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Approval Sheet

The Impact of Immune Activation on Infant BCG Vaccine Response

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Abstract Cover Page

The Impact of Immune Activation on Infant BCG Vaccine Response

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Williams College
2015

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An abstract of
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Master of Public Health
in Hubert Department of Global Health
2018

ABSTRACT

The Impact of Immune Activation on Infant BCG Vaccine Response

By: Naomi Patterson

BACKGROUND:

Tuberculosis (TB) is a major cause of morbidity and mortality in children. Bacillus Calmette Guérin (BCG) is the only vaccine currently available to prevent TB, but limited vaccine efficacy has not curbed the TB epidemic. Immune activation has been linked with decreased immune responses to live vaccines. However, the relationship between immune activation and BCG response is unknown. Using an existing cohort of Kenyan human immunodeficiency virus (HIV)-uninfected mothers and their infants, we aim to determine how immune activation among mothers and infants at birth relates to infant BCG vaccine response at 10 weeks of age.

METHODS:

Pregnant women were enrolled at Kisumu County Hospital, Kisumu, Kenya, and mother-infant pairs were followed for 10 weeks from January through December 2014. Socio-demographic and clinical data were collected from mothers upon enrollment. Whole blood samples were collected from mothers and infants at delivery and stimulated in vitro with BCG or un-stimulated for 12 hours and then cryopreserved. Later, flow cytometry was used to count and type T lymphocyte cells as CD4+, CD8+, and/or KI67+. We assessed Pearson's correlations and performed multivariable linear regression to evaluate the relationship between infant immune activation and infant BCG vaccine response at 10 weeks.

RESULTS AND DISCUSSION:

Of 395 mother-infant pairs enrolled, 46 mothers and infants had samples collected to evaluate for immune activation at delivery. Infant immune activation at birth was associated with maternal immune activation at delivery ($r=0.357$, $p= 0.015$). Maternal number of doses of sulfadoxine-pyrimethamine (SP) for malaria intermittent preventive therapy (IPT) was associated with lower infant immune activation at birth ($r=-0.323$, $p= 0.029$) and a trend toward higher BCG vaccine response at 10 weeks of life ($r=0.256$, $p=0.086$). Maternal de-worming with mebendazole or the number of tetanus boosters during pregnancy, were not associated with infant immune activation at birth. After adjusting for the number of antimalarial IPT doses, infant immune activation was not associated with BCG vaccine response at 10 weeks of life.

CONCLUSION:

We found that infant immune activation detected by the frequency of KI67+CD4+ T cells was not associated with BCG vaccine response. Maternal malaria preventive therapy was associated with infant immune activation.

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DEFINITION OF TERMS

Term	**Definition**
Mycobacterium tuberculosis	The bacterium that causes tuberculosis.
MDR-TB	Multiple drug resistant tuberculosis
Bacille-Calmette-Guérin (BCG)	Vaccine against severe TB that is given to children at birth in several countries.
Innate Immunity	Responds to all infections in a non-specific way
Adaptive Immunity	Can recognize and specifically respond to a particular antigen.
Immune Activation (I.A.)	% KI67+ CD4+ and CD8+ T cells.
CD8+ T cells	Cytotoxic T lymphocyte cells
CD4+ T cells	"Helper" T lymphocyte cells that interact with and regulate the immune system.
TH	Th is a marker to distinguish types of CD4 cells. The Th1 designation indicates that the particular T cell secretes interferon-gamma (IFNγ) to recruit CD8 cells as opposed to Th2 that recruits innate immune cells. (1)
KI67	A marker that is expressed on most cells during cell growth and proliferation, and common in cancer research. This marker is used as a proxy for active status of immune cells.
Infant BCG Response	% BCG-stimulated Th1 cytokine positive CD4 cells at 10 weeks of life

CHAPTER 1- Introduction

The Problem: Tuberculosis

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* and is a pervasive public health concern. In 2015, there were an estimated 10.4 million new TB cases, including 1.2 million HIV-infected people, and 1.0 million children (2). TB disease typically affects the lungs (pulmonary TB) but can also affect other sites in the body (extra-pulmonary TB) and is either active (contagious) or latent (dormant). In latent TB, an infected person shows no outward signs of disease. TB transmission occurs when an individual with active pulmonary TB expels bacteria into the air (by coughing or sneezing), and susceptible individuals breathe in the bacteria contained in small airborne droplets. The clinical manifestations of active TB include the common symptoms of cough, fatigue, weight loss, night sweats and fever (3). Of adults with latent TB, approximately 5-10% will develop active TB during their lifetime (2, 4); the risk of progression from latent to active TB is higher in young children or among people infected with human immunodeficiency virus (HIV) (2, 3, 5).

There are several bacteria in the genus *Mycobacterium* that cause diseases that are clinically indistinguishable from tuberculosis and therefore complicate diagnosis and treatment of TB. Tuberculin skin testing (TST) and blood tests can detect latent TB and become positive 2-6 weeks post infection (3). Active TB diagnosis requires that sputum or other clinical specimens be inspected microscopically for acid-fast bacilli (AFB) and cultured for the identification of *M. tuberculosis*, or tested with rapid diagnostic methods such as Xpert® MTB/RIF (3, 6). Therefore diagnosing TB and any type of drug resistance may be a time-consuming and labor-intensive clinical process. To further complicate

matters, sputum smears of people with TB disease and TST are often negative in HIV positive individuals and respiratory samples are more difficult to obtain in children <5 years of age (7).

Treatment and Clinical Outcomes

The death rate from untreated TB is high. Before anti-TB drug development (1944), about 70% of people with sputum smear-positive pulmonary TB died within 10 years, as did about 20% of people with culture-positive (but smear-negative) pulmonary TB (2, 3). The most common treatment against drug-susceptible pulmonary TB is a 6-month regimen of four antibacterial drugs: isoniazid, rifampicin, ethambutol and pyrazinamide (2). The treatment for TB that is resistant to one or multiple drugs involves a more protracted timeline with drugs that are also more expensive and toxic (2, 3).

Prevention

There has been a renewed effort to control tuberculosis worldwide through the United Nations' Sustainable Development Goal (SDGs). The End TB strategy within the SDG, aims for 90% reduction in TB related deaths and 80% reduction in TB incidence by the year 2030, compared with 2015 (2). The ongoing crisis of antimicrobial resistance, specifically multidrug resistant TB (MDR-TB), poses a great challenge to the End TB Strategy. In this context, prevention of initial infection is more important than ever. There is currently a vaccine available to combat TB, the live attenuated Bacille Calmette-Guérin (BCG) vaccine, which is recommended by WHO for administration at birth to all infants in TB endemic countries. Kenya is one of the top 10 countries with the highest proportion of

TB, including both a high burden of HIV-TB co-infection and MDR-TB (2). While the BCG vaccine is widely used to prevent severe complications in children, vaccine coverage in Kenya is relatively low, less than 80% (8).

Tuberculosis is a significant burden on global health systems, especially in HIV and MDR TB endemic areas. As of 2016, HIV/TB is the second leading cause of disability adjusted life years (DALYs) in Kenya, according to The Institute for Health Metrics and Evaluation (IHME) (9). This health burden contributes to social and economic challenges that can inhibit social mobility or make moving out of poverty very difficult. This is where a concerted public health effort involving a more protective vaccine can have a significant impact.

The Problem: BCG vaccine

BCG vaccine was developed nearly 100 years ago and is the most widely administered vaccine worldwide, but has limited efficacy (10). BCG vaccine is only given to children at birth, and while it does protect children from severe TB, vaccination is not sufficient to prevent TB disease after exposure, especially in adults. There are several factors that affect the immunogenicity of this vaccine, including vaccine strain, geographic location, and environmental mycobacteria exposure (10). There are currently several alternate, and presumably more effective, TB vaccines in development, however, a new vaccine is not expected in the near future (2). The quality of BCG is explored in greater detail in Chapter 2.

TB Immunity and Immune Activation

The body's response to *M. tuberculosis* infection involves both the innate and adaptive immune system. CD4+ Th1+ T lymphocytes (heretofore referred to as T cells) recruit other immune cells, including cytotoxic CD8+ T cells to help fight the infection (11). There is not a standard immunologic correlate of protection, as antibody levels and other commonly used measures of immune activation are not indicative of an effective immune response to, or protection from tuberculosis (12). For the purposes of this work, we are using the expression of Th1 cytokines (interferon gamma, TNF-alpha, IL-2) by CD4+ T cells stimulated with BCG to indicate BCG vaccine immune response (13-18).

Previously published evidence suggests that immune activation prior to vaccination leads to a lowered vaccine response, but the direct effects on the BCG vaccine response has not been evaluated (19). However, a recent study demonstrated that infants with increased T cell activation were at higher risk for TB disease (11). Therefore, we hypothesize that existing T cell immune activation (KI67+ CD4+ cells) at birth will decrease BCG vaccine immunogenicity (% BCG stimulated Th1+ CD4+ cells) at 10 weeks of life compared to infants without immune activation.

Problem Statement

While the BCG vaccine has been on the market and used for several years, it has variable efficacy. Immune activation is associated with decreased vaccine efficacy in other live vaccines, but the effect of immune activation on BCG immunity has not been defined. A better understanding of this interaction can inform scientific knowledge on the BCG

vaccine effectiveness, especially in TB endemic countries where pre-existing immune activation is expected.

Purpose Statement

We aim to evaluate whether immune activation affects BCG vaccine immune response. More information on how immune activation at birth relates to long-term immune response to the BCG vaccine will contribute to existing knowledge to inform TB vaccine development.

Research Question

1 - How does immune activation in infant cord blood (at birth) relate to infant BCG response at 10 weeks?

2 - What factors associate with infant immune activation at birth?

3 - What factors associate with immune activation at 10 weeks of age?

4 - How does immune activation in infant cord blood affect infant immune activation at 10 weeks?

Significance Statement

The improved knowledge is important in this context because there are several countries with high TB incidence and therefore this study may lead to useful additional information. Having a better understanding of the relationship between immune activation and BCG response can direct research and could potentially inform immunization research and policy. For example, if our hypothesis is correct and BCG response is greatly reduced in

infants with immune activation, this may inform the addition of booster doses so that immunized infants could potentially have more similar responses. Furthermore, a better understanding of BCG vaccine limitations could better inform features to be addressed during the development of new TB vaccines.

CHAPTER 2 - Literature Review

TB is an infectious disease that is endemic in several countries worldwide. TB is caused by the bacterium *Mycobacterium tuberculosis* and is spread through respiratory droplets. The BCG vaccine is the only available vaccine to prevent TB and is administered to infants at birth in most TB-endemic countries. However, BCG vaccine prevents only severe TB in children and has limited long-term efficacy (14, 18, 20-26). In addition, there is large variability in BCG vaccine efficacy, which is not well understood and may be affected by host genetics, geographic location, and concurrent infections (11). We aim to evaluate T cell activation in infants prior to BCG vaccine, and assess whether T cell activation at birth may reduce BCG effectiveness at 10 weeks in the TB endemic country of Kenya (27). Here we review what is known about immune activation in general, BCG effectiveness as well as the interplay between immune activation and vaccine responses.

How Vaccines Work: The Immunological Perspective

In order for vaccines to protect against a disease, there needs to be a robust immune response. When an individual is exposed naturally to a pathogen that is potentially disease causing, both the innate and adaptive immune systems are engaged to prevent infection or fight the pathogen if infection does occur, with innate immunity occurring first and adaptive immunity coming second (Figure 1). A vaccine's role is to prime the body with an altered or weakened version of a pathogen in order for the adaptive immunity to enact a faster and stronger response in the future, preventing the onset of symptoms. Innate immunity relies on non-specific inflammatory and cellular responses to set the stage for

the subsequent adaptive immune response. Adaptive immunity involves the proliferation of B cells that can produce pathogen specific antibodies, as well as the proliferation of antigen-specific CD4+ and CD8+ T cells (Figures 2, 3).

CD8+ T cells are cytotoxic and can directly kill cells, most useful against viruses, and CD4+ T cells are often referred to as helper T cells that can recruit other immune cells to make up a robust response. Vaccines are meant to expose individuals to pathogens and induce long-term memory and therefore protection without causing disease (1). Therefore vaccines, if made and administered properly, are a safe way to protect people from the morbidity and mortality of some known pathogens.

CD4+ T cells are often the first white blood cell activated in the adaptive immune response. $CD4^+$ cells act by secreting cytokines that are able to directly affect the behavior of other immune cells. Generally, $CD4^+$cells help other immune cells with their tasks and are referred to as helper T cells (1).

CD8+ T cells on the other hand have the ability to secrete cytotoxic factors to kill pathogens, or infected cells directly in addition to cytokines (1). This cytotoxicity allows the immune system to control the spread of viruses before they are able to infect more of the body (1). Therefore the concerted effort of B and T cells create a robust immune response to the current infection as well as to create memory B and T cells to protect the human body in the event of future infection, however this robust response can take several days to weeks to occur naturally and an individual may feel the ill effects of an infection.

Assessing Immune Activation

There are several markers to detect immune activation. HLA-DR is a protein that is expressed on the surface of major histocompatibility complex (MHC) that is only present in immune cells while an immune response is being stimulated (28). HLA-DR is commonly used to assess immune activation. For example, in a case-control study of the relationship between immune activation and risk of developing TB, immune activation was measured by the percentage of HLA-DR+ CD4+ T cells (14). The authors found that immune activation relates to an increased risk of TB transmission (odds ratio of 1.12 per 1 unit increase, P=0.002) (14). In addition to HLA-DR, other markers include immune proteins like CD69, CD25, antibodies and KI67 (29).

In this study context, we are using KI67 as the marker of immune activation. The strength of measuring KI67 is that it is a protein that is expressed during cell growth, and KI67 expression in T cells is an indicator of T cell growth and therefore T cell proliferation (30). However, a limitation of its use is that it is only expressed in cells that are actively dividing and therefore KI67 is likely to miss active T cells that are not dividing at the time of sample collection. Therefore by looking at CD4+ and CD8+ cells that also express KI67, both stimulated by BCG and un-stimulated, we are able to quantify immune activation and determine if said activation is related to the response of the BCG vaccine.

Immune Activation and TB Response

The body's response to infection by *M. tuberculosis* involves both innate and adaptive immunity, mediated by T cells as detailed above. CD4 Th1+ T cells recruit other immune cells, including cytotoxic CD8 T cells to help fight the infection (11). This

recruitment of the immune system to respond to an infection, specifically the replication of immune cells, is the immune activation process that we are interested in studying. Furthermore, it has been shown in several studies of TB and the subsequent immune response, that CD4+ T cells play a leading role in the fight against TB while CD8+ T cells and regulatory T cells may play important supporting roles (14, 17).

Additionally, a matched case-control study gave credence to the hypothesis that immune activation increases the risk of TB disease. In this study, infants in the Western Cape of South Africa who were HIV negative, TB negative, and given BCG at birth, were randomized to receive an experimental TB vaccine or placebo at 4-6 months of age (14). Both arms were later pooled when looking for cases and controls. Fletcher et al. (14) found that where there was immune activation (HLA-DR$^+$ CD4$^+$ T cells), there was an associated increase in TB disease risk (OR=1.828, 95% CI=1.25–2.68, *P*=0.002) (14). A recent study by Comas et al. (11), additionally demonstrated that infants with increased T cell activation were at higher risk for TB disease.

HIV status is an important factor when considering the immune response to TB and the BCG vaccine, as HIV infection induces immunosuppression and is a leading risk factor for developing TB, especially in settings where TB and HIV co-exist (Figure 4, 5). There is evidence that HIV infection impairs the body's ability to fight against latent TB and prevent re-activation or disease progression (31). A case-control study conducted in Hospital Pediátrico David Bernardino (HPDB) in Angola showed that BCG vaccine provided no protection from TB disease in HIV positive children (27, 32). As HIV infection is a serious comorbidity for TB, BCG vaccination also showed reduced efficacy for infants who were exposed to, but not infected with, HIV (27). Furthermore, HIV infection at the time of BCG

vaccination led to a higher proportion of BCG related adverse events, increased and chronic immune activation and IFN-γ that makes it more difficult to fight infections generally and TB specifically (27). HIV infected individuals tended to have more *M. tuberculosis* related CD4+ T cell death, suggesting a potentially dampened ability of *M. tuberculosis*-specific CD4+ T cells to proliferate (16).

Exploration of BCG Effectiveness

While an immune correlate of protection for tuberculosis has not been identified, measuring T cell response to BCG vaccine in vitro can approximate BCG vaccine immunogenicity. BCG effectiveness can also be estimated by TB disease outcomes in BCG-vaccinated individuals. A good immune response, involving the recruitment of the immune system, proliferation of T and B cells to recognize vaccine, infers good protection from TB transmission and generally leads to less incidence of TB in the vaccinated cohort.

In "Protection by BCG Vaccine Against Tuberculosis: A Systematic Review of Randomized Controlled Trials," P. Mangtani and colleagues (22), evaluated 211 relevant articles from 10 scientific databases. They searched between the establishment date of the database and May-October 2009 in order to establish what is currently known about the efficacy of the BCG vaccine against TB infections. For each paper, the rate ratio (RR) was estimated comparing incidence of TB between vaccinated and unvaccinated cohorts, and the vaccine efficacy(VE), where VE= 1-RR was calculated. This review showed that the "efficacy of BCG against pulmonary tuberculosis ranged from substantial protection, in the UK MRC trial (33) (RR 0.22; 95% confidence interval [CI], 0.16–0.31), to absence of clinically important benefit, in the Chingleput trial (RR, 1.05; CI: 0.88–1.25)" (22).

A case-control study was conducted with Treaty Indian TB cases living in Alberta, Canada; they concluded that the BCG vaccine conferred about 57% protection from tuberculosis (24).

In other studies, older children and adults have been studied extensively in relation to BCG. In developed countries where TB transmission is unlikely, there is evidence that BCG vaccine effectiveness (VE) wanes over time. One study found that BCG had similar VE both at 10-15 and 15-20 years after vaccination, 51% (95% CI 21, 69%) and 57% (CI 33, 72%) respectively, after which VE wanes from 25% (CI –14%, 51%) 20-25 years, and 1% (CI –84%, 47%) 25-29 years post-vaccination (23). Similarly, a retrospective cohort study conducted on Norwegian nationals suggested that the BCG vaccine gives long-term protection. VE decreased every 10 years from 61% [95% CI 24-80] to 58% [27-76] to 38% [-32 to 71]; to 42% [-24 to 73], indicating that protection in some form still existed 40 years posts vaccination (25).

In conclusion, the studies by Mangtani et al. and Nguipdop-Djomo et al., support the idea that the BCG vaccine is most effective in children and the protective effect of BCG typically decreased in trials that were conducted in locations closer to the equator where previous exposure to Mycobacteria is more common (22, 25). In an additional systematic review, the inconsistency in protective efficacy was re-iterated as well as the indication that a second dose (booster) does not increase the protectiveness of the BCG vaccine (18). However, an additional study suggested that a booster of BCG vaccine may be useful after 14 weeks of age, after memory has been established (26).

The above listed factors that influence the variation in BCG response, distance from the equator, and previous exposure to mycobacteria have not been evaluated in the context

of immune activation, leading us to consider immune activation as playing a potential role when it comes to BCG vaccine response (14, 20, 21, 24).

Immune Activation and Vaccination

In a 2014 study evaluating the effect of immune activation on yellow fever vaccine response, Muyanja and colleagues (19) compared the immune response in 50 volunteers in Entebbe, Uganda (where infection is likely), to 50 volunteers in Lausanne, Switzerland (where infection is unlikely). They found that vaccine response as indicated by yellow fever (YF) specific $CD8^+$ T and B cell responses were substantially lower in vaccinated individuals from Entebbe when compared to vaccinated individuals from Lausanne (19). The muted vaccine response in the Entebbe cohort was associated with reduced YF-17D replication and therefore reduced protection (19). Prior to vaccination, higher frequencies of activated (HLA-DR+, KI67+) T cells suggest that the Entebbe volunteers had an activated immune composition (19). This study provides evidence that immune activation prior to vaccination can impede vaccine response. Similarly, in a study of HIV infected women and response to the trivalent influenza vaccination suggests that existing CD4+ HLA-DR+ T cell activation may decrease the vaccine induced antibody response and theraefore the immunogenicity of the influenza vaccine (34).

Immune Activation and The BCG Vaccine

The various above-mentioned studies provide evidence that immune activation before vaccination may be correlated with a decrease in vaccine immunogenicity. However,

it is unknown whether immune activation will affect BCG vaccine responses, as there is a lack of published literature on this topic.

In Conclusion

The BCG vaccine has been on the market for several years although BCG vaccine efficacy is variable. Prior studies have found that immune activation at the time of vaccination was associated with decreased vaccine immunogenicity after yellow fever and influenza vaccination (19, 34). The effect of immune activation on BCG vaccination in infants in a TB endemic country has not been evaluated to date. We hypothesize that immune activation at birth may lead to reduced effectiveness of the BCG vaccine in infants in Kisumu County Kenya where previous exposure to various mycobacterium species is likely. This proposed study aims to evaluate if immune activation will affect BCG vaccine immunogenicity will provide important contribution to the literature and has implications for future TB vaccine development.

CHAPTER 3 – Methods and Results

Methods

The Maternal-infant Mycobacterial Immunity (MIMI) Study, led by Dr. Cranmer and colleagues, measures immunological responses to the BCG vaccine in HIV-1 negative pregnant women and their infants in Kisumu County Kenya in 2014 (12). Mycobacterial infection among Kenyan mothers may affect the neonatal immune system (12). This particular geographic location may have an effect on BCG response than can inform BCG understanding in other countries with a high burden of TB and as such makes this study particularly important to analyze (11).

We performed a secondary analysis of data collected for the MIMI. Mothers were enrolled during pregnancy, and maternal blood samples were taken at the time of delivery. Infant blood was collected at birth (cord blood prior to BCG administration) and 10 weeks of age (12).

Population and Sample

The MIMI Study was conducted in Kisumu County Kenya January-December 2014 (12). The Kisumu County is an urban region of western Kenya with generally low socioeconomic status, and high rates of HIV and TB, but also very good access to hospitals and two CDC backed laboratories.

Research Design: Instruments and Process of Data Collection

Visit Procedures and Demographic Data Collection:

During pregnancy, HIV negative mothers were recruited to the study if they planned to deliver at the Kisumu East District Hospital. During routine care in the 3rd trimester they were approached and informed about the MIMI Study. Through subsequent screening, the pool of eligible participants was narrowed. A survey was used for screening and enrollment purposes. Eligible participants who voluntarily agreed to be in the study provided informed consent. At the time of sample collection, (birth, 6 weeks, 10 weeks) an additional questionnaire was given. Medical records and questionnaire answers were used to collect demographic, categorical, data. This information included details of drugs taken during pregnancy including antimalarial (sulfadoxine-pyrimethamine), de-worming drugs (mebendazole), and booster doses of vaccine.

Laboratory Based Procedures:

The whole blood samples that were collected through venipuncture were analyzed using the 12-hour whole blood assay optimized by the South African Tuberculosis Vaccine Initiative (SATVI) research group to both classify and count the various cell types of interest (13). Briefly, whole blood was immediately placed in tubes stimulated with phytohemagglutinin (PHA) as a positive control, BCG, and un-stimulated. Cells were then cryopreserved and stored at -80 degrees C and transported the Fred Hutchinson Cancer Research Center in Seattle, Washington for flow cytometry analysis (12).

Immune activation was measured by the percentage of CD4 and CD8 cells expressing KI67 after BCG stimulation or un-stimulated. BCG vaccine response at 10 weeks was

measured by the frequency of Th1 cytokine positive CD4 cells at 10 weeks. The following analysis was restricted to mother-infant pairs where immune activation data was available at all time points of interest.

Statistical Analysis

We performed statistical analysis using descriptive statistics, correlations and linear regression in SAS statistical software package (version 9.4) to evaluate the cohort and any immune correlates, including the relationship between infant CD4+ T cell activation (% BCG stimulated and un-stimulated KI67+ CD4+ T cells at birth) and BCG vaccine response (% BCG Stimulated TH1+ CD4 cells). Correlation indicates if there is a direct and statistically significant (or not significant) association between two variables. Two-sided p-values < 0.05 were interpreted as statistically significant. These correlations and the above literature review were used to select variables that may influence the relationship of interest.

Confounding occurs when the relationship between the outcome and predictor is significantly and quantifiably distorted by an extraneous variable. In order to confound the relationship there needs to be reasonable evidence that this external factor is associated with both the outcome and predictor. In this analysis we used the 10% rule as the assessment for confounding. If this variable, when included in the model describing the relationship between immune activation and BCG vaccine response, causes the estimate to change by more than 10%, and it is biologically plausible, then this variable confounds the relationship and must be controlled for by inclusion in the model. If a variable does not

statistically correlate with immune activation and BCG response, there is less evidence that this relationship exists and the argument for confounding is weaker. Here we assess the above variables that may be important to consider as potential confounders (Table 3, Figure 7). The final was chosen to be parsimonious, with only variables that are plausibly associated and control for confounding.

Ethical Considerations

I performed a secondary analysis using existing de-identified data, which does not meet Emory IRB criteria for human subjects research and is exempt from IRB review. Letter of exemption is available upon request.

Limitations in Methodology

There may be alternate markers of immune activation (e.g., HLA-DR) that we did not measure. In addition, KI67 expression may vary by stimulation condition: during the 12 hours of stimulation time of the assay, un-stimulated T cells may down regulate the expression of KI67 and therefore underestimate immune activation. BCG stimulation may prevent this down regulation, therefore we assessed immune activation in both BCG stimulated and un-stimulated samples.

In terms of statistical analysis, the correlations explored are Pearson correlation coefficients and the corresponding p-values and as such are subject to the associated assumptions, including: absence of outliers, normality, linearity and absence of background "noise". These are similar assumptions for any model built using linear regression that might be in question in some way. In addition, the number of data point included in data

analysis is relatively small, which limits the generalizability of the results and our power to detect meaningful associations.

Results

Reminder of the Research Questions

1 - Does immune activation at birth influence BCG response at 10 weeks of age?

2 - What factors associate with infant immune activation at birth?

3 - What factors associate with immune activation at 10 weeks of age?

4 – Does immune activation at birth influence immune activation at 10 weeks?

Descriptive Analysis

The MIMI cohort consists of mostly married women with median age of 24 years (IQR 21, 27). Infants were generally healthy, with median birth weight of 3.4 (IQR 3, 3.5) kg. Most mother-infant pairs (93%) lived in homes without access to piped water, but most (59%) had access to electricity (Table 1). When it comes to baseline levels of immune activation, the mothers had low levels of non-stimulated CD4+ immune activation with a median of 0.094% (IQR 0.042, 0.185), and almost no CD4+BCG stimulated immune activation, median 0.000% (IQR 0.000, 0.005).

The maternal immune activation metrics at delivery are similar to infant immune activation metrics at birth. Infant CD4+ un-stimulated immune activation had a median of 0.171% (IQR 0.058, 0.396) and median of 0.000% (IQR 0.000, 0.043). (Table 1). The infants seemed to have mounted an immune response to BCG manifesting in BCG stimulated immune activaion at 10 weeks of age. The median CD4+ stimulated immune activation is 0.129% (IQR 0.038, 0.263), while the un-stimulated is 0.206% (IQR 0.102, 0.319) (Table 1).

Correlative Analysis

The variables mentioned above in Table 1 were assessed for correlation. This was done to determine what might influence immune activation at birth (Table 2a), at 10 weeks of age (Table 2b), as well as evaluate if any variables correlate with the outcome of interest, BCG immunogenicity, as measured by BCG Stimulated TH1+ CD4+ Infant T Cells and/ or the primary predictors of interest at birth (Table 2c) and at 10 weeks of age (Table 2d). The evaluation of correlations informs the model we can build to describe the relationship, establishes further ground for assessing confounding, as well as determining if any relationship exists at all where an absence of a correlation is still of interest.

There are several correlative relationships with immune activation and descriptive characteristics of this cohort. Indicators of overall infant health; one and five minute APGAR scores, birth weight, as well as maternal tetanus toxoid boosters and de-worming with mebendazole, and maternal TB exposures did not correlate with infant immune activation at birth. However the number of maternal IPT treatments for malaria (sulfadoxine-pyrimethamine)($r= -0.3229$, $p=0.029$) and maternal un-stimulated CD4+ T cells at delivery ($r=0.3570$, $p=0.015$), were associated with infant BCG stimulated CD4+ T cells at birth (Table 2a).

In evaluating correlations with immune activation at 10 weeks of age, only infant sex is significantly correlated with infant un-stimulated CD4+ T cells at 10 weeks ($r=0.3248$, $p=0.028$) (Table 2b). Also, as expected, all CD4+Th1 cells at 10 weeks that were BCG stimulated were correlated with the subset of BCG stimulated CD4+ T cells that were also KI67+ ($r=0.6160$, $p<.0001$) (Table 2b). Additionally the relationships mentioned above with BCG stimulated CD4+ T cells are no longer significant with maternal malaria

treatments (r=0.2583, p=0.083), nor maternal CD4+un-stimulated immune activation (r=0.1436, p=0.341).

In considering the direct associations with BCG Stimulated TH1+ CD4+ Infant T Cells at 10 Weeks, there was not a significant correlation with any measure of infant immune activation at birth, nor maternal immune activation (Table 2c, 2d).

Multivariable Linear Regression

The linear regression modeling describes the relationship between the percentage of BCG stimulated Th1+ CD4+ T cells at 10 weeks and BCG stimulated KI67+ CD4+ T cells at birth while controlling for relevant confounders.

The model shown in Figure 8 describes the relationship between infant immune activation at birth and immune response to the BCG vaccine at 10 weeks, including the confounder of the number of maternal malaria treatments. This model shows a suspected negative relationship where an increase in immune activation leads to reduced immune conversion to vaccine, however, this model is a poor fit for the data with an overall p=0.1983 and an r^2= 0.0725, which can impact its predictive ability.

Initial Findings

The analysis above suggests that existing immune activation at birth, indicated by expression of the marker KI67, does not directly influence response to the BCG vaccine at 10 weeks as indicated by the percentage of BCG stimulated CD4+ T cells (Table 2). Due to the fact that the number of maternal malaria treatments is correlated with infant immune activation at birth, but de-worming or the numbers of tetanus boosters in the mother do

not seem to have a direct association, this association suggests that this malaria treatment is somewhat unique immunologically. This finding calls into question the standard IPT treatment given to women during pregnancy, and is concern because many countries that are TB endemic are also malaria endemic. However, this relationship between maternal IPT for malaria and infant immune activation is no longer evident at 10 weeks of life, which may suggest that this interaction at birth has a short-lived effect. Nevertheless, this finding illuminates the need for further research.

CHAPTER 4 – Discussion, Conclusion and Recommendations

Discussion

We have found that the number of maternal malaria treatments (IPT) is statistically correlated with infant BCG stimulated CD4+ T cell immune activation at birth. Additionally, BCG stimulated CD4+KI67+ immune activation is not statistically correlated with BCG vaccine response at 10 weeks of age (Table 2c).

The number of maternal malaria treatments appears to be a confounder for the relationship between BCG stimulated immune activation at birth and BCG response at 10 weeks. In the available literature, antimalarial drugs that are deemed safe during pregnancy, "(1) chloroquine, (2) amodiaquine, (3) quinine, (4) azithromycin, (5) sulfadoxine-pyrimethamine, (6) mefloquine, (7) dapsone-chlorproguanil, (8) artemisinin derivatives, (9) atovaquone-proguanil and (10) lumefantrine," do not induce spontaneous abortion at a higher than usual level (35). It was also determined that the recommended drugs are not toxic and do not lead to cancer outcomes in infants (36). However, vaccine responses and immune activation were not assessed in the above study, and there are few human studies, due to pregnant women being classified as a vulnerable population (35, 36). In the present study in Kisumu, malaria IPT treatments refer to sulfadoxine-pyrimethamine specifically. Therefore there is a need to study all malaria IPT treatment drugs in the context of infant immune activation.

There is evidence of infant immune activation at birth before the administration of the BCG vaccine. Interestingly, in further review of Table 1, maternal BCG stimulated immune activation at delivery and infant BCG stimulated immune activation at birth have

almost identical numbers (very low level). As explored in Chapter 2, waning of vaccine effectiveness after a number of years is expected. This may indicate a decline in CD4+ KI67+ T cells that respond to BCG stimulation over time, however this connection is presumed as VE was not confirmed immunologically (23). As the median age for this cohort is 24, which presumably is 24 years post vaccination; we would expect VE to reduce to around 25%, and supposedly a similar reduction in active BCG stimulated CD4+ cells. However, an important consideration is that we observed waned activation, and it is still possible that there are CD4+ T cells that would respond to BCG stimulation, but not active at the time of data collection.

There does not appear to be any association between the level of immune activation at birth and immune activation at 10 weeks. At birth, the infants in this cohort have very low immune activation parameters, while they have more activation in general at 10 weeks (Table 1). At birth an infant has not been exposed to external pathogens, but by 10 weeks of age, the infants would have received several other vaccines as well as potentially been exposed naturally to pathogens. Therefore it is understandable that the immune activation makeup is quite different between these time points.

The number of maternal malaria treatments is associated with decreased infant immune activation, but other maternal drugs during pregnancy that may affect the immune system (anti-parasite de-worming (with mebendazole) and tetanus toxoid boosters) were not associated with immune activation at birth (Table 2a). Immune activation has not been assessed when it comes to treatments for malaria, specifically in the context of sulfadoxine-pyrimethamine (35). However, repeated malaria infections have been linked to chronic

immune activation of the individual as well as the infant of pregnant women, therefore treatment for malaria might influence this immune activation (37-40).

Helminthic infection was shown to down regulate antigen-specific IFN-γ producing T cell responses to *M. tuberculosis*, suggesting that de-worming treatments may induce the proliferation of CD4+ T cells. There was an association with maternal immune activation (Appendix A), but this association did not seem to effect infant immune activation in the way that maternal IPT did (41). In this reviewed literature, albendazole was the de-worming treatment given, not mebendazole, and pregnancy outcomes were not assessed indicating an additional area for future research. Helminth infections are also associated with chronic immune activation, perhaps suggesting that the mothers who were given de-worming treatments may have a differing immune make-up at baseline, which may inform the observed correlation with maternal immune activation (Appendix A) (42). Furthermore, studies of tetanus toxoid boosters are limited, but suggest that its mechanism of action may be B cell mediated, which was not evaluated in this study (43).

It is also interesting that all immune activation measures at birth are not at all associated with BCG response immune activation at 10 weeks. There is no indication that overall health at birth (1 or 5 minute APGAR, birth weight) is associated with immune activation. In addition, it has been established that sex/gender has a large differential influence on the immune system and on response to several vaccines with females typically responding more strongly to vaccine than males (44-46). However there was not a direct relationship between immune activation at birth and infant sex, and only an association with non-stimulated CD4+ T cells at 10 weeks (Table 2a, 2b).

We focused on BCG-stimulated CD4+ immune activation at birth for the purposes of model building. Immune responses to BCG tend to be $CD4^+$ T-cell-mediated due to the many functions they can perform, further justifying looking predominantly at CD4+ cells (14). Furthermore, based Figure 7, maternal malaria treatments is negatively associated with immune activation of infants at birth, and positively associated with BCG vaccine response. These factors together strengthen the hypothesis that infant immune activation leads to a lower BCG vaccine response as maternal malaria treatments has opposing effects for two parameters that we hypothesize to have a negative relationship.

When it comes to evaluating the model, the assumption a normal distribution for all variables is violated, perhaps necessitating log transformation of the variables in future studies (Appendix B). The variables are not independent because they come from either mother-infant pairs, which are not independent by nature or are of the same infant cohort. However, with the assessment of the immune distribution, an argument for independence can be made. This model has a poor fit, and therefore may pose the most utility as an additional tool for understanding the relationship of interest.

Limitations

The results are specific for HIV negative infants in a TB endemic setting. For most of the sub-Saharan African countries that are TB endemic, HIV is a common comorbidity. Therefore while this exclusion simplifies and strengthens the analysis, it also limits generalizability. Also, in this available data there is no good comparison group. This study could have been strengthened by also looking at a cohort of infants given the BCG vaccine in an area that is not TB endemic (a control that would be difficult to find and may require

historical data). Another option for a control would be to enroll infants from a separate location where it is possible to get immune activation numbers measured in the same way for infants before and after the administration of other vaccinations.

Another weakness is that we only used KI67 expression as the marker for immune activation. Future studies to evaluate alternate measures of immune activation, including HLA-DR, are warranted.

In addition there is a weakness of focusing the analysis on correlations that are unable to signify causality. It is always be possible that the associations found may be artifacts of the data, the non-specific quality of the BCG vaccine itself, or other unmeasured characteristics.

Immune activation was only measured at baseline and at 10 weeks, but not at the 6-week visit. If there were more time point information taken, this would allow for another snapshot of the immune activation status, potentially illuminating a relationship over time that cannot be found with only two time points.

Additionally, this study has a relatively modest sample size (n=46) which, in addition to the limited generalizability, may limit the statistical power to detect associations within the cohort.

Conclusions

As expected, there is a negative association between the percentage of BCG stimulated KI67+ CD4+ T-cells at birth (primary predictor) and percentage of BCG stimulated CD4+ T cells at 10 weeks (outcome), but it is not statistically significant, suggesting the lack of a direct relationship.

We also found that maternal immune activation, as well as use of drugs to treat malaria during pregnancy (sulfadoxine-pyrimethamine) has an association with infant immune activation at birth. However, this relationship was no longer evident at 10 weeks, suggesting that maternal immunity and drugs may not have a lasting impact on her infant. After adjusting for the effect of malaria IPT, infant IA was not associated with BCG vaccine response. Future studies to evaluate alternate measures of immune activation to evaluate its effect on BCG vaccination are warranted.

Recommendations

My results suggest there is a relationship between maternal immune activation, infant immune activation and the number of maternal malaria treatments (IPT). This relationship suggests that there may be an influence of drugs taken during pregnancy and the infant's immune activation, which can have implications not just for BCG vaccination, but also for all vaccines that are routinely administered in infancy. Available research on the impact of maternal immune activation has largely been on animal models, and conclude that maternal immune activation could lead to altered brain development (47-51). While the study of maternal immune activation is a relatively new field, there have been the established links to psychiatric disorders in offspring, but the mechanism, effect of environmental influencers, and long-term implications for offspring is still unknown (51). Furthermore, there have not been studies relating maternal immune activation to infant immune activation or vaccine response specifically, which leaves quite a lot for further study.

Further research is also needed to tease apart the relationship between immune activation and vaccine response in the context of BCG and TB prevention, as this secondary analysis does not show the direct relationship that is hypothesized. In addition, the levels of waned immunity over time may suggest that a higher dose of vaccine for TB endemic areas may be useful. Additionally BCG is one of the few vaccines given in infancy that does not have an accompanying booster dose. As reviewed in Chapter 2, the results of booster doses have been inconsistent, however recent research suggests that repeat BCG dosages might warrant further study (52). A research study with several markers for immune activation and BCG vaccine response (KI67, HLA-DR, the percentage of stimulated CD4+

and CD8+ T cells, and/or maternal antibodies) as well as evaluation of dose response, perhaps with a longer follow-up, would be a nice continuation of the work discussed here.

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FIGURES

FIGURE 1

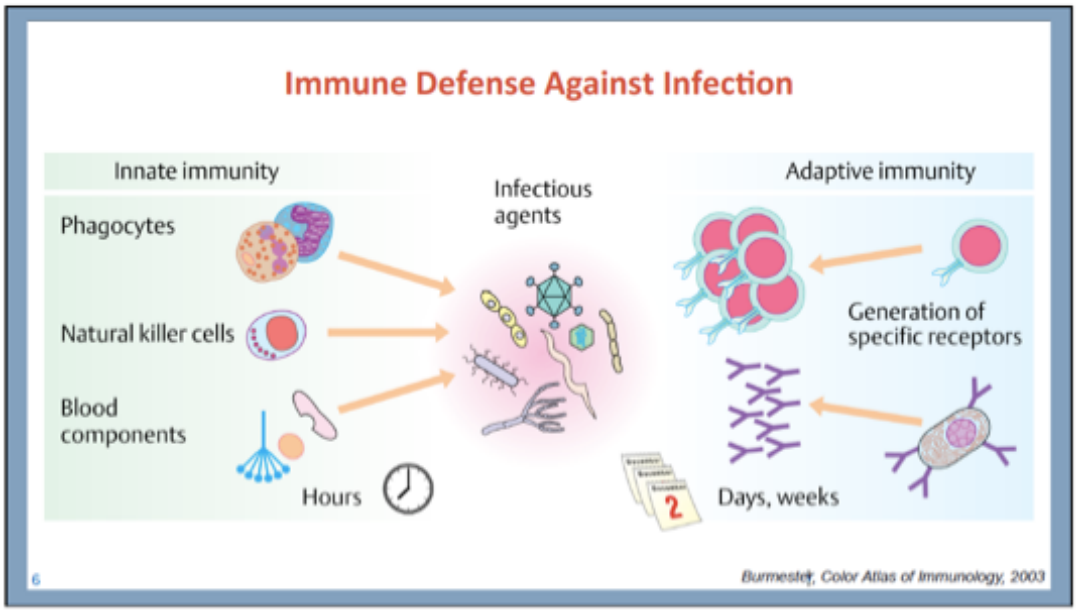


FIGURE 2

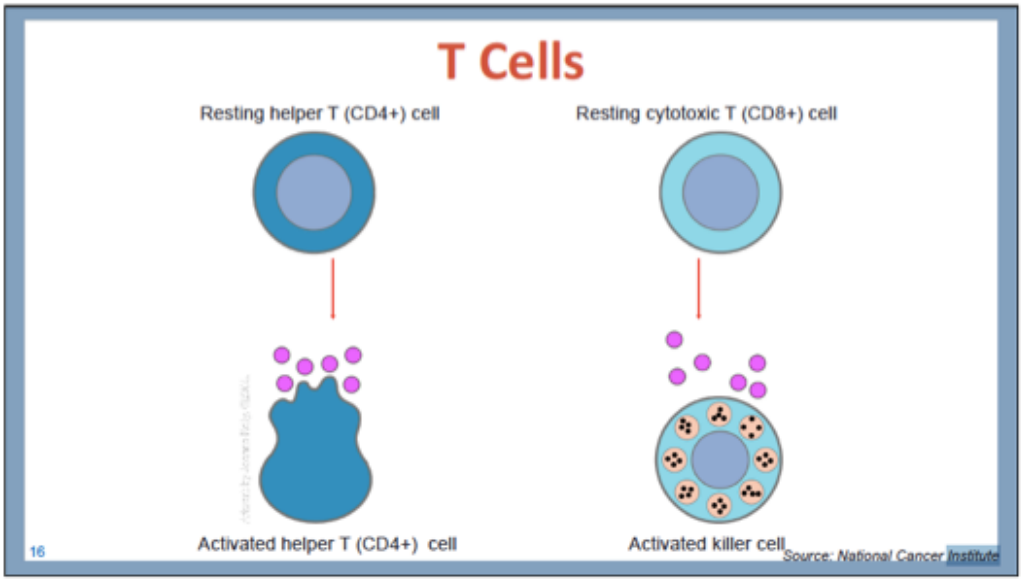


FIGURE 3

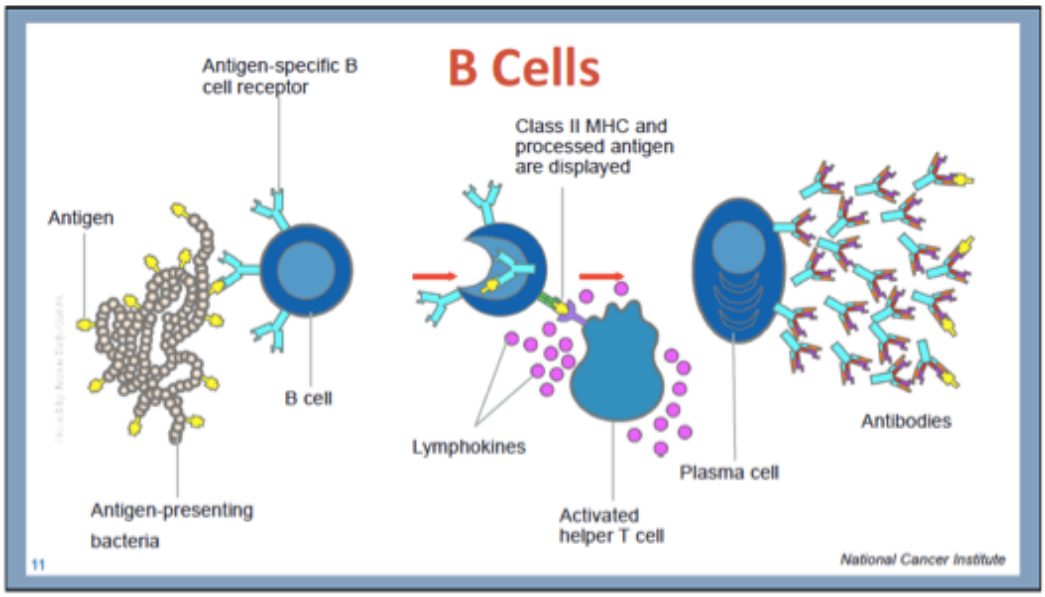


FIGURE 4

Population attributable fractions for risk factors for TB

	RELATIVE RISK FOR ACTIVE TB DISEASE[a]	PREVALENCE (%)[b] (ADULTS IN 30 HIGH TB BURDEN COUNTRIES)	POPULATION ATTRIBUTABLE FRACTION (ADULTS IN 30 HIGH TB BURDEN COUNTRIES)
HIV	21	0.9	15
Undernutrition	3.2	12	21
Diabetes	3.1	8.5	15
Alcohol misuse	2.9	4.0	7.0
Smoking	1.9	19	15
Indoor air pollution	1.4	53	17

[a] Source: Lönnroth K, Castro K, Chakaya JM, Chauhan LS, Floyd K, Glaziou P, Raviglione *M. Tuberculosis* control and elimination 2010–2050: cure, care and social change. Lancet. 2010 May 22;375(9728):1814-29. doi: 10.1016/S0140-6736(10)60483-7.

[b] Estimate of prevalence is based on a weighted average (by population size) for the 30 high TB burden countries.

FIGURE 5

Countries in the three TB high-burden country lists that will be used by WHO during the period 2016–2020, and their areas of overlap

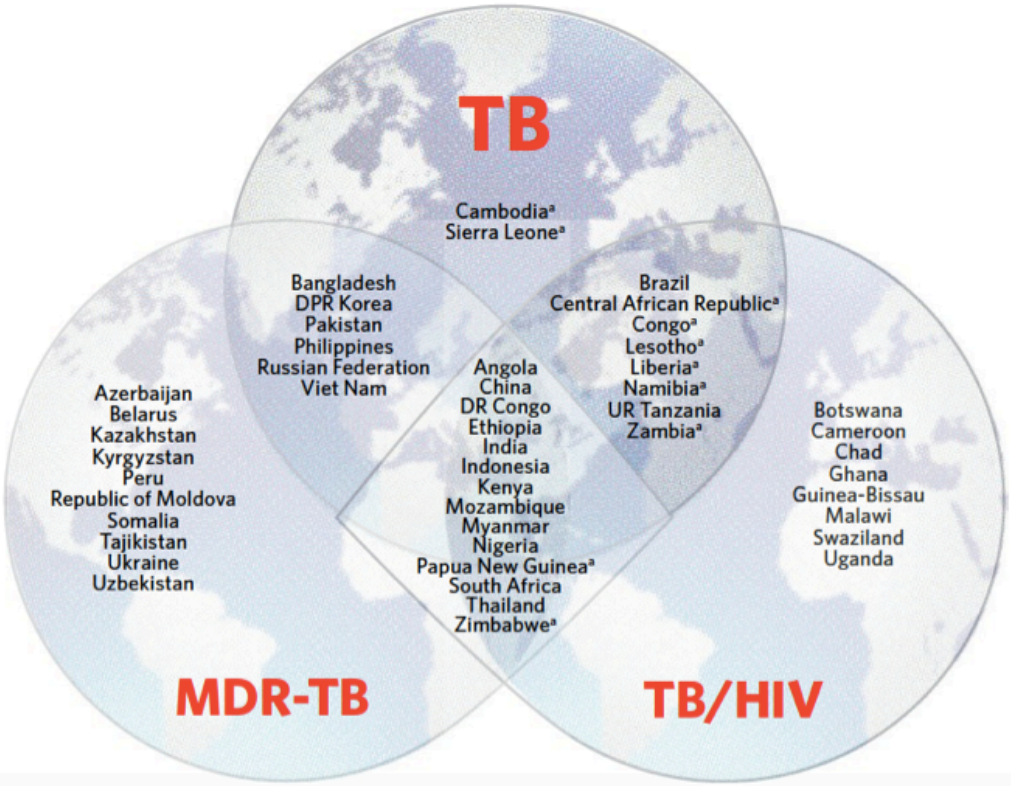


Figure 6 - Maternal-Infant Mycobacterial Immunity (MIMI) Study Subjects

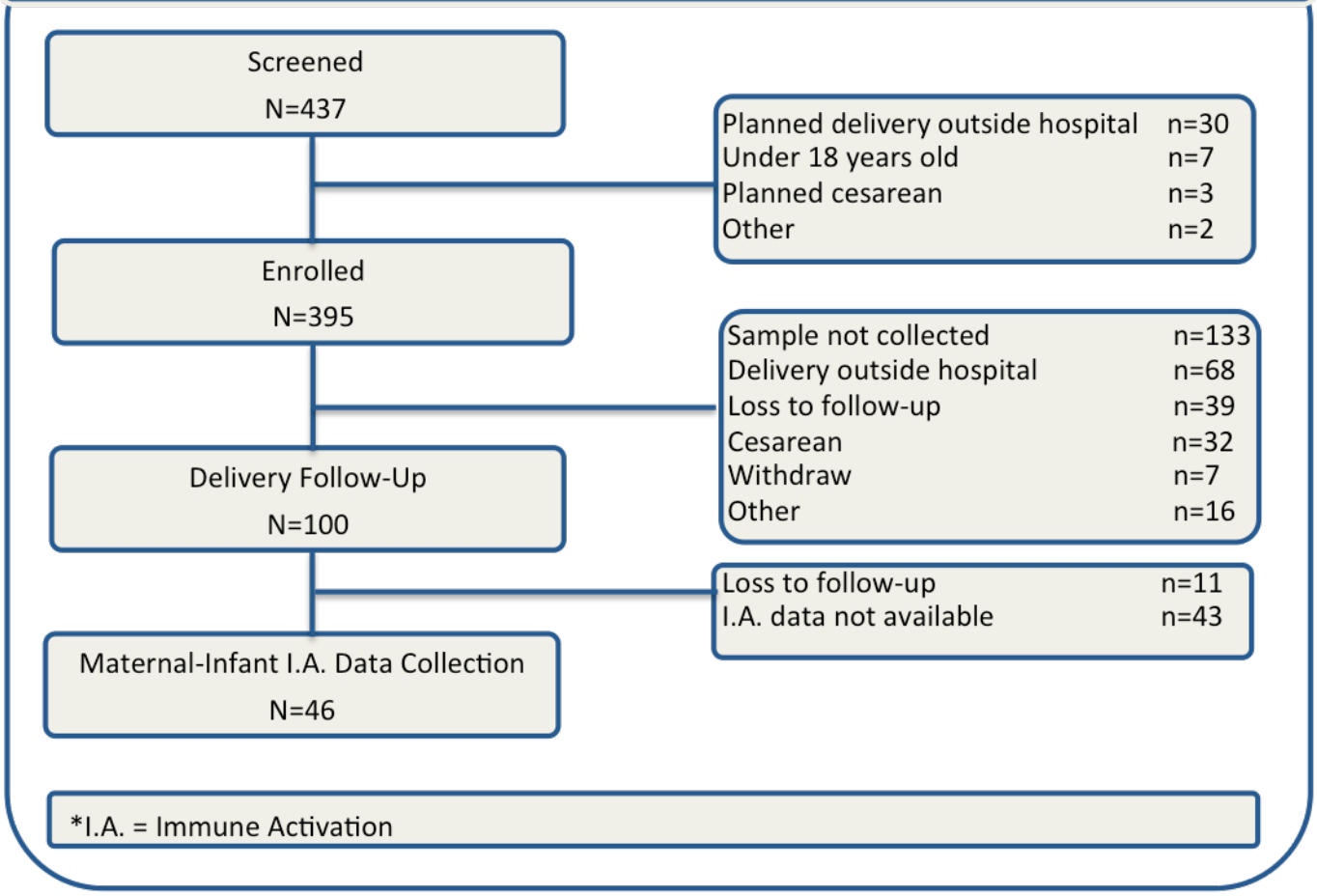
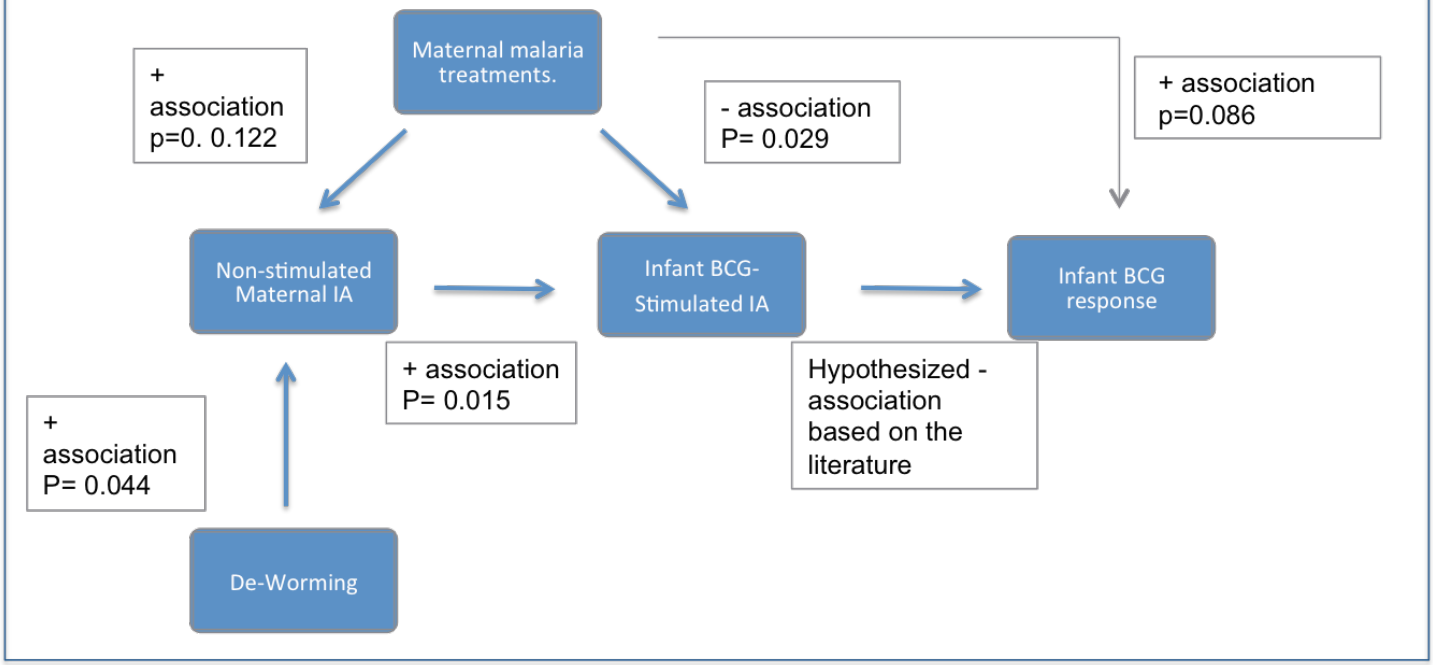


Figure 7: Diagram exploring the relationship between correlations of interest.



BCG Stimulated TH1+ CD4+ T Cells at 10 Weeks = -0.3941 + (-0.1086)(Non-Stimulated KI67+ CD4+ T Cells at Birth) + (0.2783)(Number of Maternal Malaria Treatments)

Figure 8- Model describing the relationship between immune activation at birth and BCG response at 10 weeks signified by the percentage of BCG stimulated CD4+ cell, while controlling for confounding

TABLES

Table 1: Descriptive characteristics of the MIMI cohort from Kisumu County Kenya in 2014 including both general characteristics and potential immune correlates restricted to mother-infant pairs with data for infant immune activation. N=46

Maternal Characteristics	% or Median (IQR)	Min - Max
Age in Years	24 (21, 27)	18 - 40
Married**	71%	
Years of Education	12 (8, 12)	7 - 16
No running water	93%	
No electricity at home	41%	
No TB exposure**	98%	
Number of treatment doses (SP) for malaria	3 (3, 4)	1 - 6
De-worming during pregnancy	91%	
Number of tetanus toxoid boosters	1 (1, 2)	0 - 3
Infant Characteristics at Birth		
Female Sex	48%	
Gestational Age in Weeks**	38 (38, 39)	36 - 41
Birth Weight in kg	3.4 (3, 3.5)	2.5 - 4.3
APGAR 1 minute	9 (9, 10)	3 - 10
APGAR 5 minute	10 (9, 10)	5 - 10
Infant Immune Activation at Birth		
BCG Stimulated KI67+ CD4+ T Cells	0.000% (0.000, 0.043)	0.000 - 0.441%
Non-Stimulated KI67+ CD4+ T Cells	0.171% (0.058, 0.396)	0.000 - 5.810%
BCG Stimulated KI67+ CD8+ T Cells	0.000% (0.000, 0.039)	0.000 - 0.770%
Non-Stimulated KI67+ CD8+ T Cells	0.416% (0.096, 0.605)	0.000 - 4.750%
Maternal Immune Activation at Delivery		
BCG Stimulated KI67+ CD4+ T Cells	0.000% (0.000, 0.005)	0.000 - 2.082%
Non-Stimulated KI67+ CD4+ T Cells	0.094% (0.042, 0.185)	0.000 - 0.383%
BCG Stimulated KI67+ CD8+ T Cells	0.000% (0.000, 0.014)	0.000 - 1.919%
Non-Stimulated KI67+ CD8+ T Cells	0.085% (0.041, 0.230)	0.000 - 0.511%
Infant Characteristics at 10 Weeks of Life		
Weight in kg***	5.9 (5.0, 5.9)	4.0 – 7.0
Length in cm**	58 (57, 60)	52 - 68
Head Circumference in cm*	37 (36, 41)	31 - 43
Infant Immune Activation at 10 Weeks of Life		
BCG Stimulated KI67+ CD4+ T Cells	0.129% (0.038, 0.263)	0.000 - 0.846%
Non-Stimulated KI67+ CD4+ T Cells	0.206% (0.102, 0.319)	0.000 - 1.010%
BCG Stimulated KI67+ CD8+ T Cells**	0.200% (0.041, 0.410)	0.000 - 2.859%
Non-Stimulated KI67+ CD8+ T Cells **	0.304% (0.175, 0.276)	0.000 - 4.180%
BCG Vaccine Response at 10 Weeks of Life		
BCG Stimulated TH1+ CD4+ Infant T Cells	0.253% (0.146, 0.469)	0.005 - 7.780%

*n=45 **n=44 ***n=43

Table 2: Descriptive correlation analysis explores the linear relationship between the variables of interest with both stimulated and un-stimulated immune cells in the infants enrolled in the MIMI Study n=46.

2a. What is associated with immune activation at birth?

Variable	BCG Stimulated KI67+ CD4+ T cells at birth		Non- Stimulated KI67+ CD4+ T cells at birth		BCG Stimulated KI67+ CD8+ T cells at birth		Non-Stimulated KI67+ CD8+ T cells at birth	
	r	p-value	r	p-value	r	p-value	r	p-value
Infant Sex (female)	0.0100	[0.943]	-0.1820	[0.227]	0.0860	[0.570]	-0.0644	[0.6708]
Birth Weight	-0.1150	[0.447]	-0.0742	[0.624]	-0.0758	[0.617]	-0.1443	[0.3387]
Gestational Age	-0.2607	[0.084]	0.0524	[0.732]	-0.2920	[0.052]	0.0117	[0.9393]
APGAR 1 minute	-0.1486	[0.324]	0.0745	[0.623]	-0.2237	[0.135]	-0.0301	[0.8428]
APGAR 5 minute	-0.2067	[0.168]	0.0919	[0.543]	-0.1551	[0.303]	0.0434	[0.7748]
Maternal Age	0.0148	[0.922]	-0.1355	[0.370]	-0.0512	[0.735]	-0.1580	[0.2944]
Maternal Years of Education	-0.1657	[0.271]	0.0961	[0.525]	-0.227	[0.130]	0.0837	[0.5802]
Maternal TB Exposure	-0.0658	[0.668]	-0.0429	[0.760]	-0.0604	[0.694]	-0.0612	[0.6898]
Water access	0.0263	[0.862]	-0.0466	[0.759]	0.0220	[0.884]	-0.0734	[0.6280]
Electricity access	0.0016	[0.992]	0.1071	[0.479]	-0.0703	[0.643]	0.0768	[0.6121]
Number of maternal malaria treatments	-0.3229	**[0.029]**	0.1733	[0.249]	-0.2978	**[0.044]**	0.1298	[0.390]
Maternal deworming	-0.0369	[0.808]	0.1087	[0.472]	-0.0687	[0.650]	0.1297	[0.390]
Number of tetanus toxoid boosters	0.0472	[0.756]	0.1607	[0.286]	0.0117	[0.939]	0.2139	[0.154]
Maternal Non- Stimulated KI67+ CD4+ T Cells	0.3570	**[0.015]**	0.2670	[0.073]	0.4228	**[0.003]**	0.4421	**[0.002]**
Maternal Non- Stimulated KI67+ CD8+ T Cells	0.4754	**[0.001]**	0.1875	[0.212]	0.5131	**[0.0003]**	0.3425	**[0.020]**
Maternal BCG Stimulated KI67+ CD4+ T Cells	-0.0830	[0.584]	0.2606	[0.080]	-0.0737	[0.627]	0.2260	[0.131]
Maternal BCG Stimulated KI67+ CD8+ T Cells	-0.0769	[0.612]	0.2921	**[0.049]**	-0.0584	[0.700]	0.2591	[0.082]

Bold indicates significance of less than 0.05

2b What is associated with immune activation at 10 weeks?

Variables	BCG Stimulated KI67+ CD4+ T Cells at 10 Weeks		Non-Stimulated KI67+ CD4+ T Cells at 10 Weeks		BCG Stimulated KI67+ CD8+ T Cells at 10 Weeks		Non- Stimulated KI67+ CD8+ T Cells at 10 Weeks	
	r	p-value	r	p-value	r	p-value	r	p-value
Infant Sex (female)	0.1142	[0.450]	0.3248	**[0.028]**	0.0459	[0.765]	0.1696	[0.265]
Birth Weight	0.2170	[0.148]	-0.0028	[0.986]	0.1852	[0.223]	-0.1467	[0.336]
Weight at 10 Weeks	-0.1337	[0.393]	-0.2432	[0.116]	-0.0033	[0.983]	-0.2738	[0.079]
Length at 10 weeks	-0.1507	[0.329]	-0.1719	[0.265]	-0.0916	[0.559]	-0.2403	[0.121]
Head circumference at 10 weeks	0.2767	[0.066]	-0.0157	[0.918]	0.2877	[0.058]	0.1301	[0.400]
Gestational Age	0.0832	[0.587]	-0.0749	[0.625]	0.0377	[0.808]	-0.1990	[0.625]
APGAR 1 minute	-0.0213	[0.888]	0.0581	[0.702]	0.0143	[0.926]	-0.2029	[0.181]
APGAR 5 minute	0.1260	[0.404]	0.2449	[0.101]	0.0817	[0.594]	0.0474	[0.757]
Number of maternal malaria treatments	0.2583	[0.083]	0.0422	[0.781]	0.0610	[0.690]	0.0126	[0.935]
Maternal deworming	0.1123	[0.457]	-0.1702	[0.258]	0.0750	[0.624]	-0.0527	[0.731]
Number of tetanus toxoid boosters	-0.1551	[0.304]	-0.0050	[0.974]	0.0346	[0.821]	0.1016	[0.507]
Maternal Non- Stimulated KI67+ CD4+ T Cells	0.1436	[0.341]	0.0294	[0.846]	-0.0682	[0.656]	0.1285	[0.400]
Maternal Non- Stimulated KI67+ CD8+ T Cells	0.1069	[0.480]	-0.1233	[0.414]	-0.1109	[0.468]	-0.0436	[0.776]
Maternal BCG Stimulated KI67+ CD4+ T Cells	0.0402	[0.791]	-0.0009	[0.995]	-0.0605	[0.693]	-0.0379	[0.805]
Maternal BCG Stimulated KI67+ CD8+ T Cells	0.0430	[0.777]	-0.0038	[0.980]	-0.0586	[0.702]	-0.0493	[0.748]
BCG Stimulated KI67+ CD4+ T Cells at Birth	-0.0327	[0.829]	-0.0940	[0.534]	0.0191	[0.901]	0.0561	[0.714]
Non- Stimulated KI67+ CD4+ T Cells at Birth	0.1397	[0.354]	0.0558	[0.713]	0.0615	[0.688]	0.0472	[0.758]
BCG Stimulated KI67+ CD8+ T Cells at Birth	-0.0391	[0.796]	-0.0656	[0.665]	-0.0026	[0.986]	0.0963	[0.529]
Non- Stimulated KI67+ CD8+ T Cells at Birth	0.0989	[0.513]	0.0164	[0.914]	-0.0152	[0.921]	0.0130	[0.933]
BCG Stimulated TH1+ CD4+ T Cells at 10 weeks	0.6160	**[<.0001]**	0.1542	[0.306]	0.6302	**[<.0001]**	0.0523	[0.733]

Bold indicates significance of less than 0.05

2c. Is immune activation at birth associated with BCG response at 10 weeks?		
Variable	BCG Stimulated TH1+ CD4+ Infant T Cells at 10 Weeks	
	r	p-value
Infant Sex (female)	0.2431	[0.104]
Birth Weight	0.2114	[0.159]
Gestational Age	-0.0377	[0.806]
APGAR 1 minute	-0.0970	[0.522]
APGAR 5 minute	0.0093	[0.951]
Number of maternal malaria treatments	0.2562	[0.086]
Maternal deworming	0.0356	[0.814]
Number of tetanus toxoid boosters	0.1411	[0.350]
BCG Stimulated KI67+ CD4+ T Cells at Birth	-0.0756	[0.618]
Non-Stimulated KI67+ CD4+ T Cells at Birth	-0.0371	[0.807]
BCG Stimulated KI67+ CD8+ T Cells at Birth	-0.0513	[0.735]
Non-Stimulated KI67+ CD8+ T Cells at Birth	-0.0672	[0.657]
Maternal Non-Stimulated KI67+ CD4+ T Cells	-0.0001	[0.999]
Maternal Non-Stimulated KI67+ CD8+ T Cells	-0.0532	[0.726]
Maternal BCG Stimulated KI67+ CD4+ T Cells	-0.0440	[0.771]
Maternal BCG Stimulated KI67+ CD8+ T Cells	-0.0401	[0.791]
Bold indicates significance of less than 0.05		

2d. Is immune activation at 10 weeks associated with BCG response at 10 weeks?		
Variable	BCG Stimulated TH1+ CD4+ Infant T Cells at 10 Weeks	
	r	p-value
Weight at 10 Weeks	0.1175	[0.453]
Length at 10 weeks	0.0178	[0.909]
Head circumference at 10 weeks	0.1715	[0.260]
Infant Sex (female)	0.2431	[0.104]
Non-Stimulated KI67+ CD4+ T Cells at 10 Weeks	0.1542	[0.306]
Non-Stimulated KI67+ CD8+ T Cells at 10 Weeks	0.0523	[0.733]
BCG Stimulated KI67+ CD4+ T Cells at 10 Weeks	0.6160	**[<.0001]**
BCG Stimulated KI67+CD8+ T Cells at 10 Weeks	0.6302	**[<.0001]**
BCG Stimulated KI67+ CD4+ T Cells at Birth	-0.0756	[0.617]
Bold indicates significance of less than 0.05		

Table 3- The assessment for confounding evaluates the relationship between the variables and their effects on the relationship between Non-stimulated CD4+ immune activation at birth and BCG response at 10 weeks. All variables included here have biological plausibility for confounding.

Adjustment	Estimate	95% CI	% change from the crude
Crude	-0.0479	(-0.440, 0.344)	--
Number of maternal malaria treatments	-0.1085	(-0.497, 0.280)	125.5*
Fully Adjusted	-0.1085	(-0.497, 0.280)	125.5*

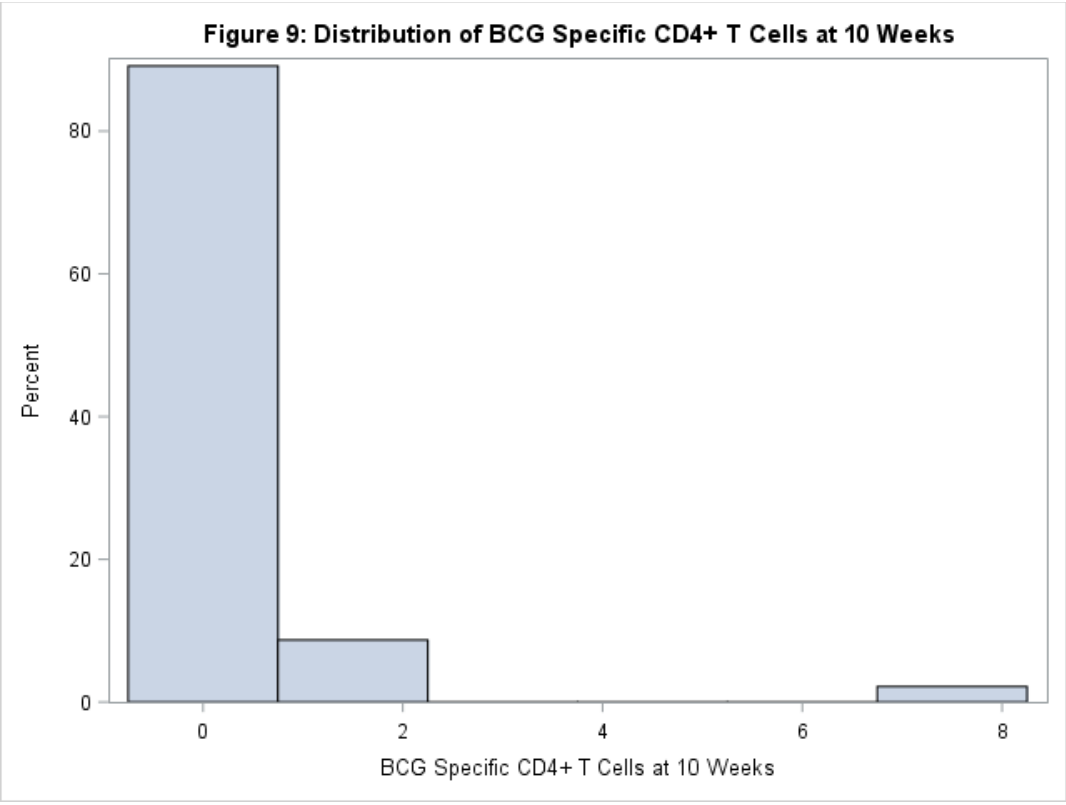
*Indicates percent change from the crude is more than 10% indicating confounding that must be adjusted for by inclusion in the model.

APPENDIX

A. Table detailing correlations between maternal immune activation and other descriptive maternal characteristics and infant characteristics at birth.

What is associated with maternal immune activation at delivery?								
Variables	BCG Stimulated KI67+ CD4+ T Cells		Non-Stimulated KI67+ CD4+ T Cells		BCG Stimulated KI67+ CD8+ T Cells		Non-Stimulated KI67+ CD8+ T Cells	
	r	p-value	r	p-value	r	p-value	r	p-value
Age	-0.0001	[0.995]	-0.1318	**[**0.383]	-0.0230	[0.880]	-0.0489	[0.747]
Years of Education	0.1959	[0.192]	-0.0613	**[**0.686]	0.1914	[0.203]	-0.1270	[0.400]
Water Access	-0.0518	[0.732]	-0.1880	**[**0.211]	-0.0455	[0.764]	-0.2005	[0.182]
Electricity Access	0.1432	[0.343]	0.0942	[0.533]	0.1685	[0.263]	0.0872	[0.565]
Number of malaria treatments	0.2770	[0.062]	0.2314	[0.122]	0.2718	[0.068]	0.0631	[0.677]
De-worming	0.0578	[0.703]	0.2979	**[0.044]**	0.0568	[0.708]	0.268	[0.071]
Number of tetanus toxoid boosters	0.0258	[0.865]	0.0980	[0.517]	0.0424	[0.780]	-0.0437	[0.773]
TB exposure	-0.0337	[0.826]	-0.0425	[0.782]	-0.0345	[0.822]	-0.0882	[0.565]
Infant Sex	-0.1726	[0.251]	-0.1248	[0.409]	-0.1714	[0.255]	-0.1053	[0.486]
Birth Weight	0.2571	[0.085]	-0.0464	[0.759]	0.2578	[0.084]	-0.1079	[0.475]
Gestational Age	0.1815	[0.233]	-0.1621	[0.287]	0.1653	[0.278]	-0.2388	[0.114]
APGAR 1 minute	0.0361	[0.812]	-0.1240	[0.412]	0.0419	[0.782]	-0.1159	[0.443]
APGAR 5 minute	0.0454	[0.764]	-0.3029	[**0.041]**	0.0446	[0.768]	-0.4169	**[0.004]**
Bold indicates significance of less than 0.05								

B.



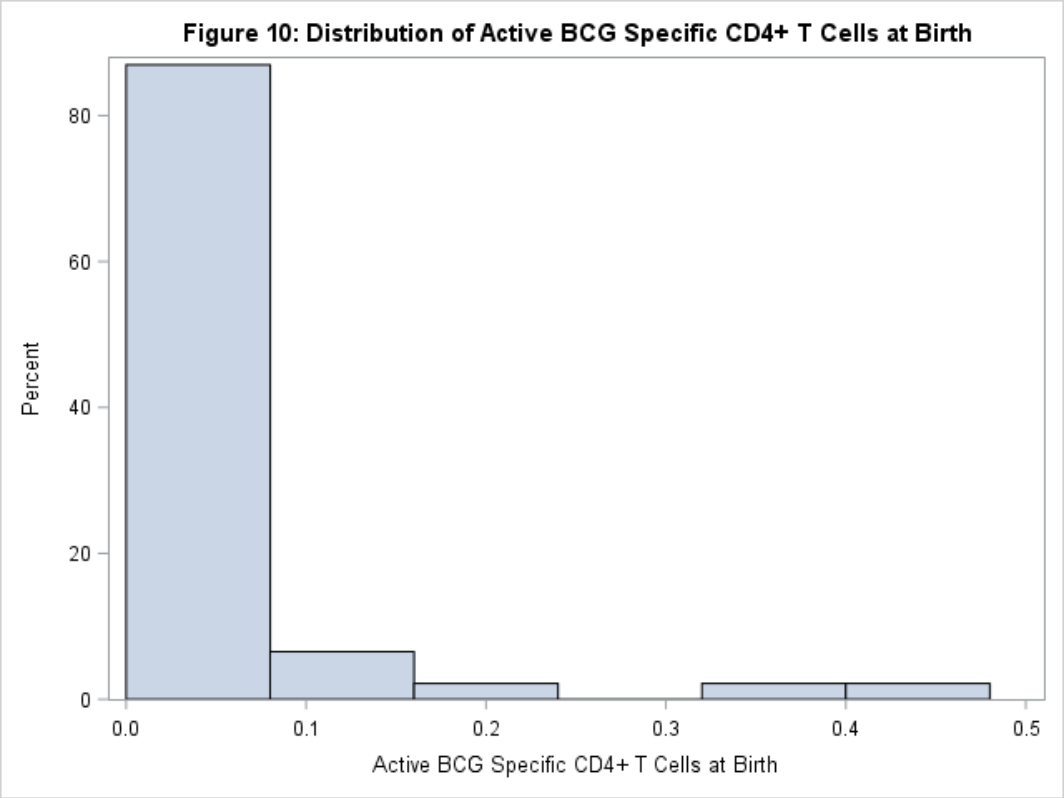


Figure 10: Distribution of Active BCG Specific CD4+ T Cells at Birth
Percent
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
Active BCG Specific CD4+ T Cells at Birth

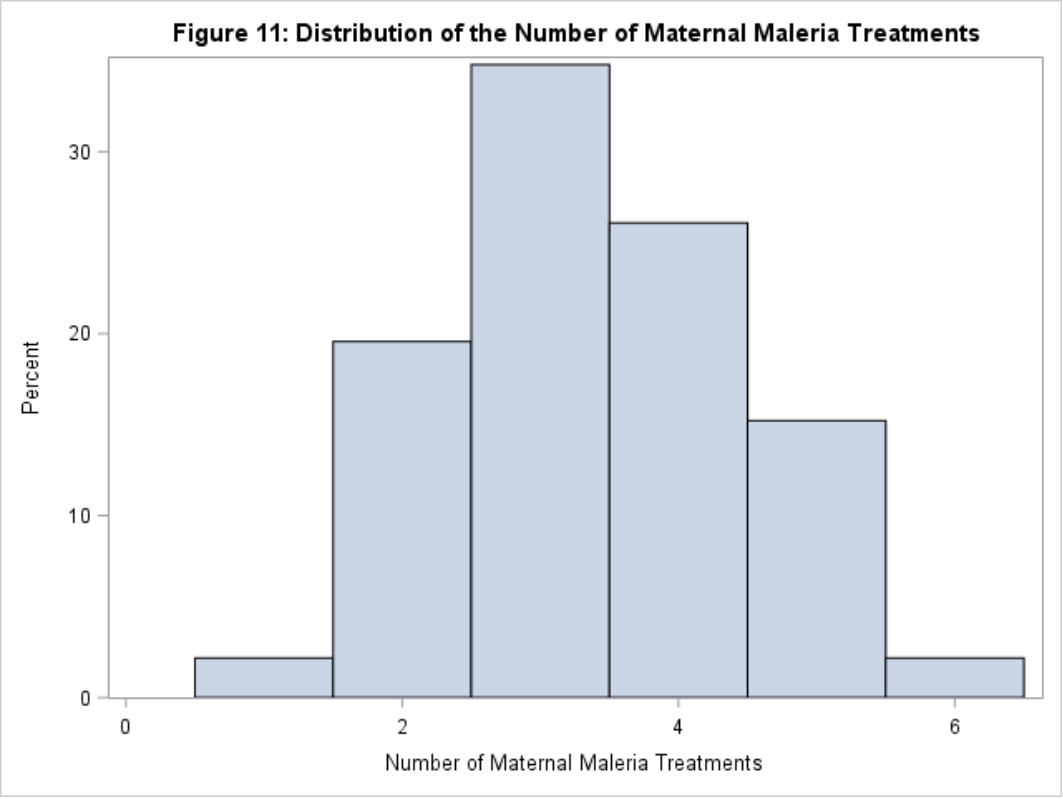
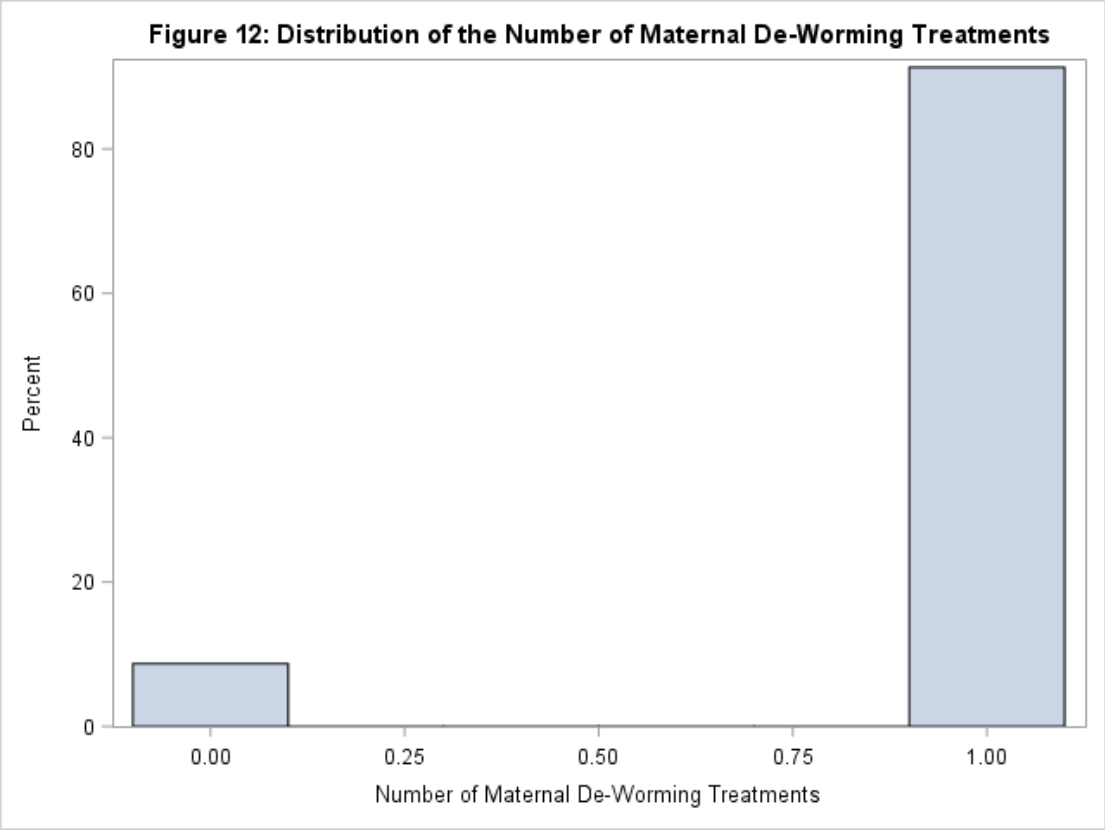
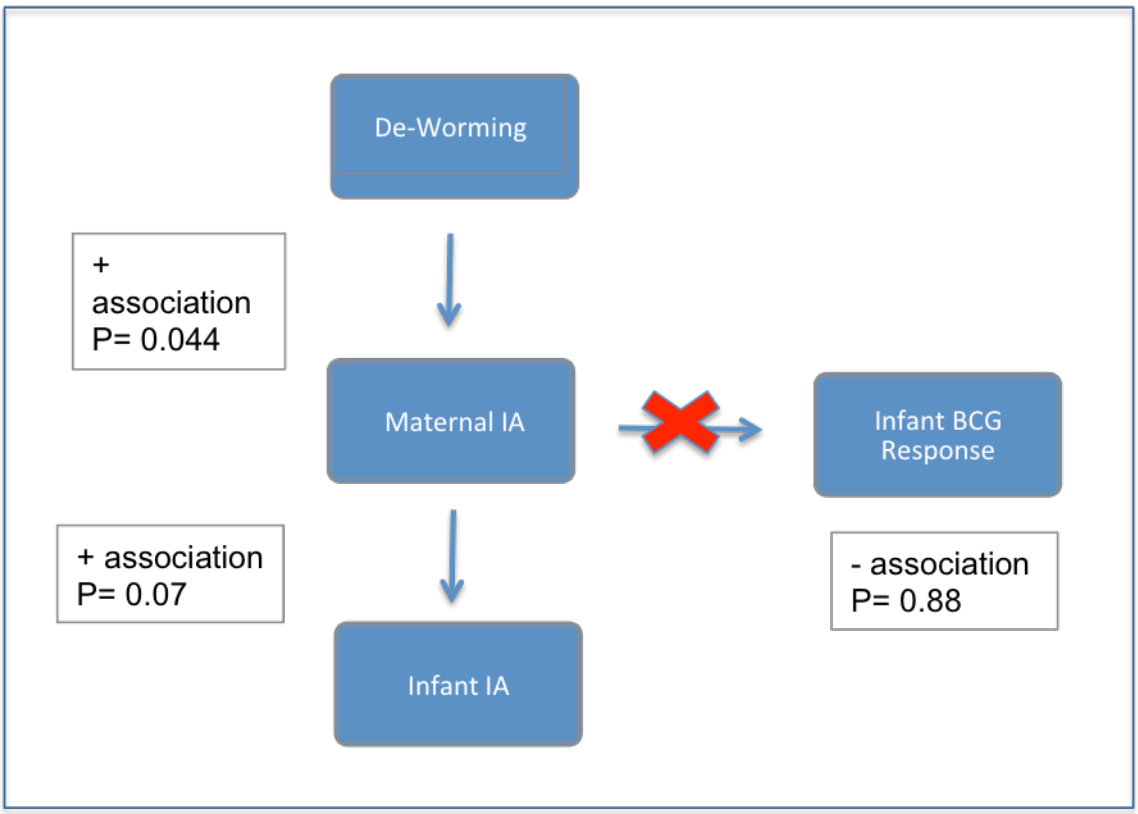


Figure 11: Distribution of the Number of Maternal Maleria Treatments
Percent
30
20
10
0
0
2
4
6
Number of Maternal Maleria Treatments



C. Description of maternal Immune Activation Relationships



D. Model describing immune activation at birth based on maternal immune activation at delivery

Assessment of confounding

The assessment for confounding evaluates the relationship between the variables and their effects on the relationship between BCG-stimulated CD4+ immune activation at birth and maternal non-stimulated CD4+ immune activation at delivery.			
Adjustment	Estimate	95% CI	% change from the crude
Crude	0.3189	(0.065, 0.573)	--
Number of maternal malaria treatments	0.4075	(0.172, 0.644)	27*
Number of maternal de-worming treatments	0.3608	(0.095, 0.626)	13*
Number of maternal tetanus toxoid boosters	0.3179	(0.060, 0.576)	0.33
Fully Adjusted	0.4243	(0.178, 0.671)	33*
*Indicates percent change from the crude is more than 10% indicating confounding that must be adjusted for by inclusion in the model.			

Model

BCG Stimulated KI67+ CD4+ T Cells at Birth = 0.1241 + (0.4243)(Maternal Non-Stimulated KI67+ CD4+ T Cells at Delivery) + (-0.03427)(Number of Maternal Malaria Treatments) + (-0.0238)(Number of De-Worming Treatments)
Model describing the relationship between BCG stimulated infant immune activation at birth and maternal non-stimulated immune activation while controlling for confounding.