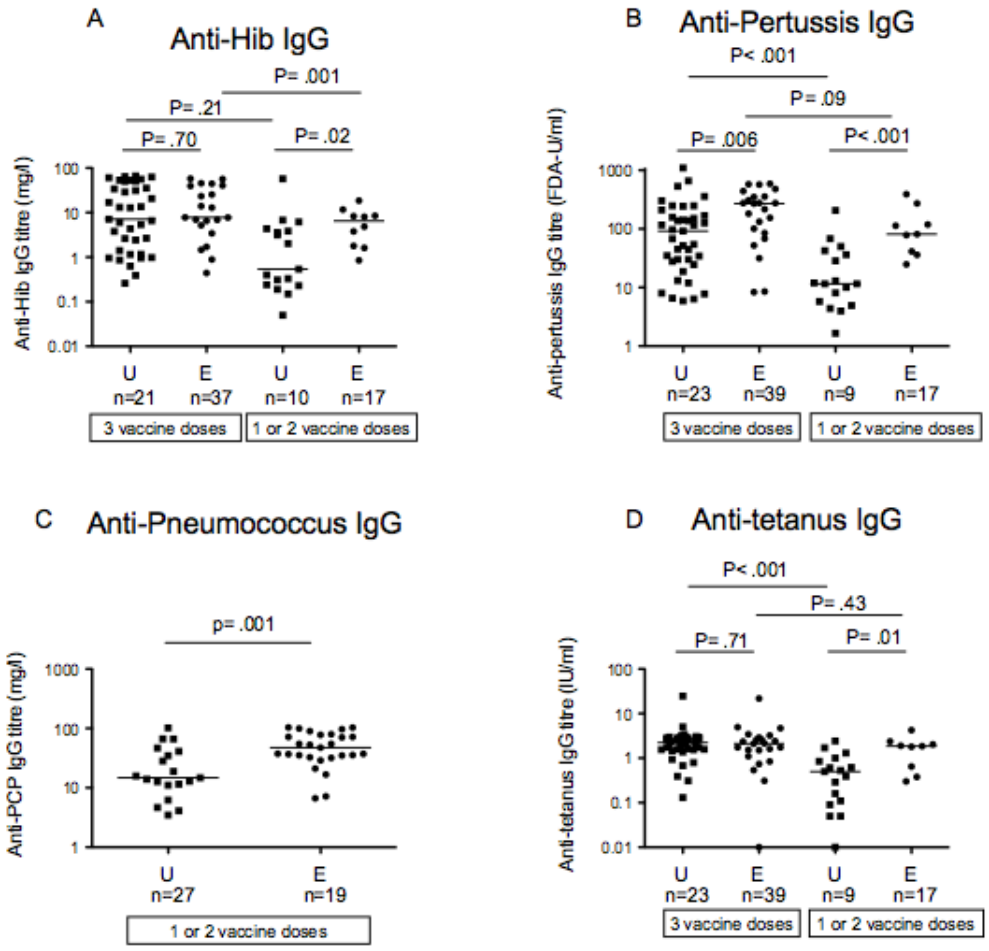


Figure 42. Specific antibody titres in HIV-unexposed infants (U) and HIV-exposed, uninfected (E) at 16 weeks of age.
Specific antibodies to (A) Hib, (B) pertussis, (C) pneumococcal capsular polysaccharide (PCP) and (D) tetanus were non-preferentially analysed on available sample volume. Horizontal lines indicate median response.

The fold-increase in antibody level before and after vaccination was significantly higher in HIV-exposed than in HIV-unexposed infants for Hib (21.15-fold increase, IQR 6.84 – 118.4, vs. 2.97-fold increase, IQR 0.71 – 16.69; P= .007), pertussis (9.51-fold increase, IQR 2.8 - 24.25, vs. 2.16-fold increase, 0.41 – 6.84; P= .002) and pneumococcus (2.06-fold-increase, IQR 0.96 – 5.70, vs. 0.31-fold increase, IQR 0.26- 1.04; P< 0.001). There was no difference in the fold-increase at pre- and post-vaccination between the 2 groups for tetanus-specific responses (14-fold increase, IQR 3.26 – 116.20, vs. 12-fold increase, IQR 2.81 – 36.35; P= .538). Figure 43 shows pre- and post-vaccination antibody levels for individual infants; infants with the lowest levels of anti-Hib, pertussis, pneumococcus and tetanus specific antibodies showed the greatest vaccine responses at 16 weeks. HIV-exposure was associated with a greater magnitude of change between birth and 16 weeks of age. The

decreased antibody levels observed in some infants between birth and 16 weeks occurred in different infants for different antibodies.

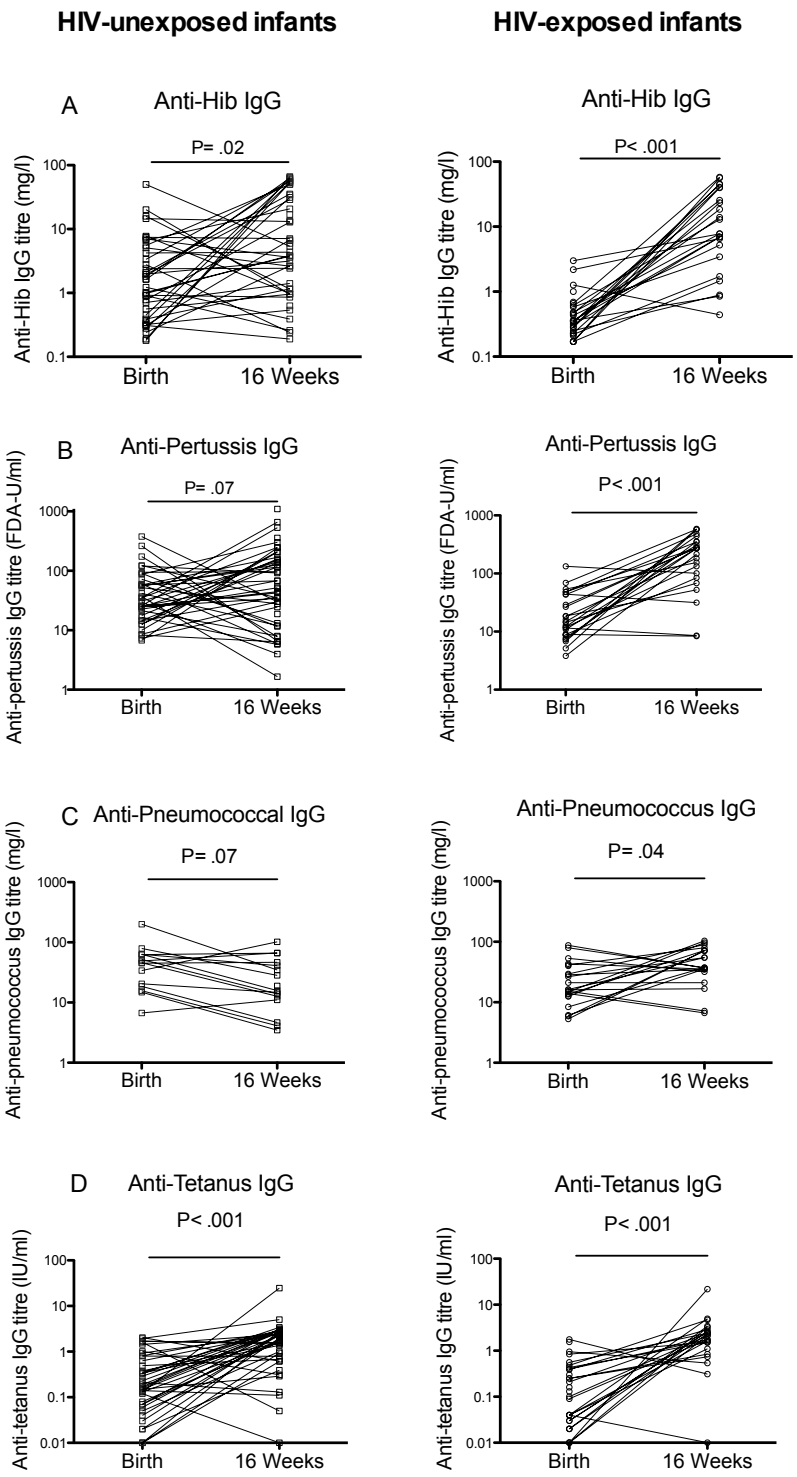


Figure 43. Change in specific antibody titres between birth and 16 weeks in HIV-exposed and unexposed infants.

Each infant is represented by a line that starts at the specific antibody level at birth and ends in the specific antibody level at 16 weeks. Each pair of graphs depicts specific antibodies to (A) Hib, (B) pertussis, (C) pneumococcal capsular protein and (D) tetanus. Results are shown for infants who received 3 doses of DTP-Hib or 2 doses of pneumococcal conjugate vaccine for whom paired samples were available.

6.6 Summary of key findings

At birth, HIV-exposed infants had lower specific antibody to Hib, pertussis, pneumococcus and tetanus compared to HIV-unexposed infants, this corresponded to a lower proportion of HIV-exposed infants with levels considered protective against Hib, pertussis, tetanus and hepatitis B. A significant proportion of unexposed infants also had low levels of antibody that may leave them vulnerable to infection with Hib and pertussis early in life.

Women infected with HIV had lower levels of specific antibody to Hib and pneumococcus compared to HIV-uninfected women, however similar levels of anti-pertussis and anti-tetanus antibody were observed. A lower proportion of HIV-infected women had levels of anti-Hib antibody considered to be protective.

There was a strong positive correlation between maternal and infant specific antibody levels.

There was significantly reduced trans-placental transfer of antibody from mother to infant in HIV-exposed infants compared to HIV-unexposed infants.

At 16 weeks of age, following vaccination at 6, 10 and 14 weeks of age, HIV-exposed infants had higher specific antibody responses to pertussis and pneumococcus, but similar levels of anti-Hib and anti-tetanus antibody compared to HIV-unexposed infants. Where fewer than the 3 scheduled doses of vaccine had been received, HIV-exposed infants had significantly higher antibody responses to Hib, pertussis and tetanus.

The fold increase in antibody from pre- to post-vaccination was significantly higher in HIV-exposed infants for Hib, pertussis and pneumococcus. The increase in response was similar for tetanus.

Discussion

Chapter 7: Discussion

Uninfected HIV-exposed infants have increased rates of morbidity and mortality compared to infants who have not been exposed to HIV *in utero*. There are numerous factors that may contribute to this, not least social considerations, which may add up to significant clinical disadvantages. A number of immunological changes have been observed in HIV-exposed infants, even in the absence of infection. Some of these changes persist into childhood, however how these might impact on clinical outcomes is not understood. Evaluation of vaccine responses represents a relatively simple way to probe the immune system for functional insights. How HIV-exposure affects the ability to respond to a selection of vaccines that activate cellular and humoral responses has not been fully examined and was therefore a main objective of this thesis.

HIV-exposed infants are more likely to be in contact with an adult who has TB due to the high prevalence of TB co-infection in HIV-positive hosts. Given the reduced CD4 counts observed in this population of infants, the ability to respond to *Mtb* might be compromised. The majority of HIV-exposed infants will receive BCG vaccination at or around birth. Even a small reduction in the protective efficacy of BCG in these infants would have large public health implications, given the large numbers of infants born to HIV-infected mothers, particularly in sub-Saharan Africa.

In utero sensitisation to other maternal infections can also prime the developing immune system. A proportion of HIV-exposed and unexposed infants display IFN-γ responses to mycobacterial antigens at birth, prior to exposure to *Mtb*, BCG vaccination or environmental mycobacteria. Whether *in utero* exposure to mycobacterial antigens might explain this has not been examined.

This mother-infant cohort study was set up to address the gap in knowledge regarding responses to BCG vaccination in HIV-exposed compared to HIV-unexposed infants born to mothers sensitised and not sensitised to *Mtb*. In addition to assessing T cell responses, this study investigated the impact of maternal HIV infection on humoral responses to other EPI vaccinations.

7.1 Clinical study

7.1.1 Clinical outcomes of HIV-exposed, uninfected infants.

We found that HIV-exposed, uninfected infants had increased rates of illness, in keeping with a number of other studies.[11,19,24,36,38] HIV-exposed, uninfected infants had significantly more attendances to primary health clinics and there was a trend towards increased admissions to hospital for infectious morbidity. A study enrolling over 14,000 infants, of which nearly 3,000 were uninfected HIV-exposed infants, in Zimbabwe found that up to the age of 2 years, HIV-exposed, uninfected infants were 1.2 times more likely to attend primary healthcare for illness.[24] We detected a similar increase in attendance at any primary health clinic (30% increased), however since our study cohort was significantly smaller this did not reach statistical significance. The rate of hospital admission for serious infectious morbidity was also higher amongst HIV-exposed, uninfected infants in our study. The Zimbabwe study also found that HIV-exposed, uninfected infants were hospitalised up to 50% more often in the neonatal period. Later in infancy rates of hospital admission were similar to HIV-unexposed infants. When only considering LRTI as the cause of admission, neonates were 2.7 times more likely to be admitted to hospital.[24] The trends observed in our study were in keeping with these results. Other smaller studies have not observed a difference.[16,43]

A number of factors may intersect to explain the reasons for increased morbidity and mortality in uninfected HIV-exposed infants and children, not least powerful socio-economic forces. In our study HIV-infected women were more likely to live in an informal housing structure with no household running water or toilet. Marinda found that lower household wealth was associated with an increased risk of death in HIV-exposed, uninfected infants.[10] We found that women living with HIV were older and more likely to have had more than one child compared to HIV-uninfected mothers. Other studies have consistently observed these differences. [10,45,62,203] The number of children in the household may impact household resources and also transmission of pathogens.

Another factor that has been considered to impact morbidity and mortality is replacement feeding of HIV-exposed, uninfected infants. In this study all HIV-exposed infants received exclusive replacement feeding, whereas all HIV-unexposed infants were breast-fed at birth and the majority were still receiving breast milk at 4 months of age.

In our study there was no difference in birth weight or subsequent growth of infants. Some studies have observed a difference in birth weight, whilst others have not found any difference.[10,44-46,204,205] However, subsequent growth has been consistently found to be similar in HIV-exposed, uninfected infants compared to unexposed infants.[43-46] In large studies the absolute difference in birth weight was minor. We deliberately excluded preterm infants and low birth weight infants (LBW, <2.5kg), therefore it is not altogether surprising that we failed to detect a significant difference.[10]On a population level, an increase in the number of LBW infants might have a significant impact since this is a risk factor for increased mortality rates.[61]

7.1.2 The effect of HIV infection on haematological parameters

HIV-infected mothers tended to have lower haemoglobin levels and had significantly reduced RBC counts compared to uninfected mothers. This is commonly observed in studies of HIV-infected individuals and is thought to be due to reduced erythropoiesis caused by inflammatory cytokines.[206,207] Maternal anaemia is associated with HIV progression and mortality.[206] We did not observe difference in haemoglobin at birth or 16 weeks amongst any groups of infants.

Our data show that HIV-infected women had lower WBC than uninfected mothers, however their infants also had similar WBC at birth compared to unexposed infants. Multiple haematological abnormalities have been observed in HIV-exposed, uninfected infants exposed to anti-retroviral drugs *in utero* or postpartum, particularly Zidovudine, in many studies and in diverse locations.[208] Anaemia is transient in most studies, however neutropenia and lymphopenia may be more prolonged. Anaemia is more pronounced in infants born to mothers receiving combination antiretroviral treatment.

7.1.3 Assessment of *Mtb* sensitization in pregnancy

Discordant results were common between TST and QFN tests, however, the agreement between tests was similar amongst HIV-infected (73%) and uninfected women (76%). Until recently there were no studies examining the performance of IGRAs in pregnancy, however very recently two studies have been published that show fair agreement between TST and IGRA in pregnancy, $\kappa = 0.26 – 0.45$, in line with the concordance found in our study, $\kappa =$

0.49.[209,210] To our knowledge, there are no published studies examining the concordance of IGRAs and TST in pregnant women living with HIV.

Although TST reactivity and interpretation are unaltered in pregnancy, both tests are well know to be affected by HIV infection, particularly with increasing immunosuppression.[211] There is little data to inform the use of IGRAs in pregnancy with one study concluding that the performance of IGRAs are comparable in pregnant and non-pregnant women.[209] Recent data suggests that pregnant women with latent TB are not more likely to progress to active disease than non-pregnant women, even in the presence of HIV infection.[212] However, active TB in pregnancy, in particular, extrapulmonary TB is associated with considerable maternal morbidity and mortality, intrauterine growth restriction, premature delivery and infant mortality.[213] Accurate identification of maternal *Mtb* sensitisation and appropriate use of isoniazid preventative therapy (IPT) to prevent progression to disease is therefore critical. IPT can be used safely in pregnancy and is effective in preventing active disease.[214] IGRAs may be a useful tool in identifying women at increased risk of active TB who would benefit from IPT. In one study, a positive IGRA was associated with a 4.5 fold increased risk of TB in the 2 years postpartum. For women with a CD4 count of less than 250 cells/µl, a positive IGRA was also associated with an increased risk of mortality and with infant TB and mortality.[215] In this study the indeterminate rate was significantly higher (15%) than the rate observed in our study (1%).

7.1.4 Clinical correlates of BCG vaccination

All infants had evidence of a BCG scar at 4 months of age, but HIV-exposed infants tended to have smaller scars. The actual difference was small and did not reach statistical significance, p= .05. In a study in The Gambia, Ota *et al* observed a tendency for HIV-exposed, uninfected children to fail to develop a BCG scar, however they examined the response at 18 months of age compared to just 4 months in our study.[216] Other studies have not found any difference in the proportion of HIV-exposed, uninfected infants who develop a scar at 3 - 4 months of age compared to unexposed infants.[73] Whilst a BCG scar is a indicator of vaccination, there is no clinical evidence that the size of the BCG scar is associated with protection against TB.[200,217] Scar size has however been correlated with immune responses to BCG. Djuardi and colleagues found that BCG scar size at 4 years of age was moderately correlated with Th2 responses to PPD at 5 months post-BCG vaccination. This response diminished with

increasing time post BCG vaccination.[85] Elliott also observed a moderate positive correlation between BCG scar size and IL-5 response to mycobacterial antigens.[218]

7.2 Cellular responses to BCG vaccination

7.2.1 Infants at birth

We observed that a small proportion of infants at birth had detectable proliferative responses to BCG antigens in both the $CD8^-$ (putative $CD4^+$) and $CD8^+$ compartments. A number of studies have detected BCG-induced IFN-γ in whole blood at birth.[73,84-87,133,219]. This is likely to be population dependent as no response was observed in newborns from Western Australia, USA or the UK.[81,87,108] Innate cells such as NK cells are likely to be the predominant source of IFN-γ at birth.[163] To our knowledge, our study is the first to detect T cell lymphoproliferation in response to BCG antigens at birth. We were able to demonstrate that exposure to HIV *in utero* did not explain this phenomenon.

There is compelling evidence that foetal HIV-exposure can 'prime' the developing immune system resulting in a more activated and mature immunophenotype in HIV-exposed, uninfected infants.[27,29-31,65,67-69] It has been postulated that HIV-exposed, uninfected infants may therefore have a higher immune 'set-point' at birth making them more responsive to unrelated antigens.[73] A previously unexplored hypothesis is that sensitisation to mycobacterial antigens *in utero* might also 'prime' the immune system such that infants have recall responses to mycobacterial antigens after birth. This has been demonstrated in the mouse model, but not explored in humans.[82] We demonstrated that maternal *Mtb* sensitisation does not result in altered proliferative responses to BCG antigens in HIV-exposed or HIV-unexposed infants at birth.

In order to analyse the *in vitro* immune response to mycobacterial antigens in more depth, we examined the intracellular expression of cytokines in $CD8^-$ and $CD8^+$ T cells. We found that the total expression of IFN-γ, TNF-α, IL-2 or IL-17 in proliferating T lymphocytes was similar amongst groups of infants at birth. We then examined T cells that produced single cytokines or combinations of cytokines, revealing interesting differences between the groups of infants. At birth, HIV-exposed, uninfected infants had higher frequencies of IFN-γ^+ TNF-α^+ $CD8^-$ T cells. Both $CD8^-$ and $CD8^+$ T cells producing TNF-α alone were increased amongst HIV-exposed, uninfected infants compared to unexposed infants. In addition, IL-2 single-positive $CD8^+$ T cells (interpreted as central memory cells) were increased compared

to unexposed infants.[220] When comparing the four groups of infants, infants born to HIV-infected, QFN-positive mothers had the highest frequencies of BCG-specific TNF-α single-positive $CD8^+$ T cells.

We went on to measure multiple cytokines and chemokines using Luminex to substantiate and extend our findings from the flow cytometry assay. In keeping with the flow cytometry data, we found increased levels of TNF-α in cell cultures stimulated with BCG antigens from HIV-exposed infants compared to HIV-unexposed infants at birth. The difference in TNF-α levels between HIV-exposed and unexposed infants was most significant in those born to *Mtb* sensitised mothers, suggesting that both maternal HIV and *Mtb* sensitisation were influential. Infants born to HIV-infected, *Mtb* sensitised mothers had the highest levels of TNF-α. TNF-α is a pro-inflammatory cytokine that is released from macrophages and lymphocytes in addition to multiple other cell types. The increase in TNF-α may be a reflection of increased immune activation in HIV-exposed, uninfected infants.

IFN-γ secretion in response to BCG antigens was lowest amongst HIV-exposed newborns of *Mtb* unsensitised mothers. HIV-exposed infants born to *Mtb* sensitised mothers had levels similar to HIV-unexposed infants. It seems therefore that exposure to HIV *in utero* is associated with impaired infant responses to mycobacterial antigens at birth, however in the presence of maternal *Mtb* sensitisation, infant responses are 'primed' such that their response to mycobacterial antigens are similar to infants not exposed to HIV *in utero*. It is possible that maternal HIV infection and *Mtb* sensitisation have a greater influence on immune cells ascribed to innate immunity, such as NK cells compared to T lymphocytes. HIV-exposed infants born to QFN-positive mothers did have marginally higher $CD8^-Ki67^+IFN\text{-}y^+$ compared to exposed infants born to QFN-negative mothers, however this did not reach statistical significance. It strengthens this argument that Watkins defined the cells producing the majority of IFN-γ at birth in response to BCG as NK cells rather than T cells.[163]

HIV-exposed infants born to *Mtb* sensitised mothers had higher GM-CSF responses to BCG antigens compared to HIV-unexposed infants born to *Mtb* sensitised mothers. GM-CSF has a myriad of immunomodulatory properties[221] with one of it functions inducing monocytes to produce cytokines, including IFN-γ, that increase the function of NK cells. This observation might form part of the mechanism by which IFN-γ levels are increased in HIV-exposed infants born to *Mtb* sensitised mothers. Increased numbers of NK cells have been observed

in HIV-exposed, uninfected infants and it is postulated that these might play a role in protection against HIV infection.[65,222] Our study did not investigate NK cells; hence we cannot draw any conclusion for the function of NK cells from our own dataset.

The mechanism by which maternal HIV infection and *Mtb* exposure influences infant responses to BCG antigens is not clear. It is possible that antigen crosses the placenta in the context of HIV infection and this primes the infant response to other antigens, indeed this is thought to be the mechanism of HIV-specific responses observed in HIV-exposed, uninfected infants. In the context of *Mtb* infection rather than TB disease, this possibility seems less likely. An alternative is that maternal cells cross the placenta to the infant. Although it is well documented that this does occur with very small numbers of cells, this also seems unlikely to be the mechanism. A further possibility is that maternal cytokine crosses the placenta and influences the immunological milieu of the developing foetus. This may be the reason for the associations between maternal and infant responses to BCG observed in this study.

7.2.2 Infants at 16 weeks

We found no difference in the proliferative response to BCG vaccination at 16 weeks of age in HIV-exposed, uninfected infants compared to unexposed infants. Akin with the approach we took at birth, we also investigated whether maternal *Mtb* sensitisation might have any bearing on responses to vaccination, a previously unexplored question. Again we found no difference in T cell proliferation to mycobacterial antigens between infants. There was no difference in total expression of intracellular cytokines between any of the groups of infants. We characterised the polyfunctional response to BCG antigens and found that the predominant response was $CD8^-$ IFN-γ^+TNF-$\alpha^+$and IFN-γ^+IL-2^+TNF-α^+ T cells. No differences were seen in any subset of $CD8^-$ cells.

We did observe differences in the frequency of BCG-specific IL-2 single-positive $CD8^+$ T cells; these cells formed a small proportion of the overall T cell response. HIV-exposed, uninfected infants had significantly higher frequencies of these cells compared to HIV-unexposed infants. Infants born to *Mtb* sensitised mothers also had significantly higher frequencies of these cells, irrespective of maternal HIV infection. Whilst this small subset of $CD8^+$ T cells may not have any bearing on the protection afforded by the BCG vaccine, these cells may reflect antigenic “memory” in infants born to mothers with HIV or TB infection.

To complement the assessment of the ability of T cells to proliferate in response to mycobacterial antigens in HIV-exposed and unexposed infants, we chose to measure levels of cytokines and chemokines secreted into cell culture supernatants after 24 hours of incubation with BCG antigens. At this early time point, this is likely to primarily provide a reflection of the function of innate cells.[85] Measurement of growth factors assesses the ability to induce proliferation of other cells whilst measurement of chemoattractants assesses the ability to organise the immune response.[202] Our analysis of the proliferative response to BCG is entirely corroborated by the results of the multiplex ELISA assay. We found no differences in any secreted cytokine or chemokine response to BCG antigens amongst 16-week old infants; maternal HIV infection or *Mtb* sensitisation did not significantly influence any of the cytokine or chemokine responses.

Our finding of comparable immunogenicity of the BCG vaccine in HIV-exposed, uninfected infants by 16 weeks is in keeping with another study performed in South Africa. The authors observed no difference in total $CD4^+$ cytokine response, total IFN-γ, IL-2 or TNF-α $CD4^+$ response, or in the IFN-γ^+IL-2^+TNF-α^+ $CD4^+$ response to BCG between HIV-exposed and unexposed infants.[127] The similarities between the two studies were found despite differing methodologies; they employed a short-term stimulation assay rather than the 6-day proliferation assay used in this study. The short-term stimulation assay gives a reflection of the effector response, whereas we choose to use a longer-term assay to give an indication of central memory responses. This is an important question given that HIV-exposed, uninfected infants have increased proportion of central memory cells and a decreased proportion of naïve cells.

A very large Ugandan study also found no difference in secreted cytokine levels in response to mycobacterial antigens amongst BCG-vaccinated HIV-uninfected infants, irrespective of maternal HIV status.[88]

Another smaller study found a tendency towards a reduced proliferative capacity in response to PPD (no measure of statistical significance reported), but similar responses to BCG in HIV-exposed, uninfected infants compared to HIV-unexposed infants.[29] A further study reported a higher proportion of proliferating $CD4^+$ T cells and lower proportion of $\gamma\delta^+$ cells in HIV-exposed infants aged 6 months to 18 months compared to unexposed infants[141]. The method used to assess proliferation in these studies may be less robust and specific;

they examined BCG-specific proliferation using forward scatter-area versus side scatter-area to gate on resting and blast lymphocytes rather than gating on cells expressing a proliferation marker such as Ki67, which was the approach we took.

An increased antigen-specific response is generally regarded as more optimal, therefore the strikingly similar quantitative and qualitative responses observed between HIV-exposed and unexposed infants suggests BCG is at least equally immunogenic in HIV-exposed infants and may confer similar levels of protection. Our data supports the WHO recommendation that HIV-exposed, uninfected infants should receive the BCG vaccine.[130] We have shown that, at least in a research setting, that it is feasible to delay vaccination until the HIV infection status of the infant is established to avoid administering the BCG vaccine to HIV-infected infants.

7.2.3 Change in immune response to mycobacterial antigens from pre- to post-BCG vaccination.

A significant strength of our study was the measurement of paired responses to mycobacterial antigen pre- and post-vaccination, since this enabled us to examine the vaccine-attributable response.

BCG vaccination was associated with a substantial increase in BCG-specific T cell proliferation. Proliferating $CD8^-$ and $CD8^+$ T cells expressing IFN-γ, TNF-α, IL-2 and IL-17 were significantly upregulated in infants at 16 weeks compared to newborn infants. This verifies that this assay can show vaccine "take". It can be assumed that the response to mycobacterial antigens detected in infants following BCG vaccination is antigen-specific and is not a result of increasing maturity of the immune system or non-specific T cell activation. This assumption is based on studies showing that the immune response to mycobacterial antigens is significantly higher in vaccinated compared to age-matched unvaccinated infants.[133,184]

We examined the change in immune response pre- and post- vaccination to determine if there was a difference in the increase in HIV-exposed, uninfected infants compared to HIV-unexposed infants. Maternal HIV and *Mtb* sensitisation had no effect on the expansion of specific T cells in response to BCG vaccination; the increase in response from pre- to post-vaccination was the same in all infants.

This is in contrast to a study by Van Rie and colleagues who found that the pattern of change in IFN-γ response to BCG vaccination was altered in some HIV-exposed, uninfected infants.[73] A proportion of HIV-exposed, uninfected infants in their study failed to show an increased IFN-γ response to mycobacterial antigens between birth and 6 weeks post BCG vaccination. The majority of infants did however show an appropriate increase of the IFN-γ response to BCG vaccination, in line with our own data.

We were able to measure a large number of cytokines and chemokines released in response to mycobacterial antigens before and after BCG vaccination. We found an increased expression of IFN-γ and IP-10 in response to BCG antigens in vaccinated 16 week old infants compared to unvaccinated newborn infants. Whilst a large number of cytokines and chemokines are released in response to BCG antigens at 16 weeks of age, the response is downregulated compared to the response to the same antigens pre-vaccination. For example, IL-10 and TNF- α were induced by BCG antigens in 16 week old infants, however the response was lower than that at birth, pre-vaccination.

This is in keeping with the results of a study carried out in South Africa examining the influence of BCG vaccine strain, route and method of administration and age of vaccination in immune responses.[133] The authors observed induction of IFN-γ and IL-10 by BCG antigens, however IL-10 responses were higher in cord blood compared to BCG-vaccinated and unvaccinated infants at 10 weeks of age. Conversely, IFN-γ levels were similar in cord blood and unvaccinated infants at 10 weeks of age.

To our knowledge, the only other study using a broad array of cytokines, chemokines and growth factors following BCG vaccination was a study by Lalor and colleagues. They showed that BCG vaccination induces pro-inflammatory cytokines (IFN-γ, TNF-α, IL-2, IL-1 α and IL-6), anti-inflammatory (IL-4, IL-5 and IL-13), Th17 (IL-17), regulatory (IL-10), chemokines (IL-8, IP-10 and MIP-1 α) and growth factors (GM-CSF and G-CSF).[86] Our study differed from this study in a number of ways; our study was able to use paired samples from a much larger cohort of infants pre- and post-vaccination, whereas in the study by Lalor *et al* only 19 vaccinated infants and 9 unvaccinated infants were included. We were also able to examine maternal responses and conduct extensive phenotyping of the cell populations. Our study was conducted in a setting with a high burden of TB disease despite universal BCG vaccine at birth compared to Lalor's study, which was conducted in a setting with a low burden of TB

disease where selective BCG vaccination of infants is practised. Whole blood was stimulated for 6 days with PPD in Lalor's study, whereas we used the BCG vaccine itself as the antigen and collected cell culture supernatants after 24 hours of culture. We chose to analyse supernatants collected after 24 hours of culture with BCG antigens as this may favour assessment of the innate response rather than the adaptive response, thus complementing the longer-term culture used for assessment of proliferative responses using flow cytometry.

7.2.4 Mothers

A further unique aspect of our study was the inclusion of mothers and infant pairs and to correlate their responses.

Mtb sensitisation amongst HIV-infected mothers, but not amongst HIV-uninfected mothers, significantly affected the *in vitro* response to BCG antigens. HIV-infected mothers who tested QFN negative had lower frequencies of BCG-induced $CD8^-$ and $CD8^+$ T cell proliferative responses compared to those tested QFN positive. Additionally, HIV-infected women without evidence of *Mtb* sensitisation had significantly lower frequencies of $CD8^-$ T cells expressing IFN-γ, TNF-α or IL-2 and $CD8^+$ T cells expressing TNF-α. Proliferative responses amongst HIV-infected, QFN positive mothers were similar to those of HIV-uninfected women. No differences were observed amongst HIV-uninfected women.

Central memory T cell responses to childhood vaccines are lost early in the course of HIV infection and remain severely impaired, even with successful control of HIV and $CD4^+$ T cell recovery during treatment.[223,224] By contrast, memory responses are detectable for many years in HIV-uninfected individuals, even in the absence of ongoing antigen-exposure.[140,224]

A positive QFN test represents a lasting immunological response to mycobacterial antigens and as such is an indirect measure of previous exposure of the immune system and does not necessarily equate to "latent infection" with *Mtb*.[225] The term "latent infection" may represent a spectrum ranging from effective elimination of infecting bacilli to persistent non-replicating bacilli or replicating bacilli that are maintained at a subclinical level by an effective immune response.[226] Persistence of live mycobacteria must occur in a proportion of individuals since progression from subclinical infection to clinical disease occurs. Individuals infected with HIV have a substantially increased risk of progression to clinical disease, as the immune system incompletely controls viable mycobacteria. This persistence

of mycobacteria may cause constant re-stimulation of the pool of memory cells. If cross-priming of memory T cells can occur by related mycobacterial antigens, this constant reminder to the immune system may mean that BCG-induced T cells are boosted. Thus, we detected an increased pool of BCG-induced proliferating cells secreting type 1 cytokines (putative central memory cells) in HIV-infected women with positive QFN tests. In women without HIV-infection the pool of memory cells is maintained and immune exposure to *Mtb* did not increase this pool further. It is possible that active *Mtb* may persist at a low level in HIV-infected women or that an increased burden of *Mtb* antigen is present, leading to boosting of the response to other mycobacterial antigens such as BCG.

Weir and colleagues have clearly shown that cross-reactivity between species of mycobacteria can occur.[107] BCG vaccinated school children in the UK showed a marked increase in IFN-γ response following BCG vaccination to a number of mycobacterial species, the size of the change in response was related to the genetic similarity to *M.bovis*.

In mothers, HIV infection and TB infection alone as well co-infection with HIV and TB had a significant effect on multiple T cell subsets. In addition to measuring the ability of T cells to proliferate in response BCG antigens, we also sought to measure the quality of the response as an additional reflection of the functional potential of T cells. We used multi-parameter flow cytometry to measure the ability of T cells to express more than one cytokine simultaneously as increased functionality is thought to reflect increased quality of response.[202] We found that amongst women with HIV infection *Mtb* sensitisation had a significant effect on the ability of T cells to produce multiple cytokines, however amongst HIV-uninfected women, this effect was less clear. HIV-infected women with positive QFN tests had significantly higher frequencies of BCG-specific IFN-γ^+IL-2^+TNF-α^+IL-17^+, IFN-γ^+IL-2^+TNF-α^+, IFN-γ^+TNF-α^+ $CD8^-$ $Ki67^+$ T cells. Within the $CD8^+$ compartment differences in polyfunctional cells expressing IFN-γ, IL-2 and TNF-α were also observed. Viral infections, such as CMV, that persist at low levels maintain multifunctional cells, however infections such as HIV with high viral replication, antigen load and high persistence are associated with a reduction in capacity of T cells to produce more than one cytokine simultaneously.[202] We propose that maternal HIV infection reduced the ability of T cells to produce multiple cytokines simultaneously, however in the context of *Mtb* infection, antigen persistence at a low level maintained the ability to produce polyfunctional cells.

Analysis of secreted cytokine and chemokine levels in response to BCG revealed that HIV-infected mothers had lower IFN-γ, IL-1Ra, MDC and IL-10 levels compared to HIV-uninfected mothers. This confirms the results of the multi-parameter flow cytometry assay, that HIV-infected women are less able to mount a pro-inflammatory (IFN-γ) response to BCG antigens. It may follow therefore that a lower regulatory or suppressor response (IL-10, IL-1ra and MDC) is required to counter-balance the reduced inflammatory response.

IL-1Ra is an antagonist of the pro-inflammatory cytokine IL-1β and its action is mediated by binding to the IL-1 receptor without transducing any signal. Higher serum levels of IL-1Ra have previously been noted in serum of HIV-infected individuals compared to uninfected controls and the level is negatively correlated with markers of immune activation and HIV disease progression.[227] MDC plays a role in the generation and amplification of type II responses and it may have anti-HIV activity.[228]

7.2.5 Association between maternal and infant responses

A number of associations between maternal and infant responses to BCG antigens were observed, suggesting that *in utero* antigenic exposure or the immunological milieu may influence post-natal responses.

In the presence of maternal *Mtb* sensitisation, there was an association between the frequency of maternal and newborn infant BCG-specific proliferating $CD8^-$ T cell frequencies. This would indicate that maternal *Mtb* sensitisation might have some effect on proliferative responses at birth; however, there was no statistically significant difference in the overall proliferative response between infants born to QFN positive or negative mothers. Specific differences in T cells producing cytokines were however observed. In particular, we found that infants who were born to *Mtb* sensitised mothers who were also exposed to HIV *in utero* had the highest frequencies of BCG-specific TNF-α single-positive $CD8^+$ T cells. This is in keeping with the positive correlation between the frequency of BCG-specific $Ki67^+CD8^-$ TNF-α^+ T cells in infants at birth and the frequency of these cells in mother.

At birth, the frequency of proliferating BCG-specific $Ki67^+CD8^-$ and $CD8^+$ IL-2^+ T cells were associated with the frequency of these cells in the mother. This correlation was not observed in the presence of maternal HIV infection. At 16 weeks, this correlation persisted in the $CD8^+$ compartment. This suggests that antigen-specific memory may be transferred

from mother to child. In the context of maternal HIV infection memory responses are severely impaired, therefore few associations are seen between the mother and infant. The exception to this is the association between $CD8^+Ki67^+IL\text{-}2^+$ T cells in HIV-infected mothers who are also *Mtb* sensitised and their infants.

When examining correlations between levels of secreted cytokines between mothers and infants, there was a strong association between maternal and newborn infant TNF-α levels, IL-1β, IL-6 and IL-10 in response to BCG antigens. Responses were background subtracted suggesting that these are BCG-antigen specific, however an association between maternal and infant TNF-α and IL-1β was also seen in response to tetanus antigens. It is possible that the immunological milieu *in utero* has an impact on the development of the foetal immune system. This is likely to be transient as there were very few associations that persisted at by 4 months of age. The mechanism of association between the maternal immune response and infant response has not be elucidated in this study, however, it is possible that cytokines are transferred across the placenta which might influence infant responses.

7.2.6 Differences in response to BCG antigens between mothers and infants.

We observed a number of differences in the immune response to BCG antigens in unvaccinated newborn infants compared to adults. Pro-inflammatory cytokines (TNF-α, IL-12p40 and IL-1β) were elevated in infants compared to adults and immunomodulatory molecules such as IL-1Ra and IL-10 were increased. Some chemokines (IL-8, MDC and MIP-1β) were also increased. MDC is involved in amplification of type 2 responses.[228] It follows that higher MDC responses were observed at birth compared to maternal responses since infant responses are weighted towards type 2 responses at birth. MIP-1β is a potent chemoattractant for regulatory T cells, in line with the increased IL-10 responses observed.[229] IL-8 is produced from macrophages upon processing of antigen to attract neutrophils to the site and as such it is a mediator of the inflammatory response.

IFN-γ responses were lower in infants than in mothers in response to BCG antigens. This is not altogether surprising given that infants had not yet received the BCG vaccine. Moreover, the published literature has shown that $CD4^+$ cells, but not $CD8^+$ cells, from newborn infants produce less IFN-γ due to hypermethylation of the IFN-γ promoter.[120] It was slightly surprising that the IL-12p40 response to BCG antigens was increased in newborn infants compared to mothers. However, others have found that neonatal peripheral blood mononuclear cells produce equivalent amounts of IL-12p40 in response to microbial

stimulation, but significantly reduced bioactive IL-12p70, which is implicated in mycobacterial defences.[230] The inability to produce significant amounts of IL-12p70 can be overcome by addition of GM-CSF.[230] Infant cells have an increased ability to produce IL-10, and this may be partly responsible for the lower Th1 responses commonly observed.[230]

7.3 Specific antibody responses

To our knowledge, this study is the most comprehensive work to date evaluating the association of maternal HIV infection on maternal specific antibody levels and infant antibody responses to routine EPI vaccines. The key findings are that HIV-exposed infants have lower specific antibody levels at birth than their non-HIV exposed peers. Similarly, a smaller proportion of HIV-exposed infants have levels deemed to be protective. This study also highlights low levels of specific antibody in HIV-uninfected women with the consequence that half of their infants may not be sufficiently protected against Hib and pertussis early in life.

The low level of specific antibody observed amongst HIV-exposed uninfected infants is due to a combination of factors: lower antibody titres to Hib and pneumococcus in HIV-infected pregnant women, and reduced transplacental transfer of Hib, pertussis, pneumococcal and tetanus specific antibody. This study was unable to directly address the question of whether the lower antibody amongst HIV-exposed infants was specific to antibody subclasses. However, different subclasses of IgG cross the placenta with different efficiency. IgG1 is preferentially transferred, followed by IgG4, IgG3 and then IgG2.177 Vaccines, such as tetanus, containing protein antigens elicit a predominantly IgG1 and IgG3 response and therefore vaccine-specific IgG is transferred with greater efficiency than specific IgG to polysaccharide vaccine antigens, such as Hib, which elicit a predominantly IgG2 response. This might explain the higher proportion of infants who achieved a protective level of antibody against tetanus compared to the lower proportion of infants achieving a protective level of antibody against Hib.

Inducing sufficient protection in early infancy is important. Infants less than 3 months of age who acquire pertussis infection are at increased risk of serious morbidity and death than older infants.[231] This study re-emphasizes that infants are incompletely protected by current vaccination schedules, HIV-exposed, uninfected infants are especially vulnerable, however HIV-unexposed are also at risk.

These findings are consistent with two studies in HIV-infected women from Kenya indicating that maternal HIV is associated with lower tetanus and measles specific antibody in cord blood and also with reduced placental antibody transfer.[232,233] Maternal tetanus specific antibody levels are lower amongst HIV-infected women in some studies; inconsistencies observed may be due to differences in vaccination practice during pregnancy.[232,233]

Whilst it is known that measles, Hib and pneumococcal vaccine responses are reduced in children infected with HIV, [179,234-237] there is a paucity of studies investigating the influence of infant HIV-exposure (in the absence of infection) on responses to vaccines. We observed higher level of antibody in HIV-exposed infants to pertussis and pneumococcus compared to HIV-unexposed infants following completion of the immunization schedule. This can be explained by the lower maternally derived antibody levels at birth. Conversely, higher levels of maternal antibody amongst HIV-unexposed infants at birth corresponded with lower responses post-vaccination. Other studies have also reported that maternal antibodies can inhibit infant response to measles, tetanus, whole cell pertussis and Hib vaccines; this effect varies considerably between different vaccines and studies.[182,238,239] The mechanisms through which maternal antibody inhibit infant responses to vaccination are not fully understood. However, a plausible explanation is that maternal antibodies mask or hide vaccine antigenic epitopes, preventing recognition and binding by infant B cells; a key determinant of infant responses appears to be the maternal antibody-to-vaccine antigen ratio.[240] The inhibition of maternal antibody can potentially be overcome by increasing the vaccine dose.[240]

HIV-exposed infants who had missed doses of vaccine prior to sampling at 16 weeks had higher antibody responses than HIV-unexposed infants to Hib and tetanus, as well as pertussis and pneumococcus. An explanation for this observation is that higher maternal antibodies, observed amongst HIV-unexposed infants, may influence the response to the first dose of vaccine, but not to subsequent doses due to the longer lag time and decline in maternal antibody titres. A study in Finland reported a similar effect; infants with high levels of maternally derived antibody had lower anti-Hib antibody after the first dose of Hib vaccination, but not after the second dose.[241]

Another other possible explanation for the higher antibody levels seen amongst HIV-exposed infants is that a more ‘active’ or ‘mature’ immune system in HIV-exposed infants

accounts for increased responses to vaccination.[27, 31, 65, 68] However, reduced inhibition of maternal antibody seems a more biologically plausible explanation.

This study results supports the evaluation of maternal and neonatal immunization strategies to augment specific antibody responses and potentially prevent infections in infants in early life, particularly in HIV-exposed uninfected infants. Maternal immunization is a proven strategy for the prevention of neonatal tetanus and influenza in infancy. Two or more doses of tetanus toxoid during pregnancy can reduce neonatal tetanus mortality by 94%.[242] Inactivated influenza vaccine delivered during pregnancy can reduce influenza illness by 63% and all respiratory illness by one third. [243] Evaluation of pneumococcal or pertussis vaccination strategies during pregnancy in settings with high prevalence of HIV is merited with potential to benefit both the mother and child. [177,244-247] Impairment of infant responses to vaccination as a result of increased maternal antibody would have to be carefully evaluated for individual vaccines; however, inhibition may be overcome with increasing vaccine dose. [240] Safety of maternal immunization is a significant concern, however, there is no evidence that maternal vaccination is associated with increased risk to the developing foetus.[248] Poor uptake of seasonal influenza vaccination amongst pregnant women in the UK illustrates the challenge of implementation of maternal vaccination programmes.[249] Barriers to implementation include incorrect advice by healthcare workers and failure to offer the vaccine and anxiety regarding safety amongst pregnant mothers.[250]

An alternative and feasible strategy is neonatal vaccination. Oral polio, hepatitis B and BCG are currently administered at birth as part of the WHO Expanded Programme on Immunisation (EPI). Vaccine coverage is high for these vaccines since many infants have contact with the healthcare system at birth. Neonatal pertussis vaccination has been shown to be safe and immunogenic, however it potentially interferes with vaccine responses to other EPI vaccines.[251] A small number of studies have also administered Hib and pneumococcal conjugate vaccine to neonates and have shown that successful priming can occur.[251]

Further evaluation of both maternal and neonatal vaccination strategies is therefore important, as each has merits and challenges.

7.4 Limitations of the study

The size of our cohort of mother-infant pairs was moderate and was set up to principally assess immunological rather than clinical outcomes. We were able to identify a trend towards increased morbidity amongst HIV-exposed, uninfected infants in keeping with the published literature from larger studies addressing clinical endpoints. The study size however, precluded us from fully assessing clinical outcomes, which was not the focus of the study.

The study was conducted in an informal peri-urban settlement. It was an ideal environment to address our study questions since there is a high prevalence of HIV infection amongst women attending maternity services coupled with a well-established PMTCT programme. The study setting does not necessarily reflect provision of care in many other high HIV burden countries of the world. One unique aspect of the study setting was the provision of free replacement feeding for HIV-exposed infants whose mothers chose exclusive replacement feeding. In many other contexts breast-feeding is the most commonly practised and most appropriate feeding method for HIV-exposed infants. Since all HIV-exposed infants received replacement feeds, we were unable to assess the effect of breast milk on immune responses.

In this study peripheral blood was collected from newborn infants and a repeat sample was collected 10 weeks after the BCG vaccine was administered. This limited the volume of blood available and therefore restricted the number of assays that could be conducted. Peripheral blood sampling was preferred over cord blood sampling in this study since it would not have been feasible to collect cord blood samples in this setting for a number of reasons. Firstly, we demonstrated that an antenatal recruitment strategy was not efficient in this mobile population, we would therefore needed to consent mothers in labour, which is not a suitable setting for truly informed consent. Secondly it was not possible to be present at the delivery of all the women from a logistics or safety point of view in a township setting, since many would have delivered out of routine working hours. All assays using whole blood required to be set up in the laboratory within a few hours of obtaining the samples.

The limited blood volumes did not allow for assessment of the memory phenotypes of proliferating cells in this study. However, it is likely that the T cells proliferating in response to antigen were central memory cells given that central memory cells show vigorous

proliferation in response to antigen, whereas effector memory cells have reduced expansion potential.[220]

Resource constraints coupled with limited blood volumes precluded the ability to use a short-term stimulation assay to complement the longer-term lymphoproliferation assay. We chose the longer-term assay over the shorter-term assay to allow a better assessment of the central memory response, since this is thought to be critical for long-term protection induced by vaccines.[252] During the 6-day incubation period, cells divide in response to antigen. This longer incubation time may therefore increase the sensitivity of the assay as cells that are "resting" or require more than a short period of stimulation to divide, can be also detected. A shorter-term assay is complementary since it allows the direct *ex vivo* quantification of antigen-induced T cells that produce cytokines and may reflect an effector memory response. However, it is more sensitive to processing delays.[252] Given that a single investigator was performing the clinical and laboratory aspects of the study, it would not have been feasible to incubate the blood within 2 hours of sample collection. In addition, Mansoor *et al* have recently used a short-term assay to compare the BCG-induced T cell response in infected and uninfected HIV-exposed infants to control infants without demonstrating any effect of HIV-exposure in uninfected infants on responses to BCG vaccination.[127] Studies have shown that results from the short-term and longer-term assays do not correlate and confirm that they assess different aspects of the immune response.[102,253]

Given unlimited resources it would have been ideal to assess the innate immune response to mycobacterial antigens in mother-infant pairs. The measurement of cytokines and chemokines in day 1 supernatants may give some insight into early innate immune response, however to further understand the dynamic at a cellular level, it would be optimal to identify which cells are producing the response.

On day 6 of incubation, PMA and ionomycin are added to assess the potential of the expanded cells to produce cytokine. This gave a measure of cell function, however it meant that it was not possible to use a CD4 marker to directly identify $CD4^+$ T cells, rather this population of cells were identified by the lack of expression of CD8. It is probable therefore that cells which were $CD8^-$ and $CD4^-$ were included in the population of putative $CD4^+$ cells,

this population would include γδ cells. These cells are likely to represent a small proportion of CD8- cells after 6 days of culture.

A viability marker was used to discriminate between live and dead cells. This was a major advantage in this study, since a number of newborn infant samples displayed high proportions of dead cells following prolonged culture. There is a high turn-over of neonatal T cells which show an increased susceptibility to apoptosis,[120] explaining the need to exclude a much higher number of newborn infant samples compared to older infant or adult samples. The exclusion of samples with high proportions of dead cells from the analysis may have meant that we underestimated the effect of maternal HIV or *Mtb* sensitisation on newborn infant responses to BCG. However, this approach should have avoided the invalid identification of differences between groups of infants since samples with high proportions of dead cells tend to produce erroneous populations of cytokine-producing T cells.

Many statistical tests were performed in the analysis of the immune responses to BCG vaccination in mothers and infants, so it is possible that some apparently significant findings could have occurred by chance. A cautious approach should be taken to results where only a small effect was shown. Further studies will be required to confirm or refute the findings of this study. In order to account for the multiple comparisons, in a number of cases adjustment of multiple testing was applied and in these cases adjusted p values are reported. We did not formally adjust for multiple comparisons in the analysis of cytokine data. Individual results should therefore be interpreted with caution, the analysis does however give an overview of patterns and we have demonstrated consistency between cytokine responses assessed using Luminex and multi-parameter flow cytometry. Polyfunctional T cells form a small proportion of the overall response to antigen and the total number of cells are low. These results should also be interpreted conservatively and they require further studies to confirm the findings.

This study assessed humoral responses to EPI vaccines in both mothers and infants. We did not collect data on maternal vaccination history, due to limitations in recall and documentation. Vaccination records in this setting are typically available for young children only. This meant that we were unable to identify the contribution of maternal vaccination versus natural exposure to infections on maternal specific-antibody responses. Women in the study had comparable ages; therefore similar maternal vaccination history between

groups could be inferred based on the date of the introduction of the EPI schedule in South Africa EPI (1973). Data regarding infant vaccination was carefully prospectively recorded.

Although antibody levels can be used to indicate potential susceptibility to infection, there is some uncertainty regarding the functional relevance of a single so-called protective level. In addition, protective levels for collective response to multiple pneumococcal serotypes are unclear and there is paucity of evidence for defining the protective levels for other antibodies such as pertussis.[254] Functional assays may give a superior assessment of the ability of the immune system to effectively clear a pathogen.

We were unable to correlate antibody levels with long-term vaccine responses or clinical outcomes in mothers or infants, since our follow-up period was limited. However, this data contributes to a potential explanation for the higher morbidity and mortality observed amongst African HIV-exposed infants. For example, the lower pneumococcal-specific antibody amongst HIV-exposed infants prior to vaccination might be associated with increased severity of pneumonia seen in this group of infants.[28] This study highlights the need for larger prospective studies to determine whether the lower antibody levels in HIV-exposed infants at birth translate into increased morbidity from vaccine-preventable infections. This study however, can begin to answer novel and relevant mechanistic questions and may inform future clinical and vaccine studies.

7.5 Future studies

In order to validate our findings, a further mother-infant study is underway to assess levels of protective vaccine-specific antibody responses in the UK population. This cohort study is being undertaken at Imperial College London and includes a group of HIV-exposed uninfected infants to determine if these infants are at increased risk of vaccine-preventable infections such as pertussis. Together these two studies may provide compelling evidence of vulnerability to infection amongst women and newborn infants in diverse settings and may highlight the increased risk amongst HIV-exposed, uninfected infants prior to immunisation.

In order to further probe the reasons underlying the low antibody response seen amongst HIV-exposed uninfected infants compared to unexposed infants, it would be interesting to measure IgG subclasses to determine whether these lower antibodies are subclass specific.

An unexplored area of interest is the effect of maternal HIV infection on levels of serotype specific GBS antibody in HIV-infected mothers and their infants. HIV-exposed infants have increased rates of invasive GBS disease, in particular late-onset disease.[36] Their mothers may have increased vaginal carriage rates of GBS, however whether they have reduced levels of specific antibody, which results in lower levels in their infants and thus increased susceptibility to disease remains to be ascertained. As an extension of this current project, serum from mothers and infants will be analysed for levels of serotype specific GBS antibodies.

Demonstration of vulnerability calls for an intervention to mitigate the risk of infection in newborn infants, both HIV-exposed and unexposed. An intervention study administering pertussis vaccination to pregnant women is being planned since this is a highly relevant pathogen in young infants and recent resurgence in disease has been seen. A clinical trial of a GBS vaccine in pregnancy is also planned.

7.6 Concluding remarks

In summary, we found an increased rate of illness in HIV-exposed, uninfected infants, consistent with the published literature. We identified increased vulnerability factors in HIV-affected mother-infants pairs compared to HIV-unaffected mother-infant pairs that might influence the increased morbidity seen in HIV-exposed, uninfected infants, including socio-economic factors. We report for the first time that the agreement between the TST and QFN test is similar amongst HIV-infected and uninfected women immediately post-partum. IGRAs may be a useful adjunct to the identification of *Mtb* infection in pregnant mothers, irrespective of HIV status. We found that all infants developed a BCG scar, however HIV-exposed, uninfected infants may develop smaller scars. It is possible that this might be related to an alteration of the immune response to BCG in these infants.

We have demonstrated that, at least in a research setting, that it is feasible to delay BCG vaccination until the HIV infection status of the infant is established to avoid administering the BCG vaccine to HIV-infected infants.

At birth, BCG-induced proliferation and total intracellular cytokine production was similar amongst all infants. Frequencies of subsets of T cells were however different amongst HIV-exposed and unexposed infants. Maternal *Mtb* sensitisation modulated the effect of infant

HIV-exposure. IFN-γ TNF-α double-positive $CD8^-$ T cells, single positive TNF-α $CD8^-$ and $CD8^+$ T cells and IL-2 single positive $CD8^+$ T cells were all higher in HIV-exposed infants compared to HIV-unexposed infants. Infants born to HIV infected, *Mtb* sensitised mothers had the highest frequencies of TNF-α $CD8^+$ T cells.

Measurement of secreted cytokines using multiplex ELISA corroborated these findings. TNF-α levels in cell culture supernatants were highest in HIV-exposed infants, particularly amongst those infants born to *Mtb* sensitised mothers. Conversely, IFN-γ levels were lower amongst HIV-exposed infants compared to unexposed infants. Maternal *Mtb* sensitisation however seemed to prime the infant response and was associated with increased infant IFN-γ levels, such that HIV-exposed infants born to mothers with positive QFN tests had similar levels compared to HIV-unexposed infants.

Following vaccination at 6 weeks of age, all infants had similar frequencies of BCG-specific T cells at 16 weeks of age. The magnitude of response and the quality of the response was unaffected by maternal HIV infection and *Mtb* sensitisation. Levels of secreted cytokines, chemokines and growth factors were also similar amongst groups of infants at 16 weeks of age. No difference in the change in BCG-specific response was found between groups of infants pre- and post-vaccination. This data suggests that HIV-exposed infants are as likely to benefit from BCG vaccination as HIV-exposed infants.

The study measured maternal responses in parallel with infant responses at birth. *Mtb* sensitisation significantly influenced the response to BCG-antigens in HIV-infected but not in HIV-uninfected mothers. This is most likely because the memory response to BCG vaccination is lost in HIV infection, but cross-priming by *Mtb* infection boosts the pool of memory T cells.

A number of associations between maternal and infants response to BCG antigens at birth were observed, however these were transient and not seen at 16 weeks of age. Infant responses at birth were distinct from maternal responses. A number of pro-inflammatory cytokines, immunomodulatory molecules and chemokines were increased whilst other cytokine responses such as IFN-γ were lower.

This study also comprehensively described specific antibody responses in mother-infant pairs with and without maternal HIV infection before and after infant vaccination, and elucidated mechanisms for reduced responses in HIV-exposed infants early in life. Robust humoral responses to vaccination were observed amongst HIV-exposed and HIV-unexposed infants. A significant percentage of HIV infected as well as HIV-uninfected women had insufficient protection against vaccine-preventable infections, which has implications for HIV endemic and non-endemic settings.

Our data confirm that HIV-exposed infants have the same potential to respond to immunisations as HIV-unexposed infants; there is a lack of evidence of immunological impairment in HIV-exposed, uninfected infants following immunisation. The increased risk of morbidity and mortality is more likely to be related to socioeconomic differences, some of which have demonstrated in this study cohort. However, we did observe concerning vulnerabilities with regards to antibody protection against vaccine-preventable diseases prior to immunization, which might apply to other infections too, which we did not probe with our assays. Larger prospective studies are required to ascertain the relationship between these observed immune responses and clinical endpoints. Targeted vaccinations strategies may be required in HIV-infected women and their infants to ensure optimum protection against vaccine-preventable diseases early in life.

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Appendices

Appendix I: Maternal and infant case report form

Maternal and Infant case report form: Antenatal and birth

Mother/infant BCG study *(Version 22/01/09)*

Dr C Jones, Dr A Hesseling, Dr B Kampmann

Study number
Mother:

Maternal information ***(obtain from mother and mother's antenatal folder for current pregnancy)***	
1. Mother's first name	
2. Mother's surname	
3. Mother's clinic folder number	
4. Mother's date of birth (DD/MM/YYYY)	
5. Mother's cell phone number	
6. Mother's land line phone number	
7. Mother's address	
8. Mother lives in: 1) brick house/other formal structure, or 2) shack 3) other	
9. Date enrolled (DD/MM/YYYY)	
10. Any known TB contact in mother? (Y/N) *If yes, fill in TB contact sheet* *	
11. Any symptoms suggestive of TB** or recent TB investigation in mother? Y / N *If yes, specify*	
12. Previous treatment for TB (Y, N)	
13. Number of years since TB treatment (<1, 1-5y, 5-10y, >10y)	
14. Maternal chronic health condition? Y/N, Specify	
15. Maternal gravity	
16. Maternal parity	
17. Antenatal maternal HIV test (Pos, Neg, Indeterminate, not done) *(Ask the mother to disclose status, test results checked with permission)*	
18. Date of HIV test (DD/MM/YYYY)	
19. If initial HIV test negative, did mother had repeat HIV test (Y, N, U)**	
20. Date of repeat HIV test in mother (DD/MM/YYYY)	
21. Repeat HIV test result (Pos, Neg, Indeterminate)	
22. **If HIV+**: Mother given AZT antenatally (Y, N, U)	
23. **If HIV+:** Date AZT started (DD/MM/YYYY)	
24. **If HIV+:** Gestation when AZT started	
25. **If HIV+:** Maternal CD4 count (abs and %)	

26. **If HIV+:** Date of CD4 count (DD/MM/YYYY)	
27. **If HIV+**: Maternal viral load (VL)? (copies / not done)	
28. **If HIV+**: date of viral load (VL) (DD/MM/YYYY)	
29. **If HIV+:** Mother HIV WHO stage at HIV testing (I, II, III, IV) *from clinical notes*	
30. Maternal weight at enrolment (kg)	
31. Maternal length at enrolment (m, 2 decimals)	
Labour and delivery details	
32. Delivery – Normal vaginal / assisted vaginal / emergency c-section	
33. List any other complications in labour / delivery eg PROM, haemorrhage	
34. Mother given NVP intrapartum (Y, N, U)	
35. Gestation of infant	
36. Name of baby (if known)	
37. Baby's date of birth	
38. Baby's gender (M/F)	
39. Baby's birth weight (g)	
40. Baby's head circumference (cm) ***Please measure this yourself if not done***	
41. **If HIV exposed:** Baby given NVP at 6-72 h birth (Y, N, U)	
42. **If HIV exposed:** Baby given AZT after birth*?* (Y, N) State number of days	
43. Feeding (1) Breast only 2) breast and bottle 3) Bottle only)	
44. Clinical exam normal (Y/N) **Fill in Clinical examination form**	
Management:	
45. Stool sample obtained from mother? (Y/N)	
46. Blood taken from infant (Y/N)	
47. Blood taken from mother (Y/N)	
48. If mother HIV negative: Rapid HIV test performed with pre-and post-test counselling to confirm status (Y/N)	
49. Result of rapid test (Pos, Neg, Indeterminate)	
50. Referral to HIV services made if mother tests positive (Y/N)	
51. TST done? (Y/N)	
52. Date of next study visit? (If HIV exposed, give date in 4 weeks, if HIV-unexposed, give date in 6 weeks)	

* **Note 1**: If **current household contact** – do not enroll and refer to TB services for screening

****Note 2:** Suggestive TB symptoms: cough of 10 days or longer, night sweats, chest pains, poor appetite
TB screening: sputum or CXR

Appendix II: Survey of maternal perceptions of postnatal recruitment

Interview Number	Postnatal recruitment acceptable? (Y/N)	Location of discussion? (bedside / other room)	Understood information? (Y/N)	Willing for maternal and infant blood sampling? (Y/N)	Wish to be present for infant bloods? (Y/N)	Other views/ thoughts
1	Y	Other room	Y	Y	Y	
2	Y	Other room	Y	Y	Y	
3	Y	Other room	Y	Y	Y	
4	Y	Bedside	Y	Y	Y	Afraid to talk openly close to maternity staff
5	Y	Other room	Y	Y	N	
6	Y	Other room	Y	Y	N	What if baby sick at W/E?
7	Y	Other room	Y	Y	N	Is this a life long study?
8	Y	Other room	Y	Y	N	What if the baby is infected with TB?
9	Y	Other room	Y	Y	N	Is this going to be a success for their child?
10	Y	Other room	Y	Y	N	

Appendix III: Study exit questionnaire

As part of taking part in this study, we would like to know more about mothers feel about their and their baby's participation. We would also like to understand more about the home circumstances of women who are taking part. All information will be kept strictly private.

1. Where do you get your drinking water from? (*Tick one only*)

I have a tap in my house ☐ I get water from a public tap ☐

Other ☐ Specify.....

2. What is the main type of toilet that your household uses? (*Tick one only*)

I have a flush toilet in my house ☐ I use a bucket toilet in my house ☐

I use public toilets ☐ I have no access to a toilet ☐

Other ☐ Specify.....

3. What is your highest level of schooling? (*Tick one only*)

No schooling ☐ Matric ☐

Primary school ☐ Further education (post matric) ☐

Secondary school, no matric ☐ Other ☐

4. Do you currently have a job? (*Tick one only*)

I have a job with regular money ☐ I do not have a job ☐

I have a job sometimes ☐ I have another source of income ☐

Specify .....

5. **Does anyone in your household have a job with regular money?** (*Tick one only*)

Yes ☐ No ☐

6. **What fuel do you mainly use for cooking?** (*Tick one only*)

Paraffin ☐ Electricity ☐

Gas ☐ Specify..... Other ☐

7. **Do you have any of these items in your household?** (*Tick all that apply*)

Cell phone ☐ Television ☐

Landline ☐ DVD player ☐

Fridge ☐ Bicycle ☐

Car ☐ Radio ☐

8. **What do you think this study is about?** (*Tick the answers that best fits the mothers reply*)

About the BCG vaccine ☐ About TB ☐

How the mothers HIV and or TB affects how the baby reacts to the BCG ☐ Other ☐

Specify.....

9. **Why did you decide to take part in the study?** (*Tick **all** the answers that fits the mothers reply*)

Thought it would benefit my baby ☐ Help people in the future ☐

Thought it would benefit me ☐ Transport costs help me ☐

Thought I would get better healthcare ☐ I wanted to help you ☐

You asked me to ☐ Don't know ☐

Other ☐ Specify.....

10. **Did you feel free to say yes or no to being a part of the study?** (*Tick one only*)

Yes, I felt free ☐ No, I felt pressured ☐

11. **Have you been satisfied with the study?** (*Tick one only*)

Very satisfied ☐ Satisfied ☐ Neutral ☐ Dissatisfied ☐ Very dissatisfied ☐

12. **What are the advantages of the study, if any?** (*Tick **all** the answers that fits the mothers reply*)

I can see a doctor if my child is sick ☐ I don't have to wait ☐

The transport money helps me ☐ Other ☐

Specify

13. **What are the disadvantages of the study, if any?** (*Tick **all** the answers that fits the mothers reply*)

It takes up my time ☐ The blood tests ☐

I find it difficult to travel to the clinic ☐ I felt stigmatised ☐

No problem ☐ Specify Other ☐

14. **How did you feel about having blood taken from you? (***Tick one only*)

I did not mind ☐ I found it very upsetting ☐

I found it a little upsetting	☐	Other, specify.....	☐

15. **How did you feel about having blood taken from your baby? (***Tick one only)*

I did not mind	☐	I found it very upsetting	☐
I found it a little upsetting	☐	Other, specify.....	☐

Appendix IV: Flow cytometry data analysis

	Group	n (n for IL-17)	$CD8^-Ki67^+$ T cells				$CD8^+Ki67^+$ T cells			
			IFN-γ^+	TNF-α^+	IL-2^+	IL-17^+	IFN-γ^+	TNF-α^+	IL-2^+	IL-17^+
Mothers	1	21 (17)	0.05 (0.00-0.18)	0.06 (0.23 - 0.34)	0.07 (0.01 - 0.32)	0.00 (0.02-0.06)	0.02 (0.00 - 0.12)	0.02 (0.00 - 0.05)	0.05 (0.00 - 0.10)	0.00 (0.00-0.04)
	2	31 (24)	0.09 (0.00-0.129)	0.11 (0.02 - 0.54)	0.12 (0.00 - 0.39)	0.00 (0.02-0.08)	0.07 (0.00 - 0.24)	0.07 (0.01 - 0.27)	0.07 (0.00 - 0.13)	0.00 (0.00-0.03)
	3	21 (19)	0.04 (0.00 - 0.16)	0.13 (0.2 - 0.30)	0.06 (0.00 - 0.13)	0.00 (0.00-0.07)	0.05 (0.00 - 0.28)	0.05 (0.00 - 0.14)	0.00 (0.00 - 0.10)	0.00 (0.00-0.02)
	4	20 (19)	0.06 (0.00 - 0.21)	0.08 (0.00 - 0.18)	0.07 (0.02 - 0.13)	0.03 (0.00-0.05)	0.08 (0.03 - 0.21)	0.07 (0.00 - 0.19)	0.03 (0.00 - 0.13)	0.14 (0.00-0.06)
p			**.89**	**.42**	**.70**	**.53**	**.42**	**.12**	**.24**	**.24**
Infants at birth	1	16 (12)	0.00 (0.00 - 0.04)	0.00 (0.00 - 0.04)	0.00 (0.00 - 0.13)	0.00 (0.00-0.09)	0.00 (0.00 - 0.03)	0.00 (0.00 - 0.03)	0.00 (0.00 - 0.02)	0.00 (0.00-0.00)
	2	26 (21)	0.00 (0.00 - 0.06)	0.00 (0.00 - 0.02)	0.00 (0.00 - 0.04)	0.00 (0.00-0.01)	0.00 (0.00 - 0.4)	0.00 (0.00 - 0.05)	0.00 (0.00 - 0.10)	0.00 (0.00-0.04)
	3	14 (12)	0.00 (0.00 - 0.04)	0.00 (0.00 - 0.11)	0.00 (0.00 - 0.02)	0.00 (0.00-0.02)	0.00 (0.00 - 0.4)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.03)	0.00 (0.00-0.00)
	4	14 (13)	0.00 (0.00 - 0.04)	0.00 (0.00 - 0.02)	0.00 (0.00 - 0.04)	0.00 (0.00-0.00)	0.00 (0.00 - 0.11)	0.00 (0.00 - 0.08)	0.00 (0.00 - 0.07)	0.00 (0.01-0.10)
p			**.98**	**.87**	**.78**	**.67**	**.99**	**.30**	**.40**	**.13**
Infants at 16 weeks	1	14 (9)	0.18 (0.00-1.23)	0.63 (0.11-2.44)	0.67 (0.10 - 2.11)	0.14 (0.06-1.32)	0.17 (0.00 – 1.72)	0.23 (0.00 – 0.47)	0.08 (0.00 – 0.31)	0.08 (0.00-0.38)
	2	24 (18)	0.92 (0.02-2.10)	1.16 (0.15-4.54)	0.70 (0.11-2.37)	0.06 (0.00-0.70)	0.17 (0.00 – 0.50)	0.21 (0.00 – 0.58)	0.18 (0.00 – 0.50)	0.00 (0.00-0.09)
	3	14 (13)	0.27 (0.03 - 1.15	0.71 (0.09 - 4.15)	0.72 (0.07-3.66)	0.09 (0.02-0.58)	0.04 (0.00 – 0.48)	0.07 (0.00 – 0.20)	0.03 (0.00 – 0.16)	0.00 (0.00-0.09)
	4	16 (16)	0.22 (0.07 - 1.19)	0.36 (0.11-2.60)	0.25 (0.13 - 1.84)	0.42 (0.00-0.42)	0.06 (0.00 – 0.22)	0.08 (0.00 – 0.41)	0.07 (0.00 – 0.41)	0.00 (0.00-0.05)
p			**.89**	**.91**	**.89**	**.39**	**.54**	**.63**	**.50**	**.59**

Table 1: Median (IQR) frequencies of Tetanus-specific $CD8^-$ and CD8+ Ki67+ T cells expressing cytokines in mothers at delivery, infants pre-vaccination at birth and post-vaccination at 16 weeks of age.

Responses were measured by intracellular cytokine staining and multi-parameter flow cytometry in a whole blood proliferation assay from four groups of mothers and infants at birth and repeated in infants at 16 weeks. Groups were defined by maternal infection status; all infants were HIV- and TB-uninfected. A reduced number of samples were available for analysis of $IL\text{-}17^+$ $CD8^+$ and $CD8^-$ T cells. Differences between groups calculated by the Kruskal-Wallis test.

Appendix V: Luminex data analysis

	Mothers		Infants at birth		Infants at 16 weeks	
	Day 1	Day 6	Day 1	Day 6	Day 1	Day 6
EGF	1.6	1.6	1.6	5.19	2.73	28.58
GM-CSF	248.34	761.28	392.89	961.71	187.65	1005.68
IL-1ra	53.03	84.92	166.84	376.89	26.25	32.6
IL-12p40	25.75	22.96	46.97	51.55	23.89	22.96
IL-17	1.17	1.7	1.17	1.17	1.04	8.9
IP-10	53.71	47.67	25.33	32.52	253.56	593.72
MCP-1	5375.88	5957.58	6692.12	6825.24	5404.73	6481.02
MDC	92.41	789.34	207.83	693.85	141.18	1639.24
MIP-1β	5053.6	3107.32	10000	15450.86	3543.55	1699.23
sCD40L	116.46	28.99	70.99	22.29	139.4	54.32
TNF-α	1873.14	1266.98	3259.09	1240.32	1413.72	1146.87
IFN-γ	7.35	76.68	3.03	18.79	74.32	900.75
IL-2	1.81	1.59	0.74	0.95	13.06	1.59
IL-10	458.09	372.88	576.1	380.34	125.13	116.83
IL-13	1.74	1.97	2.2	3.72	6.31	45.77
IL-1β	1973.47	1409.24	2990.22	2457.86	3208.22	3244.83
IL-6	6411.33	7806.25	6769.94	7037.16	4844.88	6091.45
IL-7	1.6	1.6	1.6	1.6	1.6	1.04
IL-8	11963.28	13555.08	14640.19	11901.42	10375.43	11602.34

Table 2: Optimisation of Luminex assay.
Comparison of secreted levels of cytokines and chemokines after 1 or 6 days of culturewith BCG antigens in mothers, infants at birth and infants at 16 weeks of age. Median values in pg/ml are stated; n=4 in each group.

Appendix VI: Analysis of specific antibody responses

Independent variables	Hib			Pertussis			Pneumococcus			Tetanus		
	b	SE b	P	b	SE b	P	b	SE b	P	b	SE b	P
	Infant regression model											
Maternal HIV	0.49	0.12	< .001	0.38	0.08	< .001	0.24	0.10	0.01	0.52	0.16	.002
Infant Gender	0.10	0.12	.39	0.14	0.08	.10	-0.04	0.10	.71	-0.05	0.16	.75
Infant Birth Weight	-0.12	0.15	.42	0.05	0.10	.61	-0.01	0.12	.90	-0.06	0.20	.76
Maternal Age	0.00	0.01	.98	0.02	0.10	.03	0.02	0.01	.21	0.02	0.02	.27
Maternal Gravidity	0.08	0.12	.27	-0.02	0.05	.66	-0.03	0.06	.56	0.08	0.10	.42
Housing structure	0.01	0.12	.96	0.11	0.08	.17	-0.03	0.10	.74	-0.15	0.17	.40
R^2		.20			.26			.08			.12	
Independent variables	Maternal regression model											
Maternal HIV	0.39	0.12	.002	0.16	.010	.09	0.25	0.09	0.005	0.35	0.16	0.03
Maternal Age	-0.01	0.02	.52	0.02	0.01	.16	0.01	0.01	.18	0.03	0.02	.20
Maternal Gravidity	0.10	0.07	.17	0.01	0.05	.88	-0.01	0.05	.86	.08	0.09	.39
Housing structure	-0.04	0.13	.73	0.11	0.09	.24	-0.10	0.09	.28	-0.07	0.16	.65
R^2		.11			.07			.10			.08	

Table 3. Association of specific antibody levels in mothers and infants with maternal HIV and other covariates.
b: unstandarized co-efficient, SE: standard error. Independent variables were entered simultaneously into the model and were defined as: Maternal HIV (0 = uninfected, 1 = infected); Infant gender (1 = male, 2 = female), Housing structure (Informal structure = 0, brick house = 1).

	Maternal antibody level				Placental transfer[a]			
	CD4		Viral load		CD4		Viral load	
	r (95% CI)	P	r (95% CI)	P	r (95% CI)	P	r (95% CI)	P
Hib	-0.07 (-0.36 – -0.23)	.63	0.14 (-0.19 – -0.43)	.40	-0.01 (-0.30 – 0.28)	.95	-0.19 (-0.48 - 0.13)	.24
Pertussis	0.31 (0.01 - 0.56)	.04	-0.01 (-0.33 - 0.30)	.92	-0.04 (-0.33 – 0.25)	.77	-0.31 (-0.57 – -0.004)	0.05
Pneumococcus	0.33 (0.02 - 0.57)	.03	0.06 (-0.26 - 0.37)	.72	-0.20 (-0.47 – 0.10)	0.17	-0.27 (-0.54 – 0.04)	0.08
Tetanus	0.37 (0.09 - 0.61)	.01	0.02 (-0.30 - 0.33)	0.91	-0.02 (-0.32 – 0.28)	0.88	-0.09 (-0.40 – 0.24)	0.58

Table 4: Correlation of maternal CD4 and viral load with level of maternal specific antibody and placental transfer of antibody.

	Median (IQR) level of antibody according to number of doses of vaccine received			P values
	1	2	3	
Hib (mg/l)	0.54 (0.26 - 4.11)	1.77 (0.23 – 4.22)	7.75 (2.07 – 37.52)	<0.001 † ‡
Pertussis (FDA u/ml)	10.55 (3.71 - 27.74)	41.89 (11.60 – 105.5)	129.1 (33.79 – 275.4)	<0.001 † ‡
Pneumococcus (mg/l)	30.44 (12.01 – 42.64)	37.06 (16.05 -76.32)	N/A	0.097 §
Tetanus (IU/ml)	0.44 (0.30 – 0.67)	0.82 (0.19 – 1.89)	2.13 (1.52 – 2.85)	<0.001 † ‡

Table 5: Association of level of specific antibody and number of doses of vaccine received prior blood sampling at 16 weeks.

The Kruskal-Wallis test was used to compare the level of specific antibody measured for 3 groups of infants receiving 1 (n=6), 2 (n=22) or 3 (n=65) doses of vaccine containing Hib, pertussis and tetanus antigens. Dunn's post-test was used to compare the difference between each column; * denotes a statically significant difference at the P <0.05 level between infants who received 1 vs. 2 vaccine doses, † between infants who received 1 vs. 3 vaccine doses and ‡ between infants who received 2 vs. 3 vaccine doses. § The unpaired t-test was used to compare infants who had received 1 vs 2 doses of pneumococcoal vaccine (PCV7). Only 2 doses of vaccine are routinely scheduled before 16 weeks and this vaccine was introduced during the study period, therefore only 49 infants received this vaccine (n=15 received 1 dose, n=34 received 2 doses).

Appendix VII: Published papers

1. Jones CE, Naidoo S, De Beer C, Esser M, Kampmann B, Hesseling AC. Maternal HIV Infection and Antibody Responses Against Vaccine-Preventable Diseases in Uninfected Infants. *JAMA*. 2011;305(6):576–584.

2. Jones C, Whittaker E, Bamford A, Kampmann B. Immunology and pathogenesis of childhood TB. *Paediatric Respiratory Reviews*. 2011;12(1):3–8.

3. Lindsey B, Jones C, Kampmann B. Bridging the gap: maternal immunisation as a means to reduce neonatal deaths from infectious diseases. *PathGH*. 2012;106(3):137–138.

Maternal HIV Infection and Antibody Responses Against Vaccine-Preventable Diseases in Uninfected Infants

Christine E. Jones, BMBS, MRCPCH
Shalena Naidoo, BSc
Corena De Beer, PhD
Monika Esser, MBChB, MMed (Paed)
Beate Kampmann, MBBS, PhD
Anneke C. Hesseling, MD, PhD

Context Altered immune responses might contribute to the high morbidity and mortality observed in human immunodeficiency virus (HIV)–exposed uninfected infants.

Objective To study the association of maternal HIV infection with maternal- and infant-specific antibody levels to *Haemophilus influenzae* type b (Hib), pneumococcus, *Bordetella pertussis* antigens, tetanus toxoid, and hepatitis B surface antigen.

Design, Setting, and Participants A community-based cohort study in Khayelitsha, Western Cape Province, South Africa, between March 3, 2009, and April 28, 2010, of 109 HIV-infected and uninfected women and their infants. Serum samples from 104 women and 100 infants were collected at birth and samples from 93 infants were collected at 16 weeks.

Main Outcome Measure Level of specific antibody in mother-infant pairs at delivery and in infants at 16 weeks, determined by enzyme-linked immunosorbent assays.

Results At birth, HIV-exposed uninfected infants (n=46) had lower levels of specific antibodies than unexposed infants (n=54) did to Hib (0.37 [interquartile range {IQR}, 0.22-0.67] mg/L vs 1.02 [IQR, 0.34-3.79] mg/L; $P<.001$), pertussis (16.07 [IQR, 8.87-30.43] Food and Drug Administration [FDA] U/mL vs 36.11 [IQR, 20.41-76.28] FDA U/mL; $P<.001$), pneumococcus (17.24 [IQR, 11.33-40.25] mg/L vs 31.97 [IQR, 18.58-61.80] mg/L; $P=.02$), and tetanus (0.08 [IQR, 0.03-0.39] IU/mL vs 0.24 [IQR, 0.08-0.92] IU/mL; $P=.006$). Compared with HIV-uninfected women (n=58), HIV-infected women (n=46) had lower specific antibody levels to Hib (0.67 [IQR, 0.16-1.54] mg/L vs 1.34 [IQR, 0.15-4.82] mg/L; $P=.009$) and pneumococcus (33.47 [IQR, 4.03-69.43] mg/L vs 50.84 [IQR, 7.40-118.00] mg/L; $P=.03$); however, no differences were observed for antipertussis or antitetanus antibodies. HIV-exposed uninfected infants (n=38) compared with HIV-unexposed infants (n=55) had robust antibody responses following vaccination, with higher antibody responses to pertussis (270.1 [IQR, 84.4-355.0] FDA U/mL vs 91.7 [IQR, 27.9-168.4] FDA U/mL; $P=.006$) and pneumoccocus (47.32 [IQR, 32.56-77.80] mg/L vs 14.77 [IQR, 11.06-41.08] mg/L; $P=.001$).

Conclusion Among South African infants, antenatal HIV exposure was associated with lower specific antibody responses in exposed uninfected infants compared with unexposed infants at birth, but with robust responses following routine vaccination.

JAMA. 2011;305(6):576-584 www.jama.com

INFECTIOUS DISEASES ACCOUNT FOR nearly 6 million deaths worldwide annually in children younger than 5 years.[1] Immunization against vaccine-preventable infections therefore remains essential to achieving Millennium Development Goal 4, which is to reduce childhood mortality by two-thirds.[2] Before acquisition of immunity, infants are protected by maternal IgG transferred across the placenta. Maternal antibody levels, immunization, infection, and infant gestational age can influence the efficiency of this process.[3-7] Although maternal antibody is essential to protect the infant in the first months of life, maternal-specific antibody can also interfere with the infant's own response to vaccination.[8]

The high prevalence of maternal human immunodeficiency virus (HIV) in many parts of the resource-poor world, coupled with successful programs to reduce mother-to-child transmission of HIV, has led to increasing numbers of HIV-exposed infants who are not HIV-infected themselves (ie, HIV-exposed infants).[9] These infants and children represent a vulnerable group with increased rates of lower respiratory tract infection and meningitis and up to

Author Affiliations: Academic Department of Pediatrics, Imperial College London, London, England (Drs Jones and Kampmann); Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa (Drs Jones and Kampmann); Medical Research Council (UK) The Gambia, Fajara, The Gambia (Dr Kampmann); Department of Pathology, Stellenbosch University, Cape Town, South Africa (Drs De Beer and Esser and Ms Naidoo); National Health Laboratory Services, Tygerberg Hospital, Cape Town, South Africa (Drs De Beer and Esser); and Desmond Tutu TB Center, Department of Pediatrics and Child Health, Stellenbosch University, Cape Town, South Africa (Dr Hesseling).
Corresponding Author: Christine E. Jones, BMBS, MRCPCH, Academic Department of Pediatrics, Imperial College London, St Mary's Campus, Norfolk Place, London W2 1PG, England (christine.jones@imperial.ac.uk).

4-fold higher mortality in the first year of life.[10-13] A number of factors are likely to contribute to this increased vulnerability, including socioeconomic factors, but immunological phenomena might also be important.

To design appropriate interventions for these vulnerable infants, it is important to understand how maternal HIV infection influences infant susceptibility to common pathogens. We therefore studied the association of maternal HIV infection with maternal- and infant-specific antibody levels. Because absolute levels of antibody that associate with protection against infection are poorly defined for a number of specific antibodies, we assessed how maternal HIV affects both the magnitude and putative protective levels of these antibodies.

METHODS

Study Setting

The study was conducted between March 3, 2009, and April 28, 2010, in a community health center in Khayelitsha, Western Cape Province, South Africa, a rapidly expanding urban informal settlement. In this context, all women are offered voluntary counseling and testing for HIV at antenatal care registration; the participation is consistently close to 100%.[14,15] In 2009, the HIV prevalence among women attending antenatal clinics was 32%, with reported vertical transmission of 3.3%.[14] During the study period, the Prevention of Mother to Child Transmission program consisted of dual therapy for mothers and infants, starting with the administration of zidovudine at 28 or more weeks' gestation, then zidovudine for 1 month to the infant and a single dose of nevirapine to both mother and infant. Mothers were eligible for highly active antiretroviral treatment if their CD4 count was less than 200 cells/μL. Exclusive infant feeding options were encouraged and mothers were provided with free formula for 6 months if they chose exclusive formula feeding.

The study was approved by the Universities of Cape Town and Stellenbosch, South Africa, and the National Health Service Research Ethics Committee, England. Our study was nested in a cohort study investigating the influence of maternal HIV and mycobacterial infection on infant immune responses to BCG vaccination. The BCG vaccination (Danish strain 1331, Statens Serum Institute, intradermal vaccine) was delayed until 6 weeks of age to allow for determination of infant HIV infection and to avoid BCG vaccination of HIV-infected infants and vaccine adverse events.[16-18] Infants received all other routine vaccines according to the South African Expanded Program on Immunization schedule: oral polio vaccine (Sanofi Pasteur, Lyon, France) at birth; combination diphtheria, tetanus toxoid, and pertussis vaccine, and *Haemophilus influenzae* type b vaccine (DTP-Hib; Sanofi Pasteur); hepatitis B (Heber Biotec, Havana, Cuba); and oral polio vaccine at 6, 10, and 14 weeks. From July 2009, pneumococcal 7-valent conjugate (Wyeth, Andover, Massachusetts) and rotavirus vaccinations (GlaxoSmithKline, Rixensart, Belgium) were administered at 6 and 14 weeks, and diphtheria, tetanus toxoid, and acellular pertussis vaccine combined with inactivated polio vaccine and Hib (DTaP-IPV/Hib; Sanofi Pasteur) replaced the DTP-Hib vaccine. Tetanus immunization is not routinely administered to pregnant women in this setting; therefore, no vaccines were administered to participating mothers.

Eligibility

Women were eligible if they had delivered a healthy infant at the Site B Maternal Obstetric Unit within the previous 24 hours, knew the result of the HIV test at antenatal care registration, and were willing and able to provide written informed consent for themselves and their infant. Mothers were excluded if they were younger than 18 years (2 women), planning to move away during the study period (8 women), did not intend to return to the routine Site B baby clinic for ongoing care (15 women), were unwell (2 women), had evidence of active tuberculosis or were on tuberculosis treatment (1 woman), or had a current household or other close tuberculosis contact (1 woman). Infants weighing less than 2.5 kg or estimated at less than 36 weeks' gestation (8 infants), with acute illness (1 infant), or part of a twin birth (2 infants) were excluded. Consecutive eligible women were enrolled irrespective of their HIV status. Once sufficient numbers of HIV-uninfected women were reached (approximately 50% of the sample), HIV-infected women were consecutively enrolled. A study nurse obtained written informed consent in the participants' home language.

Study Measures

A venous blood sample was collected from the mother and infant within 24 hours of delivery and transported to the laboratory within 4 hours. All infants had a further venous blood sample collected at 16 weeks. Mothers who tested negative for HIV during pregnancy had a rapid HIV test (Abbott Determine HIV-1/2, Toyko, Japan) at enrollment with pretest and posttest counseling to confirm their HIV status. The HIV-exposed infants had an HIV polymerase chain reaction (Amplicor HIV-a DNA kit, version 1.5; Roche Molecular Systems Inc, Branchburg, New Jersey) performed at ages 4 and 16 weeks. Infant vaccination status was verified from vaccination cards ("Road to Health" records). Serum was separated and stored at –80°C for analysis by standard commercial enzyme-linked immunosorbent assays by researchers blinded to maternal HIV infection status and personal information.

Laboratory Assays

Hib capsular polysaccharide and pneumococcal capsular polysaccharide specific IgG were measured using VaccZyme Human Anti-Hib and Anti-PCP Enzyme Immunoassay kits (MK016 and MK012, The Binding Site Ltd, Birmingham, England). Microwells in the pneumococcal assay were supplied precoated with pneumococcal

capsular polysaccharide antigens 1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F, and incorporated C-polysaccharide antibody absorption, which confers limited protection against pneumococcal infection.[19] Specific IgG to *Bordetella pertussis* (pertussis) and tetanus toxoid were measured using SERION enzyme-linked immunosorbent assays classic kits (ESR120G and ESR108G Serion Immundiagnostica GmbH, Würzburg, Germany). Hepatitis B surface antigen was measured using an AxSYM HBsAg (V2) kit (Abbott, Wiesbaden, Germany) in a fully automated system. All reagents, including controls, were supplied with the commercial kits and manufacturer's instructions were followed.

Anti-Hib antibody titers of more than 1.0 mg/L were regarded as protective[20]; pertussis titers of more than 30 Food and Drug Administration (FDA) U/mL were regarded as positive (defined by the manufacturer); tetanus antibody levels were classified as providing sufficient protection if more than 0.1 IU/mL[21]; and more than 10 mIU/mL was regarded as seropositive and protective against hepatitis B infection. No level of protective immunity has been established for a collective response to multiple pneumococcal serotypes.

Data Management and Statistical Analysis

Statistical analyses were completed using SPSS version 18 (SPSS Inc, Chicago, Illinois) and GraphPad Prism version 5.0a (GraphPad Software Inc, La Jolla, California). Two-sided $P<.05$ was considered significant. All comparisons were prespecified except for the comparison of infants who had not received all vaccinations, which was post hoc.

The magnitude of specific antibody response between groups was compared using the unpaired *t* test when data were normally distributed; interquartile ranges (IQRs) are shown. When the distribution was nonnormal, data were log transformed; the unpaired *t* test was used if resulting distributions were normal; and the Mann-Whitney test was used for nonnormal data. Simple correlations were assessed using Pearson or Spearman correlation in the case of normal or nonnormal distribution, respectively. A multiple linear regression model was used to assess the relationship between the magnitude of maternal and infant Hib, pertussis, pneumococcal, and tetanus responses at delivery in relation to maternal HIV status, treating maternal age, gravidity, and household type (informal structure or brick house), a proxy for socioeconomic status in this community, as covariates. Infant sex and birth weight were used as additional covariates in analyses of infant responses at birth. All independent variables were entered into the model simultaneously (forced entry method). Proportions were compared using the Fisher exact test; if any cell contained a value of zero, 1.0 was added to all cells before testing was performed. Hepatitis B specific antibody data had a binomial distribution and therefore only the proportion of participants with seroprotective levels of hepatitis B–specific antibody results were analyzed. Placental transfer was defined as the ratio of infant-to-mother specific IgG concentration at birth.[22] Missing data were excluded from analysis. We did not adjust for multiple comparisons.

Sample size was determined for the cohort study; this substudy was powered to investigate differences between antibody responses in HIV-exposed and HIV-unexposed infants of at least 30%, with the prespecified hypothesis that the magnitude of responses would be lower in HIV-exposed infants.

RESULTS

Participant Characteristics

Of 120 eligible mother-infant pairs, 11 mothers declined to participate; therefore, 109 maternal-infant pairs were enrolled (91% participation rate). Of these pairs, 47 mothers (43%) were infected with HIV and 62 (57%) were uninfected. All women testing negative for HIV at their antenatal care registration had a further repeat negative HIV test at delivery. Samples were collected from 105 mothers (96% of the maternal sample; 47 were infected and 58 were uninfected with HIV) at delivery, and from 101 infants (93% of the infant sample; 47 were exposed and 54 were unexposed to HIV) at birth. Sample volumes were insufficient for 4 women and 8 infants. One infant (1%) was determined to be infected with HIV at 4 weeks and was referred for rapid initiation of antiretroviral treatment (mother-infant pair subsequently was excluded from analysis). Follow-up samples were available for 94 infants (87%; 38 were exposed and 55 were unexposed to HIV) at a mean postnatal age of 16.4 weeks (SD, 1.7). One late follow-up sample was excluded from the analysis (collected at 28 weeks after birth). The final analysis was based on samples from 104 women and 100 infants collected at birth and samples from 93 infants collected at 16 weeks.

Characteristics of the study cohort are shown in Table 1. All HIV-infected women chose exclusive formula replacement feeding. The mean (SD) CD4 count among the HIV-infected women was 474 (252) cells/μL and the median (IQR) viral load was 800 (357-6000) copies/mL. Seven women had CD4 counts of less than 200 cells/μL; 3 of these were taking highly active antiretroviral treatment at enrollment and 4 were referred to commence highly active antiretroviral treatment following delivery.

Infant-Specific Antibody Responses at Birth

At birth, HIV-exposed uninfected infants had significantly lower specific antibody levels compared with unexposed infants to Hib (0.37 [IQR, 0.22-0.67] mg/L vs 1.02 [IQR, 0.34-3.79] mg/L; $P<.001$), pertussis (16.07 [IQR, 8.87-30.43] FDA U/mL vs 36.11 [IQR, 20.41-76.28] FDA U/mL; $P<.001$), pneumococcus (17.24 [IQR, 11.33-40.25] mg/L vs 31.97 [IQR, 18.58-61.80] mg/L; $P=.02$), and teta-

nus (0.08 [IQR, 0.03-0.39] IU/mL vs 0.24 [IQR, 0.08-0.92] IU/mL; $P=.006$) (FIGURE 1).

These lower levels observed in HIV-exposed infants at birth corresponded with a lower proportion of HIV-exposed infants with levels considered to be protective against Hib (17% vs 52%; $P<.001$), pertussis (24% vs 57%; $P=.001$), tetanus (43% vs 74%; $P=.002$), and hepatitis B (21% vs 54%; $P=.01$).

In a multiple linear regression model for factors associated with magnitude of specific antibody response at birth, HIV exposure remained associated with reduced Hib titers (unstandardized regression coefficient [b] =0.49; SE, 0.12; $P<.001$), pertussis (b=0.38; SE, 0.08; $P<.001$), pneumococcus (b=0.24; SE, 0.10; $P=.01$), and tetanus (b=0.52; SE, 0.16; $P=.002$) levels (eTable 1, available at http://www.jama.com). There was no association with maternal age, gravidity, housing structure, infant sex or birth weight for Hib, pneumococcus, and tetanus levels, but increased maternal age was associated with higher pertussis-specific antibody titers (b=0.02; SE, 0.01; $P=.03$) (eTable 1).

Maternal-Specific Antibody Responses

To investigate the mechanisms associated with infant response, we measured specific maternal antibody levels in parallel. HIV-infected women had lower specific antibody levels than uninfected women to Hib (0.67 [IQR, 0.16-1.54] mg/L vs 1.34 [IQR, 0.15-4.82] mg/L; $P=.009$) and pneumococcus (33.47 [IQR, 4.03-69.43] mg/L vs 50.84 [IQR, 7.40-118.00] mg/L; $P=.03$). No differences were observed for pertussis (22.07 [IQR, 12.48-29.67] FDA U/mL vs 23.64 [IQR, 12.87-54.68] FDA U/mL; $P=.26$) or tetanus (0.09 [IQR, 0.03-0.33] IU/mL vs 0.15 [IQR, 0.06-0.67] IU/mL; $P=.12$) between HIV-infected and uninfected women. In a multiple regression model for factors associated with level of maternal-specific antibody response, maternal HIV infection remained associated with low Hib and pneumococcal antibody levels; however, there was no significant association with maternal age, gravidity, or housing structure for any of the specific antibody responses (eTable 1).

HIV-infected women were less likely to have anti-Hib antibodies levels considered to be protective (35% vs 59%; $P=.02$). The proportion of women with protective antibody levels against pertussis (24% vs 38%; $P=.14$), tetanus (47% vs 64%; $P=.11$), or hepatitis B (26% vs 33%; $P=.52$) was similar in HIV-infected and HIV-uninfected women. The overall proportion of all women with protective antibody levels was low for pertussis (32%), tetanus (41%), and hepatitis B (30%).

In HIV-infected women, CD4 count was positively correlated with the level of antibody to pertussis ($r=0.31$; $P=.04$), pneumococcus ($r=0.33$; $P=.03$), and tetanus ($r=0.37$; $P=.01$), but not with Hib ($r=-0.07$; $P=.63$) (eTable 2). There was no correlation between maternal HIV viral load and any specific antibody level (eTable 2).

In HIV-infected women and their infants, the correlation between maternal- and infant-specific antibody responses were statistically significant for Hib ($r=0.91$; $P<.001$), pertussis ($r=0.78$; $P<.001$), pneumococcus ($r=0.86$; $P<.001$), and tetanus ($r=0.95$; $P<.001$). In HIV-negative women, the correlation between maternal and infant responses were also statistically significant for Hib ($r=0.95$; $P<.001$), pertussis ($r=0.89$; $P<.001$), pneumococcus ($r=0.80$; $P<.001$), and tetanus ($r=0.93$; $P<.001$).

Association of Maternal HIV With Placental Transfer of Specific Antibody

The proportion of maternal-specific antibody transferred across the placenta to infants was significantly reduced among HIV-infected women and their infants. Using infant:maternal anti-

Table 1. Characteristics of HIV-Infected and HIV-Uninfected Women and Their Uninfected Infants

Characteristics	No. (%) of Participants		*P* Value
	HIV-Infected Women and Exposed Infants (n = 46 at Birth)	HIV-Uninfected Women and Unexposed Infants (n = 54 at Birth)	
Maternal age, median (IQR), y	27.0 (24.0-31.3)	24.0 (20.0-27.5)	.002[a]
Maternal primigravidity	10 (21)	28 (45)	.01[b]
Female infant sex	25 (57)	33 (57)	.68[b]
Infant delivered by normal vaginal delivery	46 (100)	54 (100)	>.99
Birth weight, mean (SD), kg	3.16 (0.35)	3.23 (0.44)	.38[c]
Weight at 16 wks, mean (SD), kg[d]	6.81 (0.93)	6.60 (0.93)	.29[c]
Exclusive breast feeding at birth[e]	0	54 (100)	<.001
Exclusive breast feeding at 16 wks[e]	0	23 (42)	<.001
Household lives in informal structure[f]	36 (78)	34 (54)	.02[b]

Abbreviations: HIV, human immunodeficiency virus; IQR, interquartile range.
[a] Mann-Whitney U test.
[b] Fisher exact test.
[c] *t* Test.
[d] Weight at 16 weeks available for all infants followed up to 16 weeks (38 HIV-exposed infants and 55 HIV-unexposed infants).
[e] No breast feeding was reported at any study visit for HIV-exposed infants.
[f] An informal structure is a shack constructed of materials such as wood and corrugated iron.

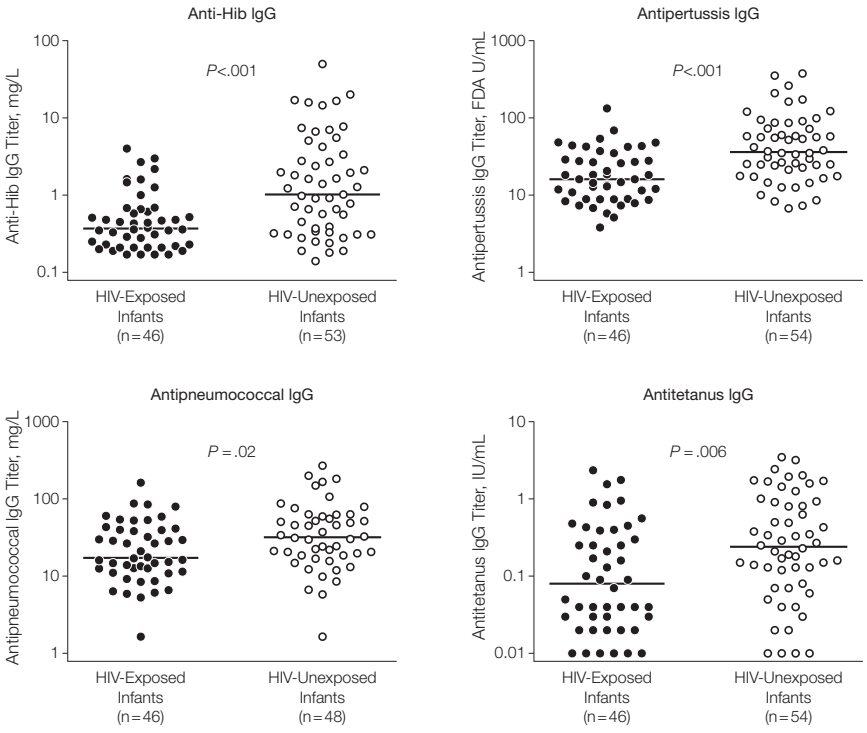
body ratios as a proxy for placental transfer, HIV-infected women had significant reductions in placental transfer of 23% for Hib, 40% for pertussis, and 27% for tetanus-specific antibodies compared with HIV-uninfected women, with a trend toward a reduction in placental transfer of pneumococcal specific antibodies (TABLE 2). Among HIV-infected women, there was no association between maternal CD4 count or viral load and placental transfer (eTable 2).

Specific Vaccine-Induced Antibody Responses in Infants at 16 Weeks

In stratified analysis for infants who had received 1, 2, or 3 doses of DTP-Hib vaccine (n=6, 22, and 65, respectively), there was no difference in antibody levels between infants who had received 1 or 2 doses (eTable 3); these groups were therefore combined for further analysis. Similarly, data were combined for infants who had received 1 or 2 doses of pneumococcal capsular polysaccharide (n=15 and 34, respectively). There was no statistical difference in the proportion of HIV-exposed and HIV-unexposed infants who received fewer than 3 doses of DTP-Hib vaccine (25% vs 16%; $P=.31$) or fewer than 2 doses of pneumococcal capsular polysaccharide (20% vs 49%; $P=.06$) before the 16-week sampling.

Despite initially lower titers at birth, HIV-exposed uninfected infants mounted robust responses following vaccination. In the group that received all 3 scheduled doses of DTP-Hib vaccine, HIV-exposed infants had significantly higher responses to pertussis (270.1 [IQR, 84.4-355.0] FDA U/mL vs 91.7 [IQR, 27.9-168.4] FDA U/mL; $P=.006$) than unexposed infants did (FIGURE 2), but had similar responses to Hib and tetanus. HIV-exposed infants also had higher levels of pneumococcal-specific antibody than HIV-unexposed infants did (47.32 [IQR, 32.56-77.80] mg/L vs 14.77 [IQR, 11.06-41.08] mg/L; $P=.001$). Among infants who had received only 1 or 2 doses of DTP-Hib vaccine, responses were higher in the HIV-exposed infants than unexposed infants to Hib (6.46 [IQR, 1.74-9.29] mg/L vs

Figure 1. Specific Antibody Titers in Uninfected HIV-Exposed and HIV-Unexposed Infants at Birth



HIV indicates human immunodeficiency virus; FDA, Food and Drug Administration. Specific antibodies to *Haemophilus influenzae* type b (Hib), *Bordetella pertussis* antigens, pneumococcus, and tetanus toxoid from serum samples collected within 24 hours of birth were nonpreferentially analyzed on available sample volume by commercially available enzyme-linked immunosorbent assays. Horizontal lines indicate median response. The Mann-Whitney U test was used to compare antibody levels at birth between HIV-exposed and HIV-unexposed infants.

Table 2. Influence of Maternal HIV Infection on Placental Antibody Transfer

Specific Antibody	Placental Transfer, Median (IQR)[a]		Reduction, %[b]	*P* Value[c]
	HIV-Infected Mother–Exposed Uninfected Infant Pairs	HIV-Uninfected Mother–Unexposed Infant Pairs		
Haemophilus influenzae type b	0.57 (0.45-0.79)	0.74 (0.61-1.00)	23	.002
Bordetella pertussis	0.91 (0.61-1.20)	1.51 (1.15-2.06)	40	<.001
Pneumococcus	0.62 (0.41-0.77)	0.73 (0.53-0.94)	15	.05
Tetanus toxoid	0.95 (0.60-1.12)	1.30 (1.03-1.86)	27	<.001

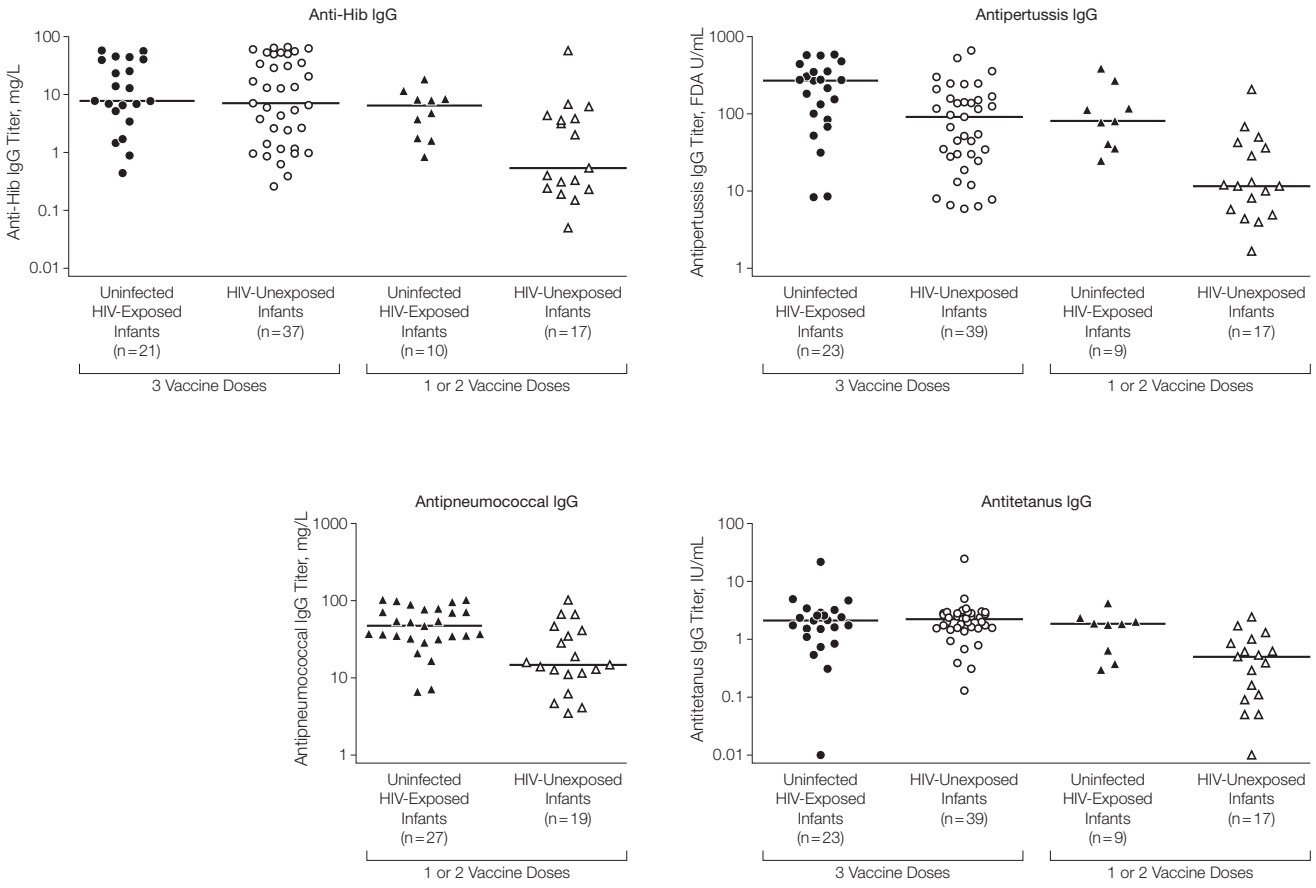
Abbreviations: HIV, human immunodeficiency virus; IQR, interquartile range.
[a] Placental transfer of antibody from mother to infant is expressed as a ratio of infant/maternal specific IgG concentration at birth.
[b] Percentage reduction in placental transfer between HIV-infected and HIV-uninfected women; calculated as the ratio of the placental transfer from HIV-infected women:placental transfer from HIV-uninfected women, subtracted from 100.
[c] Mann-Whitney U test.

0.54 [IQR, 0.24-4.10] mg/L; $P=.02$), pertussis (81.16 [IQR, 38.64-195.40] FDA U/mL vs 11.60 [IQR, 5.30-39.42] FDA U/mL; $P<.001$), and tetanus (1.86 [IQR, 0.51-2.21] IU/mL vs 0.50 [IQR, 0.10-0.93] IU/mL; $P=.01$) (Figure 2).

The fold increase in antibody level before and after vaccination was significantly higher in the HIV-exposed infants than in the HIV-unexposed infants for Hib (21.15-fold increase [IQR, 6.84-118.40] vs 2.97-fold increase [IQR, 0.71-16.69]; $P=.007$), pertussis (9.51-fold increase [IQR, 2.80-24.25] vs 2.16-fold increase [IQR, 0.41-6.84]; $P=.002$), and pneumococcus (2.06-fold increase [IQR, 0.96-5.70] vs 0.31-fold increase [IQR, 0.26-1.04]; $P<.001$). There was no difference in the fold-increase at prevaccination and postvaccination between the 2 groups for tetanus-specific responses (14-fold increase [IQR, 3.26-116.20] vs 12-fold increase [IQR, 2.8136.35]; $P=.54$).

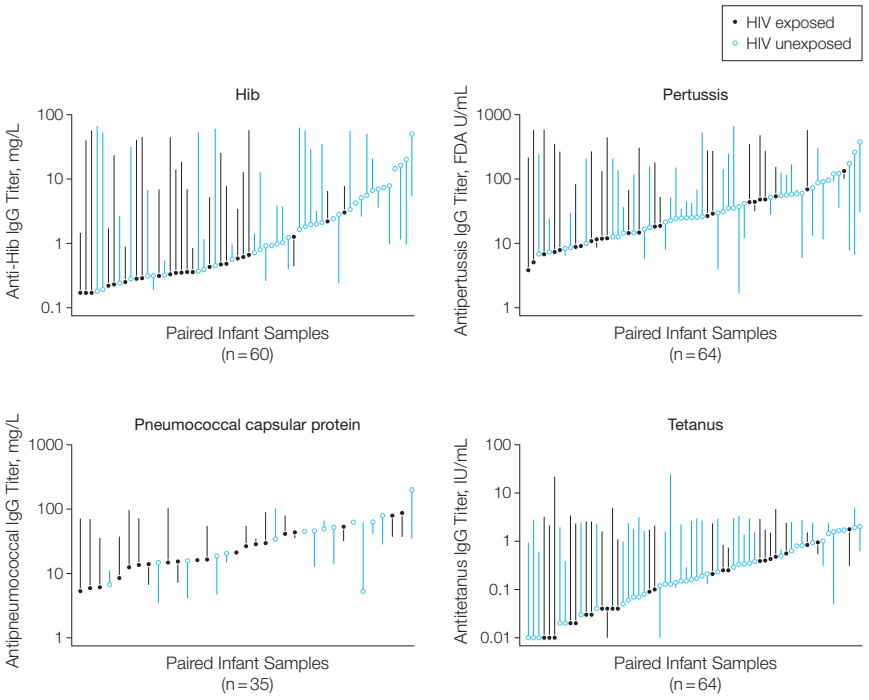
FIGURE 3 shows prevaccination and postvaccination antibody levels for individual infants. Infants with the lowest levels of anti-Hib, pertussis, pneumococcal, and tetanus-specific antibodies showed the greatest vaccine responses at 16 weeks. HIV exposure was associated with a greater magnitude of change between birth and 16 weeks.

Figure 2. Specific Antibody Titers in Uninfected HIV-Exposed and HIV-Unexposed Infants at 16 Weeks



HIV indicates human immunodeficiency virus; FDA, Food and Drug Administration. Specific antibodies to *Haemophilus influenzae* type b (Hib), *Bordetella* pertussis antigens, pneumococcus, and tetanus toxoid from serum samples collected at 16 weeks were analyzed by enzyme-linked immunosorbent assays. Three vaccine doses indicate when the vaccine schedule was complete with vaccines administered at 6, 10, and 14 weeks; and 1 or 2 vaccine doses indicate when the schedule was incomplete (except pneumococcal vaccination, for which only 2 doses were scheduled before 16 weeks). Samples were collected from 93 infants at 16 weeks (38 HIV-exposed infants and 55 HIV-unexposed infants) and antibody levels were nonpreferentially completed on available sample volume. The number of samples analyzed for each exposure group in each vaccine dose group is indicated. Horizontal lines indicate median response. For anti-Hib IgG, $P=.70$ for uninfected HIV-exposed infants vs HIV-unexposed infants at 3 vaccine doses; $P=.02$ for uninfected HIV-exposed infants vs HIV-unexposed infants at 1 or 2 vaccine doses; $P=.21$ for uninfected HIV-exposed infants at 3 vaccine doses vs 1 or 2 vaccine doses; and $P=.001$ for HIV-unexposed infants at 3 vaccine doses vs 1 or 2 vaccine doses. For antipertussis IgG, $P=.006$ for uninfected HIV-exposed infants vs HIV-unexposed infants at 3 vaccine doses; $P<.001$ for uninfected HIV-exposed infants vs HIV-unexposed infants at 1 or 2 vaccine doses; $P=.09$ for uninfected HIV-exposed infants at 3 vaccine doses vs 1 or 2 vaccine doses; and $P<.001$ for HIV-unexposed infants at 3 vaccine doses vs 1 or 2 vaccine doses. For antipneumococcal IgG, $P=.001$ for uninfected HIV-exposed infants vs HIV-unexposed infants. For antitetanus IgG, $P=.71$ for uninfected HIV-exposed infants vs HIV-unexposed infants at 3 vaccine doses; $P=.01$ for uninfected HIV-exposed infants vs HIV-unexposed infants at 1 or 2 vaccine doses; $P=.43$ for uninfected HIV-exposed infants at 3 vaccine doses vs 1 or 2 vaccine doses; and $P<.001$ for HIV-unexposed infants at 3 vaccine doses vs 1 or 2 vaccine doses.

Figure 3. Change in Specific Antibody Titers Between Birth and 16 Weeks in Uninfected HIV-Exposed and HIV-Unexposed Infants



HIV indicates human immunodeficiency virus; FDA, Food and Drug Administration. Each infant is represented by a vertical bar that starts at the specific antibody level at birth and ends in the specific antibody level at 16 weeks; the length of the bar reflects the magnitude of the vaccine response. The infants are ordered by specific antibody level at birth with specific antibodies to *Haemophilus influenzae* type b (Hib), pertussis, pneumococcal capsular protein, and tetanus. Results are shown for infants who received 3 doses of combination diphtheria, tetanus toxoid, and pertussis vaccine and Hib (DTP-Hib) or 2 doses of pneumococcal conjugate vaccine for whom paired samples were available.

COMMENT

To our knowledge, we present the most comprehensive study to date evaluating the association of maternal HIV infection and maternal-specific antibody levels and infant antibody responses to routine World Health Organization Expanded Program on Immunization vaccines. We demonstrate that HIV-exposed uninfected infants have lower specific antibody levels at birth than their non–HIV-exposed peers. Similarly, a smaller proportion of these infants have levels deemed to be protective. We show that this is due to a combination of factors: lower antibody titers to Hib and pneumococcus in HIV-infected pregnant women and reduced transplacental transfer of Hib, pertussis, pneumococcal, and tetanus-specific antibodies. Our data also highlight low levels of specific antibody in HIV-uninfected women with the consequence that half of their infants may not be sufficiently protected against Hib and pertussis early in life.

Our findings are consistent with 2 studies in HIV-infected women from Kenya, indicating that maternal HIV is associated with lower tetanus and measles–specific antibody in cord blood and also with reduced placental antibody transfer.[4,23] Maternal tetanus-specific antibody levels are lower among HIV-infected women in some studies; inconsistencies observed may be due to differences in vaccination practice during pregnancy.[4,7]

Although it is known that measles, Hib, and pneumococcal vaccine responses are reduced in children infected with HIV, there is a paucity of studies investigating the influence of infant HIV exposure (in the absence of infection) on responses to vaccines.[24-28] We observed an increased vaccine response in HIV-exposed infants to pertussis and pneumococcus compared with HIV-unexposed infants following completion of the immunization schedule. This can be explained by the lower maternally derived antibody levels at birth. Conversely, higher levels of maternal antibody among HIV-unexposed infants at birth corresponded with lower responses postvaccination. Other studies have also reported that maternal antibodies can inhibit infant response to measles, tetanus, whole cell pertussis, and Hib vaccines; this effect varies considerably between different vaccines and studies.[8,29] The mechanisms through which maternal antibodies inhibit infant responses to vaccination are not fully understood. However, a plausible explanation is that maternal antibodies mask or hide vaccine antigenic epitopes, preventing recognition and binding by infant B cells; a key determinant of infant responses appears to be the maternal antibody-to-vaccine antigen ratio.[30]

HIV-exposed infants who had missed doses of vaccine before sampling at 16 weeks had higher antibody responses than HIV-unexposed infants to Hib and tetanus, as well as pertussis and pneumococcus. An explanation for this observation is that higher maternal antibodies observed among HIV-unexposed infants may influence the response to the first dose of vaccine but not to subsequent doses. A study in Finland[31] reported a similar effect; infants with high levels of maternally derived antibody had lower anti-Hib antibody after the first dose of Hib vaccination, but not after the second dose.

A limitation of our study is enrollment at a single center with a modest number of mother-infant pairs. Sampling was however consecutive and representative of women and infants accessing care in this community setting. We did not have data on maternal vaccination history, due to limitations in recall and documentation. Vaccination records in this setting are typi-

cally available for young children only. Women in our study groups had statistically different but clinically comparable ages; therefore, similar maternal vaccination history between groups could be inferred based on the date of the introduction of the universal Expanded Program on Immunization schedule in South Africa (1973).

Although antibody levels can be used to indicate potential susceptibility to infection, some uncertainty remains regarding the functional relevance of a single so-called protective level. In addition, protective levels for collective response to multiple pneumococcal serotypes are unclear and there is a paucity of evidence for defining protective levels for other antibodies such as pertussis.[32] Functional assays may give a better assessment of the ability of the immune system to effectively clear a pathogen. Further work to address this aspect is ongoing.

We were unable to correlate antibody levels with long-term vaccine responses or clinical outcomes in the women or infants. However, our data contribute to a potential explanation for the higher morbidity and mortality observed among African HIV-exposed infants. For example, the lower observed pneumococcal-specific antibody among HIV-exposed infants before vaccination might be associated with increased severity of pneumonia observed in this group of infants.[12] Our data highlight the need for larger prospective studies to determine whether the lower antibody levels in HIV-exposed infants at birth translate into increased morbidity from vaccine-preventable infections.

Our study results also support the evaluation of novel maternal and neonatal immunization strategies to augment specific antibody responses and potentially prevent infections in infants in early life, particularly in HIV-exposed infants. In view of similar deficiencies also observed in the non–HIV-exposed group, benefits may exist for these infants too.

The implementation of vaccination programs in pregnancy, although resulting in decreased infant and maternal morbidity, is challenging because immunization in pregnancy may impair infant responses to vaccination as a result of increased maternal antibody.[21,33,34] Evaluation of pneumococcal or pertussis vaccination strategies during pregnancy, or before pregnancy, in settings with high prevalence of HIV however may benefit both mother and child.[35-39] An alternative and feasible strategy is neonatal vaccination. For example, neonatal pertussis vaccination is safe and results in early antibody responses; however, responses to Hib and hepatitis B vaccines may be affected.[40] The timing of neonatal vaccinations therefore needs to be carefully considered.[41] We recommend evaluation of both maternal and neonatal vaccination strategies, as each has merits and challenges.

In conclusion, our study describes specific antibody responses in mother-infant pairs with and without maternal HIV infection before and after infant vaccination and elucidates mechanisms for reduced responses in HIV-exposed uninfected infants early in life. A significant percentage of non–HIV-infected women also showed insufficient protection. Larger prospective studies are needed to ascertain the relationship between these observed immune responses and clinical end points. Targeted vaccination strategies may be required in HIV-infected women and their infants.

Author Contributions: Dr Jones had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs Kampmann and Hesseling contributed equally to this article.
Study concept and design: Jones, Esser, Kampmann, Hesseling.
Acquisition of data: Jones, Naidoo, De Beer, Hesseling.
Analysis and interpretation of data: Jones, Kampmann, Hesseling.
Drafting of the manuscript: Jones, Kampmann, Hesseling.
Critical revision of the manuscript for important intellectual content: Jones, Naidoo, De Beer, Esser, Kampmann, Hesseling.
Statistical analysis: Jones, Hesseling.
Obtained funding: Jones, Kampmann, Hesseling.
Administrative, technical, or material support: Jones, Naidoo, De Beer, Kampmann, Hesseling.
Study supervision: Esser, Kampmann, Hesseling.
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Mini-symposium: Childhood TB in 2010

Immunology and pathogenesis of childhood TB

Christine Jones, Elizabeth Whittaker, Alasdair Bamford, Beate Kampmann *

Academic Department of Paediatrics, 2nd Floor Wright-Fleming Institute, Imperial College London, St. Mary's Campus, Norfolk Place, London W2 1PG

EDUCATIONAL AIMS, RESEARCH DIRECTIONS

Educational Aims

- *To discuss the range of immune mechanisms involved in the control of mycobacteria*
- *To provide review of studies conducted in the human host and in children in particular*
- *To illustrate the influence of age on immune function relevant to tuberculosis*
- *To present the immune mechanisms involved as a continuum of responses rather than as strictly compartmentalised*
- *To address the interplay of both host and mycobacterial factors in eliciting immune responses*

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INTRODUCTION

Tuberculosis (TB) in children most commonly results from exposure to a household contact with active TB, and represents ongoing transmission of *Mycobacterium Tuberculosis (Mtb)* in the community.[1] Infants and young children have an increased risk of infection following exposure and progress more readily from infection to active TB disease; in the absence of intervention, infants have a 50-60% risk of disease in the first year following infection.[2,3] It could therefore be argued that the determining factor for the higher susceptibility to disease in children is prolonged, intimate contact between the child and the index case, which might lead to a larger inoculum of *Mtb*. However, there is little evidence to support this assumption, since the mycobacterial load in children is notoriously low, which lies at the root of the problem of bacteriological confirmation of primary TB. Young children more commonly present with disseminated disease and have an increased risk of death.[2] Even low bacillary loads in children can lead to acute and severe illness, be it respiratory or disseminated, especially in children younger than 2 years of age. The generally accepted assumption is therefore that qualitative and quantitative differences in the immune responses to *Mtb* infection between adults and children determine outcome. In the following review, we describe the multiple factors involved in containment of mycobacteria and review potential differences between responses in adults versus children. We have chosen to base this article primarily on studies conducted in the human host and - where available - in children. It is however obvious that crucial data on the impact of age on many of the cited factors are missing from the published literature, and we indicate where further studies would be warranted in this context.

PATHOGENESIS - A BRIEF OVERVIEW

A child in close contact with an infectious index case may inhale *Mtb* aerosolised in infected droplets. Should the bacilli be successful in traversing the physical and anatomical barriers encountered, *Mtb* bacilli are inhaled into the terminal alveoli. Once in the terminal alveoli, *Mtb* is readily phagocytosed by resident

* Corresponding author. Tel.: +44 207 594 2063; Fax: +44 207 594 3894.
E-mail addresses: christine.jones@imperial.ac.uk (C. Jones), e.whittaker@imperial.ac.uk (E. Whittaker), a.bamford@imperial.ac.uk (A. Bamford), b.kampmann@imperial.ac.uk (B. Kampmann).

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alveolar macrophages and dendritic cells. This process of internalisation results in activation of antimicrobial mechanisms, which serve to limit the growth of *Mtb* and recruit additional immune cells. Bacilli are processed and presented on the cell surface by antigen presenting cells that migrate to regional lymph nodes and present the *Mtb* antigens to T cells. Secretion of cytokines, such as IL-12, causes $CD4^+$T cells to proliferate and secrete IFNγ, which further activates macrophages to become microbicidal. The fate of *Mtb* within the macrophage leads to four possible outcomes; *Mtb* infection can either cause primary TB disease, become dormant (''latent TB'', LTBI), be eliminated or reactivate later to cause disease. Factors assigned to innate as well as acquired immune responses will determine the outcome, as well as the mycobacteria themselves. We acknowledge that the compartmentalisation of immune mechanisms into innate and adaptive immune responses is increasingly artificial, and we describe below the elements associated with both ''arms'' of this response, but also relay their close interplay.

FIRST OBSTACLES

Antimicrobial peptides, proteins and neutrophils

Following inhalation, *Mtb* is initially exposed to antimicrobial peptides (defensins, cathelicidins) and proteins (lactoferrin, lysozyme) in respiratory secretions with both bactericidal and immunomodulatory effects.[4] These peptides and proteins are produced by multiple cell types including neutrophils, monocytes, macrophages, T cells and epithelial cells.[4,5] They are present in the airways from the early neonatal period, but their relevance in paediatric tuberculosis is yet to be established and normal age-ranges for these substances do not exist.[6]

Cathelcidin can be induced by vitamin D. *Mtb* stimulates Toll-like Receptor 2 (TLR2), a pattern recognition receptor (PRR), which in turn increases the expression of vitamin D receptor and causes the conversion of vitamin D to its active form facilitating the induction of anti-mycobacterial cathelcidin.[7] The interplay between vitamin D status, TLR function and antimycobacterial peptides warrants further investigation in the context of childhood TB, particularly since clinical data show that vitamin D deficiency is very common in children with TB.[8] However, whether vitamin D deficiency is a result of TB or a contributor to susceptibility remains to be established.

Collectins

Collectins are soluble proteins which include mannose-binding lectin (MBL), surfactant protein A (SP-A) and surfactant protein D (SP-D).[9] These form a first line of defence against *Mtb* by binding to mycobacteria and thereby affecting uptake, killing and innate immune receptor expression. In particular, MBL binds *Mtb* capsular lipoarabinomannan (LAM) leading to opsonisation, complement activation and enhanced Toll-Like Receptor (TLR) signalling. Their relevance is demonstrated by increased susceptibility to *Mtb* in individuals with polymorphisms in the SP-A gene and abnormal levels of serum MBL.[10] Serum MBL peaks at 1 month of age and then decreases to adult levels by 12 years of age.[11] Low MBL has been shown to have a protective effect in Mtb infection, suggesting that age related changes in MBL levels may have an effect on patterns of disease in childhood.[12]

Complement and complement receptor

Mtb can activate complement pathway in a variety of ways and can also bind complement receptor 3 (CR3) directly thus facilitating uptake by macrophages.[13–15] Uptake via CR3 leads to a different pathway of macrophage activation compared to uptake via Fc receptors.[15] Complement components are relatively low in the neonatal period but approach adult levels by 6 months of age.[16] How these age related changes affect susceptibility to tuberculosis, or patterns of disease, is yet to be investigated.

Neutrophils

Neutrophils are abundant in the BAL fluid of adults with pulmonary tuberculosis and frequently contain mycobacteria in an active state of division.[17] They are an early feature during granuloma formation and on stimulation with mycobacterial antigens, produce a number of cytokines and chemokines, which can potentially influence adaptive immune responses.[18] The potential for neutrophil mediated mycobacterial killing is debated but has been demonstrated *in vitro* and a number of neutrophil products are mycobactericidal. In adults, the risk of latent tuberculosis infection following contact with active pulmonary TB has been shown to be inversely proportional to baseline neutrophil count.[19]

Together these observations suggest a substantial role for neutrophils in the control and/or pathogenesis of *Mtb* infection. Quantitative and functional differences in blood neutrophil populations are well recognised during the neonatal period.[20] What contribution these differences might have on the pattern of *Mtb* disease observed in children has not yet been investigated.

ANTIGEN PROCESSING AND PRESENTATION

Macrophages

Having survived these first obstacles of innate effector mechanisms, *Mtb* is engulfed by macrophages in the terminal alveoli, primarily by the binding of *Mtb* with CR3.[21] Mycobacterial cell wall products are recognized by TLR 2 and 4, resulting in activation of a cascade of signalling molecules including myeloid differentiation factor 88 (MyD88). This series of events cumulates in the activation of NFκB that ultimately triggers the synthesis of inflammatory cytokines and chemokines that elicit a specific immune response.

In addition to the recruitment of $CD4^+$ T cells by the action of IL-12 from macrophages and dendritic cells, these inflammatory cytokines recruit multiple other cell types to the locality, such as natural killer (NK) cells and γδ cells, which also secrete cytokines to activate infected macrophages (discussed further below).

Whilst children have similar numbers of alveolar macrophages by 24-48 hours of life, their function is impaired with deficient macrophage phagocytosis and recruitment during early childhood with consequences for the initiation of an antigen-specific response.[22]

Dendritic cells

Dendritic cells (DC) are highly efficient antigen presenting cells (APC). As such, they play an essential role in the initiation of the antigen-specific T cell response.[23] *Mtb* infects DCs by binding to the DC-specific C-type lectin (DC-SIGN) in addition to CR3 and mannose receptor. The process of internalizing *Mtb* results in activation and maturation of the DC as characterized by upregulation of major histocompatability complex (MHC) class II molecules, co-stimulatory molecules, CD54, CD40, and B7.1 and secretion of IL-1, IL-12 and TNF-α.[23]

Activated DCs migrate to the draining lymph nodes by a CCL19/21 dependant mechanism where they mature and present processed *Mtb* antigen on surface MHC class II to $CD4^+$T cells with the aid of co-stimulatory molecules, thereby inducing the adaptive immune response.[23]

Infants have fewer circulating DCs than adults and their functional capacity is reduced.[24] In particular, the ability of DCs to

present antigen to naïve T cells appears to be reduced until the second year of life.[22]

Once inside the lymph node, the matured DCs present MHC-peptide complexes to T cells via the T cell receptor (TCR) and, along with co-stimulatory molecules such as CD80 and CD86 and cytokines such as IL-12p70, trigger naïve T cells to proliferate and differentiate.

The capacity to produce IL-12p70 is markedly reduced at birth, and its synthesis by peripheral blood mononuclear cells continues to be impaired compared to adult levels until 12 years of age.[25,26] Whilst cord blood DC numbers are attenuated compared to DC numbers observed in adults, this alone is not sufficient to explain the marked reduction in IL-12p70 producing capacity.[26] Increased IL-10 production may play a role in the reduction in the neonatal period, however IL-10 levels measured in cell culture supernatants in childhood and adolescence are comparable to those found in adulthood.[26] The relative impairment in IL-12p70 synthesis can be overcome by the provision of DC maturational signals, GM-CSF and IL-4, suggesting that it is the immaturity of DCs which limits the synthesis of IL-12 and the initiation of a type 1 (Th1) type response rather than an intrinsic defect.[26] Other studies have further characterized this deficiency as a defect in IL-12(p35) gene expression.[25] Along with the relatively poor antigen presenting capacity of DCs, impaired IL-12 function may in turn translate into increased susceptibility to TB in children.

THE ROLE OF DIFFERENT LYMPHOCYTE POPULATIONS

CD4⁺ T cells

Both experimental and clinical evidence suggest that T cell immunity is critical for control of *Mtb* infection, in particular $CD4^+$ T cells. *Mtb*-specific $CD4^+$ T cells primarily produce Th1 cytokines, which include IFNγ, IL-2 and TNFα. Studies of human immune deficiencies associated with disseminated mycobacterial disease reveal that IFNγ is critical for optimal activation of macrophages and hence for protection against TB.[27,28] A group of disorders termed 'Mendelian Susceptibility to Mycobacterial Disease' (MSMD) all affect Th1 cytokines, i.e. the IFNγ/IL-12 pathway, are associated with severe disease caused by mycobacteria, and often manifest in childhood.

Conditions in which $CD4^+$ cells are depleted, such as HIV, are recognised to lead to increased susceptibility to TB infection and severe TB disease, but IFNγ is not an absolute correlate of protection.[29] Although immune reconstitution with anti-retroviral medication leads to normalization of $CD4^+$ numbers in HIV-infected individuals, this is not associated with significant increases in production of IFNγ in response to mycobacteria.[30] Although children have higher natural levels of $CD4^+$ T cells, these cells do not confer equal protection compared with adults, if measured by antigen specific production of IFNγ.[26] Healthy adults with LTBI demonstrate strong *Mtb*-specific IFNγ responses in comparison to adults with active TB, but children have poorer responses, particularly when suffering from disseminated disease.[31,32] Disseminated forms of TB such as tuberculous meningitis (TBM) or miliary TB have also been associated with weaker *Mtb*-specific IFNγ responses than pulmonary TB in children. However, it is not clear if this is causal or as a result of disease.

Neonatal $CD4^+$ cells exhibit reduced capacity to express Th1-effector function, partly attributed to hypermethylation of the proximal promoter of the IFNγ gene.[33] This results in a highly restricted pattern of IFNγ response to a variety of stimuli. A type 2 (Th2) response with production of IL-4, IL-10 and IL-5 appears to predominate in the neonatal period as demonstrated by antigen-specific T cell responses to diphtheria-tetanus-acellular pertussis vaccine and studies of children with congenitally acquired CMV and Herpes infection.[34] However, BCG vaccination is able to induce a robust Th1 type response already at birth.[35]

The production of IFNγ by antigen-specific T cells as a diagnostic tool has been exploited in the development of the interferon gamma release assays (IGRA), which are discussed in more detail elsewhere in this review series. Briefly, these may not be as useful in children compared to adults, possibly due to the age-related differences discussed here. In the early stages of primary TB, the pool of antigen-specific effector T cells, the read-out for the IGRA, is only beginning to be established, unlike during reactivation disease seen in adults, where an existing effector memory pool simply requires to be resurrected.[36]

While it is clear that $CD4^+$ T cells and Th1 cytokines are critical in the cell-mediated response to *Mtb*, it is also apparent that this part of the immune response alone is not enough. Due to the availability of better laboratory assays that allow the simultaneous measurements of T cell populations and cytokines in small blood samples, the role of other T cell subsets, cytokines and chemokines are now beginning to be better defined.

CD8⁺ T cells

While both $CD4^+$ and $CD8^+$ T cells produce cytokines that activate macrophages and lead to granuloma formation (IFNγ/TNFα), $CD8^+$ T cells also have a directly cytotoxic effect and express microbicidal perforins and granulysins.[37] Granulysins are produced by $CD8^+$ T cells, NK cells and γδ T cells and can kill extra-and intracellular mycobacteria, the latter in conjunction with perforin. *Mtb* specific $CD8^+$ T cells are expanded in adults with tuberculosis, however little is known of their role in children. The limited paediatric studies of $CD8^+$ T cells to date suggest that specific $CD8^+$ T cell responses may be limited in children compared with adults. For example HIV-specific $CD8^+$ T cell responses are infrequently detected in infants under 1yr.[38] One small study of 16 children demonstrated increased proportions of $CD8^+$ T cells in children with active TB but these clones were $CD8^+CD45RA^+CCR7^-$ (naïve) T cells with weak IFNγ secretion.[39] Further studies are necessary to elucidate the importance of the $CD8^+$ T cells in the paediatric immune response to TB.

Polyfunctional T cells have been associated with more effective control of murine intracellular infections including *Mtb*.[40] Recent studies have identified polyfunctional T cells in patients with TB and as part of induced responses to novel TB vaccine antigens, but further studies regarding the mechanism of induction of these polyfunctional T cells and their role as a correlate of protective immunity are required.[41,42] Whether age-related differences in these T cell populations might explain some of the increased susceptibility of younger children to TB remains to be established.

BRIDGING INNATE AND ADAPTIVE IMMUNITY

γδ, *Th17 and regulatory T cells (Tregs)*

γδ T cells are a further source of IFNγ and granulysins as part of the immune response to TB.[43] While $CD4^+$/$CD8^+$T cells recognise mycobacterial peptides in the context of the MHC Class I or II, γδ T cells recognise non-proteinaceous antigens. $V\delta2^+$ subset of γδ T cells are the dominant γδ T cell subset in healthy adults and constitute a link between innate and adaptive immunity to *Mtb*, responding rapidly by producing cytokines without extensive requirements for antigen processing and presentation. $V\delta2^+$ cell dysfunction and anergy have been described in tuberculosis, and adult studies have shown decreased function in patients with TB versus healthy controls as measured by mycobacterial antigen-specific production of IFNγ.[44] Studies in children demonstrated an increased proliferation of γδ T cells in TB cases compared to

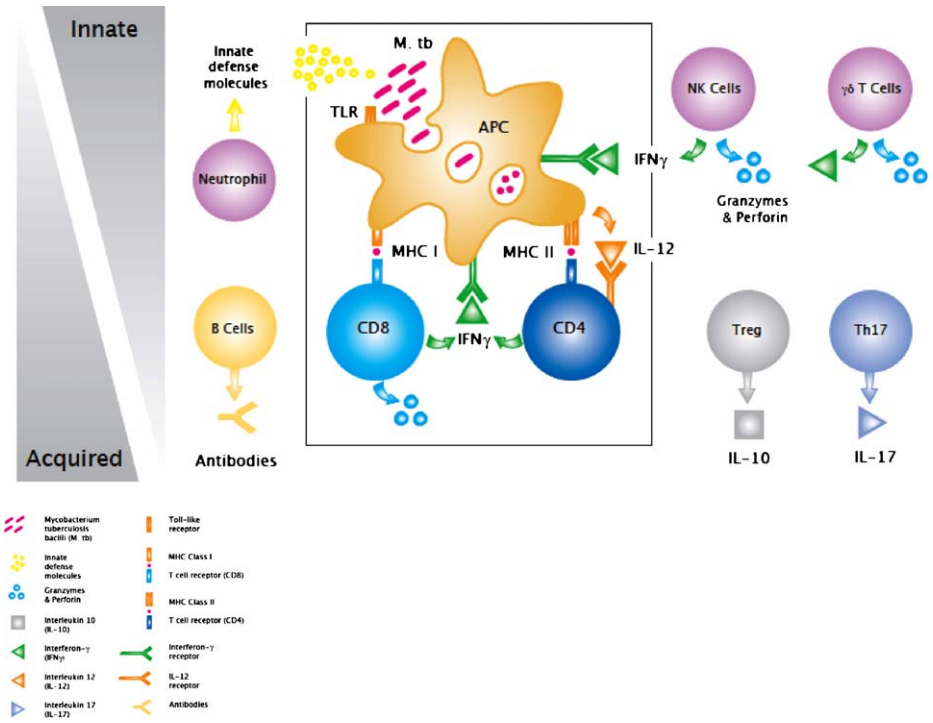

Figure 1. This figure illustrates the key components of innate and adaptive immune mechanisms in the response to Mycobacterium tuberculosis (Mtb)
Innate defense molecules in the airways facilitate the phagocytosis of Mtb by macrophages and dendritic cells (antigen presenting cells, APCs) and Toll-like receptor (TLR) signaling. Mtb is processed within the APC and presented to CD4 cells in regional lymph nodes on major histocompatability complex (MHC) class II molecules. IL-12 is secreted by APCs, which causes CD4 cells to proliferate and produce IFNγ. IFNγ, produced by CD4 cells, CD8 cells, NK cells and γδ cells activates the APC to become microbicidal. Other molecules such as perforins and granzymes, produced by CD8 cells, NK cells and γδ cells, also facilitate destruction of Mtb bacilli. T regulatory (Treg) cells, Th17 cells and B cells act to modulate the immune response to Mtb.
(Illustration © Hugh Gifford 2010).

healthy children, however this was also associated with decreased production of IFNγ and granulysin.[43]

Recent adult and murine studies have demonstrated that γδ T cells are also major producers of IL-17, in response to *Mtb*.[45] IL-17 is a potent neutrophil recruiting agent and is responsible for much of the inflammatory damage previously ascribed to the Th1 cytokine response. Recent studies have identified a distinct $CD4^+$ T cell subset, Th17, which produces IL-17 in response to mycobacterial antigens and participates in the protective immunity against *Mtb*.[46] Initial studies of adults with active TB disease compared to healthy donors, show reduced *Mtb*-specific Th17 response, possibly due to suppression by Th1 cytokines.[47] IL-17 has not been studied in the context of TB in children, but is found to be increased in children with chronic inflammation, including inflammatory bowel disease and juvenile idiopathic arthritis.[48]

Th17 cells develop from naïve $CD4^+$ T cell precursors in the presence of TGFβ and IL6. In the absence of IL6, TGFβ stimulates the development of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg).[49] Tregs are immune modulators that produce IL-10 and TGFβ, both known to suppress Th1 and Th17 responses. In adult patients, Tregs are expanded in blood and disease sites.[50] Their role in paediatric TB has not yet been fully elucidated, and further studies are ongoing.

Natural Killer cells

A further cell group linking innate and adaptive immune responses, like γδ T cells, are Natural Killer [NK] cells. The vast majority are cytotoxic and produce granulysin to lyse cells, and the remaining 5-10% are IFNγ producing. Recent evidence shows that activated NK cells reduce Treg expansion by direct lysis of MTB-specific Treg cells, favouring Th1 responses.[51] In adults with TB disease however, decreased NK cell activity and increased Treg numbers have been noted.[52] Paediatric studies measuring granulysin have identified decreased production in children with active TB disease. These levels returned to normal after chemotherapy.[53]

B cells and antibody

B cells and antibody have long been considered to be of secondary importance in *Mtb* immunology, but it is now recognised that B cells may well have a general immunomodulatory role through antigen presentation, co-stimulation and cytokine production [Figure 1]. Sub-groups of B cells with effector and regulatory function- analogous to T cells- have been postulated, although not yet in the context of TB.[54] Antibody isotype and the type of FcR involved in an immune response both affect patterns of T cell activation/inhibition and potentially influence disease outcome.[55] There are well known deficiencies in the antibody responses to T cell-independent antigens in children under 2 years of age, but whether this plays a role in relation to antibody-responses to mycobacterial antigens and the pattern of disease in childhood is yet to be explored.[56] Whether there is a place for antibody-profiling in immunodiagnostics in children remains to be established.

EVASION of the immune response- The mycobacteria matter too

Mtb and the human host have co-evolved over thousands of years. It is therefore unlikely that the outcome of mycobacterial infection is solely determined by the host response. *Mtb* has developed several strategies to evade mycobacterial control by macrophages, although few of these mechanisms have been explored in children in particular.

A described mechanism is evasion of autophagy whereby *Mtb* is able to reduce the fusion of infected phagosomes with lysosomes thereby avoiding destruction by lysosomal hydrolyases and enabling the pathogen to survive within the infected cell.[57]. The mechanisms by which activated macrophages act locally to inhibit intracellular growth of *Mtb* are as yet incompletely understood (reviewed in [58]). *Mtb* is also able to inhibit antigen presentation to CD4+ T-cells through inhibition of MHC II expression thereby subverting induction of the adaptive response and allowing it to reside within macrophages within granulomas.[59] Furthermore, suppression of T cell function via the IL-6 receptor has recently been described.[60] Interesting differences between a variety of strains of *Mtb* to induce - or downregulate- key cytokine profiles have been described.[61]

Summary notes

The increased risk of active TB, especially of disseminated disease probably results from differences in immune responses between children and adults at several levels: deficiencies in macrophages and DCs delay the recruitment of antigen-specific T cells to primary and secondary sites of infection. Less efficient antigen presentation in the lymph nodes by DCs may result in decreased production of key cytokines, which in turn limits the activation of macrophages. This may allow the organism to proliferate to a greater extent and overwhelm the primary lung immune responses. What mechanisms in detail might then lead to dissemination of disease from the lung to other organs remains to be fully established.

The described differences in immune profiles between adults and children and between children of different ages are likely to have implications for the design and use of immunodiagnostic tests for TB. More detailed studies of all components of the immune response and their relationship to age will need to inform the development of novel assays suitable for children.

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All authors declare that we have no conflict of interest.

RESEARCH DIRECTIONS

Childhood Tuberculosis

To overcome the limitations of our knowledge with regards to different immune mechanisms in tuberculosis, we require

- *To evaluate the impact of age on immune mechanisms deemed to be important for control of mycobacteria*
- *To establish normal ranges for immune parameters in healthy children of different ages (reference values)*
- *To adapt laboratory assays for use in children*
- *To include children of different ages in all aspects of tuberculosis research*
- *To evaluate primary TB in children as a model to study mechanisms of mycobacterial control or dissemination*

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Invited article

Bridging the gap: maternal immunisation as a means to reduce neonatal deaths from infectious diseases

Ben Lindsey[1], Christine Jones[1], Beate Kampmann[1,2]

[1]Academic Department of Paediatrics, Imperial College London, UK, [2]MRC Unit, The Gambia

Introduction

Although significant progress towards Millennium Goals 4 and 5 has been recorded, neonatal mortality remains a global challenge. There were an estimated 3.3 million neonatal deaths worldwide in 2009, accounting for a significant proportion of under-5-year mortality. Progress in this age group is now most urgently required.[1] Infectious diseases are the major cause of neonatal deaths and some can be prevented by vaccination, which is accepted as one of the most successful and cost-effective health interventions. However, to achieve full protection against infections requires multiple doses of vaccines given over several months in the first year of life.

During this particularly vulnerable period of early infancy, newborns remain partially protected through transfer of immunoglobulins from the mother, which carry specific antibodies against infections or vaccine antigens that the mother had previously encountered. However, maternal levels of such specific immunoglobulin (IgG) are frequently sub-optimal.[2] Maternal immunisation represents a strategy that could be employed to 'bridge the gap' in protection: the aim is to enhance the antibody levels against a particular infectious disease by giving the vaccine to the pregnant woman, who will then transmit a protective level of antibody to her infant *in utero* and through breastmilk after birth. Multiple factors can affect the transfer of IgG across the placenta, including maternal IgG concentration, the IgG subtype, gestational age and maternal co-infections, such as HIV.[3] These factors, among others, also determine the level of immunity that an infant can obtain from a maternal vaccine.

Successful examples of achieving infant protection through maternal immunisation are maternal vaccination against tetanus, which has been given in pregnancy for many years, and influenza and pertussis vaccines, which are now being recommended for use in pregnancy in some countries. Maternal tetanus vaccination has been successful in reducing the burden of neonatal tetanus deaths from 787 000 in 1988 to an estimated 59 000 in 2008.[4] This has set the precedent for future vaccine strategies and has proven the concept that maternal vaccines can be effectively delivered in low income countries. However, maternal vaccination could be used more widely to reduce the large global health problem of neonatal death.

Potential disadvantages

An important issue with maternal vaccination relates to potential inhibitory effects on an infant's future response to vaccination. This has been observed with some vaccines including those for measles, tetanus and diphtheria and has shaped the current EPI schedule to some degree. The level of inhibition of the infant response varies depending on the vaccine in question and time intervals between different vaccine doses, with some preventing any antibody response, some causing only a slight reduction with antibody titres remaining above a protective threshold, and others showing no noticeable inhibition.

Other major considerations and concerns, and also myths, regarding maternal vaccines relate to the safety for the foetus. Live attenuated vaccines are contraindicated for use in pregnancy because of the theoretical risk to the foetus. However, there has been no established link between vaccines in pregnancy and serious adverse events.

Which other vaccines might be useful?

In addition to tetanus, the influenza vaccine is recommended for use in pregnancy by the World Health Organization in order to protect against the high levels of morbidity and mortality in young children and pregnant women seen in the context of flu epidemics. Current uptake of these recommendations has, however, been poor and indicates the existence of barriers to the use of maternal vaccination in the future, which need to be further explored

Correspondence to: Beate Kampmann, Department of Medicine, Imperial College London, UK. Email: b.kampmann@imperial.ac.uk

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to make maternal immunisation strategies more widely acceptable.

Another likely candidate for maternal immunisation that could reduce the burden of neonatal mortality is the pertussis vaccine. This preventable infection is responsible for a large number of infant deaths, the majority of which occur in the first few months of life, a period before the current vaccination schedule offers adequate protection. It has yet to be shown whether maternal pertussis vaccination could reduce infant infection and mortality, however a strong suggestion of benefit can be inferred from existing data.[5] Following a significant rise in national cases of pertussis in recent years and review of safety data, the Centers for Disease Control in the USA now recommend the use of the pertussis vaccine in pregnancy.

Vaccination against group B streptococcus would represent a further opportunity. Currently, this serious infection in neonates can only be partially prevented by intra-partum prophylactic antibiotics, which do not protect against all forms of the disease and divergent recommendations exist for their use and screening of pregnant women in different countries. There is currently no licensed vaccine against group B streptococcus but vaccines are being developed actively with the aim of giving these to pregnant women in the future.

What are the obstacles?

Implementation of maternal vaccination has been particularly poor in resource-rich countries. For example, although recommended officially, the uptake of maternal influenza vaccination in the UK was only 38% in 2010.[6] Reasons for this poor uptake are multifactorial and include lack of encouragement by health care workers, refusal by mothers and some practical barriers. Multiple studies have shown that healthcare workers often have incorrect knowledge regarding maternal immunisation and do not offer the vaccines. Reasons frequently given by mothers for refusing a maternal vaccine are safety concerns or that they do not feel that the vaccine is necessary. Poor uptake of current recommendations is a serious concern for the successful implementation of future maternal vaccine programmes and research into understanding and overcoming the perceived obstacles will be important.

Detailed site-specific and cultural assessments are needed to fully appreciate the feasibility of, and barriers to, implementation of maternal vaccination programmes, particularly in low and middle income countries (LMIC) where this intervention is likely to have the greatest effect.

However, the delivery systems are largely in place, with the majority of pregnant women attending at least one antenatal clinic in 24 of 28 African countries surveyed.[7] There are a number of characteristics which suggest that maternal immunisation is suitable for LMICs, such as the high proportion of mothers who breast feed and the proven acceptability of maternal vaccines among women, according to current maternal and neonatal tetanus programmes.[7] Other barriers to implementation in many of these countries including limited funding and the high prevalence of HIV and malaria which both limit the effectiveness of maternal immunisation.

Conclusion

Maternal vaccination can protect the young infant against vaccine-preventable infections in the first months of life. This strategy is already widely implemented with tetanus and influenza vaccines and many other vaccines could be used in the future, particularly those for pertussis and group B streptococcus. However, the gap between recommendation and implementation will have to be successfully bridged if maternal vaccination is to reach its full impact and prevent neonatal deaths.

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