MAKERERE UNIVERSITY

Investigation of the mechanisms behind non-specific stimulation of memory B-cells by *Mycobacterium tuberculosis* using samples from latent tuberculosis-infected and uninfected mothers

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September 2018.

DECLARATION

I hereby declare that the work in the dissertation is my own except for citations that I duly acknowledged. The dissertation has never been submitted to Makerere University examination board for an academic award.

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Dedication

I dedicate this dissertation to my parents and friends. A special gratitude to the Rt. Rev. Dr Samuel Stephen Kaziimba and Mom Margret Kaziimba for their tremendous support through the entire school programme.

I dedicate this work to Grandma Ruth Endean and Tim Endean that have sacrificed all resources to see to it that I complete this degree programme. I appreciate everything you have contributed to my entire school life. Above all, you have supported me spiritually through prayers. Because of your prayers, I have been victorious.

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List of abbreviations

M.tb	*Mycobacterium tuberculosis*
TB	Tuberculosis
BCG	Bacillus Calmette–Guérin
LTBI	Latent tuberculosis infection
SAC	*Staphylococcus aureus* Cowan
PPD	Purified protein derivative
PBMCs	Peripheral blood mononuclear cells
PWM	Pokeweed mitogen
CpG	Cytosine phosphate guanine oligonucleotide
MDR-TB	Multidrug resistant-TB
PRRs	Pattern recognition receptors
PAMPs	Pathogen-associated molecular patterns
TLRs	Toll-like receptors
TNFα	Tumour-necrosis factor alpha
ManLAM	Mannosylated Lipoarabinomannan
TIR domain	Toll/interleukin-1 receptor domain
WHO	World health organisation
ELISPOT	Enzyme-linked immunospot
CMV	*Cytomegalovirus*
UK	United Kingdom

Abstract

Mycobacterium tuberculosis may cause non-specific immune responses as observed by higher antibodies levels to unrelated antigens in different studies. These processes may be due to polyclonal activation of memory B cells by *Mycobacterium tuberculosis.* However, the mechanism behind the non-specific activation of memory B cells by *Mycobacterium tuberculosis* is not clear. Studies show that memory B cells can be activated by polyclonal stimuli such as bystander T cell help. In this *in vitro* experimental study, we investigated whether *Mycobacterium tuberculosis*-specific T cells had a role in the non-specific stimulation of memory B cells. We stimulated peripheral blood mononuclear cell samples of latent tuberculosis-infected and uninfected mothers with purified protein derivative from *Mycobacterium tuberculosis* for three days. We harvested the supernatant, the cells, and then measured antibody levels in the supernatant by ELISA. Cells were stained and analysed by flow cytometry to determine different B cell phenotypes such as activated memory B cells, resting memory B cells, and plasmablasts. From the results, antibody level to unrelated antigen and total IgG secretion was higher in latent tuberculosis infected mothers than in uninfected. We also observed a trend in a decrease of activated memory B cell and an increase of plasmablast cell frequencies after flow cytometry analysis. The findings show that *Mycobacterium tuberculosis* may induce non-specific immune responses to unrelated antigen as observed by higher antibody levels in latent tuberculosis infected than in uninfected individuals. These findings need to be investigated further with a better sample size and enough cell counts to have a better conclusion whether *Mycobacterium tuberculosis*-specific T cells are involved in the polyclonal activation of memory B cells.

CHAPTER ONE: Introduction

1.1 Background

Mycobacterium tuberculosis (M.tb) is the causative agent of tuberculosis and it is a global health problem (1). About 2 billion people are infected and around 1.4 million deaths occur each year (2). The distribution of tuberculosis varies in different regions of the world with the highest rates (100/100,000 or higher) seen in sub-Saharan Africa, India, China, and the islands of Southeast Asia and Micronesia (3). The incidence in Uganda is rated at 201 (118–306) per 100,000 population as of 2016 (http://www.who.int/tb/publications/global_report/en/ accessed on 11th.11.2017).

Despite the available tuberculosis drug regimen, deaths still occur and drug resistance is on the rise. An effective vaccine, therefore, may reduce the incidence of tuberculosis. Bacillus Calmette–Guérin (BCG), the only available tuberculosis vaccine (4, 5), protects against tuberculosis of the meninges and disseminated tuberculosis in children but provides variable protection in adults (6) and in different geographical locations (7).

In addition to protection against tuberculosis, BCG vaccine has some beneficial non-specific immune responses (8). It has been shown that BCG-vaccinated low birth weight infants have a decreased mortality compared to the unvaccinated (9, 10). Leentjens *et al.* also showed that BCG vaccination prior to influenza vaccination resulted in increased antibody titre against *Influenza virus* (11). Furthermore, Ota *et al.* found a rise in antibody titres to *Poliovirus* in infants that received BCG vaccine post oral polio vaccination (12).

Mycobacterium tuberculosis infection also has non-specific effects similar to those seen with BCG. A study by de Paus *et al.* showed increased antibody titres to non-*mycobacteria* antigen (A/H3N2 *Influenza virus*) during active tuberculosis infection (13). Studies that were done in Nigeria and Burkina Faso also showed increased *Cytomegalovirus*-specific antibody levels in active tuberculosis infection (14, 15). Experimental studies using freund's complete adjuvant (CFA) also support the fact that *mycobacteria* may cause non-specific responses (16). The dried inactivated *M.tb* component of CFA could be behind the strong immune stimulation observed

with this adjuvant. This supports the idea that *Mycobacteria* may induce responses against heterologous pathogens.

The immunological mechanism involved in this non-specific response is not clear. A study by Bernasconi *et al.* showed that human memory B-cells can proliferate and expand into plasma cells after exposure to T cell cytokines or through a challenge by bacterial cytosine-phosphate-guanine DNA, a TLR 9 agonists.(17). Human memory B-cells are prone to polyclonal activation (18). Aaby *et al.* also speculate that BCG induced non-specific effects are due to cross-reactive immunity or stimulation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) on dendritic cells and macrophages by *mycobacteria*l products (19).

We proposed that the non-specific rise in antibody titres seen in *M.tb* infection was due to polyclonal stimulation of memory B cells probably via *M.tb* specific T cell cytokines or TLRs. We had evidence to show that *M.tb* proteins induced polyclonal activation of memory B cells (Kimuda *et al.* unpublished). However, we did not know the mechanism behind this process. This study, therefore, investigated whether *M. tb*-specific effector/memory T cells played a role in the non-specific humoral response observed in *M. tb* infection. We investigated these using samples from LTBI and uninfected mothers. We believed that samples from LTBI are rich in *M. tb*-specific effector/memory T cells.

1.2 Problem Statement

Mycobacterium tuberculosis infection or exposure may lead to increases in antibody titres to non-*mycobacteria* antigens evidenced by studies that showed higher antibody titres against *Influenza virus* and *Cytomegalovirus* (13, 15). The immunological mechanism behind this was not clear.

1.3 Hypothesis

- We hypothesized that non-specific increase in antibody titre in tuberculosis infection was due to polyclonal activation of memory B cells by direct effects of *mycobacteria* on B cells via cytokines produced either by *Mycobacterium*-specific T cells or through activation of Toll-like receptors on memory B cells.

1.4 Major objective

To determine the mechanism behind *Mycobacterium tuberculosis*-induced increases in antibody responses to unrelated pathogens

1.5 Specific objectives

i. To investigate the relevance of *M.tb* specific effector/memory T cells in the non-specific activation of memory B-cells by *Mycobacterium* antigens
ii. To investigate the relevance of toll-like receptors in polyclonal activation of memory B-cells by *Mycobacterium* antigens

1.6 Significance

The body's immune system encounters many infections. Since antibodies are critical in the fight against infections, boosting antibodies to various non-*Mycobacteria* antigens can protect individuals from such infections. If it is proved that *M.tb* can cause non-specific antibody responses to other antigens, a vaccine could be designed to cause similar effects.

1.7 Conceptual framework

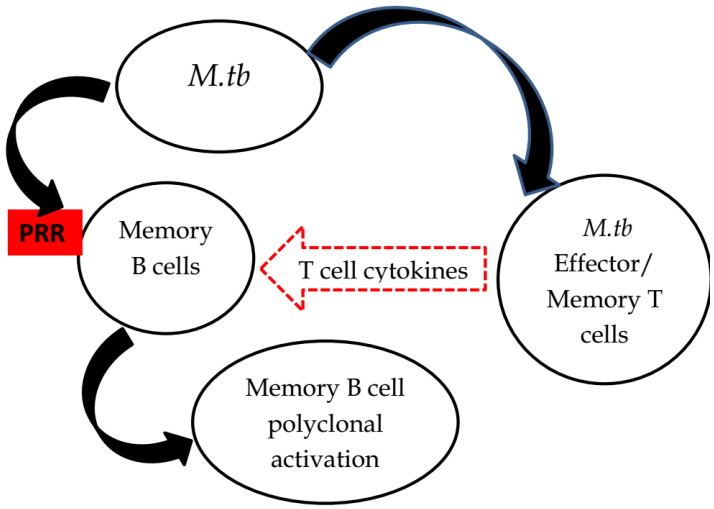


Figure 2. Showing Conceptual framework. Latent TB individuals have *Mycobacterium tuberculosis (M.tb)* specific T cells. Interaction of these cells with *M. tb* antigens cause secretion of T cell cytokines, which could cause differentiation of memory B cells into antibody-secreting cells. Alternatively, *M. tb* antigens could interact with Pattern recognition receptors (PRRs) on memory B cells to cause their differentiation into antibody-secreting cells.

CHAPTER TWO: Literature review

2.1 Pathogenesis and immune response to *Mycobacterium tuberculosis*

Tuberculosis is spread through inhalation of droplet nuclei containing *Mycobacteria tuberculosis* (*M.tb)* bacilli (20). A person with active pulmonary TB disease produces these infectious droplets when he/she coughs (21). Transmission takes place when the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs of the uninfected person (21, 22). When *M.tb* bacilli reach the lungs, they are recognized by the alveolar macrophages and dendritic cells (DCs) via their PRRs such as TLRs that recognize conserved *M.tb* molecules (PAMPs) (23). This recognition of *M.tb* antigens causes stimulation of TLRs, which initiates a signalling cascade leading to the activation of transcription factors, such as NF-κB that result in a variety of cellular responses including the production of pro-inflammatory cytokines, chemokines, and effector cytokines that direct the adaptive immune response. Inflammatory cytokines and chemokines help in the recruitment of immune cells such as monocytes, neutrophils, and lymphocytes to the infection site. However, they are unable to clear the bacteria. At this point, the bacteria resist macrophage killing by preventing phagosome-lysosome fusion and cause macrophage necrosis (24). When the bacilli are released, they multiply extracellularly and are engulfed by other macrophages. Dendritic cells that phagocytize the bacilli mature and migrate to the regional lymph node where they prime T cells (both $CD4^+$ and $CD8^+$) against mycobacterial antigens hence triggering the adaptive immune response (25). Antigen recognition by T cells causes activation and clonal expansion. The activated T cells migrate out of the lymph nodes, guided by chemokines produced by infected cells and move into infection sites in the lungs as effector T cells. In the infection sites, they secrete interferon gamma (IFNγ), which activates macrophages to kill intracellular *M.tb*. Activated macrophages and dendritic cells produce cytokines such as type 1 cytokines IL-12, IL-18, IL-23 (26), and proinflammatory cytokines like IL-1 (27) and TNF-α (28). CD8 T cells also kill *M.tb* infected cells via production of perforin, granzymes, or Fas-Fas ligand interaction to induce apoptosis (29). For a successful control of infection from spreading to other tissues, immune cells may form a granuloma around *M.tb* bacilli (30).

Granulomas consist of macrophages, T cells, dendritic cells, fibroblasts, endothelial cells, B cells, and stromal cells. These granulomas also provide the required microenvironment needed

for adequate interactions between immune cells and the cytokines they produce (31). Therefore, Granuloma formation may result in termination of infection progression. It is worth noting that some bacilli may be resistant and continue to survive despite the stressful conditions. These bacilli escape killing and enter a dormant form in which they resist elimination by the immune system (32).

In the humoral arm of the immune system, B cells and complement system also play a role in the *M.tb* immune response. B cells produce *M.tb* specific antibodies that form immune complexes to modulate the functions of effector cells such as dendritic cells and macrophages. Effector functions mediated by *M.tb* antibodies include opsonisation, neutralization of *M.tb* antigens, and antibody-dependent cellular cytotoxicity (ADCC) (33, 34). B cells can also present antigens to T cells, which result in T cell activation, polarization, and the creation of T cell memory. B cells also modulate the functions of granulomatous immune cells (35). In addition, B cells can produce cytokines such as IL-12, IFN-γ, TNF-α that promote Th1 responses (36) which have a role in anti-mycobacterial immune responses and early control of tuberculosis infection (34). In addition, anti-inflammatory B cells secrete anti-inflammatory cytokines such as IL-4, IL-33, IL-10 and transforming growth factor beta (TGF-β) that have negative effects on Th1 and Th17 inflammatory responses (37-39).

Mycobacterium tuberculosis can also activate complement system via classical and alternate pathways by binding C3 leading to opsonisation, microbial cell lysis by the formation of a membrane attack complex and recruitment of immune cells to the site of infection (40). In general, an effective immune system may completely clear the *M.tb* bacilli or contain it as it is with latent TB infection state.

Latent tuberculosis infection

Latent tuberculosis infection (LTBI) is a state of persistent immune response to stimulation by *M.tb* antigens without evidence of clinically manifested active TB (41). About 1/4 of the world's population is latently infected with tuberculosis (42) and there is a 5-10% likelihood that these will develop active TB disease in the future. The risk of TB reactivation is higher in the presence of predisposing factors such as immunosuppression observed in HIV-infected individuals (43). The tuberculin skin test (TST) and interferon-gamma release assays (IGRAs**)** are the main tests

currently available for the diagnosis of LTBI. Both tests rely on the detection of an immune response through demonstration of host sensitization to *Mycobacterium tuberculosis* (44). The two tests also depend on Memory T cell response (44-46). In the TST, delayed-type hypersensitivity responses to intradermal injection of *M.tb*-derived purified proteins indicate a positive result. Here *Mycobacterium* specific lymphocytes infiltrate the inoculation site and produce inflammatory cytokines that cause the observed induration. The IGRA measures *in vitro* IFN-γ T cell responses following stimulation of cells or whole blood with *M.tb* specific antigens (47, 48). IGRA tests measure the T-cell IFN-γ responses to *M.tb* antigens such as peptides derived from early secretory antigenic target 6-kD protein (ESAT-6) and culture filtrate protein 10 (CFP-10) (49). The secreted IFN-γ by *Mycobacterium tuberculosis*-specific T cells can be measured by ELISA (44) or T-SPOT.TB (Oxford Immunotec, Oxford, UK) (50) which uses enzyme-linked immunospot (ELISPOT) technology to quantify the numbers of activated *M.tb*–specific T cells (49, 51)

2.2 BCG non-specific effects and postulated mechanisms

Apart from the protective role of BCG against tuberculosis, this vaccine has many non-specific beneficial effects. A study by Mathurin *et al* showed that BCG vaccine can protect mice against infection with vaccinia virus (52). In an experimental model in hamsters, BCG vaccination increased the lytic activity of peritoneal cells against herpes simplex-infected cells. BCG vaccination has also been shown to enhance the antibody titres and T cell responses to other vaccines, such as hepatitis B, polio, and pneumococcal conjugate vaccines (8, 12). Ikeda *et al* also showed that administration of MDP a constituent of mycobacterial cell walls to mice protected them against *vaccinia virus* and HSV infections (53). In addition, Para *et al* demonstrated that BCG-vaccinated mice resisted malaria infection compared to unvaccinated (54). A number of studies also show how BCG vaccination is associated with a reduction in child mortality (55, 56). All these studies point to fact that there could be a possibility of cross-reactive immunity. There are postulations that try to explain these observations among which include, trained immunity (57) and heterologous immunity (58). With heterologous immunity, it is believed that the observed non-specific responses are mediated by cross-reactive lymphocytes (58) that respond against other infections.

Interestingly the non-specific observations appear to be within days, a phenomenon which cannot be clearly explained by the adaptive immune response mechanism (59) but more linked to the innate immune response where PRRs such as TLRs could play a big role. The fact that the non-specific reduction in mortality and morbidity is observed immediately following vaccination (60), suggests that the BCG vaccine confers protection by alteration of the innate immune system probably via PRRs such as TLRs.

Other live attenuated vaccines with possible non-specific effects

Examples of such vaccines include smallpox, measles, oral polio, and yellow fever vaccines. A Study in Guinea-Bissau by Aaby *et al.* demonstrated that measles vaccination was associated with a reduction in child mortality (61). Analysis of studies that compared mortality in measles-vaccinated and non-vaccinated children showed that measles vaccination was associated with mortality reduction (62). A study by Bottomley *et al*. in the Gambia children showed that vaccination against measles and yellow fever was associated with a reduction in the carriage of *H. influenzae* and *S. pneumoniae* (63) which also suggests that yellow fever vaccine too may have non-specific beneficial effects. Aaby *et al.* also speculate that vaccination with trivalent live attenuated oral polio vaccine was associated with lower mortality for children less than 5 years (64).

Findings from a study by Mayr *et al*. suggest that smallpox vaccine reduced susceptibility to a number of other infectious diseases such as scarlet fever and whooping cough (65). In addition, a study conducted in Denmark showed that smallpox-vaccinated individuals had a reduced risk of hospitalization due to infectious diseases (66). Vaccination with *vaccinia* has also been associated with a lower risk of developing melanoma and better survival in patients with malignant tumours (67, 68). These studies show that live attenuated vaccines may have non-specific immune responses. *Mycobacterium tuberculosis* may also have some non-specific responses.

2.3 *Mycobacterium tuberculosis* non-specific immune responses

Mycobacterium tuberculosis infection may also have non-specific immune responses similar to those seen with BCG and other live attenuated vaccines. A study by de Paus *et al.* showed increased antibody titres to non-*Mycobacteria* antigen (A/H3N2 *Influenza virus*) during active

tuberculosis infection (13). Studies that were done in Nigeria and Burkina Faso also showed increased *Cytomegalovirus*-specific antibody titres in active tuberculosis infection (14, 15). In addition, *Mycobacteria* have been known to exert non-specific immune stimulation evidenced by studies using complete freund's adjuvant (FCA), a *Mycobacterium*-containing preparation that is a potent immune stimulator in experimental animal models (16).

Studies proposed possible mechanisms to explain how such observations may be possible. A study by Lanzavecchia *et al.* demonstrated that memory B cells are prone to polyclonal activation (18) which could possibly explain the observed rise in antibody titres to non-*Mycobacterium* infections that were seen in active tuberculosis. A study by Bernasconi *et al* demonstrated that interaction of T cell cytokines or bacterial cytosine-phosphate-guanine DNA with human memory B-cells causes proliferation and expansion of these cells into antibody-secreting cells (17). Apart from exposure to an antigen, their study showed that memory B cells can also be activated when exposed to a polyclonal stimulant such as bystander T cell help. Hovav *et al.* demonstrated that toll-like receptor-2 may be involved in B cell non-specific stimulation by *Mycobacterium* proteins (recombinant mycobacterial 27-kDa lipoprotein) (69). In addition, it was shown that these memory B cells are able to express Toll-like receptors such as TLR10, TLR6, TLR7, TLR9 and TLR 2 which when triggered by TLR agonists causes them to proliferate and differentiate (17, 70). This was a possible explanation for the observed non-specific rise in antibody titres seen in active tuberculosis. Therefore, *Mycobacterium tuberculosis* could induce non-specific immune responses; however, the mechanism behind this effect was not clear.

We hypothesised that polyclonal activation of memory B cells was responsible for these non-specific effects and that this occurred either by the effects of *Mycobacterium*-specific T cell cytokines or direct engagement of mycobacterial proteins with B cell TLRs. We, therefore, investigated whether *M.tb* specific T cells are involved in the non-specific antibody responses observed in active tuberculosis.

2.4 Preliminary data

Findings from a study conducted at the Medical Research Council/UVRI Uganda Research Unit on AIDS using samples from Ugandan participants indicated that active pulmonary tuberculosis (APTB) was associated with higher antibody responses to several unrelated infections and/or

vaccine responses (Fig. 2; Kimuda *et al.* unpublished). This was in line with observations from de Paus *et al* and Ledru *et al.* that documented the same trend with influenza and CMV-specific antibodies respectively (13, 15).

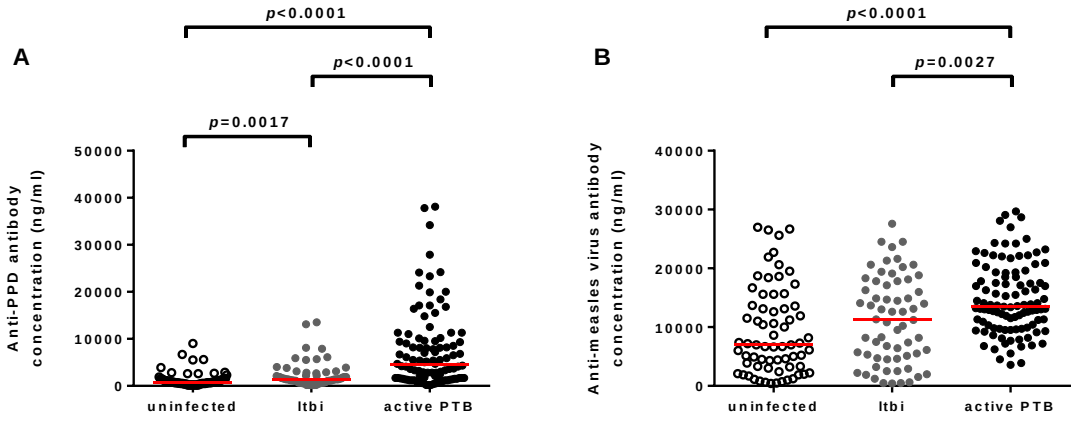


Fig 2. Showing antibodies levels in *M.tb*-infected individuals of varied status. LTBI (Latent tuberculosis infection), PTB (pulmonary tuberculosis).

More investigations were conducted to determine whether *M.tb* or mycobacterial proteins, mediated this effect via polyclonal activation of memory B cells. Preliminary findings from a small study that used healthy blood donors from Uganda observed B-cell phenotype changes after stimulation with *M.tb* purified protein derivative (PPD). This indicated that the non-specific increases in antibodies in TB infection could be due to memory B cell polyclonal activation (Fig. 3; Kimuda *et al.* unpublished). However, there was a need for more research to determine how activation occurred in memory B cells.

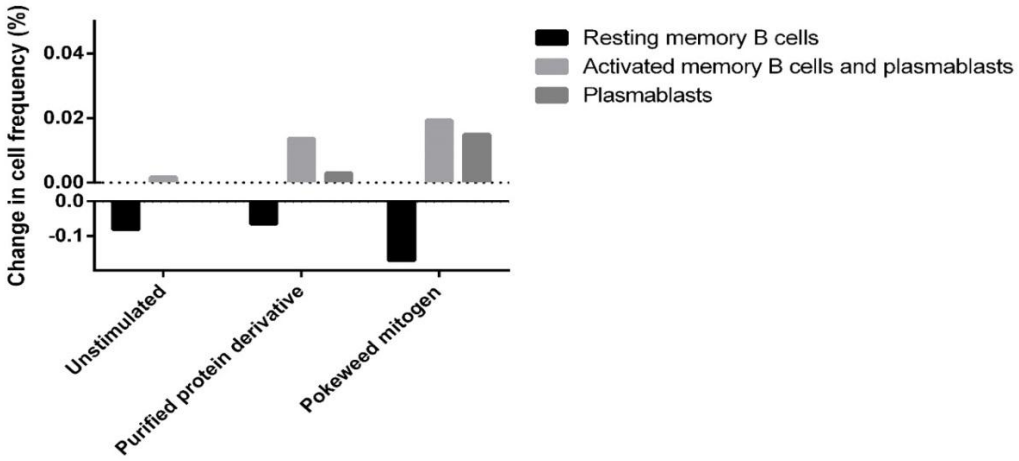


Fig 3. Showing B-cell phenotypic changes following stimulation with *Mycobacteria* antigens. Peripheral blood mononuclear cells (PBMCs) were stimulated with *M.tb* purified protein derivative and pokeweed mitogen as a positive control.

CHAPTER THREE: Methodology

3.1 Study design and population

This was an *in vitro* experimental study where we stimulated PBMCs with PPD from *Mycobacterium tuberculosis* during cell cultures. We used samples from a study that looked at the effects of maternal latent TB infection (LTBI) on infant BCG-immune responses (IBS). This observational study recruited a Ugandan cohort of 300 pregnant women. These comprised of 150 individuals with LTBI and 150 without LTBI. They determined TB infection status basing on T.SPOT.TB (Oxford Immunotec, Oxford, UK) and TST test. Individuals with LTBI were positive on both tests, while the uninfected were negative on both tests

3.2 Study site

The IBS study recruited participants from Entebbe and Kisubi hospital. We performed laboratory analysis at MRC/UVRI laboratories.

3.3 Sample size consideration

Since this was an exploratory study, we planned to use 20 PBMC samples, 10 from LTBI mothers, and 10 from uninfected mothers. This exploratory study was to provide preliminary results to guide sample size estimation for a larger study.

3.4 Variables

Independent variable

- Exposure to *Mycobacterium tuberculosis* antigens

Dependent variables

- Anti-TT antibody level
- Total or non-specific IgG secretion
- Plasmablast frequency

3.5 Sampling strategies

We used simple random selection method to obtain at least 10 samples from each group (LTBI and uninfected). Study participant ID numbers were re-arranged in a random order using Microsoft excel generated random numbers. We then chose the first 10 individuals with LTBI and the first 10 individuals without LTBI with cell counts equal or greater than $6x10^6$ cells. We then retrieved the PBMCs samples from their locations in the liquid nitrogen storage tanks.

3.6 Selection criterion

Inclusion criteria

- Stored PBMCs with a record of TST and T.SPOT TB result were included in the study

Exclusion criteria

- Samples with poor cell viability

3.7 Laboratory methods

We retrieved stored PBMCs of LTBI and uninfected mothers from the liquid nitrogen tanks and thawed them at 37°C. We then cultured the cells in presence or absence of PPD or PWM for 3 days. We performed antibody ELISA on the supernatant to measure total IgG, anti-PPD and anti-TT antibodies. The cells were stained and analysed using a flow cytometer to assess changes in B cell phenotypes. Fig. 4 below summaries the methods that were used in the entire study.

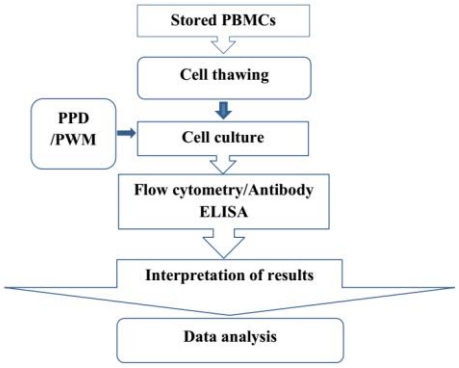


Figure 4. A flowchart showing a summary of study procedures. Peripheral blood mononuclear cell (PBMC) samples from latent TB infected (LTBI) and uninfected mothers were cultured for 3 days in presence or absence of purified protein derivative (PPD) or pokeweed mitogen (PWM) / (CpG/SAC). Thereafter, antibody ELISA and flow cytometry were done and data analysed by Flowjo and Graph Pad software. *M.tb*, (*Mycobacterium tuberculosis)*. CpG, (Cytosine phosphate guanine). SAC, (*Staphylococcus aureus* cowan).

3.7.1. Cell thawing

We thawed PBMCs from liquid nitrogen by swirling the vials in the water bath set at 37°C until a small ice pellet remained. We added an equal volume of warm (37°C) 20 % fetal bovine serum (R20) to the PBMCs in the vial drop-wise. We transferred the cell suspension to a sterile 15 ml tube containing 8 ml of R20 drop-wise and then the tube was topped up with R20 to 15 ml mark. This was centrifuged at 650g for 10 minutes, and we discarded the supernatant, and then resuspended the cell pellet in 5 ml of warm 10% fetal bovine serum (R10). We counted the cells using a haemocytometer and excluded the dead cells by trypan blue staining (see the cell-counting procedure in appendix ii). We then adjusted the volume of cell suspension to 15 ml with warm R10 and thereafter centrifuged as before. The cells were suspended in warm R10 at a concentration of 40x10^{6} cells/ml.

3.7.2. Cell culture

To a 48 well culture plate, we added 50ul (containing $2x10^6$ cells) to a 500-µl volume of culture media (10% fetal bovine serum in RPMI containing 1% Streptomycin, 1% Penicillin, 1% L-glutamine and 2.5% Hepes Buffer). We had three culture set-ups, the first was a negative control containing only culture medium, the second was a test well containing 10 µg/ml of PPD from *M.tb,* and the third was a positive control well containing 6 µg/ml CpG and 1 in 10,000 SAC or 0.5 µg/ml of PWM alone. Cell culture was at 37°C, 5% carbon dioxide for 3 days. After this period, the cell culture supernatants were harvested after centrifuging at 650g for 10 min and stored for antibody analysis using ELISA. The cells pellets were stained using monoclonal antibodies in preparation for flow cytometry.

3.7.3. Antibody ELISA

We coated the Immulon 4HBX plates (Termoscientifc, USA) with 5 µg/ml TT, 10 µg/ml PPD antigen and 0.5 µg/ml anti-human IgG (Dako Denmark A/S) diluted in carbonate-bicarbonate buffer. The plate was then incubated overnight at 2-8°C. We washed the plate with 200 µl of PBST and blocked the wells with 150 µl of 1% skimmed milk for 2h at room temperature. We discarded the blocking buffer, added 50 µl of neat supernatants to antigen-coated wells, and incubated them overnight at 2-8 °C. Plate wells were washed with 200 µl of PBST and afterwards, 50 µl of 0.5-µg/ml anti-human antibody conjugated to HRP (Dako, Denmark) diluted in 1 % skimmed milk was added and incubated for 1h. at room temperature. We washed the plates with PBS-T and added 100 µl of TMB (3', 5, 5' tetramethylbenzidine) substrate (eBioscience). The reaction was developed for 15 minutes in the dark at room temperature and thereafter stopped with 25 µl of 2M H2SO4. We measured the optical density using ELISA reader (Biotek) at 450nM.

3.7.4. Flow cytometry

Antibody panel

The monoclonal antibodies used to stain cells for flow cytometry included BV-650-A-CD19 (BD Horizon™), BV605-A-CD10 (BD Horizon™), Axfluor647-A-CD20 (Biolegend®), PE-CF594-A-IgD (BD Horizon™), BV421-A-CD27 (Biolegend®), and FITIC-A-CD21 (Biolegend®). This panel allowed us to identify B cell fraction ($CD19^+$), immature B cells ($CD10^+$), naïve B cells

($CD27^-/CD21^+/IgD^+$), activated memory B cells and plasmablasts ($CD27^+/CD21^-$), plasmablasts ($CD20^-$) (71), and resting memory B cells ($CD27^+/CD21^+$) according to Moir & Fauci (72).

Staining cell for flow cytometry

After cell culture (section 3.7.2), the PBMCs were counted using trypan blue exclusion technique (appendix ii).We resuspended the cells at a concentration of $1x10^6$ cells/ml in 1x PBS. 1 ml of cell suspension was transferred into respective labelled facs tubes. Stained the cells for viability by adding 0.25 µl of efluor780 dye (eBiosciences) into the 1 ml of cell suspension. Staining was done for 30 minutes at 2-8°C. This was washed with 3 ml of flow staining buffer at 650g x2.

FC receptor blocker (1 µl) was added after pouring off the supernatant and stained for 20 minutes at 2-8°C. Then the cells were washed with 1 ml of FACS Buffer by centrifuging at 650g for 10 minutes and pouring off the supernatant. The cells were then stained with 50 µl of the antibody cocktail for 30 minutes at 2-8°C. This was then washed with 3 ml of flow staining buffer twice at 650g for 10 minutes. The cell pellet was resuspended in 350 µl of cell fix and this was ready for flow cytometry.

3.8 Statistical analysis

Data analysis was performed by FlowJo software package, and Graph pad prism software ver.6.01. Given the sample size, non-parametric tests (paired/unpaired) were used to determine statistical significances. Mann-Whitney test was used to determine statistical differences between LTBI and uninfected. For differences between the 2 experiments such as unstimulated and stimulated wells within a group (LTBI or uninfected), we used Wilcoxon signed rank test. Data were presented as medians and interquartile range. Statistical significant differences were considered at $p<0.05$

3.9 Quality control

Antigens or products known to induce polyclonal activation were included in the experiments as positive controls. These included PWM, SAC, and CpG. Monoclonal antibodies for flow cytometry were stored correctly at the right temperatures and the optimal titre of the antibody was determined and used for analyses. Instruments such as flow cytometer (LSRII) BD were calibrated before use to ensure reliability and reproducibility of experiments. An appropriate number of events (cut-off of 100,000) were acquired to ensure reliable results. All experiments

included compensation controls to allow for calculation of spillover of dye fluorescence between monoclonal antibodies.

3.10 Ethical considerations

This study sought approval from the School of Biomedical Sciences–Higher Degrees Research and Ethics Committee (SBS-HDREC) and Uganda National Council for Science and Technology. We sought a waiver to use archived samples and we were blinded to the study participant's names in order to protect their identities.

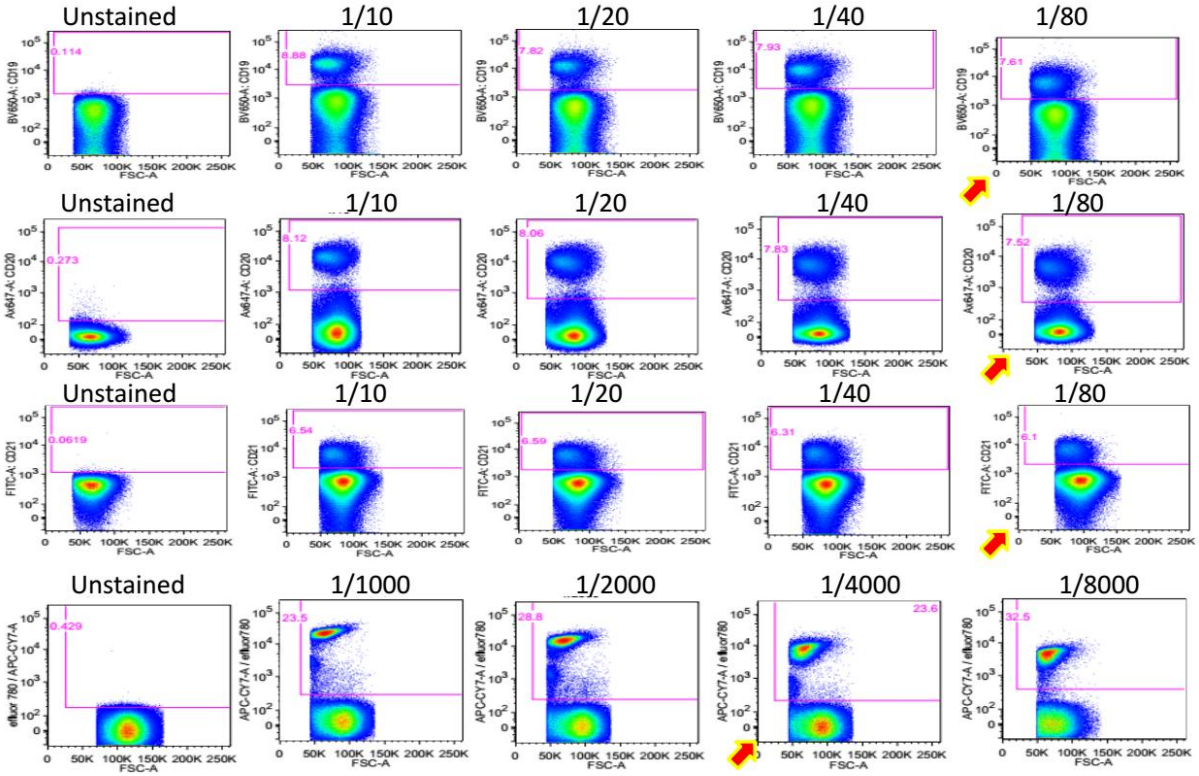
CHAPTER FOUR: Results

4.1. Optimization results

Peripheral blood mononuclear cells isolated from HIV negative blood packs obtained from Nakasero blood bank were used for all optimization experiments.

4.1.1. Titration results

We performed doubling dilutions of each antibody conjugates and the efluor780 dye in the flow-staining buffer and 1XPBS respectively. We then stained $1x10^6$ cells with 50 µl of each antibody dilution. We also stained $1x10^6$ cells with 1 ml of the different diluted efluor780 dye and thereafter, acquired the cells on LSR II flow cytometer. We chose the optimal titres according to cost-effectiveness, good cell staining, and separation of cell populations as in (fig. 5) below.



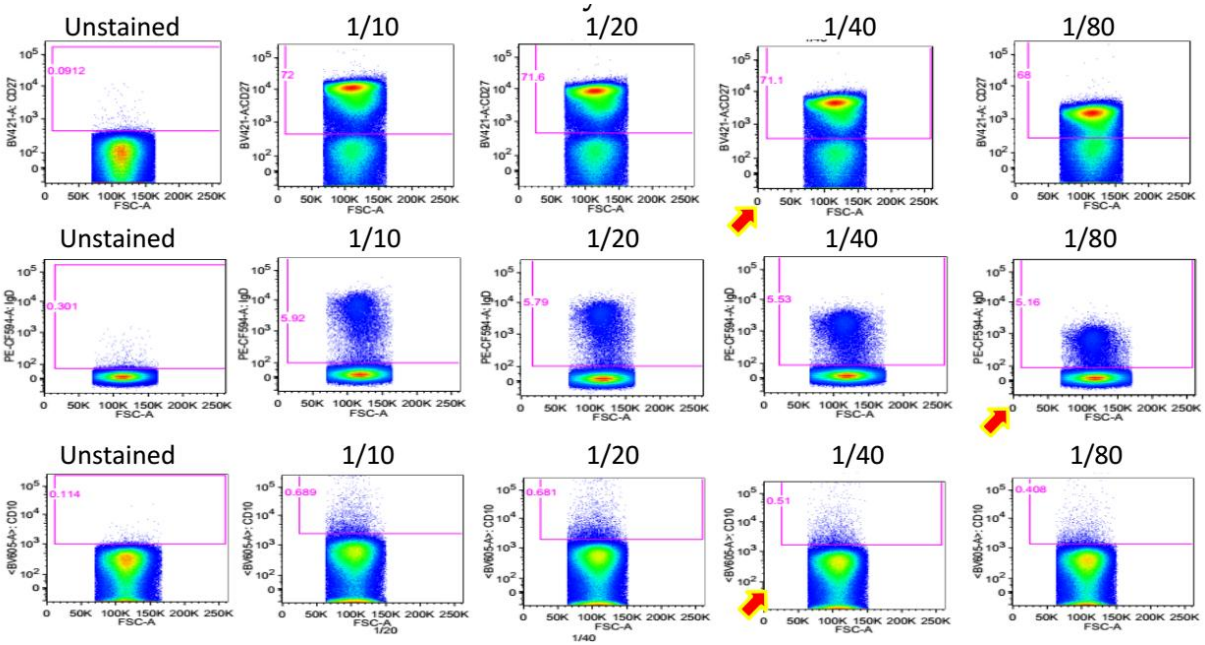


Figure 5. Showing titration results. The viability dye and the antibody conjugates were titrated by doubling dilution and thereafter, each dilution was used to stain $1x10^6$ cells respectively. These were run on the flow cytometer and analysed to determine the optimal titres. Optimal titres were chosen according to cost-effectiveness, cell population separation and staining. The titres included: Axfluor647-A-CD20 1/80, BV421-A-CD27 1/40, BV-650-A-CD19 1/80, FITIC-A-CD21 1/80, PE-CF594-A-IgD 1/80, BV605-A-CD10 1/40, and efluor780 1/4000. APC CY7 emits in the same spectrum as efluor 780 and was a default chrome on the LSRII.

4.1.2. Optimization of cell culture

We determined the cell culture density that would give optimal cell viability, supernatant antibody levels, and polyclonal activation after a 3-day cell culture. Samples were cultured as described in media alone, in the presence of PPD and in the presence of CpG/SAC as described in section 3.7.2 above. As indicated by Rakha *et al.* that higher cell concentration yield antibodies in a short time (73), these culture set-ups had varying volumes of media such as 1000 µl, 500 µl, and 250 µl. $2x10^6$ PBMCs were cultured using these media volumes providing a cell density of $2x10^6$ cells/ml, $4x10^6$ cells/ml, and $8x10^6$ cells/ml respectively as in (fig. 6) below. Cell culture took 3 days and after which we harvested and stored the supernatant at -20°C for antibody measurements. We recovered and counted the cells to determine their cell viability using trypan blue exclusion technique (appendix ii). We then stained them with monoclonal antibodies to identify the selected B cell markers for activated memory B cells, plasmablasts, resting memory B cells and naïve B cells (appendix ii). Cell viability was determined by staining

the cells with efluor780 dye (appendix ii) and analysing the proportions of live cells on LSR II flow cytometer.

Cell viability results

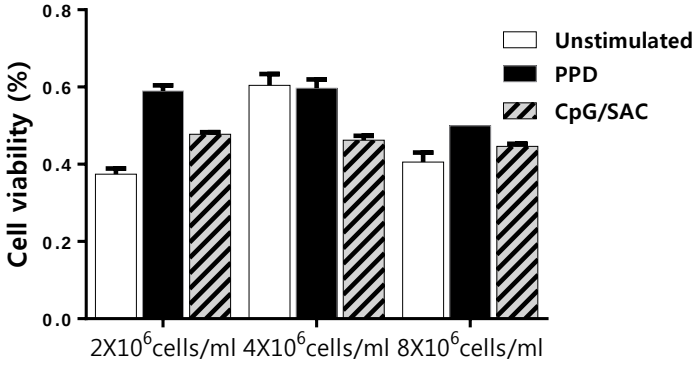


Figure 6. A bar chart showing cell viabilities using trypan blue exclusion technique. $2x10^6$ PBMCs from a blood pack were cultured in a cell culture volume of 1000, 500 and 250 µl with or without PPD or CpG/SAC. After cell culture we harvested and counted the cells by trypan blue exclusion technique to determine Cell viabilities in the different cell densities such as $2x10^6$ cells/ml, $4x10^6$ cells/ml and $8x10^6$ cells/ml. Cell counts were done in duplicates. PPD, (Purified protein derivative). CpG / SAC, (Cytosine phosphate guanine / *Staphylococcus aureus* cowan).

In the unstimulated experiment, 500 µl of culture medium ($4x10^6$ cells/ml) had the highest number of live cells at 60% viability. In the PPD stimulated experiment, higher viabilities (>50%) were observed in 1000 and 500 µl volumes of cell culture media ($2x10^6$ cells/ml and $4x10^6$ cells/ml respectively). In the CpG / SAC stimulated experiment, cell viabilities in the three different cell densities were lower than 50%; however, cell densities of $2x10^6$ cells/ml and $4x10^6$ cells/ml respectively had cell viabilities above 45%.

We performed an antibody ELISA on the harvested supernatant to determine the total IgG secreted into cell supernatant. Fig. 7 below shows the IgG absorbances as measured from different cell concentrations.

Antibody concentration in the supernatant

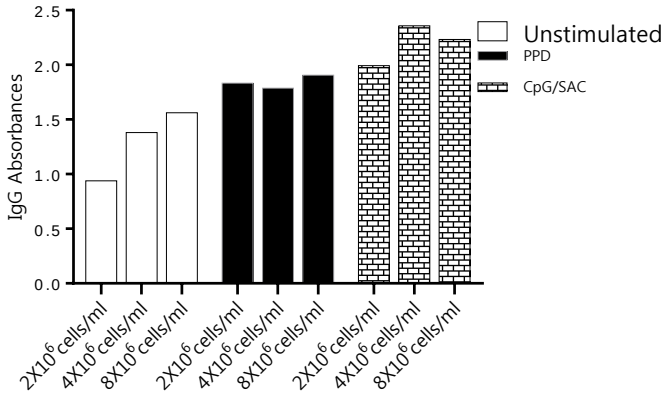


Figure 7. A bar chart showing total IgG secretion following PBMCs stimulation at different cell culture densities. PBMCs were isolated from a blood pack and $2x10^6$ cells were cultured in cell culture volume of 1000, 500 and 250 µl respectively for 3 days. Thereafter, the supernatant was used to determine IgG level by running an antibody ELISA. The outcome of this experiment was IgG absorbance. These were done in triplicates. PPD, (purified protein derivative). CpG/SAC, (Cytosine phosphate guanine / *Staphylococcus aureus* cowan).

The results in Fig. 7 show that antibody secretion increases with increasing cell density. Antibody levels were higher in stimulated wells than in unstimulated wells. This gave an indication that PPD stimulation drives the differentiation of B cells into antibody-secreting cells. The highest antibody levels were observed with the polyclonal stimulants, CpG/SAC, the positive controls. This agreed with the preliminary results by Kimuda *et al. unpublished* (fig. 3). In this experiment, the optimal cell density was $4x10^6$ cells/ml. This is because we could obtain enough supernatant for antibody ELISA analyses and still achieve high antibody production with this cell concentration. The harvested cells were stained for flow cytometry analysis and (fig. 8) below shows the gating strategy used to identify the different B cell phenotypes.

Gating strategy

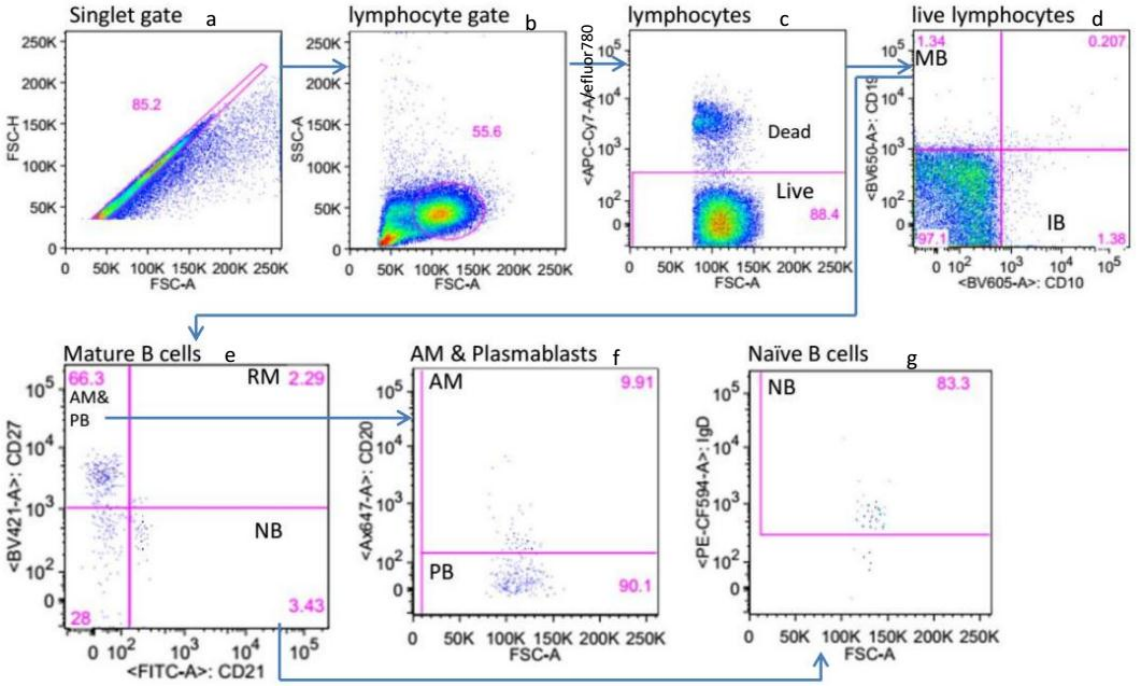


Figure 8. Showing gating strategy. Sample data (a-g) shows how B cell population were gated. Fig. 8b lymphocytes were gated on SSCA/FSCA plot. Dead lymphocytes were excluded using efluor780 and live lymphocytes were further gated to determine mature B cells (MB), immature B cells (IB), activated memory B cells (AM), resting memory B cells (RM), plasmablasts (PB), and naïve B cell (NB) populations (d-g). APC CY7 emits in the same spectrum as efluor 780 and was a default chrome on the LSRII.

The CpG /SAC stimulations had procedural errors; the responses observed with these polyclonal stimulants were not consistent. Therefore, the data presented in (fig. 9) show unstimulated and PPD stimulated cell culture results without CPG/Sac stimulations findings. For that reason, pokeweed mitogen was considered to be used in experiments with the study samples, since this had been used in the previous experiments by kimuda *et al.unpublished (*fig. 3). The major outcome for the measure of polyclonal B cell activation by flow cytometry was activated memory B cells and plasmablasts (fig. 9). After gating live lymphocytes, we separated out the mature B cells ($CD19^{+}CD10^{-}$). In the mature B cell population, we gated the activated B cells such as activated memory B cells and plasmablasts ($CD27^{+}CD21^{-}$), naïve B cells ($CD27^{-}CD21^{+}$) and resting memory B cells ($CD27^{+}CD21^{+}$). We used CD20 to differentiate plasmablasts from activated memory B cells because the expression of this protein is reduced on plasmablasts (71). Naïve B cells ($CD27^{-}CD21^{+}$) were confirmed by their expression of IgD.

Change in B cell phenotypes after varying cell culture densities.

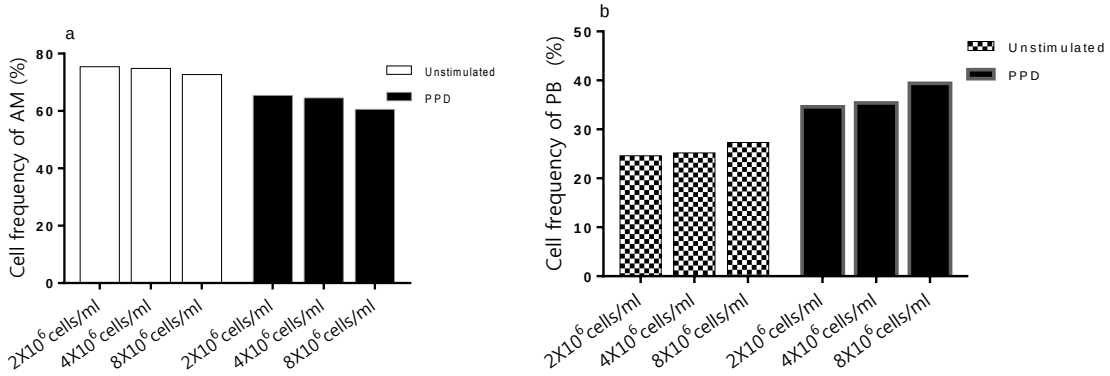


Figure 9a & b. Bar charts showing cell frequencies of AM and PB in different cell density PBMC stimulations. PBMCs from LTBI and uninfected mothers were stimulated in presence or absence of PPD antigen for 3 days. Thereafter, the cells were harvested and stained for different B cell phenotypic markers to identify activated memory B cells and plasmablasts. Flow cytometry analysis was done in order to determine the different B cell phenotypic frequencies such as activated memory B cells (AM) and plasmablasts (PB). Fig. 9a &b show the decrease of AM and increase of PB respectively. These were done in triplicates. PBMCs- peripheral blood mononuclear cells.

As shown in fig 9. Activated memory B cells decrease with increase in cell densities. Activated memory B cells were lower in PPD stimulated wells than in un-stimulated wells. The decrease in activated memory B cells in the PPD stimulated experiment corresponds to the increase in plasmablasts. The lowest frequencies of AM were observed in $4x10^6$ cells/ml and $8x10^6$ cells/ml and this was accompanied by the observed increase in plasmablasts respectively. This trend could be due to differentiation of activated memory B cells to plasmablasts. In addition, the proportions of live cells were gated by using the viability dye (efluor780). This dye stains dead cells and not live cells. The percentages of live cells are indicated in (fig. 10) below.

Cell viability from flow cytometry data

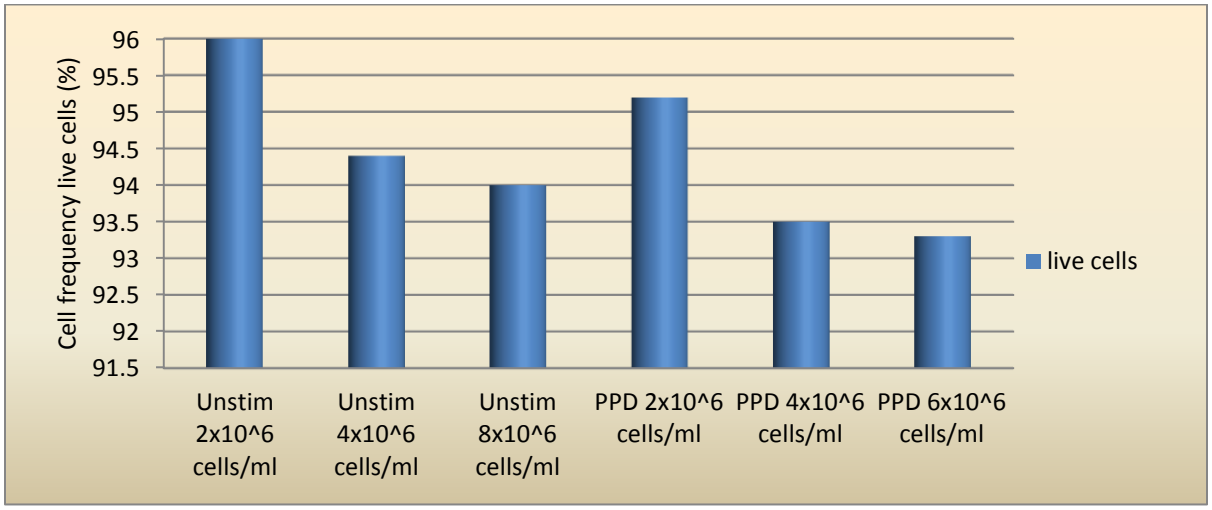


Figure 10. A bar chart showing cell viabilities after efluor780 viability dye staining. $2x10^6$ PBMCs isolated from a blood pack were cultured in the respective 1000, 500 and 250 µl of cell culture volume in presence or absence of PPD or PWM for 3 days. Afterwards, the cells were harvested, stained with eflour780 viability dye, and analysed by flow cytometry. Cell concentration of $2x10^6$ cells/ml had the highest cell viabilities. The experiment was done in triplicates. PPD, (purified protein derivatives). PWM, (Pokeweed mitogen). Unstim, (unstimulated experiment).

Flow cytometry data showed that more live cells were in cell densities of $2x10^6$ cells/ml and $4x10^6$ cells/ml than $8x10^6$ cells/ml. Both flow cytometry data (fig.10) and trypan blue exclusion technique (fig. 6.) indicated that cells Viabilities were poor in cell density of $8x10^6$ cells/ml.

4.2. Research results

We planned to use 20 PBMC samples; however, we managed to obtain 15 samples with a record of cell counts equal or greater than $6x10^6$ cells as this was part of our inclusion criteria. Then after cell culture from thawed cells, we were only able to get cell numbers above our quality control threshold/cut-off of 100,000 from 8 of the 15 samples tested. This was due to the poor cell viabilities. Due to time constraints, we only focused on one specific objective (relevance of *Mycobacterium tuberculosis*-specific T cells in the polyclonal activation of memory B cells).

4.2.1. Antibody levels in the supernatant after PPD stimulation of samples from individuals with and without LTBI

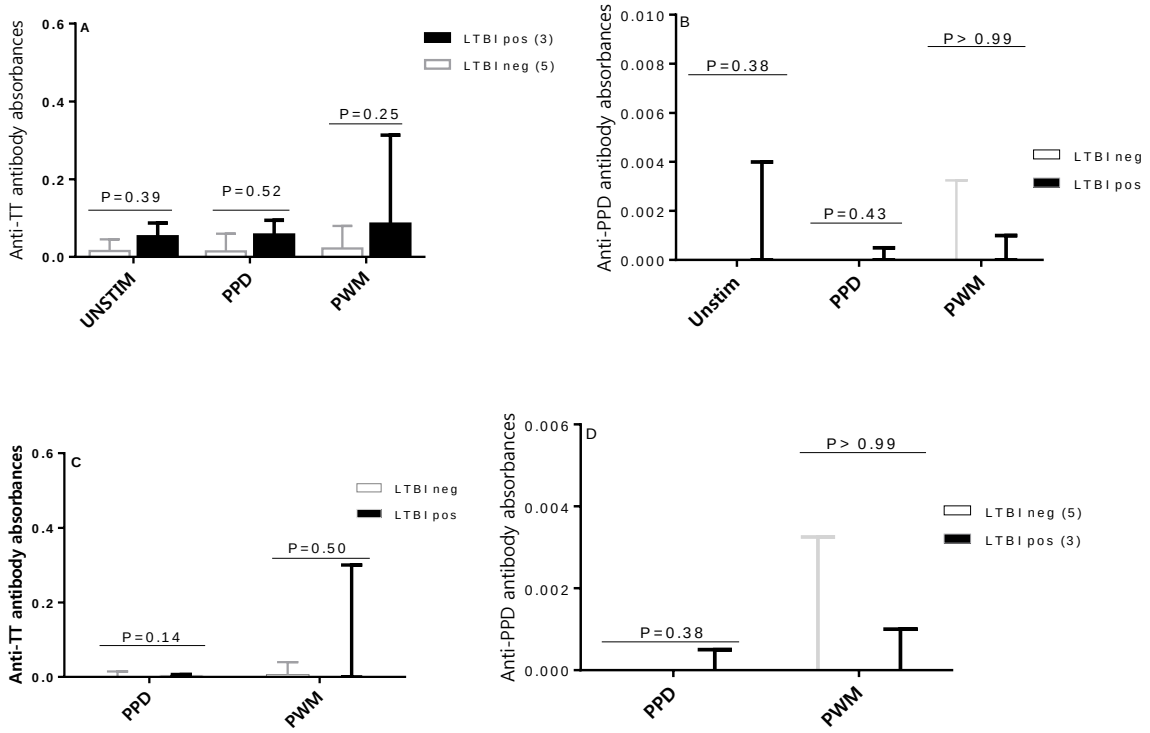


Figure 11. Showing anti-TT and PPD antibody levels in LTBI and uninfected samples. Sample data presented as medians and the interquartile range. PBMCs from LTBI and uninfected mothers were cultured in presence/ absence of PPD or PWM for 3 days. We harvested the supernatant and determined anti TT and PPD antibody levels by ELISA. Results are expressed as antibody absorbance. Fig. A & B represent data before background subtraction while C & D show data after background subtraction. We used Mann Whitney test to determine statistical differences between LTBI and uninfected PPD / PWM stimulations. The statistically significant difference was considered at P value <0.05. n=8. Unstim, (Unstimulated experiment). PPD, (Purified protein derivative). PWM, (Pokeweed mitogen). TT, (Tetanus toxoid antigen).

Fig. 11A above shows that antibody responses were not statistically different. However, there was a trend suggesting higher antibody level to TT antigen in latent TB infected individuals than in uninfected. After background subtraction (fig. 11c), the median levels were above background (unstimulation) in PPD stimulated cells although these were small responses. This trend was observed in individuals regardless of TB infection status in the case of anti-TT antibodies fig.11c and only seen in LTBI positive individuals in the case of anti-PPD antibodies (fig. 11D). PWM

stimulated cells produced higher levels of antibody above background. TT and PPD specific antibodies were not statistically different between LTBI pos and LTBI neg individuals although there was a trend toward higher anti-TT & anti-PPD antibodies among those with LTB infection.

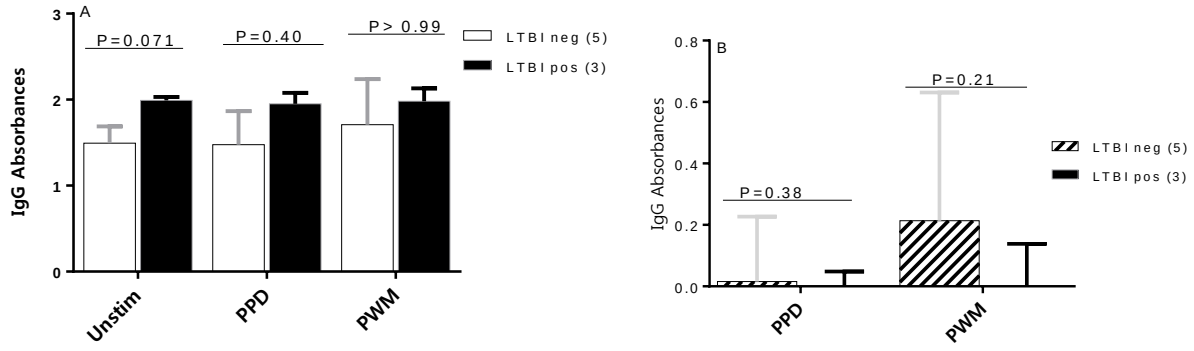


Figure 12. Total IgG concentration in Latent TB infected and uninfected. PBMC samples from LTBI and uninfected mothers were cultured in presence or absence of PPD or PWM for 3days. The supernatant was harvested and used to run an antibody ELISA to determine total IgG Concentration. The measured outcome was IgG absorbance and data was presented as medians and the interquartile range. Fig. A is data with no background subtraction and (fig. B) with Background subtraction. Mann Whitney test was used to compare statistical differences. Significant differences were considered at P values less than 0.05. n=8

Results from fig.12 show a trend suggesting a higher antibody level in latent TB infected individuals than TB uninfected (fig. 12A). After subtraction of background responses (fig. 12B), median levels were above background (unstimulation) in PPD stimulated cells although small in comparison to those induced by PWM. There was no significant difference between antibody levels induced in the latent TB negative and latent TB positive individuals following PPD and PWM stimulation, although there was a trend towards higher antibodies in the former group than the latter.

4.2.2. B cell phenotypic changes after PBMCs stimulation with PPD antigen.

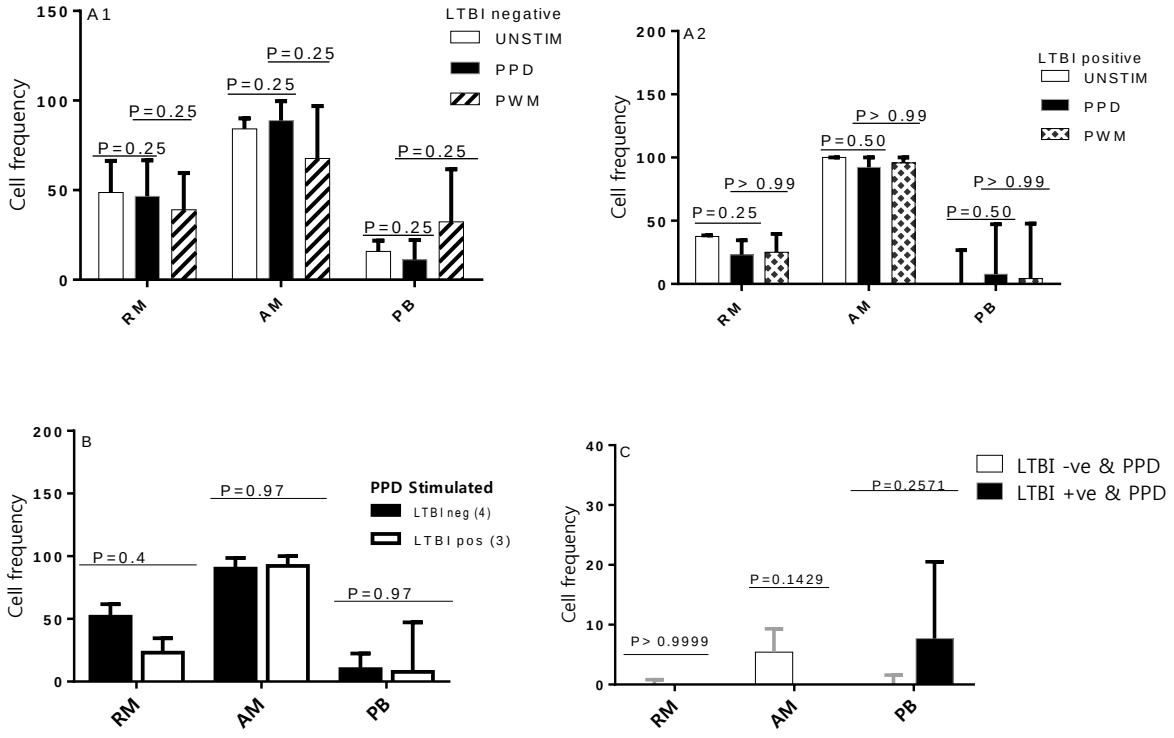


Figure 13. Showing different B cell phenotypes after stimulation of PBMCs with PPD antigen. PBMCs from LTBI and uninfected mothers were cultured in presence or absence of PPD or PWM for 3 days. Thereafter, the cells were harvested and stained for flow cytometry to identify resting memory B cells (RM), activated memory B cells (AM) and plasmablasts (PB). Polyclonal activation was based on a decrease in RM, AM and an increase in PB. Data are presented as medians and the interquartile range. Fig. A & B show data with no background subtraction. Fig. C shows data after background subtraction. Median levels were above background (unstimulation) in PPD stimulated cells although low plasmablast cell frequencies were observed more especially in the latent TB negative individuals. There was no significant difference between plasmablast frequency induced in the latent TB negative and latent TB positive individuals following PPD PWM stimulation, however, there was an observed trend towards higher plasmablast frequency in latent TB infected individuals than the uninfected. The observation in the latent TB individuals could have been due to the effect of *Mycobacterium tuberculosis*-specific T cells on memory B cells. A comparison within a group between stimulations such as negative control and PPD stimulation wells, Wilcoxon signed rank test was used. For comparison between the 2 groups (fig. B & C), the Mann-Whitney test was used to compare if the

differences were statistically significant. Statistical significance was considered at P values less than 0.05. LTBI neg: latent TB negatives. LTBI pos: latent TB positives. n=7

From fig. 13A & B above, resting memory B cells (RM), activated memory B cells were higher in latent TB negative group accompanied by a lower plasmablast frequency. In comparison, RM & AM frequencies were lower in latent TB positives with a higher plasmablast cell frequency. This trend was maintained even after subtraction of background responses (fig. B &D).

CHAPTER FIVE: Discussion

In this study, we measured antibody levels and polyclonal memory B cell activation by antibody ELISA and flow cytometry respectively to define the mechanism behind the polyclonal activation of Memory B cells by *Mycobacterium tuberculosis*. We successfully optimised cell culture conditions for the optimal detection of B cell polyclonal activation, antibody production, and optimal cell viability. We also described the impact of TB exposure/infection as determined by the presence of M. tb specific T cells on B cell polyclonal activation in a limited number of samples (3 LTBI positive and 5 LTBI negatives). The study showed a higher trend of polyclonal activation in LTBI positive individuals than in the uninfected.

From the cell culture optimization experiments, we determined the cell concentration that would yield good cell viabilities, antibody concentration, and B cell polyclonal activation. We observed that cells might have undergone proliferation and differentiation to produce antibody-secreting cells. Increase in cell concentration resulted in higher antibody concentrations, which correlated with the observed higher antibody-secreting cells (plasmablasts) (fig. 9b). This finding correlated with that by Rekha *et al.* that demonstrated the same trend that higher cell concentration results in higher antibody secretion in a short time (73). For good cell proliferation, Cell viabilities are critical and this could both have an effect on antibody production and cell proliferation. Cell concentration of $2x10^6$ and $8x10^6$ cells/ml had the poorest cell viabilities. This may indicate that cell viabilities are affected in low or higher cell concentrations. Cell viability was poor in a cell concentration of $8x10^6$ cells/ml probably because cells proliferated faster and multiplied in numbers, thereby consuming all available nutrients hence their increased death (74). Flow cytometry cell viability results contradicted with trypan blue exclusion technique at a cell concentration of $2x10^6$ cells/ml. This could have been due to procedural differences in the two techniques. This trend needed a further investigation that was not done.

From the three-optimisation experiments, we chose a cell density of $4x10^6$ cells/ml as the optimal cell density for investigation of B cell activation. This was because we achieved better cell viabilities, antibody level, and enough amount of supernatant for use in antibody analyses. In addition, it showed signs of B cell activation as determined by the proportion of activated memory B cells and Plasmablasts.

Preliminary data (fig. 2B) showed that *Mycobacterium tuberculosis* might induce production of non-specific antibodies probably via polyclonal activation of memory B cells (fig. 3). However, the mechanism how *Mycobacterium tuberculosis* induces this process is unknown. In this study, we investigated whether *Mycobacterium tuberculosis*-specific T cells may play a role in the non-specific stimulation of memory B cells. Our results fig. 11A indicated that antibody levels in latent TB infected individuals were higher than in uninfected. This may agree with the preliminary data fig. 2B that demonstrated higher non-specific antibody in tuberculosis-infected individuals. In this case, the anti-TT antibody was higher in latent TB Mothers than in uninfected. After subtraction of background response fig. 11c, PPD stimulation did not significantly contribute to the observed responses. Presumably, it might have been that PBMCs were already activated *in vivo* hence PPD stimulation did not cause a significant difference in the cell activation. The observed higher anti-tetanus toxoid antibody in LTBI than uninfected shows that *Mycobacterium tuberculosis* may play a role in this non-specific immune response. These results agree with a study conducted by Kimuda *et al* that indicated that *Mycobacterium tuberculosis* might be associated with high non-specific antibody responses. Fig. 11C shows that PPD may act as a polyclonal stimulant; however, these were small responses. Bernasconi *et al.* demonstrated that memory B cells proliferate and differentiate into antibody-producing cells in the presence of bystander T cell help (17). In this regard, our findings show that PPD stimulation did not significantly cause induction of bystander T cell help, although there was an observed trend in higher antibody level and polyclonal activation in LTBI positive than uninfected and the median levels were above background.

In individuals with LTBI, the cells are sensitized by exposure to *Mycobacterium tuberculosis in vivo* (49); therefore, these cells produce more anti-PPD (mycobacterial) antibodies and cytokines compared to TB uninfected individuals. Cytokines are believed to be polyclonal stimulants for memory B cells (17). Therefore, these cells might have provided bystander T cell help, and this had a role in the polyclonal activation of memory B cells. Polyclonal activation of memory B might have led to the observed higher antibody levels to the unrelated antigen such as Tetanus toxoid antigen (fig. 11A), this agrees with a study by Bernasconi *et al.* that documented the same trend (17). This is also in line with other studies that observed an increase in antibody levels to the non-*Mycobacteria* antigen such as A/ H3N2 influenza and cytomegalovirus respectively (13, 15). In addition, in a study by Olaleye *et al.*; a higher anti-CMV antibody level was observed in

tuberculosis cases (14). This shows that *M. tb* may induce non-specific effects to other antigens. A study by Bernasconi *et al*. also demonstrated that T cell cytokines could trigger polyclonal activation of memory B cells (17), which is a probable reason for the observed higher anti- TT antibody level in the latent TB infected group. We presume that *Mycobacterium tuberculosis*-specific T cells from latent TB infected group were activated *in vivo* and produced cytokines during cell culture experiments that could have caused polyclonal activation of memory B cells. Activated memory B cells could have differentiated into antibody-secreting cells hence the observed higher antibody levels in the latent TB group than in the uninfected.

Since cell cytokines play a role in the activation of memory B cells(17), in this study, we show that antibody level to the unrelated antigen is higher in the latent TB infected group than in the uninfected.

The observed higher non-specific antibody level in the latent TB group supports the fact that *Mycobacterium tuberculosis* may induce polyclonal activation of memory B cells. Basing on the question whether *Mycobacterium tuberculosis*-specific T cells are involved in the non-specific polyclonal activation of memory B cells, our results show that non-specific antibody level and B cell polyclonal activation were higher in the latent TB infected group than in the uninfected (fig. 11, 12&13). Latent TB infected individuals have *M. tb* specific T cells since they are infected by *Mycobacterium tuberculosis.* Since PBMCs from the latent TB individuals are sensitised by *Mycobacterium* antigen, during cell cultures, these might have produced a response that had an effect on the memory B cells to cause their differentiation into antibody-secreting cells.

Altogether, these results indicate that *Mycobacterium tuberculosis* may induce non-specific immune responses and this process could be due to polyclonal activation of memory B cells. A probable role of *Mycobacterium* specific T cells in the non-specific stimulation of memory B cells cannot be totally ignored. A higher B cell differentiation into antibody-secreting cells such as plasmablasts was observed in the latent TB infected group than in the uninfected. Additionally, total IgG secretion was higher in latent TB infected group than in the uninfected, which also supports the fact that *Mycobacterium tuberculosis* may induce non-specific antibody responses to other antigens.

5.1. Limitations

Our study was limited by a small sample size. The trends we observed may have been statistically significant if we had larger numbers.

Most of the samples had poor cell viabilities. After cell culture from thawed cells, we were only able to get cell numbers above our quality control threshold/cut-off of 100,000 from 8 of the 15 samples tested. Altogether, this was a challenge for us to achieve the planned sample size.

CHAPTER SIX: Conclusion

We compared polyclonal activation and antibody response to unrelated antigens in both latent TB infected and uninfected mothers. Exposure to *Mycobacterium tuberculosis* may induce antibody production to other unrelated antigens. Antibody secretion and polyclonal activation was higher in latent TB infected group compared to uninfected. *Mycobacterium tuberculosis*-specific T cells' role in the non-specific stimulation of memory B cells needs further investigation. *Mycobacterium tuberculosis* T cell cytokines remain a probable mechanism of how *Mycobacterium tuberculosis* induces polyclonal activation of memory B cells. This study provides the basic information that can be used for further investigation of *Mycobacterium tuberculosis*-induced non-specific immune responses.

6.1. Recommendations

Other antigens apart from measles and Tetanus toxoid antigen should be investigated to see if they show similar responses. We recommend that other approaches other than antibody and polyclonal activation be investigated as well, such as gene expression of certain antibodies or cytokines. Other probable mechanisms such as TLR signalling should be investigated as well since TRL have been also implicated in these processes (17, 75). More research should be carried out using different antigens other than *Mycobacterium tuberculosis* to find out if they possess these presumable non-specific immune responses as well.

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APPENDICES

Appendix i

Equipment and reagents

1.0 Equipment

- Carbon dioxide humidified incubator
- Water bath
- Centrifuge
- Biosafety cabinet
- BD LSR II Flow Cytometer
- Microscope
- Haemocytometer

1.1 Reagents

ITEM DESCRIPTION	Manufacturer	Clone	Catalog #	Comment
Fetal Bovine Serum	Sigma		F7524	cell culture
MultiScreen-IP Filter Plate, 0.45 µm, clear, sterile	Merck Millipore		MAIPS4510	cell culture
Immulon 4HBX ELISA plate				Antibody ELISA
2M Sulfuric acid				
Mouse Anti-human IgG				Antibody ELISA
TMB (3', 5, 5' tetramethylbenzidine) substrate				Antibody ELISA
Skimmed milk				Antibody ELISA
BV650 Mouse Anti-Human CD19	BD Biosciences	SJ25C1	563226	Flow cytometry
FITC anti-human CD21 Antibody	BioLegend	Bu32	354910	Flow cytometry
Alexa Fluor® 647 anti-human CD20 Antibody	BioLegend	2H7	302318	Flow cytometry
Brilliant Violet 421 anti-human CD27 Antibody	Bio Legend	O323	302824	Flow cytometry
PE-Cy™7 Mouse Anti-Human CD38	BD Biosciences	HB7	335790	Flow cytometry
BV605 Mouse Anti-Human CD10	BD Biosciences	HI10a	562978	Flow cytometry
PE-CF594 Mouse Anti-Human IgD	BD Biosciences	IA6-2	562540	Flow cytometry
PE Mouse Anti-Human IgG	BD Biosciences	G18-145	555787	Flow cytometry
PE-Cy™5 Mouse Anti-Human IgM	BD Biosciences	G20-127	551079	Flow cytometry

Appendix ii

Experimental procedures

2.0 PBMCs thawing procedure

- PBMCs were retrieved from liquid nitrogen to -80°C before thawing.
- One vial was swirled at a time in the water bath set at 37°C just to leave a small ice pellet
- The vial was disinfected with 70% ethanol before getting it to the safety cabinet
- Warm 20% fetal bovine serum (R20) was added drop-wise to PBMCs and then transferred drop-wise to 8 ml of warm R20
- This was topped up with warm R20 and cells washed by centrifuging at 600g for 10 minutes.
- The supernatant was poured off and the cell pellet resuspended in 10 ml of warm 10% fetal bovine serum (R10)
- Then 10 µl of the cell suspension was pipetted and mixed with 40 µl of trypan blue to stain them in preparation for cell counting.
- Then a 10 µl of the stained cells was transferred to a counting chamber as the cell suspension was topped up with 50 ml of warm R10 and centrifuged at 600g for 10 minutes.

2.2 PBMC counting

- A light microscope was used to count dead and live cells
- Number of cells counted = (average number of live cells counted X dilution factor X volume of cell suspension) X10^4
- After centrifugation, cells were resuspended to a volume of warm R10 at a concentration of 2x10^6/50 µl

2.3 Antibody ELISA

Procedure for measuring antibody concentration in the cell culture supernatant samples

Required items are:

Supernatant samples, Bicarbonate coating buffer, Immulon 4HBX plates, 1 % skimmed milk, anti-human immunoglobin G, TMB substrate, T20, 1X PBS, TT stock 0.3 mg/ml, PPD stock 1 mg / ml.

DAY 1

- The Plate was coated in triplicate with 50 µl of diluted antigen into each well including the blanks. The wells for non-specific binding were not coated with the antigen.
- For each sample, we included two wells for non-specific binding. I.e. the wells were coated with 50 µl of 0.01% skimmed milk diluted in carbonate bicarbonate buffer.
- We included blanks for each antigen in triplicates.
- Covered the plate with plate sealer and kept at 2-8°C overnight.

DAY 2

- We washed the plate with PBST X3 200 µl.
- Blotted and added 150 µl of blocking buffer (1% skimmed milk in PBST)
- Left setup at room temperature for 2hrs.
- We discarded off the blocking buffer and added 50 µl of sample to each antigen coated well including wells for non-specific binding but never added samples to the blanks. 50 µl of blocking solution were added to blank wells
- We then incubated overnight at 2-8°C

DAY3

- We washed the plate x4 with PBST 200 µl.
- Added 50 µl of 0.5 µg / ml of ant-human immunoglobin HRP diluted in blocking solution (1% skimmed milk) per well. The skimmed milk (1 %) was dissolved with PBST.
- We then incubated for 1hr and washed the plate x4 200 µl

- Then we added 100 µl of TMB substrate. This was made by adding equal volume of TMB substrate A to B.
- We developed the reaction in the dark for 20 minutes
- Then we stopped the reaction by adding 25 µl of H_2SO_4 2M to each well.
- Then we read the results at 450nM using ELISA plate reader.

Flow cytometry

3.0 Procedure for staining cells for flow cytometry

- Cells were resuspended at a concentration of $1x10^6$/50 µl after cell culture and counting
- I then Transferred 50 µl to each flow tube and washed with 1 ml FACS Buffer by centrifuging for 10 minutes at 650g X2
- I added 2 µl of FC blocker after pouring off the supernatant and stained for 20 minutes at 2-8°C
- I washed with 1 ml of FACS Buffer by centrifuging at 650g for 10 minutes and pouring off the supernatant.
- Staining with 50 µl of the antibody cocktail was done for 30 minutes at 2-8°C
- Cells were washed with 3 ml of flow staining buffer twice at 650g for 10 minutes each spin
- The cell pellet was be resuspended in 350 µl of cell fix and this will be ready for flow cytometry

3.1. Staining cells with the viability dye (efluor780)

- PBMCs were counted using trypan blue exclusion technique (refer to PBMCs counting procedure).
- We resuspended the cells at a concentration of $1x10^6$ cells / ml in 1x PBS.
- 1 ml of cell suspension was then pipetted into respective facs tubes.

- Cells were stained for viability by adding 0.25 µl of efluor780 dye into the 1 ml of cell suspension.
- Staining was done for 30 minutes at 2-8°C.

3.2. Antibody titration protocol

- We took note of the vendor, catalog number and the lot number of the antibody being titrated.
- I determined the cell concentration of about $1x10^6$ cells for each tube. Five tubes for each antibody was considered; one unstained and four dilutions. We Kept the cells on ice until ready to spin
- I made dilutions of antibody in staining medium such as 1:40, 1:80, 1:160, 1:320

  Below is an example of how antibody-doubling dilution was done.

- I spun the tubes of cells at 650g for 10 minutes 4°C.
- Discarded off the supernatant and resuspended the pellet
- Added 50 µl of staining medium to UN stained cells then 50 µl of each antibody dilution to the remaining four tubes.
- Flicked to mix cells and stain
- Put tubes in the dark 2-8°C for 30 minutes.
- Added 3ml of flow staining buffer to each tube and washed by centrifuging at 650g for 10 minutes.

- Poured off the supernatant and resuspended the cells in 300 µl of cell fix.
- Then cells were ready to be acquired on LSR II flow cytometer

Appendix iii

Raw data

1.0. Optimizations

1.1. Cell viability optimisation results from trypan blue exclusion technique

raw data												
	unstimulated				PPD Stimulated				CpG / SAC Stimulated			
	live	dead	total cells	viability	live	dead	total cells	viability	live	dead	total cells	viability
1000 µl	4.50E+05	7.50E+05	1.20E+06	37.50%	1.00E+06	7.00E+05	1.70E+06	58.82%	5.50E+05	6.00E+05	1.15E+06	47.83%
500 µl	6.00E+05	4.00E+05	1.00E+06	60.00%	1.10E+06	7.50E+05	1.85E+06	59.46%	6.50E+05	7.50E+05	1.40E+06	46.43%
250 µl	4.50E+05	6.50E+05	1.10E+06	40.91%	5.50E+05	5.50E+05	1.10E+06	50.00%	1.05E+06	1.30E+06	2.35E+06	44.68%

1.2.0. Optimizing Antibody concentration in the supernatant

1.2.1. Total IgG in the supernatant of varying culture media

	unstim 10	unstim 50	untim 250	ppd 1000 µ	ppd 500 µl	ppd 250 µl	CpG/SAC 1	CpG/SAC 5	CpG/SAC 2
TOTAL IgG	0.9375	1.3795	1.562	1.8325	1.787	1.905	1.9935	2.3565	2.233

	1	2		3	4		5	6	7	8		9	10		11	12	
A	0.004	0.084		-0.015	-0.024		-0.021		-0.002	0.004		-0.016	-0.008		0.002		Blank 450
B	-0.014	-0.02		-0.021	-0.031		0.082		-0.022	-0.011		-0.017	-0.011		0.006		Blank 450
C	1.018	0.848		1.394	1.35		1.557		1.599	2.058		1.65	1.918		1.901		Blank 450
D	-0.013	-0.028		-0.025	-0.026		-0.028		-0.027	-0.025		-0.008	-0.016		-0.002		Blank 450
E	-0.009	-0.019		0.002	0.004		0.026		1.112	2.559							Blank 450
F	-0.012	-0.023		-0.023	-0.019		-0.018		0.951	0.471					-0.003		Blank 450
G	1.8	2.184		2.409	2.318		2.231		2.638	2.407							Blank 450
H	-0.007	-0.017		-0.015	-0.006				0.02	0.019						0.003	Blank 450

	1	2	unstim 1000	3	4	unstim 500	unstim 250	6	7	8	ppd 1000	9	10	ppd 500	ppd 250	12	
A	0.006	-0.002	0.002	-0.011	-0.013	-0.012	-0.015		0.002	0.008	0.005	-0.012	-0.005	-0.0085	0.006		DS1-DS2
B	0	-0.014	-0.007	-0.016	-0.016	-0.016	0		-0.015	-0.005	-0.01	-0.014	-0.009	-0.0115	0.011		DS1-DS2
C	1.022	0.853	0.9375	1.395	1.364	1.3795	1.562		1.605	2.06	1.8325	1.655	1.919	1.787	1.905		DS1-DS2
D	-0.011	-0.023	-0.017	-0.02	-0.022	-0.021	-0.022		-0.022	-0.021	-0.0215	-0.006	-0.011	-0.0085	0.003		DS1-DS2
			CpG/SAC 1000			CpG/SAC 500	CpG/SAC 250										
E	-0.005	-0.014	-0.0095	0.006	0.01	0.008	0.027		1.116	2.561							DS1-DS2
F	-0.008	-0.019	-0.0135	-0.018	-0.015	-0.0165	-0.014		0.953	0.471							DS1-DS2
G	1.801	2.186	1.9935	2.41	2.303	2.3565	2.233		2.639	2.408							DS1-DS2
H	-0.009	-0.016	-0.0125	-0.016	-0.013	-0.0145			0.022	0.02							DS1-DS2

1.2.2. Change in B cell phenotypes after varying cell culture densities.

Sample	AM & PB	AM	AM IgG+	PB	RM	NB
Unstim 1000	2.91	75.4	91.9	24.6	24.2	92.6
Unstim 500	2.83	74.8	90.7	25.2	23.9	92.5
Unstim 250	2.28	72.7	94.1	27.3	25.7	90.3
PPD 1000	2.78	65.4	86.4	34.6	24.5	84.9
PPD 500	2.52	64.6	87.6	35.4	24.1	86.6
PPD 250	2.48	60.6	88	39.4	26.7	88.9
	Singlet/Lymp	Singlet/Lymp	Singlet/Lymp	Singlet/Lymp	Singlet/Lymp	Singlet/Lympl
AM, activated memory B cells. PB, Plasmablasts. RM, Resting memory B cells. NB, Naïve B cells						

1.3.0. Live cells using efluor780

Sample	Singlet/Lymphs/Live,Freq. of Parent
1: Specimen_001_unstained.fcs	99.6
2: Specimen_001_PPD 250.fcs	93.3
3: Specimen_001_PPD 500.fcs	93.5
4: Specimen_001_PPD 1000.fcs	95.2
5: Specimen_001_CpG,2f,SAC 250.fcs	92.4
6: Specimen_001_CpG,2f,SAC 500.fcs	93.8
7: Specimen_001_CpG,2f,SAC 1000.fcs	89.7
8: Specimen_001_Unstim 250.fcs	94
9: Specimen_001_Unstim 500.fcs	94.4
10: Specimen_001_Unstim 1000.fcs	96
Mean	94.2
StdDev	2.55

2.0. Research raw data

2.1.0. ELISA plate one

1	2	3	4	5	6	7	8	9	10	11	12	
2.417	2.382	1.368	1.388	0.076	0.073	0.107	0.101	0.089	0.092	0.005	0.017	DS1-DS2
1.338	1.41	1.406	1.401	0	0	0	0	0	0	0	0	DS1-DS2
2.372	2.301	2.353	2.235	1.647	1.478	1.781	1.375	1.697	1.72	0.013	0.014	DS1-DS2
0.08	0.087	0.052	0.057	-0.005	-0.005	-0.006	-0.005	0	0	0	0	DS1-DS2
0.044	0.063	0.058	0.058	0.051	0.048	0	0	0	0	0	0	DS1-DS2
0	0	0	0.001	0	0	0	0	0	0	0	0.003	DS1-DS2
2.079	1.982	2.127	2.031	1.998	1.964	0.019	0.016	0.016	0.017			DS1-DS2
0	0	0	0	0	0	0	0	0	0.014			DS1-DS2

2.1.1. ELISA plate two

1	2	3	4	5	6	7	8	9	10	11	12	
0.088	0.087	0.096	0.093	0.088	0.084	0.01	0.009	0.007	0.005	0.009	0.023	DS1-DS2
0.008	0	0	0	0	0	0	0	0	0	0	0	DS1-DS2
2.144	1.839	1.883	2.02	1.98	1.937	1.198	1.101	1.253	1.27	1.481	1.494	DS1-DS2
0	0	0	0	0	0	0	0	0	0	0	0	DS1-DS2
0.014	0.012	0.014	0.014	0.344	0.283	2.135	2.184	1.334	1.35	0	0	DS1-DS2
0	0	0	0	0.002	0	1.443	1.351	1.468	1.465	0		DS1-DS2
1.564	1.576	1.568	1.547	2.177	2.084	2.521	2.359	2.344	2.392			DS1-DS2
0.001	0	-0.001	-0.003	0	0.004	0.078	0.148	0.096	0.139	0.004		DS1-DS2

2.1.2. ELISA plate three

1	2	3	4	5	6	7	8	9	10	11	12	
0.017	0.014	0.016	0.013	0.022	0.022	0.004	0.009	0.008	0.004	0.005	0.134	DS1-DS2
0	0	0	0	0	0	0	0	0	0	0	0.013	DS1-DS2
1.678	1.949	2.207	2.103	2.266	2.133	1.009	1.001	1.024	1.002	1.123	1.114	DS1-DS2
0	0	0	0	0	0	-0.003	-0.004	0	0	0	0.003	DS1-DS2
0.017	0.015	0.015	0.016	0.021	0.021	2.653	2.668	1.577	1.576	0.005		DS1-DS2
0	0	0	0	0	0	1.662	1.625	1.821	1.827	0		DS1-DS2
1.444	1.545	1.502	1.45	2.301	2.255	2.895	2.903	3.017	2.863			DS1-DS2
0	0	0	-0.002	0	0	0.071	0.048	0.06	0.048	0		DS1-DS2

2.2.0. Anti-PPD antibodies

		Anti-PPD antibodies		
	Unstimulated	**PPD**	**PWM**	**Plate**
982	0	0	0	One
1864	0	0.0005	0	One
158	0.004	0	0	Two
159	0	0	0	Two
2502	0	0	0.001	Two
1865	0	0	0	Three
10	0	0	0.0065	Three
930	0	0	0	Three

2.2.1. Anti-TT antibodies

		Anti-TT antibodies		
	Unstimulated	**PPD**	**PWM**	**Plate**
982	0.0745	0.104	0.0905	One
1864	0.0535	0.058	0.0495	One
158	0.0875	0.0945	0.086	Two
159	0.0095	0.006	0.016	Two
2502	0.013	0.014	0.3135	Two
1865	0.0155	0.0145	0.022	Three
10	0.0065	0.006	0.0695	Three
930	0.016	0.0155	0.021	Three

2.2.2. Total IgG

		Total IgG		
	Unstimulated	**PPD**	**PWM**	**Plate**
982	1.5625	1.578	1.7085	One
1864	2.0305	2.079	1.981	One
158	1.9915	1.9515	1.9585	Two
159	1.1495	1.2615	1.4875	Two
2502	1.57	1.5575	2.1305	Two
1865	1.8135	2.155	2.1995	Three
10	1.005	1.013	1.1185	Three
930	1.4945	1.476	2.278	Three

2.3.0. Flow cytometry data

Sample	Singlet/Live/L	Singlet/Live/Ly	Singlet/Live/Ly	Singlet/Live/Ly	Singlet/Live/Ly	Singlet/Live/Ly	Singlet/Live/Lymphocytes/Q1: CD10–, CD19+/Q3: CD21+, CD27–/IgD+,Freq. of Parent
1: Specimen_001_0010 PPD.fcs	0.38	0	•	•	•	32.6	93.7
2: Specimen_001_0010 PWM.fcs	0.383	0	•	•	•	25	92.4
3: Specimen_001_0010 unstim.fcs	0.377	1.56	25	100	75	21.1	92.3
4: Specimen_001_158 PPD.fcs	3.35	0.816	52.8	52.6	47.2	23	91.2
5: Specimen_001_158 PWM.fcs	1.57	22	52.3	64.7	47.7	10	85.2
6: Specimen_001_158 unstim.fcs	1.92	1.08	73.3	36.4	26.7	29.8	98.6
7: Specimen_001_159 PPD.fcs	1.26	3.19	75	83.3	25	51.1	81.9
8: Specimen_001_159 PWM.fcs	1.15	8.12	46.8	72.7	53.2	38.2	67.4
9: Specimen_001_159 unstim.fcs	1.32	6.24	77.1	83.8	22.9	54	80.1
10: Specimen_001_930 PPD.fcs	10.5	2.06	100	64.1	0	64.8	87.5
11: Specimen_001_930 PWM.fcs	22.8	8.61	89.6	69.1	10.4	52.2	88.3
12: Specimen_001_930 unstim.fcs	10.2	3.04	91.5	65.1	8.51	63.7	88.9
13: Specimen_001_982 PPD.fcs	4.77	3.33	94.6	43.4	5.36	52.5	90.1
14: Specimen_001_982 PWM.fcs	3.84	3.58	95.9	46.8	4.08	55.4	73.5
15: Specimen_001_982 unstim.fcs	4.81	2.16	85	58.8	15	53.9	68.5
16: Specimen_001_1864 PPD.fcs	21.5	1.61	92.3	50	7.69	20.6	90.4
17: Specimen_001_1864 PWM.fcs	18.1	3.27	95.8	75.4	4.17	39.5	92.4
18: Specimen_001_1864 unstim.fcs	17	3.08	100	82.5	0	38.4	91.9
19: Specimen_001_1865 PPD.fcs	5.9	2.28	85.4	50	14.6	17.8	83.1
20: Specimen_001_1865 PWM.fcs	5.58	5.86	38.2	77.9	61.8	10.5	80.3
21: Specimen_001_1865 unstim.fcs	3.75	1.99	83.1	61.2	16.9	23	89
22: Specimen_001_2502 PPD.fcs	42.2	0.695	100	76.5	0	34.6	83.7
23: Specimen_001_2502 PWM.fcs	57.4	0.495	100	94.4	0	25	75.8
24: Specimen_001_2502 unstim.fcs	43.4	1.12	100	66.7	0	37.6	86.3
	CD10–, CD19+	CD21–, CD27+	CD27+/CD20+	CD27+/CD20+/I	CD27+/CD20-	CD21+, CD27+	CD27–/IgD+

2.3.1. Summary of flow cytometry data

Infection status		Sample	AM&PB	AM	AM IgG+	PB	RM	NB			
P	PPD	158 PPD	0.816	52.8	52.6	47.2	23	91.2			
P	PWM	158 PWM	22	52.3	64.7	47.7	10	85.2		TB status	
P	UN	158 unstim	1.08	73.3	36.4	26.7	29.8	98.6		982	Neg
N	PPD	159 PPD	3.19	75	83.3	25	51.1	81.9		1864	Pos
N	PWM	159 PWM	8.12	46.8	72.7	53.2	38.2	67.4		158	Pos
N	UN	159 unstim	6.24	77.1	83.8	22.9	54	80.1		159	Neg
N	PPD	930 PPD	2.06	100	64.1	0	64.8	87.5		2502	Pos
N	PWM	930 PWM	8.61	89.6	69.1	10.4	52.2	88.3		1865	Neg
N	UN	930 unstim	3.04	91.5	65.1	8.51	63.7	88.9		10	Neg
N	PPD	982 PPD	3.33	94.6	43.4	5.36	52.5	90.1		930	Neg
N	PWM	982 PWM	3.58	95.9	46.8	4.08	55.4	73.5			
N	UN	982 unstim	2.16	85	58.8	15	53.9	68.5			
P	PPD	1864 PPD	1.61	92.3	50	7.69	20.6	90.4		AM, activated memory B cells	
P	PWM	1864 PWM	3.27	95.8	75.4	4.17	39.5	92.4		PB, Plasmablasts	
P	UN	1864 unstim	3.08	100	82.5	0	38.4	91.9		RM, Restsing memory B cells	
N	PPD	1865 PPD	2.28	85.4	50	14.6	17.8	83.1		NB, Naïve B cells	
N	PWM	1865 PWM	5.86	38.2	77.9	61.8	10.5	80.3			
N	UN	1865 unstim	1.99	83.1	61.2	16.9	23	89			
P	PPD	2502 PPD	0.695	100	76.5	0	34.6	83.7			
P	PWM	2502 PWM	0.495	100	94.4	0	25	75.8			
P	UN	2502 unstim	1.12	100	66.7	0	37.6	86.3			
			Singlet/Li	Singlet/Li	Singlet/Li	Singlet/Li	Singlet/Li	Singlet/Live/Lymphocytes/Q1: CD10–, CD19+/Q3: CD			

2.3.2. School of Biomedical sciences Higher Degrees Research and Ethics committee Approval

MAKERERE UNIVERSITY

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICAL SCIENCES
HIGHER DEGREES RESEARCH AND ETHICS COMMITTEE

27th Nov 2017

File: SBS-498

To: Mr. Lugwana Samuel
Principal Investigator
Department of Microbiology
Makerere University

Category of review
[X] Initial review
[ ] Continuing review
[ ] Amendment
[ ] Termination of study
[ ] SAEs

Decision of the School of Biomedical Sciences Higher Degrees Research and Ethics Committee (SBS-HDREC) following its 74th REC meeting held on 19th Oct 2017.

In the matter concerning the review of a research proposal entitled **"Investigation of the mechanisms behind non-specific stimulation of memory B-cells by Mycobacteria TP" SBS-498**

The investigator has met all the requirements as stated by the **SBS-HDREC** and therefore, the protocol is **APPROVED.**

The approval granted includes all materials submitted by the investigator for the **SBS-HDREC** review unless otherwise stated; and is valid until **18th Oct 2018.**

Any problems of a serious nature related to the execution of the research protocol should be promptly reported to the **SBS-HDREC**, and any changes to the research protocol should not be implemented without approval from the **SBS-HDREC**, except when necessary to eliminate apparent immediate hazards to the research participant(s)

Please note that the annual report and the request for renewal where applicable should be submitted to the **SBS-HDREC** office at least six (6) weeks before expiry date of approval.

You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

MAKERERE UNIVERSITY
SCHOOL OF BIOMEDICAL SCIENCES
APPROVED
VALID UNTIL
18 OCT 2018
RESEARCH & ETHICS COMMETTEE
P.O. BOX 7072 KAMPALA

Signed. .....

Dr. Jackson Mukonzo
Vice Chairperson, School of Biomedical Sciences Higher Degrees Research and Ethics Committee.