In vitro model of *Mycobacterium* tuberculosis (*Mtb*): Impact of donor BCG vaccination and T regulatory cells on host immune responses and *Mtb* growth

by

Sudha Bhavanam

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Abstract

Tuberculosis (TB) is a chronic pulmonary disease caused by *Mycobacterium tuberculosis* (*Mtb*). *Mtb* spreads by aerosol and infects through the airways. *Mtb* is phagocytosed by macrophages in the lung, and is able to replicate inside these cells. The initial infection of *Mtb* with macrophage results in the recruitment of other innate and adaptive immune cells resulting in the formation of a granuloma, the hallmark of *Mtb* infection. Maintenance of the granuloma is thought to be depended on the host immune system. A Bacille Calmette-Guérin (BCG) vaccine was developed in 1920 to prevent *Mtb* infection and is still the only available vaccine. The efiicacy of this vaccine is variable in terms of protection against pulmonary TB infection. This thesis focuses on the impact of BCG vaccination history of donor human peripheral blood mononuclear cells (PBMCs) on the interaction between the host immune cells and *Mtb*.

This work is the first study to investigate BCG vaccination history as a potential confounding variable when evaluating the immune responses in an *in vitro* PBMC model of early *Mtb* infection using the attenuated strain *Mtb* H37Ra. After validating an *in vitro* PBMC-collagen matrix culture model of *Mtb* infection, I characterized the immune responses to *Mtb* infection in PBMCs from 10 donors. Aggregates of PBMCs were initially observed on day 3 and the size of aggregates continued to increase until day 8 post-infection when macrophages and T cell subsets were identified to be present. The levels of cytokines secreted by PBMCs infected with *Mtb* peaked on day 3 and decreased on days 5 and 8. I then stratified the data by donor prior BCG vaccination history. Mycobacterial load was significantly lower in infected PBMCs from BCG vaccinated donors compared to unvaccinated donors. The proinflammatory cytokines secreted by T helper 1 cells (Th1): INF-γ, TNF-α, IL-6, IL-4, and IL-17 in the supernatants were higher in BCG vaccinated donors compared to BCG unvaccinated donors. In contrast, the secretion of one anti-

inflammatory cytokine secreted by T helper 2 cells (Th2) as well as Tr1 cells: IL-10, was significantly lower in *Mtb*-infected PBMCs from BCG vaccinated donors compared to BCG unvaccinated donors. These results demonstrated that prior BCG vaccination led to a greater Th1 than a potential Th2 immune response and there by increased the suppression of *Mtb* growth.

Tregs are a subset of T lymphocytes whose role is to suppress the immune response to infection. The role of Tregs in the suppression of *Mtb* growth was studied in donor PBMCs with or without Tregs (depleted by antibody- coated magnetic bead separation). This study is the first report to investigate the role of Tregs in an early *in vitro* PBMC model of *Mtb* H37Ra infection cultured for 8 days. Cell aggregates were unexpectedly smaller in PBMCs without Tregs compared to PBMCs with Tregs at day 8 post-infection, suggesting that Tregs promoted rather than impaired the formation of cell aggregates. The number of *Mtb* colony forming units (CFUs) increased in PBMCs without Tregs compared to PBMCs with Tregs at days 3, 5 and 8, suggesting that Tregs may play a role in suppressing the growth of *Mtb*. Levels of IL-17 and proinflammatory Th1 cytokines IFN-γ (at days 3 and 5), TNF-α and IL-6 (at day 3) were lower in PBMCs without Tregs compared to PBMCs with Tregs. In contrast, anti-inflammatory Th2 cytokines IL-10 and IL-4 were higher at day 3 in PBMCs depleted of Tregs compared to PBMCs with Tregs demonstrating that depletion of Tregs led to an unexpected decrease in Th1 to a potential Th2 cytokine response.

This study highlights the possibility that BCG vaccination may confound results that utilize human PBMCs to study *Mtb* infection. The unexpected finding that depletion of Tregs increased *Mtb* growth and decreased Th1 immune responses to infection suggests further investigation is needed to understand the role of Tregs in early *Mtb* infection.

Preface

A portion of the literature review described in Chapter 1 has been published as "Understanding the pathophysiology of the human TB lung granuloma using *in vitro* granuloma models". I share the first authorship of this publication with Dr. Gina R. Rayat, Dr. Monika Keelan, Dr. Dennis Kunimoto, and Dr. Steven J. Drews S.J. I wrote the first draft of the manuscript and I performed the entire literature search. The co-authors each reviewed and edited the manuscript.

The studies presented in Chapters 2 has been published as "Characterization of immune responses of human PBMCs infected with *Mycobacterium tuberculosis* H37Ra: Impact of donor declared BCG vaccination history on immune responses and *M. tuberculosis* growth". I share the first authorship of this publication with Dr. Gina R. Rayat, Dr. Monika Keelan, Dr. Dennis Kunimoto, and Dr. Steven J. Drews S.J. I wrote the first draft of the manuscript and I performed the entire literature search. The co-authors each reviewed and edited the manuscript.

Chapter 3 were conceived in consultation with Dr. Drews. I cultured the *Mtb* strain used for infection, performed all experimental tests, data analysis, and data interpretation. Drs. Rayat, Keelan, and Kunimoto ~~s~~ also contributed to the study design. Chapter 3 has not yet been published. These studies received research ethics approval from the University of Alberta Research Ethics Board, under the Project Name "Immune responses and survival strategies of *Mycobacterium tuberculosis* in an *in vitro* granuloma model" No. Pro00057636, on February 10, 2016.

Dedication

To my husband Sagar, my son Amit and my daughter Archa, for all their support and love

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List of abbreviations

AFB	Acid-fast bacilli
AIDS	Acquired immunodeficiency syndrome
AG	Arabinogalactan
AS	Auramine stain
BCG	*Bacillus Calmette Guérin*
CX	Chest X-ray
CR3	Complement receptor 3
CT	Computerized tomography
CFP-10	Culture filtrate protein 10
CTLs	Cytolytic T cells
DTH-IV	Delayed-type hypersensitivity reaction-IV
DCs	Dendritic cells
DST	Drug-susceptibility testing
ESAT-6	Early secretory antigen 6 kDa
ELISA	Enzyme-linked immunosorbent assay
EMB	Ethambutol
XDR-TB	Extensively drug-resistant TB
ECM	Extracellular matrix
FDG	Fluorodeoxyglucose
FM	Foamy macrophages
FoxP3	Fork head box P3
GPCR	G protein-coupled receptor
γδ	Gamma-delta
HIV	Human immunodeficiency virus

IGRAs	Interferon Gamma Release Assays
IFN-γ	Interferon-γ
IL-1β	Interleukin-1 beta
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-17	Interleukin-17
IL-4	Interleukin-4
IL-6	Interleukin-6
INH	Isoniazid
LTB	Latent TB infection
MTB	*M. tuberculosis*
MXF	Moxifloxacin
MTD	*Mtb* direct test
MDR	Multidrug-resistant
MDR-TB	Multidrug-resistant tuberculosis
MGC	Multinucleated giant cells
Mtb	*Mycobacterium tuberculosis*
MA	Mycolic acids
NKT	Natural Killer T cells
iNOS	Inducible nitric oxide synthase
NAA	Nucleic acid amplification
PRRs	Pattern recognition receptors
PG	Peptidoglycan
PPD	Protein derivative

PZA	Pyrazinamide
QFT	QuantiFERON®TB Gold In-Tube
RD1	Region of Difference 1
Tregs	Regulatory T cells
RIF	Rifampicin
TLR	Toll-like receptors
PET	Positron emission tomography
TGF-β	Transforming Growth Factor β
TB	Tuberculosis
TNF-α	Tumor Necrosis Factor-α
WHO	World Health Organization
ZN	Ziehl-Neelsen

Chapter 1: Literature Review*

*A portion of this chapter has been published as" Understanding the pathophysiology of the human TB lung granuloma using in vitro granuloma models". Future Microbiol. 2016 Aug;11:1073-89. doi: 10.2217/fmb-2016-0005. Epub 2016 Aug 9.

1.1 Introduction

The World Health Organization (WHO) has estimated that there are between 8 to 9 million new cases each year, of whom 200 million people develop active tuberculosis (TB) during their lifetime, and that 30 million will die from TB over the next decade (1). The situation is worsened by the increasing incidence of multidrug-resistant (MDR) strains, and the deadly combination of TB with acquired immunodeficiency syndrome (AIDS) (2,3). TB is caused by *Mycobacterium tuberculosis* (*Mtb*), a bacterium that is primarily transmitted by the airborne route from humans with active pulmonary TB disease to other humans (2). In more than 95% of individuals with primary infection, there are no clinical symptoms (2,4). After primary infection, TB bacilli enter a latent or dormant state and this state may last six months or can be maintained for the lifetime of the infected person (2,4). Latent TB infection (LTBI) causes no symptoms and is not contagious (4). Usually, the only detectable abnormality is a positive tuberculin skin test (4). The WHO has estimated that close to two billion people have LTBI, and in human immunodeficiency virus (HIV)-negative patients, approximately 10% will reactivate over their lifetime and develop active TB (5,6). In HIV positive patients, 30% will reactivate over their lifetime and develop active TB (5). A key feature of LTBI infection is the formation of an organized histological structure called a granuloma (7). Although the important function of the granuloma is to contain the infection, some bacilli can survive inside granuloma for a long time in a dormant state (7). Due to a variety of reasons, the dormant bacilli will reactivate, escape the granuloma and spread throughout the body, giving rise to clinical disease (8). An understanding of the roles of the host immune system in the formation of and stability of granulomas as well as the survival and reactivation of the bacilli within these structures is critical for the design of new vaccines and anti-tuberculous drugs.

1.2 Tuberculosis Infection

TB disease is caused by the *Mtb* complex, which is comprised of several human and animal associated species and sub-species (8,9). The *M. tuberculosis* complex (MTBC) comprises *M. tuberculosis, M. africanum, M. canettii, M. bovis* (including the attenuated vaccine strain Bacillus Calmette–Guérin [BCG])*, M. microti, M. orygis, M. caprae, M. pinnipedii, M. suricattae* and *M. mungi* (9). In humans, TB is primarily caused by *Mtb* and *M. africanum* (9). TB is acquired by the inhalation of aerosolized droplets containing the bacilli from individuals with active TB (6,10). The droplets with an approximate size of 1-3μm containing approximately 3 bacilli per particle can reach the lung alveolar (10). The number of bacteria that are required to establish an infection in humans varies depending on the genetic background of the infected individual, the strain of the bacteria and host immune responses to the infection (10,11). The shift between latent and active TB infection requires an understanding of host and bacterial dynamics within the milieu of a granuloma (7). A granuloma is an organized structure of cellular accumulation, which plays a key role in host defense against *Mtb* infection (7,11). These structures sequester and contain *Mtb* preventing active disease, while long-term maintenance of granulomas leads to latent disease (7,11). Thus, it is no surprise that the disease is more prominent in populations of immunosuppressed individuals (e.g., AIDS patients) (5).

Despite the development of effective chemotherapy and vaccine for TB over the last 50 years, there remain several obstacles to controlling TB infection (12). The current vaccine BCG was developed by the French scientists Calmette and Guérin in the 1920s and is the only licensed vaccine for TB (13). BCG has been used as a vaccine for more than 70 years and has been given to more people than any other vaccine (12). BCG reduces the incidence of miliary and meningeal TB in young children and has tolerable side effects (14-16). In contrast, BCG fails to protect against

pulmonary TB in adults (14,16,17). Data regarding the protective efficacy of BCG in adults range from 0% to 80% (16). Anti-tuberculosis drugs included in the directly observed treatment program (isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), streptomycin, and ethambutol (EMB)) must be taken for 6 months or longer (3). A combination of drugs is required to avoid the development of drug-resistant TB. These obstacles severely affect patient compliance, which can lead to the emergence of MDR and extensively drug-resistant (XDR) strains (18-21). The need for immunotherapeutic vaccines and new anti-mycobacterial agents highlights the importance of studying *Mtb* in the context of an infection model (22-24).

1.2.1 Tuberculosis – a Global Problem

TB ranks as the second leading cause of death from a single infectious agent, after HIV. It is estimated that one-third of the world's population is infected with TB. According to the reports of the WHO it is estimated that 8.9–10.4 million incident cases and approximately 2 million deaths from TB have been recorded for the year 2015. Even though the number of TB cases in the world has significantly declined in the past few decades, the number of new cases continues to be considerable. Over 90% of the global TB cases are in developing countries. In 2017, according to the WHO India, Indonesia, China, Nigeria, Pakistan and South Africa account for 60% of the total number of TB cases in the world. Asia accounts for 61% of all new TB cases in the world, followed by Africa at 26%.

1.2.2 Human Immunodeficiency Virus and Tuberculosis Co-Infection

Another public health issue connected with the spread of the TB is its coincidence with HIV infection (5). HIV-infected persons with LTBI have a much higher risk of developing active TB (estimated at 3-16% per year) than the general population (estimated at <10% in a lifetime) (5,8). According to the WHO, co-infection of TB and HIV together is responsible for 0.4 million deaths

globally in 2015. The risk of developing TB is estimated to be 20 to 30 times greater in people living with HIV than people without HIV infection (25-29). In 2015, there were 10.4 million new cases of TB, of which 1.2 million were among people living with HIV. The burden of HIV / TB co-infection is highest in Sub-Saharan Africa constituting 81% of all global cases (29). This co-infection with *Mtb* and HIV represents a major barrier to the control of TB.

1.3 *Mycobacterium tuberculosis*

The *Mycobacterium* species belong to the order *actinomycetales*, family *Mycobacteriaceae* and genus *Mycobacterium* (30). The major human pathogens are *Mtb* (Tuberculosis or Koch's disease, Koch, 1882), *M. leprae* (leprosy or Hansen's disease, Hansen, 1874) and rapidly growing *Mycobacteria spp* such as *Mycobacterium avium* complex and other non-tuberculous mycobacteria (NTM) (31,32). Mycobacteria are aerobic, slender, non-motile, non-encapsulated, non-spore forming and rod-shaped bacilli (33). The bacilli measure 2-4 micrometers (μm) in length and 0.2-0.5 μm in width (30). These intra-cellular pathogens infect alveolar macrophages and are found within membrane-bound particles or 'phagosomes' (34). Mycobacterium is resistant bacilli accomplished by adapting to different environmental conditions (35). The bacilli are resistant to phagocytosis and remain dormant for decades within phagocytic or non-phagocytic tissue cells with the ability to re-activate (34,35). The lipid-rich cell wall makes the bacilli resistant to bacterial standard stains such as Gram staining (36). Acid-fast stain such as Ziehl-Neelsen (ZN stain) is used to identify *Mtb* (36) (Figure 1.1).

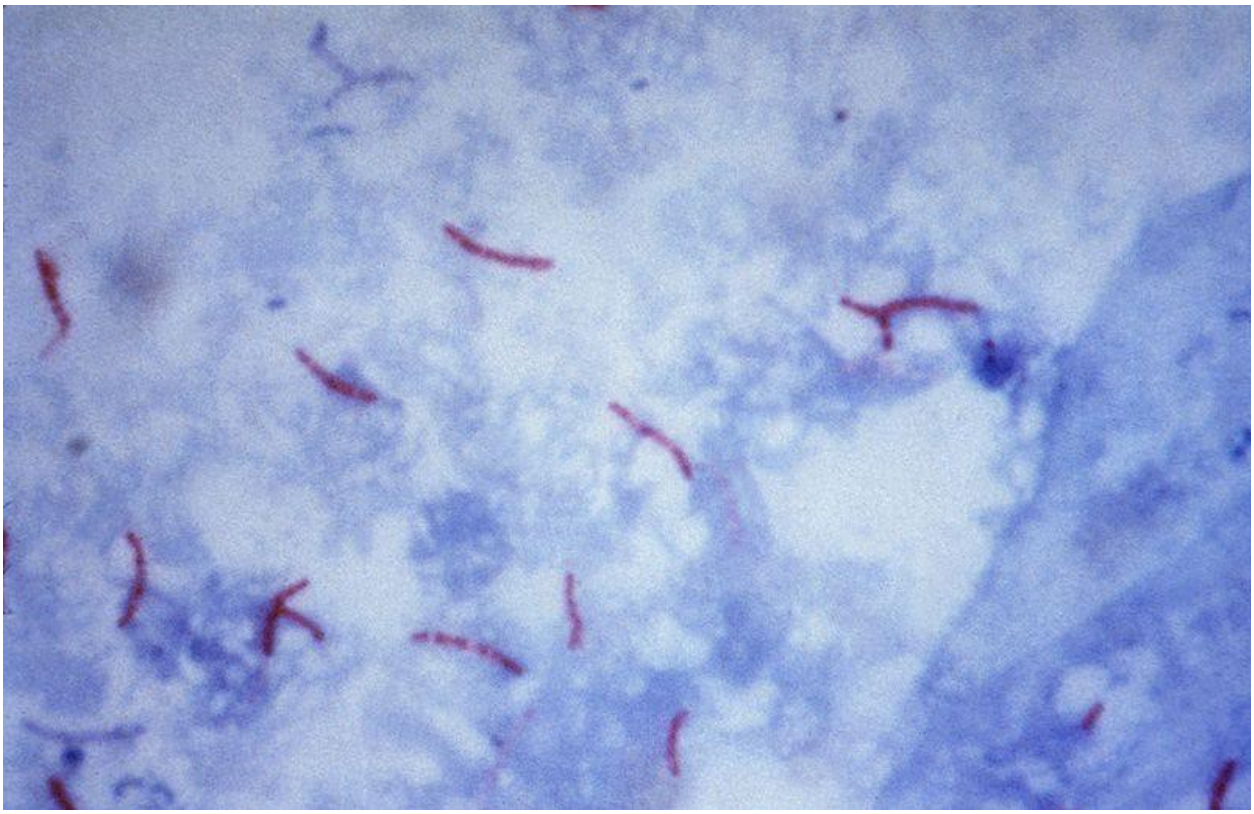

Figure 1.1 Acid-fast staining of *Mtb*

Mtb (red bacilli) stained using Ziehl- Neelsen stain in a sputum sample. Image obtained from Centers for Disease Control and Prevention. Image taken by Dr. George P. Kubica and has no copyright restrictions to use the image.
http://en.wikipedia.org/wiki/File:Mycobacterium_tuberculosis_Ziehl-Neelsen_stain_02.jpg

Mtb is a slow-growing micro-organism with a generation time of 15 and 20 hours (37) which is slow compared to other bacteria (for example *Escherichia coli* can divide every 20 minutes) (38). A single airborne droplet nucleus from an infected individual may contain between 1-3 bacilli (10). Bacilli can remain suspended in the air for several hours, sufficient to infect alveolar macrophages and establish respiratory pulmonary *Mtb* infection in humans (39). The key determinants for *Mtb* transmission are; a number of bacilli inhaled, duration of exposure and *Mtb* virulence.

1.3.1 The *Mycobacterium tuberculosis* genome

It has been over 20 years since the genome of the virulent strain of *Mtb* H37Rv was completely sequenced (40). The size of *Mtb* H37Rv genome is 4,411,529 bp (40,41). Genome analysis revealed an efficient DNA repair system with nearly 45 genes related to DNA repair mechanisms (41). Moreover, despite over 10,000 years of evolution, minimal variation was observed in 16 genetically diverse clinical strains examined (42). *Mtb* H37Rv and its attenuated *Mtb* H37Ra strains have been widely used as reference strains for studying virulence and pathogenesis of *Mtb* since the 1940s (43,44). The *Mtb* H37Ra genome is similar to that of *Mtb* H37Rv but is 8,445 bp larger than *Mtb H37Rv* because of 53 insertions and 21 deletions in H37Ra (43). Genomic analysis revealed that attenuated strains, such as the BCG vaccine or the *Mtb* H37Ra, have mutations in PhoPR two-component system resulting in a loss of virulence (43,45,46).

There are also some phenotypic characteristics in *Mtb* H37Ra that are different from virulent strain of *Mtb* H37Rv. For example, (1) a raised colony morphology (47); (2) loss of cord formation (48); (3) loss of neutral red dye binding (49); (4) decreased survival under anaerobic conditions or inside the macrophages (49,50); (5) impaired ability to disrupt phagosomal membranes (51); and (6) loss of virulence in guinea pigs (52) and mice (53,54).

1.3.2 Cell Wall

The cell wall is a key to the survival of mycobacterium (Figure 1.2) (55). The mycobacterial cell wall consists of an inner layer and an outer layer that surround the plasma membrane (55). The outer layer consists of lipids and proteins (55). The inner layer consists of peptidoglycan (PG), arabinogalactan (AG), and mycolic acids (MA) covalently linked together to form an insoluble complex and is the essential core of the mycobacterial cell wall (55). PG plays an important role in the maintenance of the bacterial shape and protecting bacilli from osmotic pressure (56). AG is

important for cell wall integrity and for attaching the impermeable MA layer to the PG layer (56). MA are strong hydrophobic molecules that are critical for the survival of *Mtb* and contributes to the drug resistance (56). Another component of the cell wall is lipoarabinomannan, a major lipoglycan involved in virulence of *Mtb* and modulating the host response during infection (56).

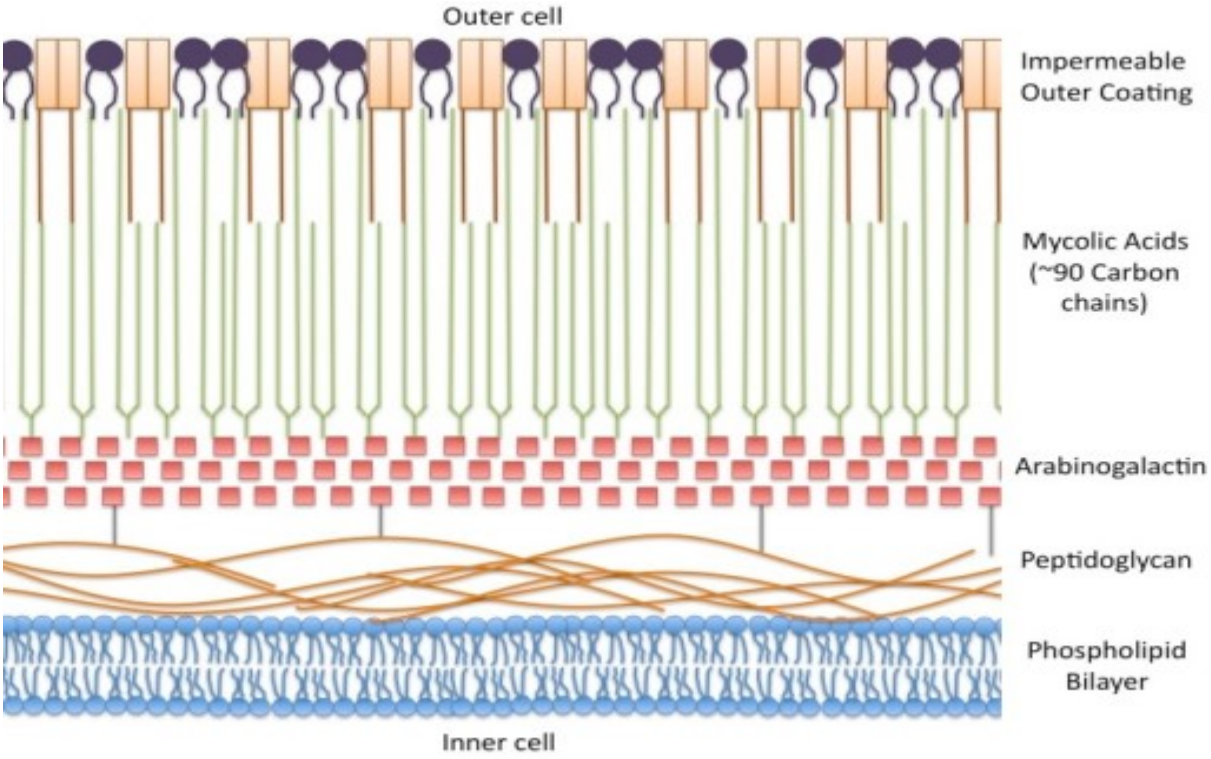

Figure1.2 *Mtb* cell wall basic structure. The cell wall of *Mtb* is comprised of four layers. The outer layer of mycolic acids surrounds inner layers of arabinogalactin and peptidoglycan. These surround the phospholipid bilayer of the cell. Image reproduced from from reference (55) with with permission from Yale Journal of Biology and Medicine.

1.3.3 Virulence determinants

Although *Mtb* does not produce any toxins, its structural and physiological properties have been recognized for mycobacterial virulence and pathology to hosts (57). *Mtb* have developed strategies to avoid or modulate the immune response in their favor (57). For example, the slow generation time of *Mtb* may reduce detection by immunosurveillance and avoid the triggering of anti-*Mtb* immune responses (37).

Other virulence factors of *Mtb* are culture filtrate proteins (found in the culture medium in which *Mtb* grows) (58); cell envelope proteins (including cell wall proteins, lipoproteins and secretion systems, proteins inhibiting antimicrobial effectors of the macrophage, gene expression regulators (including two component systems, sigma factors and other transcriptional regulators) and other proteins, including the ones of unknown function (57,59) (Table 1.1).

Table1.1 Virulence factors of *Mycobacterium tuberculosis*

Virulence factor	Protein type	Role	Virulence characterization	Reference
Antigen 85 complex	Exported	Fibronectin-binding	Tubercle formation	(56)
HbhA	Adhesin	Heparin-binding hemagglutin	*Mtb* binding to epithelial cells	(60)
ESX-1	Secretion	Virulence proteins	Long term survival of *Mtb*	(61)
WhiB3 protein	Transcription regulator	Sensing oxygen tension and redox state	Adaptation of mycobacteria to changes in oxygen tension	(62)
Acr1 (hspX*)*	α-crystalline protein homolog	Dormancy	Inhibition of antimicrobial effectors of the macrophage	(63)
19-kDa protein	Lipoprotein antigen	Blockage of IFN-γ signaling through a TLR-2 dependent mechanism	Inhibition of MHC-II antigen processing and presentation in macrophage	(64)
Sigma factors	Gene expression regulators	Help in the regulation of expression of specific genes during stress or morphological development	Adaptation to the changing environment within the host	(65)
STPKs	Serine-threonine protein kinases	Regulation of cell shape	Host-pathogen interactions and developmental changes through signal transduction using reversible phosphorylation of proteins	(66)

1.3.4 Tuberculosis pathology & pathogenesis

There are three stages in the progression of TB (1) elimination (immune detection and destruction of phagocytic cells), (2) equilibration (latent infection), and (3) escape (development of primary or post-primary tuberculosis with/without lymph node or distant sites involvement) (67). Following inhalation of bacilli, the bacilli have three potential outcomes; (1) bacilli may be killed by the immune system, (2) bacilli may multiply and cause primary TB or (3) bacilli can become dormant and remain asymptomatic as LTBI (68). Dormant bacilli can proliferate after a latency period in relation to endogenous reactivation or exogenous reinfection (8). The immunological response to *Mtb* to maintain LTBI is a complex phenomenon, the host is in constant battle with the microbe at the granuloma level to control infection and to prevent activation (8). Re-activation of *Mtb* may progress to active TB disease or cause a secondary infection (69).

1.3.4.1 Three main disease phases of TB disease

Infection with no symptoms is when the person infected with *Mtb* has mild or no symptoms (in most cases) (70). The bacterium lives in a dormant state. A small scar on the lung may be observed on a chest X-ray (71). This is referred to as LTBI (4). It is estimated that one-third of the world population have LTBI representing a large reservoir for TB disease. In the absence of active TB disease, infected people cannot spread the infection.

Active TB infection **t**his occurs in less than 5% of cases (72) where *Mtb* can multiply actively at the site of infection and the high bacillary concentrations are enough to generate spontaneous mutations and induce drug resistance (73). This is common in immunocompromised people, malnourished people and people living in poverty or poor health environments. Active infection becomes an acute infection and expels high numbers of *Mtb* bacilli (73).

Re-activated (secondary) infection is when dormant bacilli can cause active TB infection after months or years after the primary infection. The "walled off'' bacteria within the granulomatous scar, multiply and cause a secondary infection. Reactivation commonly occurs in the lungs (72). Re-activation is associated with a failing immune system commonly found in a number of high-risk individuals (for example; HIV/AIDS, malnourished or individuals taking immune-suppressive drugs) (74). *Mtb* virulence directly affects the rate of re-activation (75).

In 90% of immune-competent individuals, the host cellular immune response is elicited through cytokines and chemokines that contain or limit infection to the primary site of infection by forming granulomatous lesions named ''Ghon complex or Ghon focus'' (76). The presence of epithelioid cells indicating granulomatous lesions with or without caseation (caseous necrotic tissue) is the best diagnostic criteria for a TB infection (73). In 10% of cases, active bacterial replication results in lesions including tissue necrosis and cavitations (73,76). Infected tissue maintains stable latent bacilli and extends the production of foamy macrophages, which enable latent bacilli to escape and regrow within the alveolar spaces forming new granuloma (73). Cavitations correlate with bacillary loads. The estimated bacillary load in the cavitary wall is 10^7 to 10^{10} in contrast to 10^2 to 10^4 in caseous necrotic areas (73). TB pathogenesis is associated with bacillary loads, which increases infection rate and disease severity (73).

Four weeks post-infection, neutrophils accumulate at the site of infection surrounded by lymphocytes leading to the formation of ring-like appearance (77). External to the ring foamy macrophages (FM) accumulate to clear the infection in the alveolar spaces (78). FM ingest bacilli and are filled internally with respiratory cells, surfactant, bacilli and lipoid vacuoles; they then leave the alveolar spaces *via* the main respiratory airway tracts and are removed by the stomach swallowing reflex or by coughing (73). Re-infection occurs during the chronic phase when the FM

leave the granulomatous tissue and move towards the alveolar spaces (78). During active immunity, macrophages that are already infected and the newly arrived macrophages become foamy and filled with acid-fast bacilli which are easier to detect than in the chronic stage of infection (78).

1.4 Diagnosis of Pulmonary Tuberculosis Disease

The main symptoms of an active TB include a bloody cough, chest pains, night sweats, fever, weakness, and weight loss (79). TB diagnosis is important for differentiation between TB and other common respiratory infections and active *vs* LTBI (80). Diagnosis of pulmonary TB plays a crucial role in preventing transmission of the disease. Similar to TB infection the lower respiratory tract infections (e.g. pneumonia) also cause fever with productive/purulent sputum (81). Pulmonary tuberculosis should always be included in the differential diagnosis of persons with pulmonary signs or symptoms.

1.4.1 Radiological diagnostic tests

The preliminary diagnosis of TB is made through radiological diagnostic tests such as a chest x-ray (CXR) and computerized tomography (CT) (82).

1.4.1.1 Chest X-ray (CXR)

Although, the majority of healthy individuals do not show a radiographic pattern of LTBI, WHO recommends chest radiography to detect chest and lung lesions (79). Primary TB is characterized by lymphadenopathy, pleural effusion, lower or mid lung zone infiltrates (83). TB re-activation lesions are typically located in the lower part of upper lobes or the upper part of middle and/or lower lobes with characteristic cavitary lesion (71). CXR for TB diagnosis is categorized into five groups: (1) normal or minor findings (non-related to TB), (2) granulomas with or without calcification considered likely due to remote TB infection (LTBI) and/or is defined as possible active TB. Another three findings are considered as active TB (3) multiple non-calcified nodules,

(4) mass in the parenchyma, hilum, or mediastinum and (5) multiple parenchymal lung infiltrate with or without cavitations (71). A CXR is the major diagnostic test for examining lesions in the chest, can deliver a fast test result and is a relatively cheap procedure. However, the disadvantages of CXR are; (1) very subjective that depends on readers experience for differentiation between image findings. (2) has very poor specificity for the diagnosis of latent or active TB and (3) the inability to relate the CXR results to sputum smear results.

1.4.1.2 Computerized tomography scans (CT scans)

CT scans were introduced in the 1970s as medical imaging methods to generate three-dimensional images (84). A CT is performed in normal or inconclusive CXR. A CT determines TB disease activity and complications e.g. 1-3 mm small size nodules (85). CT scans can detect both acute and chronic changes in the lung parenchyma (85). Cavitation is a common sign of LTBI and TB disease or military TB spread (hematogenous-disseminated small nodules occurs in 2-6% of primary TB) (86). Other common CT findings of pulmonary TB re-activation are centrilobular small nodules, large nodule more than 8 mm and patchy or lobular (85,86). The main advantage of CT is achieved by reducing super imposition of structures outside the lungs but due to the hazards of radiation doses and the expensive cost CT usage is limited.

1.4.2 Tuberculin skin test (TST) or Mantoux Skin test

Since the late 19th century, no changes have been made to the TST test (87). The test is exactly the same as described first by Robert Koch in 1890 and as modified by Charles Mantoux, a French physician who developed Koch's work for use in the diagnosis of both active and latent *Mtb* infections (87). TST is an *in vivo* skin test to measure an individual's immune inflammatory response caused by a localized delayed-type hypersensitivity reaction-IV (DTH-IV) versus the injected Purified protein derivative (PPD) (88). A PPD mixture contains tuberculin's composed of a

crude mixture of heat-denatured proteins derived from cultures of *Mtb,* of more than 200 antigens that are also shared by other mycobacteria (89).

The TST reactivity has been studied in different groups such as individuals with different forms of disease, different races and ages, and from different countries (90). The average TST reaction measured 14-18 mm (90). However in 15-50% of the study population, false negative reactions occurred. The conditions that were associated with reduced TST reactivity was in advanced TB disease, malnutrition and old age (90). A study conducted in Malaysia showed a sensitivity of 86% in people with active TB and the false positive result in this study was 42%. In another study, the false-positive rate was 43% in people with no history of TB and 60% in non-TB pulmonary diseases. In contrast, in those with confirmed pulmonary TB, false-negative rate was 20.5% and in patients with TB lymphadenitis, the false negative rate was 11.7%. Over all the sensitivity and specificity of TST in extrapulmonary TB is reported as 47% and 86% (91).

In a meta-analysis of TST tests results, it was demonstrated that people who received BCG vaccination were more likely to have a positive result. The relative risk was 2.12 (95% CI:1.50 to 3.00) compared to people without BCG vaccine. The effect of BCG was less after 15 years of vaccination. A cut-off measurement of skin indurations of >15 mm is more likely to be the result of tuberculous infection than due to previous BCG vaccination (90).

Since there is no gold standard rule for TST test result interpretation, an indurations size of more than 5 mm diameter is considered as a positive TST reaction in BCG non-vaccinated individuals and more than 15 mm is a sign of LTBI in low-risk TB populations (89). In TB endemic regions TST test is considered positive if the induration is more than 10 mm e.g. China or even more than 20mm in India. TST is a simple and cost-effective (89). It is also used as a confirmatory test for BCG vaccination. However, TST is inadequate for a confirmed diagnosis of TB or

exclusion of LTBI infection. TST sensitivity is affected by several factors such as exposure to *M. avium*, *M. paratuberculosis,* and environmental mycobacteria and by skin tuberculosis (89).

1.4.3 Interferon Gamma Release Assays

To overcome the limitations of the TST, Interferon Gamma Release Assays (IGRAs) laboratory blood tests have been developed. IGRAs measure IFN-γ release in response to *Mtb* specific antigens present in the Region of Difference 1 (RD1) locus, which is absent in most of the environmental mycobacteria and BCG, thus avoiding the false positive results observed with the TST (92). Two commercially available IGRAs are the QuantiFERON®TB Gold In-Tube (QFT) assay and the T-SPOT®TB assay (93). The QFT assay is an enzyme-linked immunosorbent assay (ELISA) which is a whole-blood test and T-SPOT®TB assay is an enzyme-linked immunosorbent spot used for diagnosing LTBI (93) (Table 1.2).

Table1.2 Comparison of skin and blood tests for active or latent *Mtb* infection with standard sputum smear microscopy.

Characteristic of test	Tuberculin skin test (TST)	Quantiferon-TB Gold In-Tube test	T-Spot.TB assay
Test/Assay	Purified protein derivative injected intradermally	Enzyme-linked immunosorbent assay using whole blood	Enzyme-linked immunosorbent spot test using peripheral blood mononuclear cells
Antigens	Tuberculin proteins	ESAT-6, CFP-10, TB7.7	Mixtures of synthetic peptides (ESAT-6 and CFP-10)
Measurement	Size of skin induration	Interferon-γ	Interferon-γ producing cells
Sensitivity (*vs.* standard sputum smear microscopy)	80% to 95%	70% to 91%	84% to 91%
Specificity (*vs.* standard sputum smear microscopy)	80%	95% to 99%	95% to 97%
Affected by BCG vaccination	Yes (wanes over time)	No	No

1.4.4 Laboratory Microbiological Tests

To identify active TB infection caused by *Mtb*, acid-fast bacilli (AFB) are stained with ZN stain or Auramine stain (AS) (94). Bacteria can be grown in TB growth culture using liquid media (Middle Brook) or solid media (95).

1.4.4.1 Sputum smear microscopy

Sputum smear microscopy is used to diagnose pulmonary infection with MTBC and nontuberculous mycobacteria (NTM) (96). Sputum smear samples are prepared by placing 1 to 2 drops of sputum on the slide that are smeared on a slide for the purpose of microscopic examination (97). Acid-fast stain ZN is used in clinical mycobacteriology laboratories to identify active TB infection caused by *Mtb* (94). The acid-fast bacilli are rod-shaped bacteria that can be seen under the conventional light microscopy following a staining procedure in which the bacteria retain the color of the stain after an acid wash (82,98). Alternatively, fluorescence stains (auramine or auramine-rhodamine) can also be used for the detection of mycobacterium (82,98).

The sensitivity of smear microscopy is influenced by various factors, such as; the severity of TB disease, the type, the quality of the specimen collected, the number of mycobacteria present in the specimen (1,000–10,000 CFU/ml required for reliable detection), the method of processing the specimen (concentrated or not), the staining technique, and the quality of the examination (97,99-101). The major disadvantage of acid-fast stains is that they cannot differentiate between mycobacteria species (82,98). The overall clinical sensitivity of sputum smear staining is 22–80% depending on the burden of mycobacteria, the type of acid-fast stain used, and experience of the laboratory technician (82,98). Although acid-fast microscopy identifies bacilli, other approaches, such as culture or direct molecular detection, are still required to confirm the genus or species of *Mtb.*

1.4.4.2 Culture

Currently, *Mtb* by culture is still a standard test that is required for comprehensive antimicrobial susceptibility testing and most currently used strain-typing approaches (96). However, culture may not detect some pulmonary infections due to a variety of reasons including specimen collection timing, specimen type, laboratory processing and bacterial growth kinetics in the host (98). Key disadvantages to culture also include the long culture time-more than 2 - 4 weeks for liquid culture, 6 - 8 weeks for solid culture and the requirement of level 3 laboratories (98). Due to the long incubation times, contamination in liquid culture is also a concern in diagnostic laboratories (98).

1.4.4.3 Identification of Mycobacterium tuberculosis

Positive mycobacterial cultures are identified based on colony morphology, sequencing of key genes, or other molecular approaches from culture (82). To identify definitive mycobacterial isolates from culture, molecular methods are used (102). Molecular methods such as nucleic acid hybridization probes, line probe hybridization assays, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, and DNA sequencing allow for rapid species identification of mycobacteria (102,103). Since traditional molecular methods rely on the culturing methods, the slow growth rates of mycobacteria delay the diagnosis of *Mtb* infection (103,104). The key factors to consider in the diagnosis of *Mtb* are improved test accuracy and the short detection times. Replacement tests have been proposed in order to address these shortcomings and limitations of the current diagnostic tests described above for the detection of *Mtb* (103,104).

Nucleic acid amplification (NAA) tests detect *Mtb* complex directly from patient specimens in as little as two hours (105-108). Two NAA tests have been approved by the US Food and Drug Administration (102,108) and they are the Amplified *Mtb* direct test (MTD) and the Xpert *Mtb*

/RIF® test (93,102). These tests target MTBC specific rRNA and DNA sequences by transcription-mediated amplification and real-time PCR methods (92,109). The sensitivity for the Amplified MTD test from smear-positive respiratory specimens is 87.5–100%, while for the smear-negative samples the specificity is 63.6–100% (89,93,109). The sensitivity for the Xpert *Mtb* /RIF® assay is 90–99% from smear-positive samples, and 66–74% sensitivity for smear-negative samples compared to the standard smear microscopy and culture methods whose sensitivity of sputum acid-fast staining is 22–80% depending on the burden of mycobacteria (93,109).

1.5 Drug treatment for TB

1.5.1 First line therapy

Treatment of TB disease involves the use of multiple drugs in combination to avoid resistance to treatment and to target the persistent bacteria (110). The current first-line anti-TB therapy recommended by WHO includes a two months long intensive phase: a combination of four drugs such as INH, RIF, PZA and EMB (79,111). Following the first 2 month regime, two drugs INH and RIF are used for 4-6 months-and this phase is called the continuation phase of treatment (112).

1.5.2 Second line therapy

The second line of treatment is recommended for patients who are infected with an *Mtb* strain resistant to drugs RIF and INH, i.e. Multidrug-resistant tuberculosis (MDR-TB) (112). Globally in 2015, the WHO estimated that 3.9% of new cases and 21% of previously treated cases of TB were MDR-TB (79). An estimated 250,000 deaths from MDR-TB have occurred in 2015 (79). The countries with the largest number of MDR-TB cases (45% of the total) are from China, India and the Russian Federation (79). The treatment of MDR-TB usually lasts 20 months and includes five drugs such as pyrazinamide and four core second-line medicines: one from group A (levofloxacin, moxifloxacin, and gatifloxacin); one from Group B (amikacin, capreomycin,

kanamycin, and streptomycin) and at least two from Group C (ethionamide / prothionamide, cycloserine / terizidone, linezolid, and clofazimine). In addition to the treatment with a combination of drugs, the treatment also includes injections of aminoglycoside, like kanamycin or amikacin for 8 months (79) (Table 1.3).

Table1.3 Drugs for TB infection

Groups	Drugs	Duration of therapy
First line drugs	Isoniazid Rifampicin Pyrazinamide Ethambutol	6 months
Second line drugs **Group A** **Group B** **Group C**	 Levofloxacin Moxifloxacin Gatifloxacin Amikacin Capreomycin Kanamycin Streptomycin Ethionamide / Prothionamide Cycloserine / Terizidone Linezolid Clofazimine	Combination of: one drug from group A + one drug from group B + at least 2 drugs from group C for 20 months
Injectable drugs	Amikacin or Kanamycin	8 months

1.5.3 Treatment of extensively drug-resistant TB

Extensively drug-resistant TB (XDR-TB) is the most severe form of drug resistance in *Mtb* (111). Globally in 2015, the WHO estimated that 9.5% of MDR-TB cases have XDR-TB and are

resistant to INH, RIF, and any fluoroquinolone and at least one second-line injectable drug (kanamycin, amikacin, capreomycin) (79). The intensive phase of treatment for XDR-TB involves capreomycin for eight months followed by the treatment with at least five to six drugs in combination (111). The regimens for the treatment of XDR-TB is based on the conventional drug-susceptibility testing (DST) (112).

A new anti-tuberculosis drug, bedaquiline, has been approved in an interim recommendation by the WHO for patients where regimen contains four effective second-line drugs that cannot be constructed or in patients where there is MDR-TB plus documented resistance to a fluoroquinolone (pre-XDR-TB) (113,114). However, it should be noted that the WHO recommends the addition of bedaquiline to the treatment regimen only in adult patients with pulmonary MDR-TB (79).

1.6 Prevention of Tuberculosis

Difficulties in diagnosis and treatment of the TB disease along with the development of drug-resistant strains of *Mtb* and HIV-TB co-infection are all barriers to global control of TB. Another barrier to the control of the disease is the lack of an effective vaccine. A better understanding of *Mtb* infection and the host immune response is essential to the development of better diagnostics, treatments and effective vaccines.

1.6.1 Vaccination for TB

The only currently available vaccine for *Mtb* infection is the BCG vaccine. The BCG vaccine was originally derived in 1921 following serial passage of *M. bovis* strain isolated from a cow (115). There are currently six main strains that account for over 90% of the BCG vaccine and they are BCG Japanese, BCG Danish, BCG Glaxo, BCG Connaught, BCG Pasteur, and BCG Tice with no standard production methods shared between producers (115). The BCG vaccine lacks the RD1 locus which encodes proteins important for virulence of mycobacteria and also for T cell

responses including early secretory antigen 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-10) (116,117). The protective effects of the BCG vaccines are characterized by the joint responses of innate and adaptive immune responses to the *Mtb* infection (117).

As mentioned earlier, the BCG vaccine has variable efficiency in terms of geography and age group (117-119). Case-control studies have shown that the vaccine is effective at reducing disseminated forms of TB, including miliary TB and TB meningitis in children. However, randomized controlled trials of BCG has shown limited efficacy against adult pulmonary TB (ranging from 0-80%) (120-126). The variability in the efficacy can be explained by the difference in the geographical location between the study sites (127). For example, the UK clinical trials showed protection against adult pulmonary TB, but little protection was observed in the Malawi clinical trials (128).

In general, BCG vaccination has shown some effectiveness among children against *Mtb* infection (129). A systematic review and meta-analysis by Roy et al. indicated that BCG has a 19% efficacy in terms of protecting children from *Mtb* compared to children who received no BCG vaccination. These results are in agreement with other cross-sectional studies that estimated the association between BCG vaccination and the prevalence of TB (129,130).

Although BCG vaccination is suboptimal for protection against an adult pulmonary disease, BCG vaccination seems to have a limited effect in addressing the high number of TB cases in the world, particularly among adults and the aging population (131-134). Several factors have been proposed to explain the variability in the efficacy rate of BCG vaccine. First, the use of different strains of BCG may lead to different levels of protection (135). Four main BCG strains used for vaccination are the Pasteur 1173 P2 strain, the Danish SSI 1331 strain, the Glaxo 1077 strain, and the Japanese Tokyo 172 strain (135). The induction of an immune response differs between BCG

strains and this may impact the efficacy of the vaccine (136). Henao-Tamayo et al. (2016) noted that the new Beijing family strain of *Mtb* can induce the emergence of $Foxp3^+$ $CD4^+$ regulatory T cells (Tregs), which can reduce the protective effects of the BCG vaccine (137). Another proposed reason for the variation in the efficacy of the BCG vaccine is the exposure to environmental mycobacteria, which have been reported in regions with low BCG vaccine efficacy. It is proposed that the protective responses by BCG vaccination are inhibited by the exposure to environmental mycobacteria (138,138-141). Overall, it appears that a key barrier to control TB disease is the lack of an effective vaccine. A better understanding of *Mtb* infection and the host immune response is essential to the development of an effective vaccine.

1.6.2 Immune responses following BCG vaccination

Following intradermal BCG vaccination, epidermal macrophages interact with BCG *via* pattern recognition receptors (PRRs), including complement receptor 3 (CR3) and Toll-like receptor 2/4 (TLR2/4) (142,143). Since the lipid portion of the cell wall of BCG is very similar to *Mtb*, it has been speculated that infection of tissue macrophages is similar to *Mtb* (144,145). However, BCG first contact is with epidermal macrophages, whereas *Mtb* encounters with alveolar macrophages (146,147). Differences in the mechanisms of antigen (Ag) recognition, Ag uptake, Ag processing, and Ag presentation between these two types of macrophages remains unclear and this may be one of the reasons why BCG is not fully protective.

BCG vaccination can induce Th1 cells identified by the production of IFN-γ, and Th17 cells identified by the production of IL-17A, although IL-4 producing Th2 cells can also be generated (148). Other cell subsets such as Tregs and CD1-restricted T cells also arise following BCG vaccination, although to a lesser extent. BCG also induces cytotoxic T cells, whose main function is to lyse infected cells through osmotic disruption (149).

The importance of CD4+ Tregs to BCG vaccination has been studied in the Tregs depletion models. The diversity of Tregs is discussed later in chapter 4. It has been demonstrated that Tregs depletion decreases *Mtb* load in BCG-vaccinated mice (182). Similarly, BCG vaccination boosted with an Ag85 (*Mtb* protein) significantly decreased the number of Tregs and their depletion reduced bacterial load in the lung of *Mtb* infected mice (183). Furthermore, in BCG vaccinated human adults it has been reported that BCG immunization that induced local skin inflammation, showed increased levels of multifunctional CD4+ T cells (184). However, in BCG vaccinated individuals who developed mild local skin inflammation showed increased levels of CD8+ regulatory T cells (184). Thus, factors that influence vaccination induced inflammation could affect the development of Treg cells. Since the above studies highlight that, a vaccine booster (BCG or other) may not help in the development of immunity where Tregs may play a role (185) it is critical to understand the exact role of Tregs during BCG vaccination. Understanding their role could be critical in inducing optimal immunity to *Mtb* infection. BCG immunization in humans induces CD1-restricted CD8+ T cells that recognize BCG infected dendritic cells (DCs) (186). Studies in guinea pigs demonstrated that BCG vaccination induces humoral and CD1-restricted cytotoxic T cell-mediated immune responses (187) and are important in preventing disseminated TB and TB meningitis. The absence of group one CD1 molecules (CD1a, CD1b, and CD1c) in mice (188) has made it difficult to research the exact role of the CD1-restricted CD8+ T cell population to BCG immunity and during *Mtb* infection

The role of T cells as the main effector cells following BCG vaccination has been demonstrated in mouse knockout models (150-153). The transfer of $CD4^+$ or $CD8^+$ T cells from BCG vaccinated mice into rag1−/− mice (deficient for both B and T cells) show that $CD4^+$ T cells reduce bacterial load in the lung and spleen, while $CD8^+$ T cells reduce bacterial load in the spleen.

This data suggest that $CD4^+$ T cells are the main effector cells generated by BCG vaccination in the lung, and $CD8^+$ T cells help in preventing dissemination (miliary TB and TB meningitis) of the bacteria (154-157). In addition, it has been reported in a mouse model that BCG vaccination followed by *Mtb* challenge in the absence of $CD4^+$ T cells reduced *Mtb* bacterial burden in the lung post-infection, supporting the importance of $CD8^+$ T cells during the later phases of the disease (154,158). It is clear that BCG vaccination can stimulate both $CD4^+$ and $CD8^+$ T cells and that $CD4^+$ T cells are not as efficient as $CD8^+$ T cell in clearing mycobacterial infection in a tissue-specific environment. This indicates that the distinctive environment of the lung makes it difficult for $CD4^+$ T cells to perform their functions (156,157). It is possible that *Mtb* possesses many virulence factors that are not present in BCG that could inhibit $CD4^+$ T cells, but not $CD8^+$ T cell responses (157). Since the effector function of $CD8^+$ T cells following BCG vaccination has been characterized to a lesser extent than $CD4^+$ T cells the mechanism by which $CD8^+$ T cells contribute to the efficacy of BCG (for example cytotoxic function, stimulation of other cells *via* cytokines, chemokines, or microbicide molecules) remains to be determined.

T cell responses in human studies show that BCG vaccination efficacy is linked to the age at which BCG is administered (159). BCG is commonly administered at the time of birth, as infants are exposed to various antigens when born; it is understood that T cells are biased toward a Th2 response to prevent excessive inflammation (160). Since Th2 response can be unfavorable to mycobacterial immunity, BCG vaccination of neonates may not as good in protection against *Mtb* infection (160-164). However, studies in human newborns and infants demonstrated that Th1 biased immune response following BCG vaccination similar to adults immunized with BCG (165,166). These studies reported a high number of $IFN\gamma^+CD4^+$ T cells (167,168), and also a significant component of $CD4^+$ T cell population negative for IFNγ but positive for TNF and IL-2

(169) indicating that BCG generates different immune responses required to contain *Mtb* in infants. Hence, it seems that the efficacy of BCG against *Mtb* may not be due to the failure of generating Th1 cellular immunity at the time of vaccination. In contrast, it has been reported that cytokine and chemokine production differs in children vaccinated with BCG in Malawi and the United Kingdom (170,171). Malawian infants produced more cytokines associated with Th17 and Th2 immunity compared to infants from the UK (171). An explanation for this could be the environment surrounding the BCG vaccinated infants. For example, parasitic coinfections that dampen protective immune responses (172), or environmental mycobacteria (173).

Th17 responses in the lung are associated to increased protection and exacerbated pathology against *Mtb* infection (174,175). Previous work shows that repeated BCG vaccination exacerbates the influx of neutrophils into the lung in an IL-17A dependent manner leading to extensive immunopathology (176). In contrast, IL-17A is required to sustain IFN-γ responses by $CD4^+$ T cells in the lung. In mice, it has been reported that BCG vaccination stimulates Th17 responses within the lung (176). The presence of IL-17A in the lung may be important in generating an effective immune response that benefits the host (bacterial control with limited inflammation) or an immune response that ultimately damages the host (initial bacterial control with too much inflammation that subsequently leads to uncontrolled bacterial growth). The role of IL-17A in the immune response to *Mtb* was primarily studied during the initial granuloma formation (177,178). High concentrations of IL-17A were correlated with the presence of IL-10 (179). Although the relationship between IL-17A and IL-10 remains unclear, IL-10 has been shown to have an immunosuppressive role during the generation of BCG immunity (174,180). Similar to IL-17A (174,180), IL-10 plays an important role during the initial stages of *Mtb* infection *in vivo* (181). The role of Th17 cellular responses to BCG vaccination still remains to be understood.

Overall, these findings reveal that BCG vaccination generates effective, long-lasting immunity that prevents *Mtb* infection and the development of TB. $CD4^+$ T cells and $CD8^+$ T cells are important in maintaining the chronic phase of the disease and appear to be particularly important in the dissemination to peripheral organs.

1.7 Initiation of infection: the basics

Mtb is spread in aerosolized droplets from the sputum of an infected person. Once it enters the lung, the bacterium is deposited in the alveoli, but can also infect lung epithelial cells and activate mucosal-associated invariant T cells (182). Macrophages in the alveoli are the primary target for *Mtb* (72). The immune responses that are generated by the infection are adequate to control TB disease but are unable to clear the infection, with many individuals developing LTBI (183). A study of Italian Healthcare workers exposed to culture-confirmed cases of TB indicated that the post-exposure annual rate of TB infection was 26 per 100 person year (95% CI 13.6-50) (184). *Mtb* has the ability to manipulate the host immune responses that can facilitate the development of TB disease, which enables release of bacteria and transmission to other hosts or progress to the development of granulomas (185). Figure 1.3A illustrates how inhaled droplets enter the lung alveoli, where the bacteria are phagocytosed by AMs (186-188). The interaction between *Mtb* and AMs decides the subsequent progression of infection (Figure 1.3B). If the mycobacteria has evolved effective strategies to evade the immune response, the infection is preserved in a latent condition in the form of granuloma (Figure 1.3C). Alternatively, if the host fails to eradicate the mycobacteria, the mycobacteria is replicated and transformed into active TB disease (Figure 1.3D). A granuloma is an active lesion that has the capacity to influence T cell migration both locally and distally.

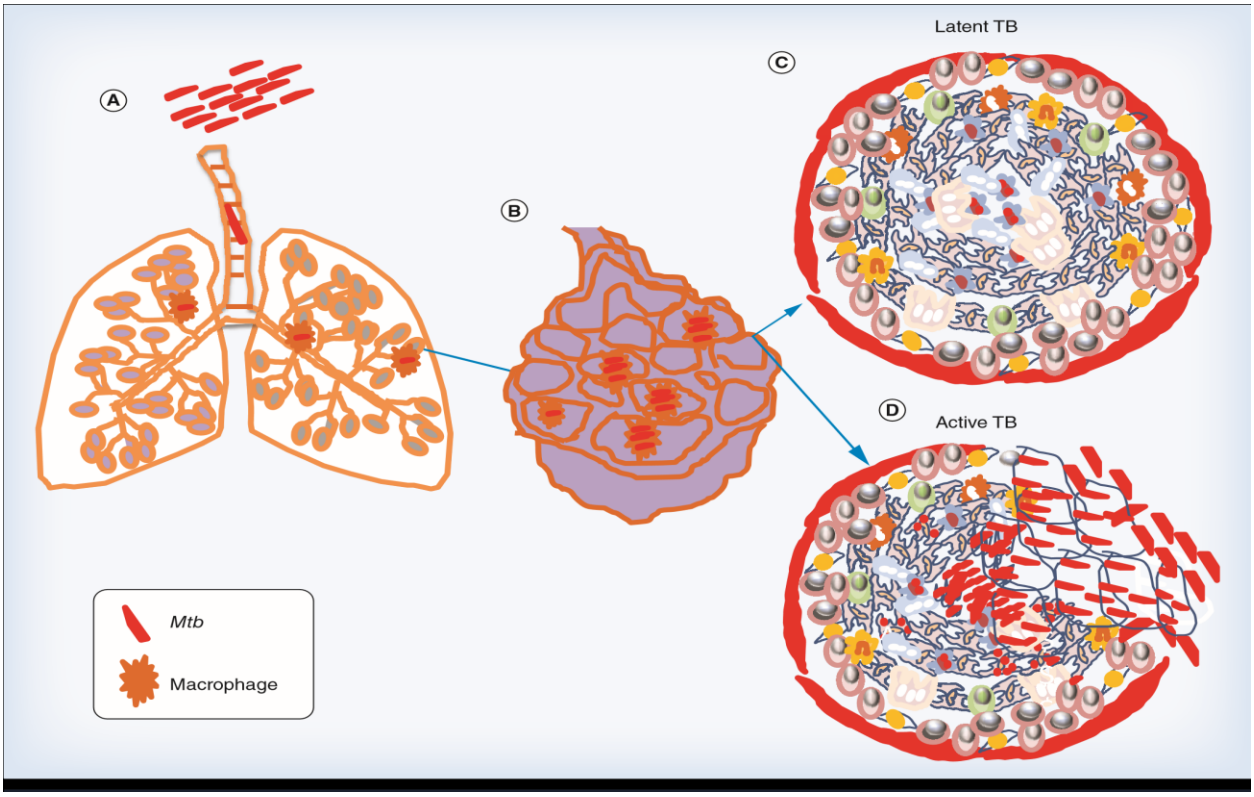

Figure1.3 Pathogenesis of tuberculosis. (A) *Mtb* enters the host when inhaled droplets are transmitted to the lungs. (B) the bacteria are phagocytized by alveolar macrophages and eliminated by different mechanisms, which include apoptosis and autophagy. (C) when *Mtb* growth is contained inside granulomas, it is preserved in a latent condition seen in 90–95% of infected individuals and infection does not transform into the disease. (D) *Mtb is* replicated in the macrophages and transformed into active tuberculosis with symptoms of tuberculosis and can also be disseminated to other tissues and organs as seen in 5-10% of cases.

1.7.1 The Granuloma

Granulomas in the lungs are the defining pathological characteristics of TB (4). Granulomas, which allow for infections to be controlled but also thrive, are the lesions that contain the mycobacteria (189,190). In granuloma structures, both the immune cells and the infection co-exist (190). Human TB granulomas are composed of a central mass of infected macrophages, neutrophils and DCs (191-193). Macrophages within the granuloma have differentiated into multinucleated

giant cells, epithelioid cells and FMs (194). The granulomas are surrounded by a central core of innate immune cells surrounded by T and B cells. The fibroblasts create a peripheral fibrotic capsule around the immune cells (192).

1.7.1.1 The classic understanding of pulmonary granuloma formation

After initial infection, DCs and monocyte-derived macrophages phagocytose the bacillus (186,195,196). *Mtb* can survive inside macrophages by inhibiting the fusion of phagosomes with the lysosomes thereby preventing the formation of a phagolysosome, the mature form of a phagosome (195). During the initiation of the granuloma formation, there is an early recruitment and clustering event involving inflammatory macrophages. New macrophages and other immune cells are then recruited to the site of infection, and develop into granulomas, the characteristic lesions of TB. After the initiation of the acquired immune responses, T cells migrate from the circulation into the parenchyma of the lung and then to the site of the infection, composed largely of macrophages and DCs (197-199). Mature granulomas form as multicellular structures composed of infected and uninfected macrophages, epithelioid cells, giant cells (multinucleated cells derived from fused macrophages), T cells and B cells (200-202) that can contain the bacilli and prevents spread of the infection. Inward migrating DCs express IL-12p40 (203), and the receptor IL-12Rβ1 (204). Major histocompatibility complex (MHC) class II expressing DCs are required for T-cell activation but outward migrating DCs also end up transporting bacteria to local draining lymph nodes (205). Inhibition of growth or death of *Mtb* is in part due to enhanced macrophage activation and the creation of an oxygen and nutrient deprived environment (206,207).

As shown in Figure 1.4, a spectrum of granulomas is observed in humans (208). Figure 1.4A describes solid granulomas, where mycobacteria are most likely dormant (35). Figure 1.4B describes necrotic granulomas, which are present in the early stages of active TB. Figure 1.4C

describes caseous (cheesy like) granulomas found in end-stage or severe TB. The solid non-caseating granuloma consists of $CD4^+$ and $CD8^+$ T cells, B cells and macrophages harboring few tubercle bacilli (Figure 1.4A)**.** A layer of fibroblasts surrounds this specific granuloma, with fibrosis occurring as the granuloma controls the infection and the inflammatory process is limited. In a latently infected individual, one or more granulomas controlling the infection can sometimes be observed; these granulomas can also be calcified (191,209). As shown in Figure 1.4B**,** necrosis first occurs in the center of the structure and may further develop into a caseous necrosis of human lung tissue (194). Figure 1.4C illustrates that caseous granulomas can progress to form cavities within the lung, leading to erosion of the granuloma into a bronchus, and the subsequent release of bacteria into the airways (206). They have a central necrotic area that contains extracellular bacteria surrounded by macrophages and phagocytes and contains lymphoid-like structures that are rich in T and B cells, as well as macrophages that contain tubercle bacilli. Immune cell responses to TB will be described in detailed in section 1.17.3.

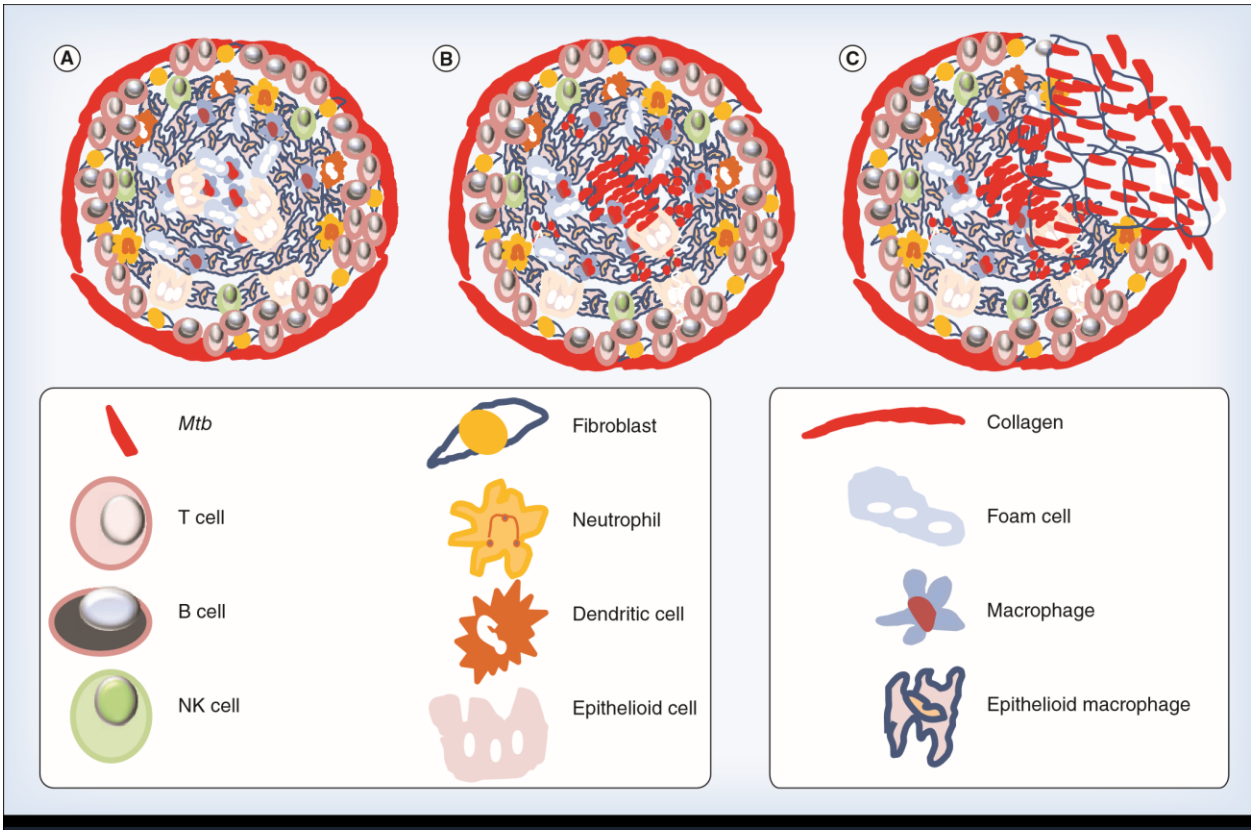

Figure1.4 Changing stability of the tuberculosis granuloma. The tuberculous granuloma is a compact, organized aggregate of epithelioid macrophages with tightly interlink cell membranes of epithelioid cells and adjacent cells. Depending on the immune response at the time of infection, the granulomas can either be solid, caseating or necrotic. In a solid granuloma, the infected macrophages are in the middle surrounded by other immune cells, such as $CD4^+$ and $CD8^+$ T cells, as well as macrophages that fuse to form multinucleated giant cells or have differentiated into foamy cells. A solid non-caseating granuloma (A) is seen during latent infection where the bacteria can be dormant and may have survived for decades. In a necrotic granuloma (B), the bacteria have multiplied and promoted the death of macrophages. In a caseous granuloma (C), the center of the granuloma is liquefied, which ultimately results in the dissemination of the bacteria to other parts of the body. The bacteria are also transmitted to other individuals due to their release *via* droplets.

1.7.1.2 Independent developmental trajectories of lesions within an individual host tissue

There is growing evidence that within the lung, each granuloma may have its own course rof fate, and that the lung should not be considered an environment where all granulomas are synchronized to a common state. The use of [18F]-fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT scanning in human lungs during a linezolid treatment study indicated diversity in the local inflammatory response leading to variability in the size of lesions and FDG avidity (210). This has been cited as evidence that lesions within the lung are controlled not only by a systemic host immune response but also by local factors that would determine the fate of individual lesions (211). Furthermore, in nonhuman primate models, molecular tracking has been used to indicate the variability in the fate of multiple lesions within a host, possibly due to differences in host-mediated killing at the individual lesion level (P. L. Lin et al., 2014). Finally, a recent mathematical model proposes that there may actually be no steady state for granulomas and that instead, there is a continuous progression of disease with each granuloma progressing at a different rate over time (212).

One hypothesis is that independent lesion fates would require locally acting agent(s) or factor(s) to modulate the pathophysiology of the granuloma environment in response to the pathogen. It is still not known what these local agents might be. It is possible that locally acting agents are the product of lipid metabolism at the site of an infection or other pathophysiologic process as described elsewhere in other organ systems (213). These could include pro- and anti-inflammatory eicosanoids (lipid mediators derived from arachidonic acid, prostaglandins, lipoxins, and resolvins), those that modulate lesion resolution (213-215), or drive the lesion towards disease exacerbation and the development of necrotic foci (215). It might be possible that these agents

provide fine-tuning to systemic changes driven by cytokines and could possibly change the direction of lesion development within the host lung (211,216).

1.7.1.3 Lesion trajectories are controlled by host & bacterial factors

The fate of an individual granuloma is most likely controlled by a variety of bacterial and host factors. Apart from the mediators described above, these driving forces may also be impacted by the factors described in the following sections.

1.7.1.4 Is the RD1 region of *Mtb* required for the creation of a granuloma?

A variety of genes have been proposed to play a role in the persistence of *Mtb* in hosts (217). Complete genome sequencing has been carried out on a variety of *Mtb* complex strains including H37Rv, CDC1551, H37Ra and BCG. It is clear that there are differences in both the presence and the expression of specific gene products amongst members of the *Mtb* complex (43,92). DNA sequence analysis identified the RD1 as an approximately 9.5-kb region of *Mtb* (92) responsible for virulence that is absent in BCG (218). The RD1 region contains genes that encode two secretory proteins, *EsxA* coding for ESAT-6 and *EsxB* coding for CFP-10, as well as the *rv3877* gene, a putative translocation pore in the cytoplasmic membrane; all are located within the ESX-1 locus that encodes a secretory type VII system (218,219). RD1 is associated with the pathogenesis of TB and contributes to the secretion of specific pro-inflammatory cytokine (e.g., IL-1) (202,219). RD1 may also be driving nonspecific cell damage (e.g., mitochondrial damage): a mouse macrophage cell line RAW264 cell infection models showed that strains of H37Rv lacking RD1 have less measurable mitochondrial damage and did not have depleted ATP levels when compared with cells infected with wild-type H37Rv (202). The soluble RD1 component ESAT-6 may also play a role in tissue remodeling and cell recruitment to support granuloma formation. ESAT-6 has been shown to induce Matrix metallopeptidase 9 (MMP-9) production in the epithelial cells and the recruitment of

macrophages to the site of infection (220). MMP-9 might drive tissue remodeling that would allow for intact solid granuloma production (221-224). However, sequence analysis alone is not enough to determine the differences between strains as seen with *Mtb.* H37Ra, which contains the RD1 region but has noticeable downregulation of the RD1 gene products CFP-10 and ESAT-6 proteins (225).

1.7.1.5 Host genetic polymorphisms may impact the balance of granuloma stability instability & the progression to active disease

Some host genetic polymorphisms, such as the polymorphism in the promoter (-403G/A and -28C/G) and intron (In1.1T/C) regions of the *Ccl5* gene, may play a role in host resistance to *Mtb* infection (226,227). However, once infection has been established, several human polymorphisms may play a role in increasing patient risk for a destabilized granuloma and active disease. A functional promoter polymorphism in the -2518A>G of the monocyte chemoattractant protein-l (MCP-1) gene *Ccl2* (chromosome region 17q11.2) has been associated with increased susceptibility to *Mtb i*nfection in some non-BCG vaccinated populations (228). The proposed model is that the polymorphism is associated with increased levels of MCP-1, which in turn is associated with decreased levels of IL-12p40 and greater likelihood of progression to active disease (228). Although, IL-12p40 is a chemoattractant for macrophages and promotes the migration of bacterially stimulated DCs, IL-12p40 is important in the activation of cytokines IL-12 and IL-23 (229). These cytokines have specific roles in the initiation, expansion and control of the cellular response to tuberculosis (230). Another functional polymorphism is found in the promoter region of *Mmp1* (chromosome region 11q22.2), an insertion of a guanine at position 1607 (-1607_1608insG), creates an Ets-1 transcription factor binding site (228,231)and enhances gene expression (232). It is

thought that polymorphisms in *Ccl2* and *Mmp1* may jointly act to increase the chance of active disease in the hosts (232).

1.7.1.6 The granuloma provides a distinctly different environment for drug-induced bacterial killing than within normal lung tissue or blood

Growing evidence suggests that bacteria within the granuloma are not subject to the same host and environmental pressures as bacteria within the normal lung or within the blood. It has been proposed that the concentration of anti-mycobacterial drugs in the blood represents the drug concentration in the lesions of the lung. However, initial work in rabbits indicates that drug penetration into lesions varies among individuals, and among drugs, and differs from the penetration into the lung in general, as estimated by penetration coefficients. Work by Kjellsson et al. has indicated that there may be no numerical differences for drug penetration across lesion type for RIF and INH, while for PZA and moxifloxacin (MXF) there were modest numerical differences in the penetration for the suppurative and coalescing lesions compared with caseous and solid types of lesions (233). Within lesions, other work has reported a lower concentration of MXF in the caseous *vs.* the cellular fraction of the granuloma (234). Penetration into the lesions and activity of anti-*Mtb* drugs in the lesions is different from what occurs in normal lung tissue. Antibiotics are frequently below effective concentrations inside granulomas and this can lead to bacterial growth between doses and prolonged treatment times. Antibiotics may also be distributed along concentration gradients within granulomas, with lower antibody concentrations toward their centers (234).

1.8 Immune response to *Mtb*

Mtb is not easily cleared by the immune system due to its immune evasion mechanisms and therefore a chronic disease develops (235). Control of TB infection depends on the development of efficient innate and adaptive immune responses at the site of infection (236). However, *Mtb*

requires an immunological balance, insufficient immune responses, as well as too much inflammation, can be harmful to the host (190). Many of the immune cells during *Mtb* infection have multiple roles in a network of immune reaction. In an active TB infection, *Mtb* is able to manipulate immune response to provide a suitable environment for growth while promoting the growth required for transmission of disease.

1.8.1 Innate immune response in TB infection

The ability of the innate immune system to recognize and react to invading microbes is essential for infection control (237). PRRs on macrophages and other leukocytes recognize and respond to microbial components (238). The PRRs recognize a diverse set of molecules generally classified as pathogen-associated molecular patterns (PAMPs) (238). In particular, the mammalian Toll-like receptors (TLR) PRRs play an important role in innate immunity by recognition of PAMPs that initiate the activation of NF-κB and other intracellular signaling pathways through the adapter protein, MyD88 (239,240). TLRs detect the presence of infection and induce activation of inflammatory and antimicrobial innate immune responses (239,240). *Mtb* primarily infects macrophages and control the macrophages bactericidal action to create a suitable environment for bacterial survival and replication (241). Innate immune response to *Mtb* is an important factor in determining the disease outcome as only a few individuals exposed to *Mtb* become actively infected (241). The innate immune cells respond to *Mtb* infection by producing pro-inflammatory cytokines leading to intercellular communication and cell recruitment to the site of infection (236). PAMPs trigger the activation of diverse PRRs and innate mediators involved in the phagocytosis of mycobacteria and induction of signaling pathways that trigger production of inflammatory cytokines such as interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) (238,242). *Mtb* enter host macrophages *via* binding to multiple PRR receptor types

simultaneously (238). These receptors belong to four main classes: opsonizing receptors (e.g. FcγR and complement receptors), scavenger receptors (e.g. CD36 and MARCO), C-type lectin receptors (e.g. mannose receptor, dectin-1 and 2, and DC-SIGN) and innate immune sensors (e.g. TLRs and NODs) (239).

TLRs are essential for recognition of microbes by antigen presenting cells (APCs) such as macrophages and DCs. In *Mtb* infection TLR2, TLR4 and TLR9 play an important role (239). Polymorphisms in TLR2 and TLR9 have been associated with increased susceptibility to *Mtb* hence confirming the key role of TLRs in the host defense against mycobacteria (239). *Mtb* has developed different strategies to interfere with TLR activation and the induction of inflammation. Upon *Mtb* infection in the lung, innate immune cells involved are the phagocytic cells such as resident alveolar macrophages, pulmonary DCs, monocytes, and neutrophils (243). *Mtb* can also bind and interact with non-specialized phagocytic cells such as alveolar epithelial cells (244). Inhaled mycobacteria are engulfed by alveolar macrophages that will become activated (206). Activated macrophages produce reactive nitrogen intermediates (RNI) as well as antimicrobial peptides that act as the first line of defense to limit bacterial replication (245). Neutrophils are also known to phagocytose and kill *Mtb* (243). Death of the macrophage is essential for the mycobacteria to escape and infect new cells (34,246). Different modes of host cell death play different roles in host defense and microbial survival during microbial infections (247,248). Apoptosis is a programmed cell death that is dependent on the induction of caspases, while necrosis is a passive form of cell death that is induced by different types of stress, inflammation or microbial infection (247). *Mtb* has developed mechanisms to limit macrophage apoptosis and promote cell necrosis, which can prevent cross-presentation of *Mtb* antigens by DCs that could inhibit and delay T cell priming

(248). Infection of macrophages with virulent *Mtb* has shown to reduce the viability of the host cells similar to necrosis rather than apoptosis (249).

1.8.2 Adaptive immune response in TB infection

Control of *Mtb* infection is dependent on the induction of cell-mediated immunity, which involves close interactions between innate and adaptive immune responses (70). Recognition of *Mtb* antigens by TLRs and other PRRs expressed on DCs triggers activation of DCs that leads to the initiation of antigen-specific adaptive immune responses (237). DCs take up the *Mtb* antigens in the lung and migrate to the draining lymph nodes to initiate activation of T cell responses (250). For the initiation of adaptive immune responses after *Mtb* infection in the lung, it takes 7-9 days for the antigens to be carried to the draining lymph nodes and an additional 6-10 days for antigen-specific T cells to become activated and migrate to the primary site of infection (251). Slow development of adaptive immune response allows *Mtb* to establish before effective immune elimination of bacteria can occur (251). Initiation of T cell responses that is insufficient to eliminate *Mtb* bacteria may be due to the slow growth of the *Mtb* bacteria (252). Moreover, early induction of Tregs with suppressive functions may be capable of delaying the priming of effector $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes (252). For the development of effector T cell responses, bacterial antigens must be presented by DCs (253). The function of $CD4^+$ and $CD8^+$ T cells in TB is to activate phagocyte killing of bacteria or to induce direct killing of *Mtb* infected cells *via* the expression of cytolytic effector molecules, but also to regulate the inflammatory environment to limit tissue damage (254). The major effector mechanism of cell-mediated immunity is the activation of infected macrophages by interferon-γ (IFN-γ) produced by $CD4^+$ T cells (255). The production of IFN-γ is regulated by IL-12, which is released by activated DCs as well as macrophages (256). IFN-γ with TNF-α activates microbicidal mechanisms in macrophages that are

responsible for the control and elimination of *Mtb* (257). In addition, the killing of *Mtb* infected macrophages is done by $CD8^+$ cytolytic T cells (CTLs) expressing granule-associated effector molecules (258). Induction of effective adaptive immune response is important for *Mtb* control inside granulomatous lesions (253). The importance of $CD4^+$ T cells in anti-TB immunity has been extensively demonstrated by the observation that virus-mediated loss of $CD4^+$ T cells in HIV-infected patients increased the susceptibility to TB (259). It has also been reported that inherited deficiencies in the IFN-γ-receptor result in a deficiency of IFN-γ signaling that leads to an increased susceptibility to mycobacteria (260).

1.8.3 Host immune responses to *Mtb* infection and the production of a granuloma

As discussed in section 1.16, a typical hallmark of TB is the formation of granulomas at the site of *Mtb* infection (70). Early granuloma formation is dictated by macrophages infected with *Mtb* (261). Macrophage cells are the key immune cells involved in the granuloma formation and control of intracellular *Mtb* replication (261). *Mtb* infected macrophages recruit uninfected macrophages and other immune cells of both myeloid and lymphoid origin to respond to TB infection (Figure 1.5).

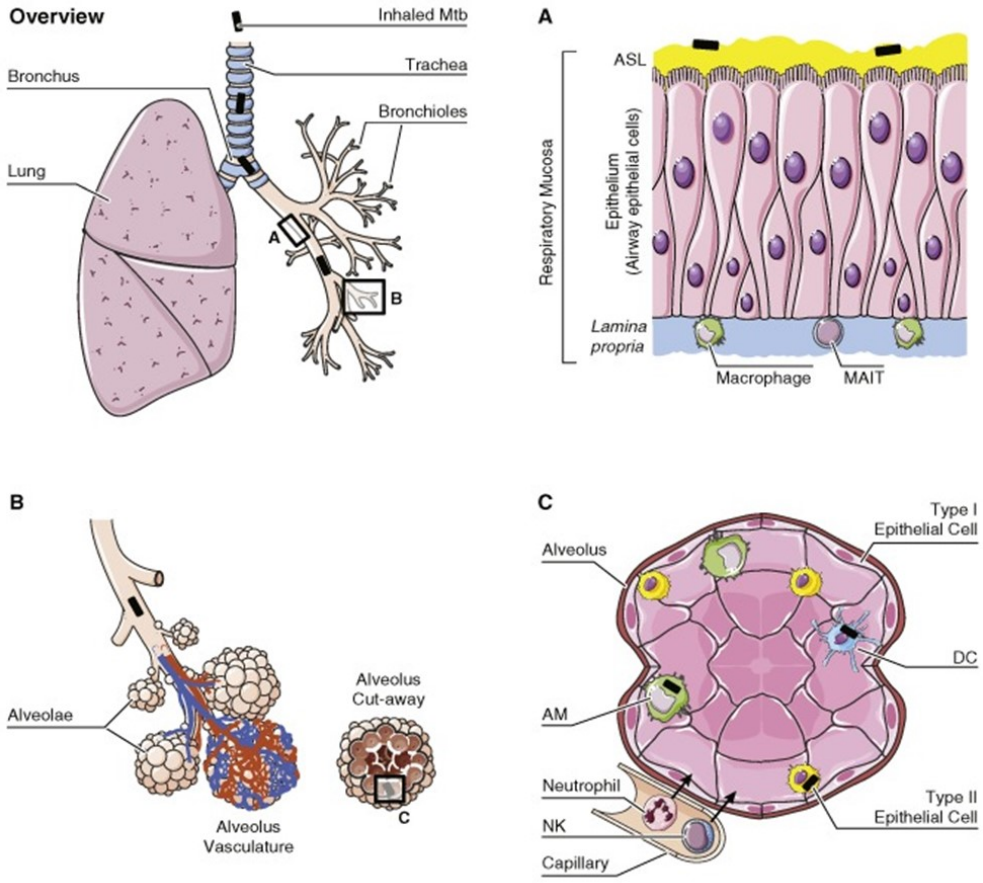

Figure1.5. Cells involved in response to TB infection. Upon inhalation into the lung, *Mtb* (black rod) travels along the trachea, bronchus, and bronchioles to the alveoli. Lining the airway is the respiratory mucosa (A). This consists of a layer of AECs that provide a tight barrier to prevent *Mtb* from invading the tissue and they have many receptors to detect *Mtb*. AECs control the composition of ASL, a substance containing mucus, anti-microbial peptides, antibodies and cytokines/chemokines. The lamina propria supports the epithelium and also contains immune cells such as macrophages and MAIT that respond to infection. *Mtb* eventually reaches the alveolar (B), which are surrounded by a network of capillaries to facilitate gas exchange. The alveolus (C) is structurally formed from type I epithelial cells, and type II epithelial cells are often found at the cell junctions. Type II cells secrete a variety of anti-microbial substances including a pulmonary surfactant. AMs and DCs are the primary resident defenders of the alveolus. They are effective phagocytes and have a range of intrinsic anti-microbial capacities. In addition, neutrophils and NKs are recruited from the surrounding capillaries to bolster the host defense. Image reproduced from from reference # 284 with permission from Wiley Global Permissions.

1.8.3.1 Myeloid cells in TB infection

1.8.3.1.1 Macrophages

After initial infection of alveolar macrophages in the lung, monocytes migrate to the site of *Mtb* infection from the blood (262). Monocytes can differentiate into tissue macrophages at the site of infection. The majority of the macrophages involved in granuloma formation are the epithelioid cells, which are activated macrophages (263). Two different macrophages CFS-1 and GM-CSF have a contrasting role in TB infection (264). The CFS-1 subset is known as classically activated macrophages with bactericidal properties that are induced by Th1 cytokines (i.e. IFN-γ) (265). CFS-1 macrophages express inducible nitric oxide synthase (iNOS), which is an enzyme that catalyzes the synthesis of the potent antimicrobial compound nitric oxide (NO) (266). Hence, the production of iNOS is strongly induced by IFN-γ. Alternatively, GM-CSF activated macrophages have anti-inflammatory properties that are induced by Th2 cytokines (i.e. IL-4, IL-13) (265). GM-CSF macrophages express Arginase type 1 (Arg-1) an enzyme that competes with iNOS for the use of arginine as a substrate (266). iNOS uses arginine as a substrate to generate toxic NO, Arg-1 use arginine as a substrate to generate ornithine (266). Ornithine promotes collagen deposition and contributes to the formation of epithelioid granulomas and tissue fibrosis (267). GM-CSF macrophage activation allows the bacteria to survive in infected macrophages partly by preventing NO synthesis (268). GM-CSF macrophages also produce anti-inflammatory cytokines including IL-10 and transforming growth factor-β (TGF-β) that could further suppress a bactericidal function of the macrophage (269). TLR signaling triggered by mycobacteria induce the production of Arg-1 *via* IL-10, IL-6 and granulocyte colony stimulating factor (G-CSF) and inhibition of Arg-1 expression has been shown to be beneficial for host survival (270). The ratio between CFS-1 and GM-CSF macrophages in the granuloma may be an important determinant for the outcome of TB infection;

the presence of both subsets is required to maintain a balance between pro- and anti-inflammatory responses (190).

Chronically activated macrophages fuse to generate MGCs, which is a characteristic of human TB (261). The cell-to-cell fusion process is caused by mycobacterial lipomannan or by IL-4 (271). MGCs release high concentrations of lytic enzymes, which destroy the surrounding tissue (271). The importance of MGCs during granulomatous inflammation remains to be determined.

1.8.3.1.2 Dendritic cells (DCs)

Mtb cannot infect DCs, but they help to keep the bacteria in a non-replicating state (272). DCs are not very effective at killing *Mtb* but the key role is the presentation of *Mtb* antigens in the initiation of *Mtb* specific T cell responses (273). DCs containing live mycobacteria stimulate T cells but also are used by *Mtb* as a tool for spreading (272,273). DCs present peptide and lipid antigens to MHC-II and CD1molecules present on various APCs, such as B cells and monocytes. However, *Mtb* induced DC maturation leads to an uncoordinated presentation of peptide antigens for subsequent T cell activation (274). DCs are important in the cross-presentation of *Mtb* antigens as DCs can engulf apoptotic vesicles from *Mtb* infected cells to prime T cells (275). An IL-12 cytokine produced by DCs is essential in the induction of IFN-γ and a Th1-mediated immune response that is required for immune protection in TB (276). *Mtb* infection may fail to induce effective production of IL-12 by DCs, which could further disrupt the induction of Th1 response (203). DCs present in the granulomatous lesions in the lungs have down-regulated MHC-II and co-stimulatory molecules and therefore DCs isolated from the granulomas fail to activate of *Mtb* specific $CD4^+$ T cells (277).

1.8.3.1.3 Neutrophils

Neutrophils can become infected and are activated by *Mtb* products, such as LAM (278). Neutrophils kill *Mtb* via the production of reactive oxygen species (279), however virulent strains of *Mtb* have evolved mechanisms to escape oxidative killing (280). Neutrophils are attracted to the site of infection by secretion of chemokine CXCL8 and recruit other leukocytes in the early granuloma formation through the secretion of CXCR3-signaling chemokines (280). Neutrophils in combination with human DCs promote DC activation with less IL-10 production and better antigen presentation compared to mycobacteria and DCs alone (280).

1.8.3.2 Lymphoid cells in TB infection

Multiple components are involved in controlling the development of a granuloma including immune cells. $CD4^+$ T cells, $CD8^+$ T cells, regulatory T cells, B cells, γ/δ T cells, NK cells, as well as NKT cells are involved in the control of TB infections (Figure 1.6).

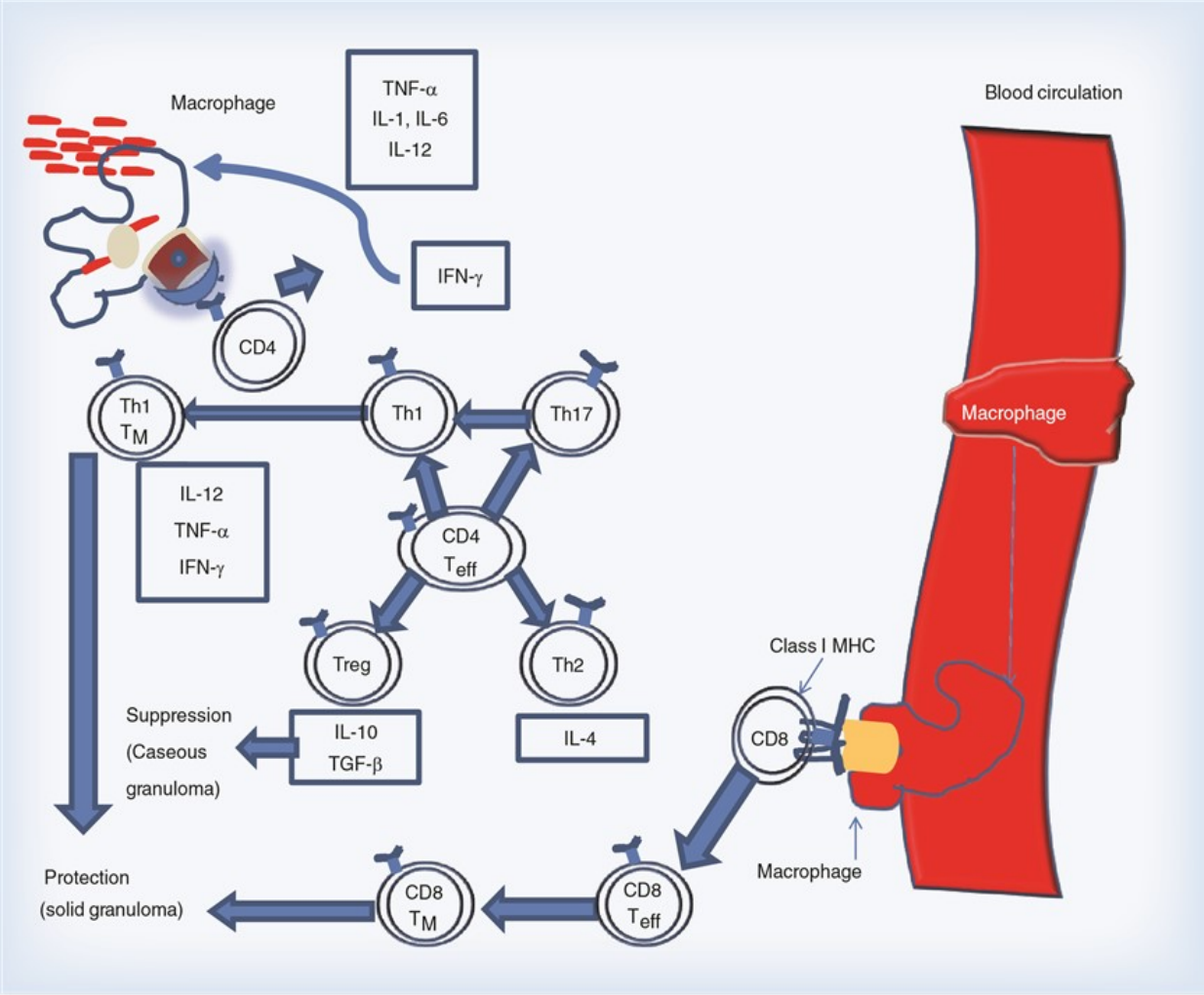

Figure1.6. Immune cells, cytokine secretion and the type of granuloma. In the presence of $CD4^+$ Th1 immune response and $CD8^+$ T cells (CTL), the solid granuloma is formed. In the presence of Tregs, a caeseous granuloma is formed.

1.8.3.2.1 $CD4^+$ T helper 1 (Th1) cells in TB infection

$CD4^+$ T cells are recognized as the most important cells in the response to *Mtb* infection (281). Their role in the clearance of *Mtb* infection has been extensively studied in both mice and humans (282). Initial studies in mouse models demonstrated that adoptive transfer of $CD4^+$ T cells from infected donors to T cell deficient recipients provided protection against *Mtb* (283). Later,

studies using CD4 deficient mice or depletion of $CD4^+$ T cells using a monoclonal antibody showed that $CD4^+$ T cells were important for protection against *Mtb* (284-286). Furthermore, mice deficient in MHC II ($CD4^+$T lymphocytes) have increased susceptibility to *Mtb* (284).

In humans, the importance of $CD4^+$ T cells in the control of *Mtb* infection is supported by reports of the immunocompromised (e.g. HIV positive patients), who are highly susceptible to *Mtb* infection and reactivation, particularly those with low $CD4^+$ T cell counts (287,288). It has been suggested that *Mtb* specific $CD4^+$ T cells are preferentially depleted in HIV-positive patients thereby increasing susceptibility to TB (289). Bacterial loads are controlled when $CD4^+$ T cells interact directly with the infected macrophages (290). Th1 cells produce IFN-γ and TNF-α which synergize to activate microbicidal mechanisms in human macrophages (257). In addition to IFN-γ and TNF-α, IL-2 is also produced by the Th1 cells that support the proliferation and clonal expansion of antigen-specific T cells (291). Th1 cytokines are required for the activation and differentiation of $CD8^+$ cytotoxic T lymphocytes (CTLs) in the killing of *Mtb* infected cells (292). A deficiency in the Th1 pathway that occurs either as a consequence of HIV infection, cancer, immunosuppressive drugs or genetic defects increases the susceptibility to mycobacterial diseases (293). APCs stimulate $CD4^+$ T cells *via* T cell receptor and co-stimulatory molecules such as CD40-CD40L and CD80/CD86-CD28 (294,295).

Activated APCs produce IL-12, which is a key cytokine that stimulates the polarization of Th1 through the production of IFN-γ (296). The early $CD4^+$ T cell production of IFN-γ is important for the effective control of TB infection (282). In addition, *Mtb* specific $CD4^+$ T cells secreting combinations of cytokines IFN-γ/TNF-α or IFN-γ/IL-2 have been detected in patients with TB/HIV co-infection (297,298). It has been demonstrated that effective treatment of TB leads to a shift from IFN-γ producing positive $CD4^+$ T cells to IFN-γ^+ /IL-2^+ producing double-positive $CD4^+$ T cells,

which suggests that polyfunctional $CD4^+$ T cells may be involved in immune protection to *Mtb* infection (298).

1.8.3.2.2 $CD4^+$ T helper 2 (Th2) cells

The Th1 response is important in the control of TB disease; in contrast, active TB disease has been associated with increased activation of Th2 cells *in vivo* (299). Th2 cells produce IL-4, IL-5, and IL-13, which contribute to the initiation of a humoral immune response (300,301). Since *Mtb* is an intracellular microbe, antibody-mediated immunity may not be protective against *Mtb* (301). It has been reported that Th2 polarization could affect disease susceptibility in TB (302,303). A 100-fold increase of IL-13 and IL-4 has been reported in patients with active TB compared to age and gender-matched controls (304). It has also been demonstrated that progressive TB disease might be due to preexisting Th2 immune responses that induce toxicity of TNF-α and/or impair bactericidal functions (305). Importantly, progressive TB disease is not only related to the absence of a Th1 response but may be due to co-existing Th1/Th2 or excessive Th2 immunity (305-308).

1.8.3.2.3 $CD4^+$ T helper17 (Th17) cells

Th17 cells are pro-inflammatory cells that mediate antimicrobial immunity against bacteria and fungi, particularly at mucosal surfaces (309). Th17 cells produce a variety of cytokines including IL-17, IL-22, and GM-CSF (310). These cells are important in generating immune responses to infection with extracellular bacteria and fungi (311,312). Studies have demonstrated that Th17 cells appear in the early phase of adaptive immunity and contribute to host defense against *Mtb* (309). Th17 cells produce IL-17, IL-21, IL-22 and IL-23 (175,313). In addition to the Th1 response, a Th17 response is considered protective in mycobacterial infection (314). Th17 cells can trigger the production of chemokines CXCL9, CXCL10, and CXCL11 which recruit IFN-γ producing $CD4^+$ T cells to the site of *Mtb* infection and restrict the growth (309). Th17 cells also

regulate the production of antimicrobial peptides and are essential in the formation of mature TB granulomas (309). The exact role of Th17 in the protection of TB still needs to be evaluated.

1.8.3.2.4 $CD8^+$ T cells

$CD8^+$ T cells are a subset of T cells that are required to control TB infection in humans (315). $CD8^+$ CTL are specialized $CD8^+$ T cells that kill *Mtb* infected cells by the release of granules containing cytolytic and bactericidal effector molecules, but can also be activated to produce Th1 cytokines that contribute to the immune protection (316,317). $CD8^+$ T cells participate in the control of *Mtb* infection by lysis of *Mtb* infected macrophages combined with the killing of intracellular bacteria and also by the expression of effector cytokines (318,319). $CD8^+$ CTLs anti-mycobacterial activity is mainly by granule-mediated cytotoxicity (320). However, death receptor-ligand can also induce apoptosis which may be less efficient in killing intracellular *Mtb* (321). *Mtb* infected cells in humans were shown to kill mycobacteria by the pore-forming protein perforin in combination with the antimicrobial peptide granulysin (318,319). Evidence from human TB suggests that $CD8^+$ effector memory T cells expressing high levels of perforin and granulysin can mediate antimicrobial activity (315,320). Reduced numbers of $CD8^+$ effector T cells were detected in patients with rheumatoid arthritis after treatment with anti-TNF-α inhibitors, which resulted in an increased susceptibility of patients to develop active TB (321).

Polyfunctional *Mtb* specific $CD8^+$ Tcells that produce double-positive cytokines IFN-γ^+/IL-2^+ has been shown to correlate with host protection and a beneficial response to anti-TB treatment (315). In contrast, $CD8^+$ T cells which produce the chemokine XCL1 (lymphotactin) negatively regulates IFN-γ production by $CD4^+$ T cells. $CD8^+$ T cell repertoires in children with TB have been identified as the clonal expansion of terminally differentiated $CD8^+$ effector T cells in severe forms of the disease (315,322). Hence, $CD8^+$ T cells may also serve as targets of immune evasion by *Mtb*.

1.8.3.2.5 Subsets of T cells in TB infection

Circulating T cells mostly have a resting or naive phenotype which can be characterized by cell markers $CD45RO^-$, $CD45RA^+$ and $CCR7^+$ (323). When a naïve T cell encounters an antigen (Ag), an adaptive immune response along with the induction of memory cells develops (254,323). Adaptive immune responses will recognize the same Ag, upon the second encounter (254). In the first phase naïve T cell encounters an Ag, a massive proliferation and clonal expansion of Ag-specific T cells is followed by a phase of contraction (324,325). The cells in this phase are called effector T cells and these cells are eliminated by apoptosis at the end of an immune response (254,326). In the second phase memory T cells develop and are maintained for prolonged periods due to retention of Ag. Memory T cells are a pool of effector T cells that can rapidly respond to later encounters with the pathogen (254).

The characterization of subsets of memory T cells are detected by surface markers (326). Memory T cells are characterized based on the presence of cell marker CD62L (326). Central memory (T_{CM}) cells are identified by the presence of the CCR7 cell marker ($CCR7^+$). These cells home to secondary lymphoid tissues and produce high amounts of IL-2 but low levels of other effector cytokines (327). The effector memory (T_{EM}) cells are identified by the absence of the CCR7 cell marker ($CCR7^-$). These cells home to the peripheral tissues and produce high levels of cytokines (327). It has been demonstrated that the telomeres are longer in T_{CM} cells than T_{EM} cells and T_{CM} cells are capable of generating T_{EM} cells *in vitro*, but T_{EM} cells don't generate T_{CM} cells (327).

Several studies have identified that polyfunctional T cells (T cells with multiple effector functions) induce immune protection to *Mtb* infection (328-331). Earlier studies in human TB have reported that polyfunctional T cells produce IFN-γ in combination with IL-2 (332-336). Later

studies identified that a subset of cells simultaneously produces IFN-γ, TNF-α, and/or IL-2 in patients with active TB disease compared to latently infected individuals (315,337-339). This subset of T cells decreased after anti-TB treatment. In another study, $CD4^+$ T cells expressing cytokines IFN-γ, TNF-α, and IL-2 were found simultaneously in adults with active TB disease compared to LTBI individuals (338). In addition, they also identified that IFN-γ single and IFN-γ/IL-2 dual secreting $CD4^+$ T cells dominated the anti-mycobacterial response (338,339). Therefore, the presence of multifunctional $CD4^+$ T cells in TB patients was related to the bacterial loads and they decrease after completion of anti-TB chemotherapy (338,339). This demonstrates that the presence of multifunctional $CD4^+$ T cells is related to active TB disease rather than a protective role. In contrast, several other studies have reported a contrasting finding that polyfunctional T cells were reduced in patients with active TB disease compared to LTBI individuals, and that the T cells recovered with anti-TB therapy (332,340). It has also been demonstrated that increased antigen-specific T_{EM} cells and a decreased T_{CM} $CD4^+$ T cells have been found in patients with active TB (341,342) compared to LTBI individuals (332). Similarly, dual IFN-γ/IL-2-producing T cells were reported in patients with anti-TB therapy (343,344). In addition, increased antigen-specific T_{EM} cells and a decreased T_{CM} $CD4^+$ T cells have been found in patients with active TB (341,342) compared to LTBI individuals (332).

Marin et al. reported that polyfunctional T cells produce IFN-γ, TNF-α, IL-2, and IL-17 after short-term (1 day) and long-term (6 days) *in vitro* stimulation using different antigens (CFP-10, PPD, or *Mtb*) (332). Further, they identified an increase in single and double $CD4^+$ T cells in long-term *in vitro* stimulation compared to short-term *in vitro* stimulation in LTBI subjects and a significant increase of single $CD4^+$ T cells in patients with active disease (332). *Mtb* stimulation increased single and triple producing $CD4^+$ T cells in LTBI subjects in 6 days compared to 1 day *in*

vitro stimulated cells (332). This suggests that different mycobacterial antigens induce distinct T cell functional signatures in LTBI subjects and in patients with active disease, and the possibility to identify CD4 T cells that correlate with the state of infection could be used as indicators of the disease (343). Petruccioli et al. have reported that RD1-proteins specific $CD4^+$ T cells with a T_{EM} phenotype correlate with active TB disease, while RD1-proteins specific $CD4^+$ T cells with a T_{CM} phenotype were associated with cured TB and LTBI (343). Therefore, this suggests that different expression of the memory and effector T cells may be used to monitor treatment efficacy (343). Similarly, Lalvani et al. reported the same trend of memory and effector T cells as demonstrated by Petruccioli, but in response to different antigenic stimulation, (PPD and RD1-peptides) (320,343). Moreover, these studies also identified that HIV infection did not influence the number of *Mtb* specific $CD4^+$ T effector cells, but was dependent on the stage of TB disease (320,343). Therefore, characterization of distinct phenotypes of T cells with several functional properties such as activation, memory, migratory and inhibitory receptors would be helpful in evaluating TB disease.

1.8.3.2.6 Regulatory T cells (Tregs)

Natural Tregs constitute about 5% of peripheral T cells which constitutively express CD25, the transcription factor fork head box P3 (FoxP3) (345) and T cell inhibitory receptors, Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and the Glucocorticoid-Induced TNFR family related gene (GITR) (294). Inducible Tregs develop from conventional $CD4^+$ T cells (345). Important functions of Tregs are to prevent autoimmunity, maintain self-tolerance and suppression of immune responses during infections (346-348). The $CD4^+CD25^+FoxP3^+$ Tregs are increased in patients with active TB (349-351). The presence of $CD4^+Foxp3^+$ Tregs was observed in the granuloma of humans, non-human primates, and mice (352-354). The immunosuppressive functions of natural and induced Tregs depend on negative signals induced by CTLA-4, GITR or Programmed Cell

Death-1 (PD-1) upon binding to their appropriate ligand on APC and soluble factors such as IL-10 and TGF-β (355). Expansion and accumulation of Tregs have been shown to decrease Th1 cell responses in patients with TB (356). Sharma et al. reported that the ratio of Tregs: effector T cells is significantly higher in the tissues of individuals with disseminated TB (miliary TB) compared to individuals with pulmonary TB (123). Similarly, in the macaque monkey model, macaques exhibited low numbers of T reg cells during early infection in the blood, but increased numbers of Tregs were observed in the lung (357).

In addition, it has been reported that immunosuppressive cytokines that impair immunity to *Mtb* can be produced by both Tregs and anti-inflammatory M2 macrophages (358,359). Increased numbers of *Mtb* specific Tregs restrict and delay T cell effector responses in TB infection. Tregs delay priming, expansion, and mobilization of both $CD4^+$ and $CD8^+$ effector T cells that leads to an enhanced *Mtb* growth (316). Tregs also counteract Th17 cells, thus inhibiting the early recruitment of IFN-γ-producing $CD4^+$ T cells to the site of infection. Immature DCs and anti-inflammatory macrophages that are infected by *Mtb* have shown to be capable of inducing Tregs (175). The role of Tregs in *Mtb* infection is unclear, but an observational study by Burl et al. suggested that induviduals who are exposed to *Mtb* have reduced number of Treg cells in the peripheral blood (360). In addition, other observational studies reported the correlation between the severity of the disease and the number of Tregs during active TB infection; increased numbers of Tregs were reported in the lungs and blood (314,349,361,362). Further, histological analysis of tissue samples revealed that caseating granulomas contain significantly greater numbers of Treg cells than other granulomas (363). Overall, human studies have discovered remarkable correlations that suggest Tregs may play a critical role in disease pathogenesis, but the exact function of Tregs (harmful *vs* beneficial) during active TB needs to be evaluated.

The role of Tregs in human TB has been demonstrated *in vitro*. Tregs depletion from PBMCs resulted in increased IFN-γ production and T cell proliferation, demonstrating the suppressive function of Tregs (349). Further studies have reported that Tregs can suppress T cell responses to some *Mtb* antigens such as heparin-binding hemagglutinin but not to *Mtb* antigen ESAT-6, suggesting that Tregs may be specific for some but not all epitopes (364). However, it is possible that the selective suppression of specific epitopes may be due to the differences in the relative affinities of T cells for their associated antigen.

In mouse models of TB infection, a low dose aerosol infection leads to the proliferation of Tregs, and increased numbers of activated T cells in the lung. The functional role of Tregs during *Mtb* infection has been demonstrated after depleting Tregs in the lung using anti-CD25 antibodies. Mice treated with anti-CD25 antibodies and subsequently infected with *Mtb* exhibited slightly enhanced Th1 effector cell proliferation and production of IFN-γ, but no change in the lung bacterial burden was observed (365). Kursar et al. transferred effector T cells ($CD4^+CD25^-$) with or without Tregs ($CD4^+CD25^+$) into T cell-deficient mice (Rag deficient), and subsequently infected with *Mtb* (366). A decrease in bacterial loads was observed in mice that received effector T cells alone compared to mice that received effector T cells with Tregs suggesting that $CD25^+$ Tregs may inhibit the protective immune response to *Mtb* (366). However, effector T cells can also express CD25 (367); hence it is not inevitably clear whether Tregs were capable of specifically suppressing immunity against *Mtb* infection.

1.8.3.3 B cells

Antibody production is one of the most important effector functions of the B cells (368). Humoral immune responses are important for protection against many pathogens (368). However, B cells also carry out a variety of other effector and regulatory functions during the course of an

immune response (369). B cells mediate protection from pathogens *via* antigen presentation, co-stimulation, and antibody secretion, production of cytokines that regulate humoral and T cell responses (370). Different effector functions of B cells are tightly controlled and they are functionally and phenotypically distinct effector and regulatory B cell subsets that are sub-divided based on their cytokine profile (371,372). Effector B cell subsets differentiate the development of T cells as Th1 or Th2, *via* production of IFN-γ and IL-4 (371)(373). Lately, it has also been demonstrated that regulatory B cells producing IL-10 can maintain $FoxP3^+$ Tregs and limit Th1 and Th17 responses in healthy individuals, but not in patients with an autoimmune rheumatoid arthritis (373). The frequency of regulatory B cells in the peripheral blood of TB patients was inversely correlated with Th17 cells (374). In contrast, it has been reported that people with active TB have higher frequencies of regulatory B cells with suppressive activity compared to healthy controls (374).

The protective immune response in TB infection is mostly based on cellular immunity, but the role of B cell-mediated immunity in TB remains controversial (375,376). It has been demonstrated that B cells had an impact on the inflammatory response and TB disease in a murine model challenged with aerosol (377). In addition, it has been reported that B cells were present in granulomatous TB lesions in the lung, which were involved in the organization and development of the TB granuloma (378-380). Thus, B cells may potentially use different functions to control other immune cells present in the granulomas including macrophages and T cells.

1.8.3.4 Other immune cells involved in TB infection

1.8.3.4.1 Gamma-delta T cells (γδ T cells)

γδ T cells are non-conventional T cells that play important roles in antimicrobial immunity as well as in chronic inflammatory reactions (381). The early source of IL-17 is from γδ T cells, which bridge innate and adaptive immunity that are important in the early host defense against *Mtb* (382). Mice deficient in γδ T cells had increased bacterial loads and decreased survival in an intravenous model of *Mtb* infection (383). In contrast, aerosol infection of *Mtb* in mice deficient in γδ T cells did not impact the control of bacterial load or survival but exhibited increased inflammation (384). Thus the role of γδ T cells in the response to *Mtb* infection is unclear.

1.8.3.4.2 Natural killer (NK) cells

NK cells are important in the innate defense against pathogens (385). It has been demonstrated that human NK cells can recognize and lyse *Mtb* infected macrophages using the NKp46 receptor (386). In addition to their role in the granule-mediated killing of infected cells, NK cells also produce significant amounts of cytokines (387). $CD3^{-}CD56^{+}$ NK cells produce IL-22 when exposed to autologous monocytes and gamma-irradiated *Mtb* in the presence of cytokines IL-15 and IL-23 (387). The contribution of NK cells to immune defense against *Mtb* through the production of IL-22 has been studied in the *Mtb* infected macrophages (387). *Mtb* infected macrophages showed increased phagolysosomal fusion and reduced *Mtb* growth, suggesting that NK cells can contribute to immune defenses against *Mtb* through the production of IL-22 (387).

1.8.3.4.3 Natural Killer T cells (NKT)

NKT cells recognize antigens presented by CD1 (388). The role of NKT cells in the immune response to *Mtb* is unclear. Reports on the role of NKT cells in *Mtb* are based on observational studies in humans (389-391). NKT cells are reduced in active TB patients compared to healthy uninfected controls (392). Infection of human PBMCs with mycobacteria led to a phenotypic shift,

resulting in an increased relative proportion of $CD4^+$NKT cells compared to $CD4^-CD8^-$ (double negative) cells (389-391). The same phenotypic shift was also observed in the mouse model of TB infection (393). Although NKT cell contribution at the site of infection is unknown, it is suggested that NKT cells that express both T-cell receptor (TCR) and NK cell markers, and γ/δ T cells that secrete IL-17 act as intermediaries between the innate and adaptive immune responses (394,395).

1.8.4 Cytokine responses to *Mtb* infection

1.8.4.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions (269). The role of these cytokines in TB infection is detailed in Table 1.4.

Table 1.4 Pro-inflammatory cytokines in TB infection

Cytokine	Receptor/Signal	Main producers	Role in *Mtb* infection
TNF-α	TNFR1, TNFR2 JNK, p38, NFκB	Airway epithelial cells, alveolar Type II pneumocytes, NK T cells, macrophages, alveolar macrophages, DCs, neutrophils, $CD4^+$ (Th1) and $CD8^+$ T cells	**Positive**: induction of chemokines, activation of macrophages, granuloma formation **Negative**:Over production caused tissue damage
IL-1α/IL-1β	IL-1R1, IL1RAcP MyD88,IRAK4,NFκB	DCs	**Positive**: recruitment and activation of phagocytic cells, Th17 polarization
IL-6	IL-6R, gp130 JAK, STAT3, MAPK	Macrophages, DCs	**Positive**: Th17 polarization
IL-12 p40,p35	12Rβ1, IL-12Rβ2 JAK2, TYK2, STAT4	DCs	**Positive**: Th1 polarization **Negative**: Over production is toxic
IFN-γ	IFNGR1, IFNGR2 JAK/STAT	Airway epithelial cells, alveolar Type II pneumocytes, NK T cells, $CD4^+$ (Th1) and $CD8^+$ T cells, γ/δ T cells, CD1-restricted T cells	**Positive**: activation of macrophage, induction of NO synthesis and bacterial killing **Negative:** Overexpression pathogenic

1.8.4.1.1 Tumor Necrosis Factor-α (TNF-α)

TNF-α is a pro-inflammatory cytokine that is required for the control of acute *Mtb* infection (242). TNF-α is produced by a variety of cells such as macrophages, DCs and T cells (269,396). C57BL/6 mice deficient in TNF-α, or the 55 kDa TNF receptor (TNFR), or where TNF-α was neutralized by using monoclonal antibody during infection, all displayed increased susceptibility to

Mtb infection (397,398). Other observations from these studies are decreased survival, increased bacterial loads and disrupted granuloma formation (397,398). TNF-α acts as a mediator of macrophage activation, apoptosis and granuloma formation (399). TNF-α, with IFN-γ, induces the production of Nitric Oxide Synthase 2 (NOS2) expression (400). In the absence of TNF-α, the NOS2 expression is delayed in response to *Mtb* infection and the granulomas formed are disorganized, with the presence of few activated or epithelioid macrophages, demonstrating that TNF-α affects cell migration and localization of *Mtb* infection (397). In addition, it has been demonstrated that blockade of TNF-α during the chronic phase of experimental *Mtb* infection in a cynomolgus macaque model led to the exacerbation of primary infection and reactivation of latent infection. Bacterial loads and pathology was increased and decreased dissemination of infection to the extra-pulmonary sites and increased pro-inflammatory response, was observed which suggests that TNF-α may also exert an anti-inflammatory effect (401).

The importance of TNF-α in human TB was first demonstrated in patients treated for rheumatoid arthritis with TNF-antagonists, which led to the reactivation of latent tuberculosis (402). The risk of developing active TB in these patients was 4-10-higher compared to the patients that were untreated for rheumatoid arthritis (403-406). The TB disease pattern in these patients is characterized by a high rate of extrapulmonary (56-62%) and disseminated TB disease (24-28%) (402,405). In addition, it has also been reported that anti-TNF treatment resulted in a decreased frequency of a subpopulation of memory $CD4^+$ T cells and also of $CD8^+$ effector memory T cells, which confirms that this treatment has severe effects on *Mtb*-specific T cell responses (321).

1.8.4.1.2 Interleukin-1 beta (IL-1β)

Numerous human genetic studies have reported an association of polymorphisms in the IL-1 or IL-1R genes altering TB disease progression and susceptibility (407-411). In contrast, treatment of autoinflammatory disease and rheumatoid arthritis by the drug Anakinra that blocks IL-1 did not aggravate TB disease or reactivate latent infection (410). Thus, it is not clear whether IL-1 has a critical role in the control of *Mtb* infection in humans.

1.8.4.1.3 Interleukin-6 (IL-6)

IL-6 exhibits both pro- and anti-inflammatory properties and has multiple roles in the immune response including inflammation, hematopoiesis, and differentiation of T cells (242,310). It is produced at the site of infection during the early stage of mycobacterial infection (242). The role of IL-6 in TB infection is controversial and little is known about IL-6 during *Mtb* infection in humans. It has been reported that patients with cavitary TB disease express lower levels of IL-6 in their bronchial alveolar lavage (BAL) fluid compared to TB patients without cavitary disease. There were no correlation between cytokine expression in BAL fluid and serum (412). In contrast, a study identified elevated blood plasma levels of IL-6 from TB patients with developed lung lesions (413).

In mouse models, the importance of IL-6 during *Mtb* infection depends upon the route and dose of infection. Neutralization of IL-6 either by antibody treatment or by gene deletion increased susceptibility to high dose intravenous challenge of mycobacteria in mice (414,415). In contrast, IL-6 deficient mice with a low dose aerosol infection of *Mtb* exhibited a modest increase in the bacterial load in the lungs compared to wild-type mice (416). In the low and high dose challenge models, increased IL-4 delayed T cell responses, and IFN-γ expression was observed (415,416). *In vivo* data support a protective role for IL-6 mediated through IFN-γ (415,416). It appears that from

animal studies and the human data, IL-6 may be associated with expression of immunity in the lung during the early stages of infection.

1.8.4.1.4 Interleukin-12 (IL-12)

IL-12 is a key player in the host defense against infection with *Mtb* and has a critical role in the stimulation of IFN-γ production. IL-12 can be detected in lung infiltrates, and granulomas, and is associated with pleurisy and lymphadenitis (417). The importance of IL-12 in TB is illustrated in an individual's deficient with IL-12p40. Deficiency in IL-12p40 increases susceptibility to *Mtb* infection (418-423). Further, patients with Mendelian susceptibility to mycobacterial disease (MSMD) exhibit susceptibility to *Mtb* due to the deficiencies in IL-12Rβ1, IFNγR1, and IL-12p40 and develop disseminated BCG infection (BCGosis) following BCG vaccination (195,424-426).

In vivo animal models suggest that IL-12 is expressed within the lung during *Mtb* infection (417). Treatment of mice with exogenous IL-12 during *Mtb* infection increased survival of mice, resistance to *Mtb* infection, and increased production of IFN-γ by splenocytes (397,427). In contrast, the same effect was not observed in the IFN-γ deficient mice, suggesting that the protective effect of IL-12 is IFN-γ dependent (397). This was supported by studies in IL-12p40 deficient mice which displayed increased susceptibility to *Mtb* infection, defective granuloma formation and reduced IFN-γ production levels by splenocytes (428,429). Further, IL-12p40 homodimers are required for optimal DCs trafficing and T cell activation suggesting a role of IL-12 in the maintenance of Th1 response during *Mtb* infection (203). However, comparison of IL-12p35 and IL-12p40 deficient mice revealed that IL-12p35 deficient mice were less susceptible to *Mtb* infection and were capable of inducing an IFN-γ response, albeit at a reduced level compared to IL-12p40 deficient mice (428).

1.8.4.1.5 Interferon-γ (IFN-γ)

IFN-γ is a key cytokine in the control of *Mtb* infection. It is primarily produced by $CD4^+$ and $CD8^+$ T cells (283,430) but also can be produced by T cells expressing γδ receptors (γδ T cells) and CD1-restricted T cells (431,432). IFN-γ activates macrophages, controls mycobacterial growth, and also induces production of inducible nitric oxide synthase (iNOS), an enzyme that synthesizes reactive nitrogen intermediates (RNIs) and NADPH-oxidase which synthesizes reactive oxygen species (ROS) (433). Deletion of the NOS2 gene in mice resulted in increased susceptibility to *Mtb* infection (434-436). The role of NOS in humans is less clear (437). Reports on the role of NADPH in the mouse model of *Mtb* are conflicting (433,434,438,438). One mechanism by which *Mtb* survives intracellularly in macrophages is through inhibition of phagosome maturation leading to a less acidic environment (439). IFN-γ has shown to increase the acidification of phagosome by the production of RNIs and ROS (35,431,432). In addition to activating macrophages, IFN-γ also play a role in the control of immunopathology, and increased survival of neutrophils (440-443).

1.8.4.2 Anti-inflammatory cytokines

The anti-inflammatory cytokines are molecules that control the pro-inflammatory cytokine response. Their role in TB infection is outlined in Table 1.5.

Table 1.5 Anti-inflammatory cytokines in TB infection

Cytokine	Receptor/Signal	Main producers	Role in *Mtb* infection
IL-10	IL-10 receptor-1 and IL-10 receptor-2	Neutrophils, Macrophages, DC, Tregs	**Positive:** Anti-inflammatory **Negative:** inhibits $CD4^+$ T cell responses, APC presentation, the function of macrophages and DCs, to produce IL-12
TGF-β	MMP-9, MMP-2	Macrophages, DC, Tregs	**Positive:**aAnti-inflammatory **Negative:** inhibits several T-cell activation and function, tissue damage
IL-4	IL4R	Th2 cells, Activated mast cells, basophils and γ/δ T cells	**Positive:** anti-inflammatory **Negative:** suppress IFN-γ production and macrophage activation
IL-17	IL-17RC, IL-17RA	$CD4^+$ (Th17) T cells, γ/δ T cells	**Positive**: recruitment of neutrophil, activation of macrophage **Negative**: drives pathology via neutrophils

1.8.4.2.1 Interleukin-10 (IL-10)

IL-10 is secreted by macrophages, neutrophils, DCs, B cells, and T cells (444). IL-10 directly inhibits $CD4^+$ T cell response. IL-10 can also inhibit APC function of macrophages, DCs, and the production of cytokines such as IL-12, thus inhibiting the development of Th1 responses against mycobacterial infections (445). The role of IL-10 during *Mtb* infection has been controversial, with many conflicting reports possibly due to the differences in the genetic

background of the mice, and the strain of *Mtb* used for infection (446). Early studies suggested that mice deficient with IL-10 had no effect on bacterial load (447) and IL-10 is not responsible for suppressing a Th1 response (448). Later studies suggested that IL-10 only suppressed protective responses early in infection (449). Furthermore, it has been shown that IL-10 inhibited the formation of mature, fibrotic granulomas capable of containing the infection, but depletion of IL-10 did not enhance protection against *Mtb* (450). An *in silico* model of non-human primates showed that IL-10 is detrimental for granuloma sterilization but at the expense of caseation (451). This was further supported by reports that virulent strains of *Mtb* induce higher levels of IL-10 (452,453). For example, *Mtb* strain H37Rv and the clinical isolate CH produce different levels of IL-10 upon infection of human monocyte-derived macrophages (453). The hypervirulence of *Mtb* HN878 may be due to the production of higher levels of IL-10 producing $Foxp3^+$ T cells upon aerosol infection in mice (452).

A role for IL-10 in contributing to the pathogenesis of *Mtb* infection is also suggested by studies in humans (446). Elevated levels of IL-10 are reported in the lungs (454), serum (455) and sputum (456) in active TB patients (446) (446). IFN-γ and IL-10 were produced by $CD4^+$ T cells isolated from BAL from active TB patients compared to the healthy controls (457). Furthermore, the suppressive role of IL-10 was demonstrated by neutralizing IL-10 with anti-IL-10. In the absence of IL-10, production of IFN-γ by PBMCs from TB patients was increased when cultured in the presence of *Mtb* and the expression of IL-10 correlated with bacterial load in active TB patients (458).

1.8.4.2.2 Transforming Growth Factor β (TGF-β)

TGF-β is produced by human monocytes and DCs they are present in the granulomatous lesions of tuberculosis patients (459,460). TGF-β inhibits T cell responses to *Mtb* infection and can

deactivate macrophages by inhibiting IFN-γ-induced NOS2 production (445). The role of TGF-β *in vivo* suggests that TGF-β suppress cell-mediated immunity, IFN-γ production, antigen presentation, production of pro-inflammatory cytokines, and cellular activation (459). In addition, TGF- β is involved in tissue damage and fibrosis loss (459).

1.8.4.2.3 Interleukin-4 (IL-4)

The role of IL-4 is to suppress IFN-γ production and macrophage activation. IL-4 is associated with Th2 responses, induced in response to helminth infections and contributes to diseases such as asthma and allergy (461-464). In a mouse model, infection with *Mtb* or reactivation of latent infection is associated with increased production of IL-4 and overexpression of IL-4 aggravates tissue damage (465). *Mtb* infection in IL-4 gene-disrupted mice demonstrated similar bacterial load and susceptibility to TB infection as wild-type mice. There were no significant increase in the bacterial load observed in the IL-4 gene-disrupted mice (447). Production of IL-4 does not promote cellular immunity to *Mtb* infection, suggesting that IL-4 may not play a significant role in the development of TB disease (447). In contrast, a study in IL-4 knockout mice reported increased granuloma sizes, increased production of pro-inflammatory cytokines, excessive tissue damage and increased *Mtb* load compared to the control mice (466).

1.8.4.2.4 Interleukin-17 (IL-17)

Th17 cells produce a variety of cytokines including IL-17, IL-22, and GM-CSF (310). These cells are important in generating immune responses to infection with extracellular bacteria and fungi (311,312). IL-17 is primarily produced by Th17 cells but other cells, such as γδ T cells and NKT cells are also able to produce IL-17 (467). During *Mtb* infection, γδ T cells are the major source of IL-17 production (468). The role of IL-17 in primary *Mtb* infection is unclear. IL-17 deficiency in the mouse models was not reported to impact the control of *Mtb* infection; however, it

is proposed that IL-17 plays an important role in the formation of granulomas following BCG infection (177,469). During *Mtb* infection, IL-17 may play a role in the control of infection through CXCL13 in the induction and localization of T cells within the granuloma (470). However, the same study reported that IL-17 was dispensable for protection against the *Mtb* strain H37Rv and a less virulent clinical isolate CDC1551 (470). In contrast, it has been reported that IL-17 was required for control of bacterial loads following intra-tracheal infection with H37Rv (177). Thus, it seems that IL-17 may contribute to protection against *Mtb* infections but the precise role of IL-17 in the pathogenesis of TB infection is unknown.

1.8.5 Chemokines in TB

CC chemokines are a sub-group of chemokines that include 27 members (471). In addition to their well-characterized roles in the regulation of inflammation and immune homeostasis, some members of this chemokine family such as chemokine CCL2 have been associated with increased susceptibility to pulmonary TB infection (472). High concentrations of CCL2 have been shown to inhibit the production of IL-12 in cultures of *Mtb* stimulated monocytes (228). CCL5 is a chemokine produced by a variety of cells including macrophages, fibroblasts, eosinophils, endothelial cells, and platelets. CCL5 exerts chemotactic activity on NK cells, T cells, DCs and macrophages to inflamed or infected tissues (473). The expression and functional activities of CCL5 have been studied in experimental models of granulomas, elicited by *M. bovis*. These results demonstrated higher levels of CCL5 in granulomas (474). Consequently, the recruitment of APCs and T cells and also the formation of granulomas induced by *Mtb* have been shown to be reduced in CCL5 knockout mice (475). CCL5 is especially important in the early responses to *Mtb* due to its role in the recruitment of IFN-γ-producing T cells to form granulomas at the site of infection (475).

CXC chemokines are a sub-group of chemokines that includes 17 members (471). It has been demonstrated that neutralization of TNF-α in *Mtb* infected macrophages resulted in a reduction of inflammatory chemokines such as CCL5, CXCL9, and CXCL10 (476). CXCL8 is a proinflammatory chemokine that recruits neutrophils and T cells in response to infection (477). It has been shown that IL-4 reduces CXCL8 levels, but has no effect on CXCL10 levels. The down-regulation of CXCL8 secretion is important to prevent inflammation in human TB, but reduce cell-mediated immune responses, since CXCL8 is required to induce neutrophil and T cell migration (478). CXCL9 and CXCL10 are induced by IFN-γ during infection, injury or inflammation. The main role is leucocyte trafficking by $CD4^+$, $CD8^+$ T cells and NK cells (479). CXCL10 secretion is induced by *Mtb* stimulated macrophages (480) and attracts T cells but not neutrophils (481)

1.9 Models Used to Study the Tuberculosis Granuloma

1.9.1 *In vivo* experimental models to study the TB granuloma

There are several nonhuman models that can be used to study the pathophysiology of the TB granuloma, (1) the humanized murine model, (2) the guinea pig model, (3) the rabbit model; and (4) the primate model. The mouse model has been very useful in obtaining the information on the cytokine and immune cell responses for granuloma formation. The benefits of the mouse model include the inexpensive nature of the assay system, the ease in handling mice, the different variant strains available and reagents available for the perturbation of the system (482,483). However, significant differences exist between the granulomatous response to *Mtb* infection in the lung between mice and humans (378). Necrosis is lacking or difficult to achieve in murine models (Figure 1.7B) and may not represent the necrosis seen in human granulomas (Figure 1.7A) (191,484-487). Finally, although latency can be studied, there is no standardized model of latency in the murine model (263). In guinea pig and rabbit models, the benefits include ease of handling and the ability to produce necrosis within the granuloma (488,489). There are now latent models of

infection that have been created in the guinea pig host but these have not been widely tested (490). The rabbit model is the only animal model that can represent the progressive disease as seen in human infection (491,492). However, drawbacks to both the guinea pig and rabbit models include the limited variety of reagents to study pathophysiology when compared with human or murine models. Finally, primate models of infection allow for granuloma production that is similar to humans and latency can be established (189,493), with necrosis also produced in granulomas (401). Drawbacks to using primates for this type of work include the expense, difficulty in handling and ethical concerns in primate research.

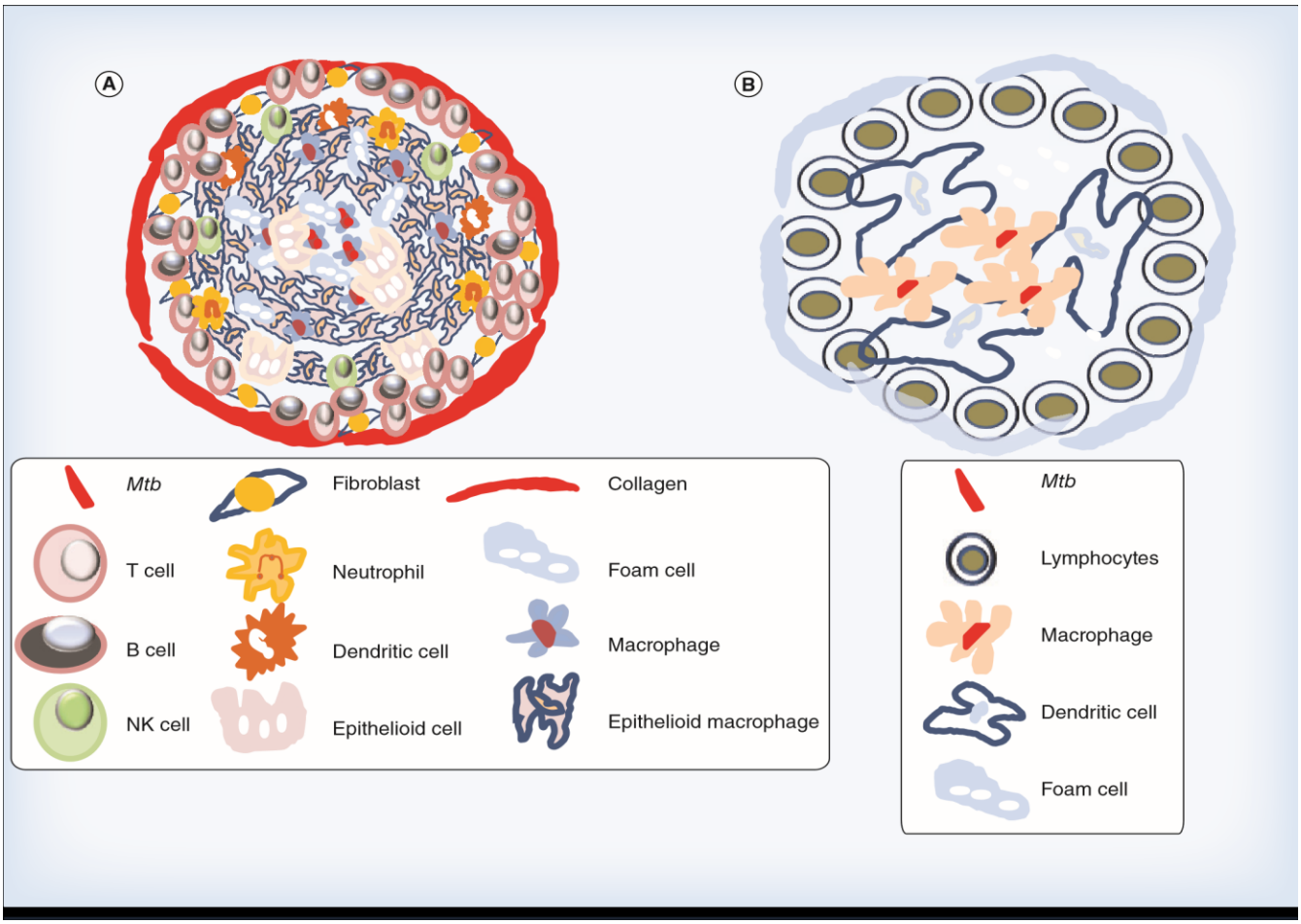

Figure1.8. Differences in granuloma structure between mouse and human granulomas. (A) In human granulomas, the infected macrophages are in the middle surrounded by other immune cells such as $CD4^+$ and $CD8^+$ T cells, as well as macrophages that fuse to form multinucleated giant cells or differentiated into foamy cells. This type of granuloma is seen during latent infection where the mycobacteria can be dormant and survive for decades. (B) granulomas in mice are comprised of loose non-necrotic aggregates, surrounded by lymphocytes and macrophages that have fused and differentiated into foamy cells. *Mtb: Mycobacterium* tuberculosis.

1.9.2 Mathematical & computational models

Mathematical and computational models have been used to predict the granulomatous response in TB infection based on experimental observations and the available information about the disease (212,494-500). Mathematical models are inexpensive and allow investigators to test a

variety of new hypotheses and incorporate a number of complex parameters without the cost and time issues encountered in wet laboratory experiments (501,502). These models have been used to address questions in TB that are difficult to approach experimentally. For example, differential equation (DE) based models describe a relationship between numbers of cells and concentrations of molecules and their rates of change in the granuloma and time (212,499,500). In contrast to DE-based models, individual-based models, or agent-based models (ABMs), are rule-based models that capture the events occurring in the immune systems (e.g., immune cells, bacteria, environmental factors) using a 2D grid representing a section of lung tissue (495-497). Although these models have been useful in answering complex questions, they are highly dependent on the parameters chosen, and require previous observations in different systems to extrapolate the results. As a result, they can miss unknown factors.

1.9.3 *In vitro* granuloma models

1.9.3.1 The experimental human lung tissue model

Previously, an early granuloma model was established using human lung tissue (503), which used a previously described 3D tissue model of the human lung mucosa (504). Macrophages were infected with *Mtb* strains prior to infection of the model and then co-introduced with PKH26 red fluorescent dye-labeled monocytes into the lung model. Using confocal microscopy, only virulent strain infections (e.g., H37Rv) and not avirulent strains (H37Ra, BCG, ΔRD1, and ΔESAT-6) were associated with monocyte/macrophage clustering at the infection sites (503). As an indicator of necrosis, another group stained for HMGB-1 protein, a marker released from cells undergoing necrosis (505). They observed a significantly higher level of HMGB-1 staining in tissues infected with H37Ra than in uninfected areas or in tissues infected with avirulent or ΔRD1 or ΔESAT-6 strains (503). However, it should be noted that this is a very preliminary model which uses indirect

indicators (e.g., macrophage, monocytes clustering and HMGB-1 protein production) and only involves macrophages, monocytes, fibroblasts and lung-specific epithelial cells (503). A key message in this study is that mycobacterial factors may be important in initiating early phases of granuloma production, as well as the development of necrosis.

1.9.3.2 Peripheral blood mononuclear cell models

Peripheral blood mononuclear cell (PBMC) granuloma models allow for the systematic determination of bacterial and host factors that drive granuloma formation and pathophysiology (209). Over the last 10 years, a variety of approaches have been used. In early models, proof of principle studies used *Mtb* antigens attached to agarose beads instead of viable mycobacteria. These antigen-bead complexes, when incubated with PBMCs, induced the production of the granuloma-like structure. The composition of immune cells within these structures was similar to that found in natural *Mtb* granulomas. Later studies used cyanogen bromide (CNBr)-activated Sepharose beads coated with purified protein derivative (PPD) (506). In a study by Puissegur *et al.*, blood samples were collected from healthy BCG-vaccinated, PPD-reactive nontuberculous control individuals (507). Monocyte-like cells were recruited to the bead surface on day one. By day four, lymphocyte-like cells were recruited and were seen to bind to the attached monocyte-like cells. By day five, bead surfaces were completely covered by recruited cells and multilayers of monocytes and lymphocytes were formed with pseudopodia (indicating cell differentiation) identified (508). Staining at day nine identified CD68-positive staining cells that might represent multinucleated giant cells (MGC). These MGC-like cells were also surrounded by CD3- stained lymphocytes (508). Potential macrophages stained strongly positive for CD168, and possible epithelioid cells were weakly stained for CD168.

Other PBMC models have avoided antigen-coated beads and utilized viable BCG to create granuloma-like structures. This approach also showed that PBMCs from healthy donors were able to form granuloma-like structures around BCG within nine days (509). These granulomas included activated lymphocytes in tight contact with macrophages, as well as multinucleated giant cells. BCG-containing phagosomes were also present in what appeared to be epithelioid cells.

However, it is not clear how BCG generates a granuloma in the absence of RD1 and how these models would vary from other *Mtb* models. BCG granulomas are possible as BCG pulmonary granulomas have been described in highly immunocompromised patients (509). The growing number of models using human donor PBMCs suggest that the immune status, previous PPD or BCG exposure, previous *Mtb* infection status and health (including acute infections) of the PBMC donor must be accounted for when *in vitro* granuloma models are studied (508).

Concerns remain about these *in vitro* PBMC granuloma models. Are these models in fact just cell aggregates with similar cellular characteristics to human granulomas? Perhaps they have some key characteristics that may improve our knowledge about *Mtb* infections. They allow us to study *Mtb* infections of cells in a mixed multicellular environment. They may also account for PBMC donor factors that might have a downstream impact on how *Mtb* infections occur, and how granulomas are formed. These models can be quite dynamic and can be used to study the proliferation of specific cell types during a mock infection.

1.9.3.3 *In vitro* granuloma models are amenable to microdissection approaches

New developments in microdissection (510) have raised the possibility that individual granulomas within an infection model could be interrogated. The previous PBMC granuloma models described above have all been approached as a single system with no attempt to dissect individual granulomas. Given the previous discussion that each granuloma may be on its own

developmental trajectory, it is possible that dissection approaches could provide more information on how individual granulomas develop within a larger system. Microdissection studies have previously been used to study the expression of host cell genes in lung tissue from *Mtb* infected mice (511). Tissue from microdissection has also been used for qPCR, and immunohistochemistry from *M. bovis* induced granulomas in cattle.

1.9.3.4 Dormancy models of TB infection

As described earlier, host cell-free *Mtb* culture experiments have often been used to mimic the conditions encountered by the bacteria within host granulomas (512-514). These have then been applied to simple infection models with some, for example, using a hypoxic-induced environment model, it was shown that *Mtb* accumulates triacylglycerides and goes into a dormant state but can regrow after re-exposure to oxygen (514). When this information was applied to a lipid loading THP-1 infection model, the *Mtb* was found to be dormant (513,514). This work has been useful in improving our understanding of the metabolic adaptation of *Mtb* during processes resembling dormancy. However, these models have been unable to demonstrate resuscitation under conditions that mimic immune suppression, which is a key element in *Mtb* pathophysiology leading to active TB. To achieve this goal, 3D *in vitro* models of granulomas have been studied.

1.9.3.5 3D *in vitro* models

Seitzer and Gerdes were the first to report the 3D granuloma model using PBMCs infected with *Mtb* (515). In their studies, PBMCs were infected with *Mtb strain* H37Rv at different multiplicity of infection (MOI) (number of bacteria/cell) and seeded into agarose-coated wells. After four days of incubation, the lowest MOI in their study (1:150) produced host cell aggregates. However, infection at a higher MOI did not result in a large aggregate formation but rather numerous very small aggregates. An increase in the number of dead cells was also observed. By

histological analysis the aggregates were confirmed to have similar phenotypical characteristics (e.g., cell population type) to natural *Mtb* granulomas. Another study combined human PBMCs, autologous macrophages and *Mtb in* ultralow attachment tissue culture plates. The non-adherent PBMCs were added on day two and five after infection to mimic the natural infection process in which additional lymphocytes are recruited to the infection site (200). The formation of granulomas was observed and acid-fast bacilli were observed within the host cells composing the granuloma. Birkness et al., also showed that the addition of IL2, IFN-γ and/or TNF-α, enhanced the formation of the granuloma and host cell recruitment. Although these models have provided researchers with important information about granuloma cell differentiation, they were unable to establish dormancy and resuscitation inside the 3D-generated granulomas.

Kapoor et al. developed a 3D *in vitro* granuloma model in which *Mtb* progresses to dormancy and subsequently resuscitates under conditions that mimic weakening of the immune system (516). PBMCs were mixed at room temperature with an extracellular matrix (ECM). *Mtb* H37Rv was added to the ECM at MOI of 1:10. After 8 days of incubation, micro granulomas, and an aggregation of lymphocytes around infected macrophages were observed, as well as the presence of multinucleated giant cells. The cytokines IFN-γ, TNF-α, IL-12p40, and IL-10 were detected in the culture supernatants. Dormancy was demonstrated by the loss of acid-fastness, accumulation of lipid bodies, development of rifampicin tolerance, and gene expression changes. Treating the granulomas with immunosuppressant anti-TNF-monoclonal antibody reactivated the dormant *Mtb.* Several other studies used the 3D granuloma model to study other mycobacterial diseases, such as *M. leprae* (517), *M. bovis* (515) and *M. avium* (200). These studies had the advantage of showing *Mtb* going into dormancy and subsequently resuscitating under conditions that mimic weakening of

the immune system, these studies were not able to mimic the different cell types in a lung tissue exposed to *Mtb* infection (200,508,516,517).

Recently, Braian et al. developed a cell-based *in vitro* human lung tissue model (518). In this model, the human lung-specific fibroblasts were cultured in a matrix of collagen. A transwell insert matrix was layered over the top of the membrane. Monocytes or macrophages isolated from the PBMC were infected with *Mtb* and added to the matrix layer, and human lung-specific epithelial cells were added to the culture. The culture was then exposed to air to initiate the production of extracellular matrix proteins, mucus secretion, and stratification by the epithelium. The advantage of this 3D tissue model is that it displays the characteristic features of human lung tissue, including epithelial cells integrated with macrophages, the formation of extracellular matrix, stratified epithelia and mucus secretion (518). Although this model has several advantages over other *in vitro* models, it has some limitations. This model consists of only monocytes and macrophages, besides lung-specific cells. It lacks neutrophils and lymphocytes which are also known to be in TB granulomas. This model can be used to study both active infection and latent states. Continued work is needed to determine if this model is representative of true dormancy.

In summary, 3D *in vitro* models are comparable to granulomas observed in clinical specimens. Potential applications of the *in vitro* models include the study of functional characterization of individual cell types, of cell surface expression, cytokine and chemokine secretion, development of dormancy, and resuscitation under conditions that suppress the host immune response. These models could potentially provide insights into the mechanistic aspects of host defenses, such as phagosomal maturation, autophagy, and antimicrobial peptides. Interestingly, 3D *in vitro* models allow manipulation of one or more cells types and provide a relevant tissue microenvironment, potentially a platform for testing vaccine and drug candidates against dormant,

as well as active mycobacteria. These models could also potentially provide insights between mycobacteria and host–cell interactions within granuloma structures, which animal models have limited ability to address.

1.10 Rationale for thesis research

The main defense mechanism against *Mtb* infection is the formation of granulomas. An understanding of the complex host-pathogen that takes place within the TB granuloma is critical for the design of new TB drugs and vaccines. The animal models of *Mtb*, through the use of knockout and transgenic animals as well as antibody depletion, has been helpful for understating the mechanisms of the immune response that are required for bacterial containment. However, this approach is limited in understanding the loss or gain of an immune component at a systemic level. *In vitro* granuloma models allow for the study of defined cellular and soluble host factors, as well as specific natural strains and acellular mycobacterial components. This ability to work with simpler systems involved in granuloma formation, while still maintaining a sense of complexity, allows for the creation of well-focused scientific hypotheses in a model that is more representative of the natural host. *In vitro* granuloma models will also allow the exploration of genetic and immunologic variables contributing to TB including the metabolic state of mycobacteria contained inside human granulomas. For example, studies in an animal models identified that BCG vaccination generates effective, long-lasting immunity that prevents *Mtb* infection and the development of TB. However, the exact mechanism by which the immunity fails in the lung is not completely understood and whether BCG vaccination is good or bad is still a debatable question. The role of adaptive immune cell subsets (Th1, Th2, Th17 and Tregs) on BCG vaccination remains to be evaluated in terms of the variability between the hosts (i.e, why some individuals develop immunity and some do not) and is key in controlling the infection. Understanding BCG vaccination

mediated adaptive immune responses to *Mtb* in animal models is problematic because they do not exactly recapitulate *Mtb* infection in humans. *Mtb* has evolved to primarily infect humans and thus using human models to study the mechanisms that are responsible for clearance of mycobacteria is very important. In doing so, our insight of the human *Mtb* granuloma response will be greatly improved, thus new ideas for biomarkers, therapy and development of effective vaccines against TB infection. These studies address an important question in human *Mtb* infection: is the formation of granuloma dependent on BCG vaccination and the variability between the hosts is simply a Th1 or inflammatory response, or is a more balanced immune response necessary for granuloma formation and containment of infection that may be valuable.

1.11 Overall study hypotheses

Host BCG vaccination history and/or types of of host immune cells (Tregs) will affect immune cell recruitment, *Mtb* growth and cytokine responses in a human PBMC-derived *in vitro* model of early *Mtb* H37Ra infection.

1.11.1 Objectives and hypothesis

Objective 1: To investigate if infection of human PBMCs with an attenuated strain of *Mtb* H37Ra forms early host immune cell aggregates (Chapter 2).

Hypothesis: Infection of PBMCs with *Mtb* H37Ra will form early host immune cell aggregates.

Objective 2: To evaluate the impact of BCG vaccination on mycobacterial load and immune responses in a human PBMC-derived model of early *Mtb* H37Ra infection (Chapter 2).

Hypothesis: Infection of BCG vaccinated donor PBMCs suppress bacterial replication and Th2 cytokine responses

Objective 3: To examine mycobacterial load and immune responses in a Tregs depleted PBMC-derived model of early *Mtb* H37Ra infection (Chapter 3).

Hypothesis: Tregs suppress bacterial replication and Th2 cytokine responses

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Chapter 2: Characterization of immune responses of human PBMCs infected with *Mycobacterium tuberculosis* H37Ra: Impact of donor declared BCG vaccination history on immune responses and *M. tuberculosis* growth*

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2.1 Introduction

Tuberculosis (TB) infection affects approximately one in three people in the world and causes approximately 1.5 million deaths worldwide each year (1). The disease is caused by *Mycobacterium tuberculosis* complex (*Mtb*), which is comprised of several human and animal associated species and sub-species (2). Following infection of the lung, the *Mtb* bacillus is phagocytosed by dendritic cells (DCs) and monocyte-derived macrophages where the bacillus survives within these cells (3-6). The host cellular immune response to *Mtb* infection includes the recruitment of new macrophages (7-10) and T cells from the circulation to the site of infection within the parenchyma of the lung. These recruited immune cells interact with the pre-existing macrophages and DCs in the lung in support of the immune response against *Mtb* infection (6). This series of events leads to the formation of a mature granuloma, a multicellular structure composed of infected and uninfected macrophages, epithelioid cells, giant cells (multinucleated cells derived from fused macrophages), T cells and B cells to contain the bacilli and to prevent the spread of the *Mtb* infection (11-13).

A portion of Chapter 1 has been published that reviews a variety of *in vitro* approaches to better understand the development of a granuloma and to control the pathophysiology of *Mtb* (14). Due to the limited access to human biopsy samples of granulomas, several three-dimensional *in vitro* models have been used to study the structure and function of granulomas. In particular, the three-dimensional *in vitro* granuloma model of *Mtb* infection which consists of donor PBMCs in a collagen matrix (15) has allowed the study of host factors that drive the formation of a granuloma (13,16,17). Human PBMCs infected with members of the *Mtb* complex formed aggregates of bacteria and monocyte-derived macrophages as well as T cells, which may represent an early granuloma formation (14,15,17-21).

In this study, a three-dimensional *in vitro* granuloma model of *Mtb* infection was used to characterize the human immune response to attenuated *Mtb* H37Ra. Although the use of attenuated strains in infection models may not mirror infection with wild-type virulent strains, use of attenuated strains allow us to assess the impact of potential confounders on experimental models using tools that are outside of a Biosafety Level 3 laboratory (22). One key confounder of infection model experiments is postulated to be Bacille Calmette-Guérin (BCG) vaccination history of PBMC donors. Some evidence suggests that a history of BCG vaccination may influence results of studies using *in vitro* granuloma models by generating protection, albeit variable, against *Mtb* infection, and would be a significant confounder of *in vitro* studies (23). Given the sparse literature in the field, this study was initiated with two aims. The first was to characterize the early host immune responses in human PBMCs infected with an attenuated *Mtb* H37Ra strain, as well as the growth of this strain during infection. The second aim was to determine the impact of BCG vaccination history of PBMC donors on the immune and bacterial responses in a three dimensional *in vitro* granuloma model of *Mtb* infection.

2.2 Materials and methods

Materials were obtained from Fisher Scientific, Ottawa, Ontario unless stated otherwise.

2.2.1 Ethics statement

This study was approved by the University of Alberta Health Research Ethics Board (Pro00057636) and all methods were performed in accordance with institutional guidelines and regulations. Informed written consent was obtained from all study participants.

2.2.2 PBMC donor enrollment

A questionnaire approved by the institutional ethics board was used to assess potential confounders in blood donors. Potential donors were asked 1) what is their age and country of birth,

2) for female donors, if they were pregnant, 3) if they recalled a prior BCG vaccination or exposure to someone with active TB, 4) if they had previously tested positive with a tuberculin skin test (TST) / interferon-gamma release assay (IGRA), 5) if they had a history of latent TB infection, 6) if they had a recent gastrointestinal or respiratory illness, 7) if they were vaccinated with a live or attenuated vaccine within the previous four weeks, and 8) if they were taking immunosuppressive drugs (Fig 1). In regard to donor TST history, each donor was asked about TST history on the donor history form. This included if the donor had any previous TST test done ("yes" or "no"). If the donor responded "yes" to a history of a TST, then the donor was asked if the TST test was positive, negative or if the donor was not sure about the TST result (Figure 2.1).

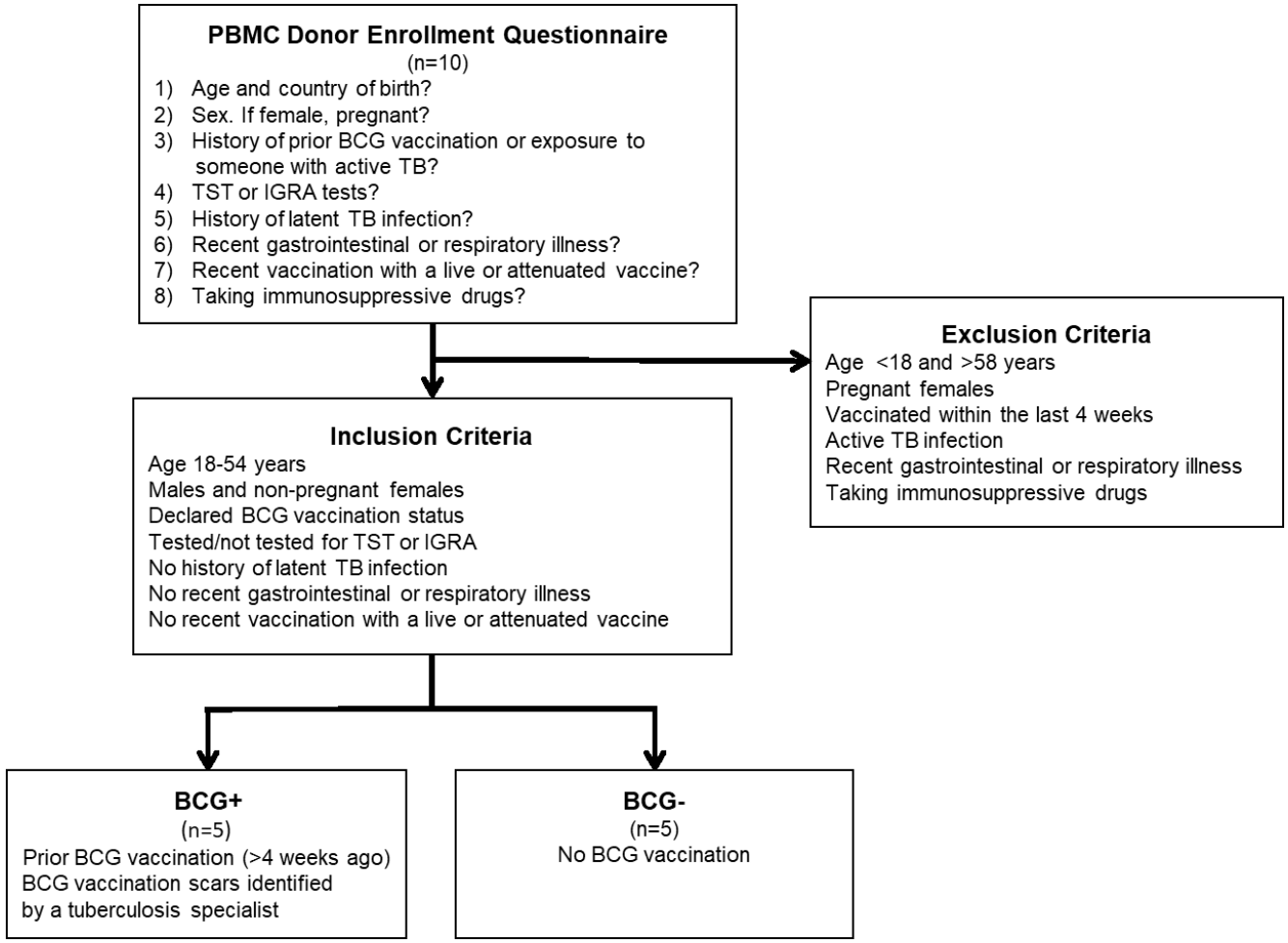

Figure2.1. Flow diagram of donor selection criteria

2.2.3 Donor inclusion criteria

Healthy males and non-pregnant females between the ages of 18-54 years with no history of latent TB infection or recent gastrointestinal or respiratory illness or recent vaccination, and not taking immunosuppressive drugs with or without the potential confounders listed above were included in this study.

2.2.4 Donor exclusion criteria

Males and females under the age of 18 years, pregnant females, individuals who were vaccinated within the last 4 weeks with the live or attenuated vaccine, individuals with active TB infection, individuals with a recent gastrointestinal or respiratory illness, or individuals who were taking any immunosuppressive drugs were excluded from the study. Individuals over age 54 were excluded from the study to avoid any age-related changes that may skew the results.

2.2.5 Presence of BCG vaccination scars

Volunteer blood donors who declared a BCG vaccination history had both arms inspected for scars consistent with BCG vaccination by DK. a tuberculosis specialist for more than 25 years. Testing of donor PBMCs with an IGRA to confirm BCG vaccination could not be done because all the donor cellswere used in the experiments.

2.2.6 Isolation of PBMCs from human blood

Volunteer donors gave informed written consent to collect 50 ml blood to isolate and use their PBMCs for research purposes. Blood was collected in vacutainers containing sodium heparin anticoagulant by phlebotomists at the Alberta Diabetes Institute Clinical Research Unit of the University of Alberta. PBMCs were isolated from whole peripheral blood following our standard protocol using density gradient centrifugation. The blood was diluted 1:1 in sterile saline and layered onto Lympholyte®-H cell separation media (Cedarlane, Burlington, Ontario)

and centrifuged at 870 x g, for 30 min at room temperature (IEC Centrifuge Model CRU-5000, O'Fallon, Missouri) without brakes. The interphase between the upper phase (plasma, thrombocytes) and the lower phase (Lympholyte®-H) containing the PBMCs was carefully transferred to a fresh tube using a Pasteur pipette and washed twice with phosphate-buffered saline (PBS). Each wash consisted of resuspending the cells in 50 ml PBS followed by centrifugation at 200 x g, for 5 min at 4°C to pellet the cells and to remove the supernatant (Beckman Coulter Allegra X-15R centrifuge, Mississauga, Ontario). A hemocytometer was used to count the total number of live and dead cells and cell viability by 0.4% Trypan Blue exclusion dye (Sigma-Aldrich, Oakville, Ontario). For cryopreservation, the PBMCs were adjusted to a final concentration of 1.5-2.0 x 10^7 cells/ml by slowly adding appropriate volumes of ice-cold cryopreservation medium, which consisted of 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 90% heat-inactivated fetal bovine serum (FBS). One ml aliquots of the cell suspension were transferred into 1.2 ml Nunc cryopreservation vials and stored at -80°C overnight and then in liquid nitrogen (-196°C).

When needed, the vials were removed from liquid nitrogen storage and thawed for 2 min in a 37°C water bath (Innova 3100 Water Bath Shaker, New Brunswick Scientific, Edison, New Jersey). Cells were immediately removed from the vials with a sterile pipette and diluted in 20 ml of fresh culture medium consisting of Roswell Park Memorial Institute medium (RPMI) (Sigma-Aldrich) supplemented with 1% Penicillin-Streptomycin and 10% FBS. The cell suspension was centrifuged for 5 min at 72 x g to allow removal of the cryopreservation medium. The cells were resuspended in RPMI culture medium, 20 µl of DNase solution (Sigma-Aldrich) was added and then incubated for 30 min in a 37°C water bath. The cells were then

filtered through Falcon™ Cell Strainers to remove the cell debris, pelleted by centrifugation at 200 x g for 5 min at room temperature.

2.2.7 Characterization of donor PBMCs

PBMCs were prepared for flow cytometry to characterize different subsets of cell populations (e.g. T cells, B cells, Treg cells, macrophages, dendritic cells (DCs) and Th1, Th2 and Th17). Cell pellets were resuspended in FACS buffer (PBS supplemented with 10% heat-inactivated FBS). The final concentration of each cell suspension was adjusted to 1 x 10^6 cells/ml in ice-cold FACS buffer and transferred to Falcon™ round bottom polystyrene tubes. Cells were washed two times, where each time cells were resuspended in cold PBS, then centrifuged at 200 x g for 5 min and the supernatant discarded. Cell pellets were resuspended in 100 µl of Human BD Fc block (BD Biosciences, Mississauga, Ontario), diluted 1:50 in FACS buffer and incubated for 30 min on ice to avoid any non-specific binding and background fluorescence. Each panel of cells was stained with a cocktail of antibodies directly conjugated to fluorochromes by adding an appropriate concentration of antibody as per manufacturer's recommendation and incubated for 30 min on ice in the dark. Cells were then washed 3 times, where each time cells were resuspended in cold PBS, centrifuged at 525 x g for 5 min and the supernatant discarded. The cells were then resuspended in 400 µl of ice-cold FACS buffer and stored at 4°C in the refrigerator until analysis on the same day. Since multiple fluorochromes were used for each panel, no live or dead stain is included in the analysis compensation tubes (BD Biosciences) were used to distinguish each fluorochrome and to avoid any spectral overlap. Compensation beads were stained as described above. To confirm the viability of the cells a hemocytometer was used to count the total number of live and dead cells and cell viability by 0.4% Trypan Blue exclusion dye (Sigma-Aldrich, Oakville, Ontario). The viability of the cells were above 90%.

2.2.8 Flow cytometry analysis and data acquisition

Data was acquired by running the samples on the BD FACSCanto™ II system (BD Biosciences) with 10,000 events collected for each tube and analyzed with the BD FACSDiva™ software (BD Biosciences). First, the cells were gated using the forward scatter (FSC) and side scatter (SSC) to find viable single cell events. Gating excluded events with low FSC and high SSC. Using a bivariate histogram, four different populations of cells were analyzed: double-positive, single positive for each antibody, and negative for both. The percent of each cell population queried was automatically generated by the software in each quadrant and was compared among the samples. No viability staining was done to asses the live and dead cells.

2.2.9 Growth of bacterial strains used in the study

An aliquot of *Mtb* H37Ra (ATCC 25177) was obtained from the American Type Culture Collection (ATCC) (Cedarlane). Bacteria were fast-thawed in a 37°C water bath (Innova 3100 Water Bath Shaker). The bacteria were centrifuged at 3000 x g for 20 min and were cultured in 15 ml conical Pyrex tubes containing 5 ml Difco 7H9 broth Middlebrook supplemented with 10% ADC (albumin, dextrose, sodium chloride, catalase) enrichment and 0.05% Tween 80 (BD Biosciences) at 37°C without agitation for 3-4 weeks. Optical density was measured at 600 nm on 1 ml aliquots of bacterial suspension removed from the culture every 3-4 days (Pharmacia Biotech Ultrospec 3000 UV/Visible Spectrophotometer, Scinteck Instruments, Manassas Park, Virginia) until it corresponded to the log phase (OD_{600} = 0.4-0.5) of bacterial growth (24). Once the bacteria reached log phase, they were harvested by centrifugation for 5 min at 1200 x g and then resuspended in an equal volume of fresh Difco Middlebrook 7H9 medium. Ten-fold serial dilutions of each suspension were plated onto Middlebrook 7H11 Selective Agar (BD Biosciences) and incubated at 37°C with 5% CO_2 between 21 and 28 days. The bacterial

concentration (CFUs/ml) was determined by dividing the number of CFUs by the product of the dilution and the volume of the plated dilution. *Mtb* H37Ra were then centrifuged at 3000 x g for 20 min to pellet the bacteria. The bacteria were resuspended in PBS and cryopreserved in PBS.

Escherichia coli (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were obtained from the culture collection of the Provincial Laboratory for Public Health, Edmonton, Alberta. Bacteria were cultivated in Luria broth (Fisher Scientific) at 37°C with vigorous shaking (120 rpm) for 12-18 h in a New Brunswick Scientific shaker incubator 126 (Eppendorf Canada, Mississauga, Ontario). Cells were harvested by centrifugation for 5 min at 525 x g and then resuspended in RPMI media. The concentration of the bacteria was determined by measuring the optical density of the suspension at 600 nm; where OD_{600} = 0.2 in the exponential growth phase.

2.2.10 Infection of human PBMCs for cell aggregate formation

Cryovials containing frozen PBMCs from liquid nitrogen were transferred to a 37ºC water bath. The cells were quickly thawed by holding the cryovials on the surface of the water bath with an occasional gentle flick. Immediately the cells were transferred into a 15 ml Falcon™ tube, with room temperature RPMI growth medium. Cells were pelleted at 525 x g for 5 minutes. Cell number and viability were determined by 0.4% Trypan Blue exclusion assay. The final concentrations of the cells were adjusted to 10 x 10^6/ml at room temperature.

The extracellular matrix (ECM) used to infect the PBMCs was prepared as described by Kapoor et al. (15)]. Briefly, ECM was prepared by mixing 0.95 ml Purecol® collagen solution (Cedarlane) with 50 µl 10x Dulbecco's phosphate buffered saline (DPBS) (Sigma Aldrich), 4 µl fibronectin (Sigma Aldrich) and 10 µl 1 N NaOH (Sigma Aldrich) per ml of matrix solution and kept on ice (pH 7.0). Then, 5 × 10^5 PBMCs at room temperature were mixed with 50 µl ECM per well of a 96 well plate. For *Mtb*-infected samples, 5 x 10^6 *Mtb* H37Ra were added to the

PBMCs-ECM mixture for a multiplicity of infection (MOI) of 0.1 (1:10). The MOI relative to the monocyte population was 10 (10:1) based on an estimate of 10% PBMCs are monocytes. *Mtb* infects monocytes, not lymphocytes (25) and infection stimulates monocyte differentiation to macrophages (26). Uninfected control wells were either left untreated or treated with Concanavalin A (ConA) (Sigma Aldrich) at 1 μg/ml to account for any T cell proliferation. For non-*Mtb* infection controls, PBMCs were infected with *S. aureus* or *E. coli* at MOI of 0.05 (1:20) since Gram-positive and Gram-negative bacteria should not form cell aggregates. All infected and uninfected samples were incubated at 37°C for 45 min, prior to overlaying with RPMI medium containing 20% human serum (Cedarlane) the samples were then incubated at 37°C with 5% CO_2 for 8 days (Forma Scientific CO_2 Water Jacketed Incubator, ThermoFisher Scientific).

2.2.11 Definition of a host immune cell aggregate structure

Host immune cell aggregates were defined as dense opaque structures that increased in size to 100 μm by day 8 of incubation when viewed under a light microscope at 10x magnification. To confirm aggregates were actual biological phenomena, the following criteria were required within the infection well: 1) evidence of intracellular infection with *Mtb* H37Ra are determined by transmission electron microscope (TEM) from preliminary experiments performed by Dr. Nasser Tahbaz and, 2) recruitment of T cells and macrophages that were differentiated from monocytes upon infection with bacteria into cell aggregates and confirmed by immunofluorescence staining (see above). TEM studies indicated that *Mtb* H37Ra could be internalized inside macrophages for up to 8 days post-infection, after which we observed extensively damaged and lysed host cells. Therefore, we used day 8 as the final endpoint in our

studies and observed the aggregation of T cells and macrophages. *S. aureus* and *E. coli* served as negative controls.

2.2.12 Screening for host immune cell aggregate structures using light microscopy

For detection of a granuloma-like structure from days 0 to day 8 of infection, samples were visualized under the Zeiss Axio Scope A1 light microscope (Carl Zeiss Canada Ltd, Toronto). Images were acquired using a Zeiss Axiocam camera (Carl Zeiss Canada Ltd) at 100x magnification.

2.2.13 Preparation of histological sections of host cell aggregates for staining

On day 8 of incubation, the medium was removed from the wells, and replaced with 4% paraformaldehyde (Sigma Aldrich) in which the samples were left overnight. ECM was carefully removed from the wells, samples were paraffin-embedded and sectioned at a histology core lab Alberta Diabetes Institute (University of Alberta). Sections of samples were deparaffinized by immersing the sample sections in three repetitions of xylene for 5 minutes each, followed two washes of 100% ethanol for 10 minutes each preparation, two washes of 95% ethanol for 10 minutes each and three washes of 70% ethanol for 10 minutes each. The sections were then washed twice in double distilled water (ddH_2O) for 5 minutes each. Deparaffinized sections of samples were microwaved in 0.1 M sodium citrate buffer for 10 min at 98°C, cooled, and blocked with 5% FBS in PBS for 30 min.

Deparaffinized sample sections were incubated with mouse anti-human CD3 antibody (T cell marker) conjugated to FITC and mouse anti-human CD14 antibody (macrophage marker) conjugated to Texas red for 1 hour at room temperature. Sections of samples were washed 3 times with 1% FBS in PBS and incubated with 4',6-diamidino-2-phenylindole **(**DAPI) stain (Sigma Aldrich) for 10 minutes at room temperature. The sections were again washed 3 times

with ddH_20, mounted with 50% glycerol in ddH_20 (Sigma Aldrich) and viewed using Zeiss Axio Scope A1 fluorescent microscope. Images were acquired with a Zeiss Axiocam camera and Axion Vision software using the Texas red filter setting (excitation wavelength, 515-560 nm; emission wavelength > 590 nm). FITC filter setting (excitation wavelength, 450-500 nm; emission wavelength, > 528 nm), and the DAPI filter setting (excitation wavelength, 600-625 nm; emission wavelength, > 568 nm) (Carl Zeiss).

2.2.14 Determination of *Mtb* H37Ra colony forming units in infected PBMCs

To measure the bacterial number, on day 0, 3, 5 and 8 post *Mtb* H37Ra infection, culture supernatants were removed from triplicate wells per condition, pooled and stored at -70°C for later measurements of cytokines. The ECM was digested by adding 50 µl of 0.1% collagenase solution (Sigma-Aldrich) to each sample well followed by incubation at 37°C with 5% CO_2 for 3 h. Infected cells were then lysed in 0.1% Triton X-100 (Sigma Aldrich, Oakville, Ontario). Ten-fold serial dilutions of lysed cells were prepared in PBS and plated on Middlebrook 7H11 Selective Agar (BD Biosciences, Mississauga, Ontario) and incubated at 37°C with 5% CO_2 for 21-28 days. The number of CFU for each incubation day was divided by the product of the dilution, and the plated volume averaged and reported as CFUs/ml.

2.2.15 Cytokine concentrations in cell culture supernatants

Cell culture supernatants were harvested on days 0, 3, 5 and 8 from triplicate wells per condition, pooled and stored at -70°C until use. Supernatants were subsequently thawed on ice for cytokines IL-10, IL-17, IL-4, IL-6, IFN-γ and TNF-α by enzyme-linked immunosorbant assay (ELISA) using commercially available kits as per the manufacturer's recommendations (BioLegend, San Diego, California). To determine the precise concentrations, dilutions of the supernatants were tested in triplicate. The lower detection limits of the assays were 3.9 pg/ml for

IL-10, IL-17 and IL-4; 7.8 pg/ml for IFN-γ; and 31.25 pg/ml for TNF-α and IL-6. Absorbance was measured at 450 nm using an EnVision™ multilabel plate reader model 2104 (PerkinElmer, Guelph, Ontario). Cytokine concentrations in the test samples were calculated based upon standard curves generated with known concentrations of recombinant human cytokines.

2.2.16 Statistical analyses

All experiments were performed in triplicate for each donor. Data were analyzed using SPSS software version 13.0 (IBM Analytics, Armonk, New York). The normal distribution of the data was tested by the Shapiro–Wilks test. All values were reported as mean ± standard deviation. Statistically significant differences among the groups were determined using two-way ANOVA, followed by a Bonferroni post hoc test. Values were considered significantly different when $p<0.05$.

2.3 Results

2.3.1 Donor Histories

A total of 10 blood donor volunteers were recruited based on their answers to the study questionnaire. Five donors declared a prior BCG vaccination history, while five donors declared no BCG vaccination history. BCG+ donors (n=5) were from India, China, and South America where BCG vaccination is typically administered at the time of birth. All donors who declared a BCG vaccination also had an identifiable BCG scar. One BCG+ donor also had a positive TST history. All BCG- donors (n=5) were born in Canada where routine administration of BCG vaccine was stopped in the 1970's and is currently not available. Two BCG- donors declared a negative TST history. Thus, only three donors declared a TST history.

2.3.2 *Mtb* H37Ra infection induced the formation of large immune cell aggregates

Infection of PBMCs with *Mtb* H37Ra resulted in the formation of cellular aggregates at day 3 post-infection (Fig 2.2a) compared with day 0. However, these cellular aggregates were

initially small in size (less than 50 μm in diameter) and became larger as incubation progressed to 5 and 8 days post-infection (Fig 2.2a). The aggregates grew more compact in appearance, forming sphere-like structures of approximately 100 μm in diameter on average. Control PBMCs obtained from the same donors that were not infected with *Mtb,* or cultured in the presence of ConA, *E. coli* or *S. aureus* did not form large sphere-like cell aggregates (Fig 2.2b), indicating that this type of aggregate formation was specific to infection with *Mtb* H37Ra.

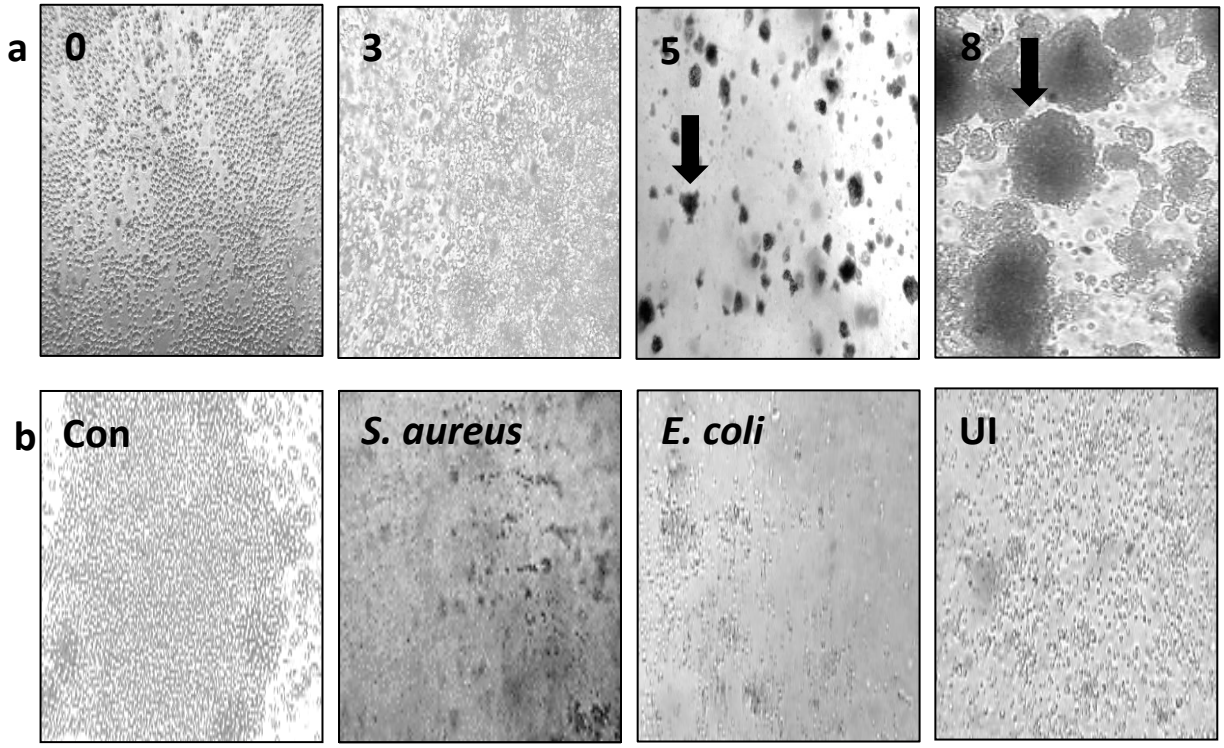

Figure2.2 Infection of human PBMCs with *Mtb* H37Ra resulted in the formation of cell aggregates. Microscopic examination of (a) human PBMCs infected with *Mtb* H37Ra at culture days 0, 3, 5 and 8, and (b) four control conditions after 8 days culture: uninfected PBMCs treated with ConA, PBMCs infected with *S. aureus* or *E.coli*, and uninfected (UI) cells. Arrows indicate the location of a representative cellular aggregate.

2.3.3 Cell aggregates were composed of T cells and macrophages

To identify the cellular components of any cell aggregates, immunostained samples were prepared using fluorescent markers for CD3 (T cell marker), CD14 (macrophage marker), and nuclei were stained with DAPI. T cells and macrophages were observed in both uninfected (Figure 2.3a) and *Mtb*-infected PBMCs (Figure 2.3b). However, cell aggregates of T cells and macrophages were only observed in the *Mtb*-infected PBMCs (Fig 3b). The following experimental conditions did not produce cell aggregates: PBMCs treated with ConA (Figure 2.3c), PBMCs infected with *E. coli* (Figure 2.3d), and PBMCs infected with *S. aureus* (Figure 2.3e).

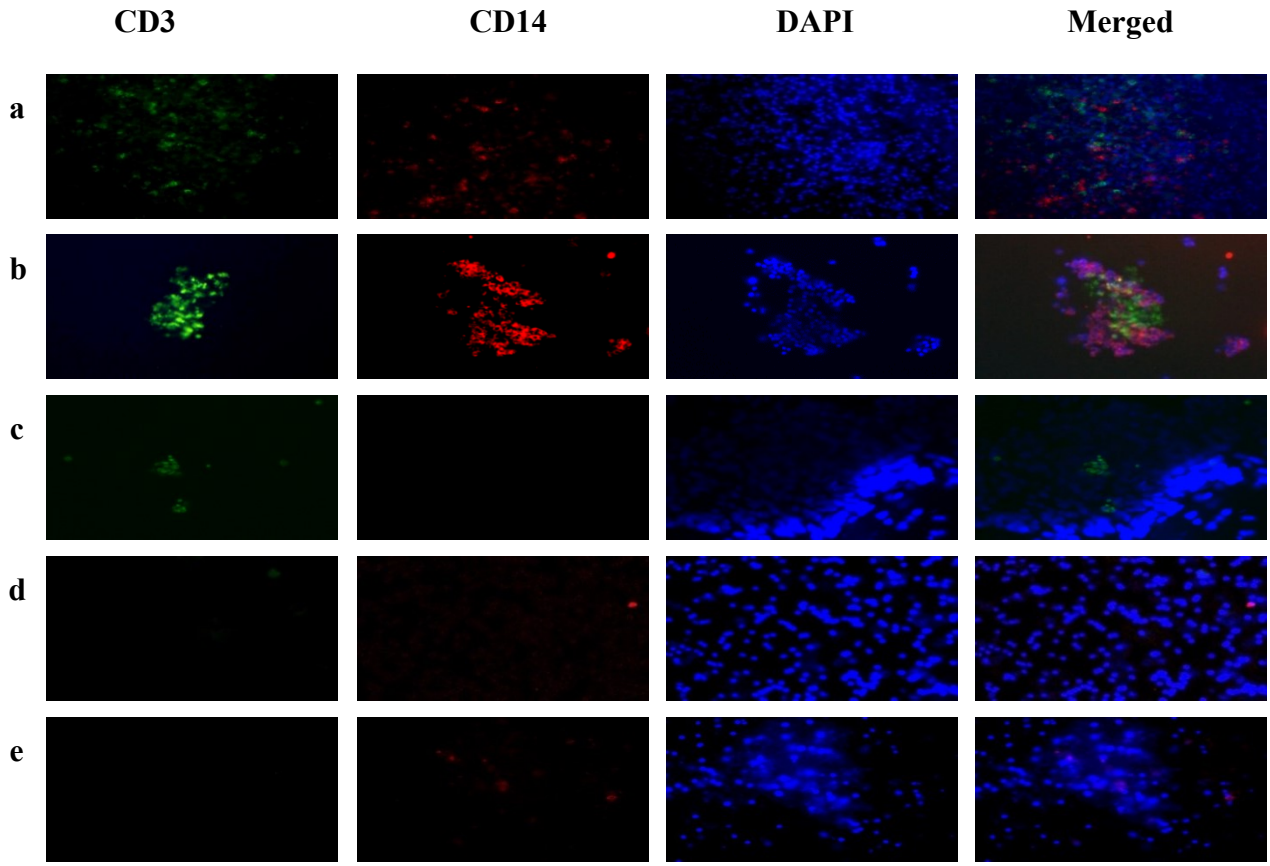

Figure2.3. Co-localization of CD3 and CD14 in cell aggregates. Fluorescent stained samples of 8 day cultures in ECM with: (a) uninfected PBMCs (no bacteria control), (b) PBMCs infected

with *Mtb* H37Ra, (c) PBMCs treated with ConA (T cell stimulation control), (d) PBMCs infected with *E. coli* (Gram-negative bacteria control), (e) PBMCs infected with *S. aureus* (Gram-positive bacteria control). CD3 (T cell marker, green), CD14 (macrophage marker, red) and nucleus (blue). One representative data set is shown for each of the 10 PBMC donors.

2.3.4 *Mtb* H37Ra load in infected PBMCs increased over time

The number of mycobacterial colony forming units (CFUs) / well were quantified at days 0, 3, 5 and 8 post-infection. Mycobacterial growth between days 0 and 3 post-infection was not significantly different (p=0.965). However, at days 5 and 8, the number of CFUs/well were significantly increased (p=0.009) compared to day 0 post-infection (Figure 2.4).

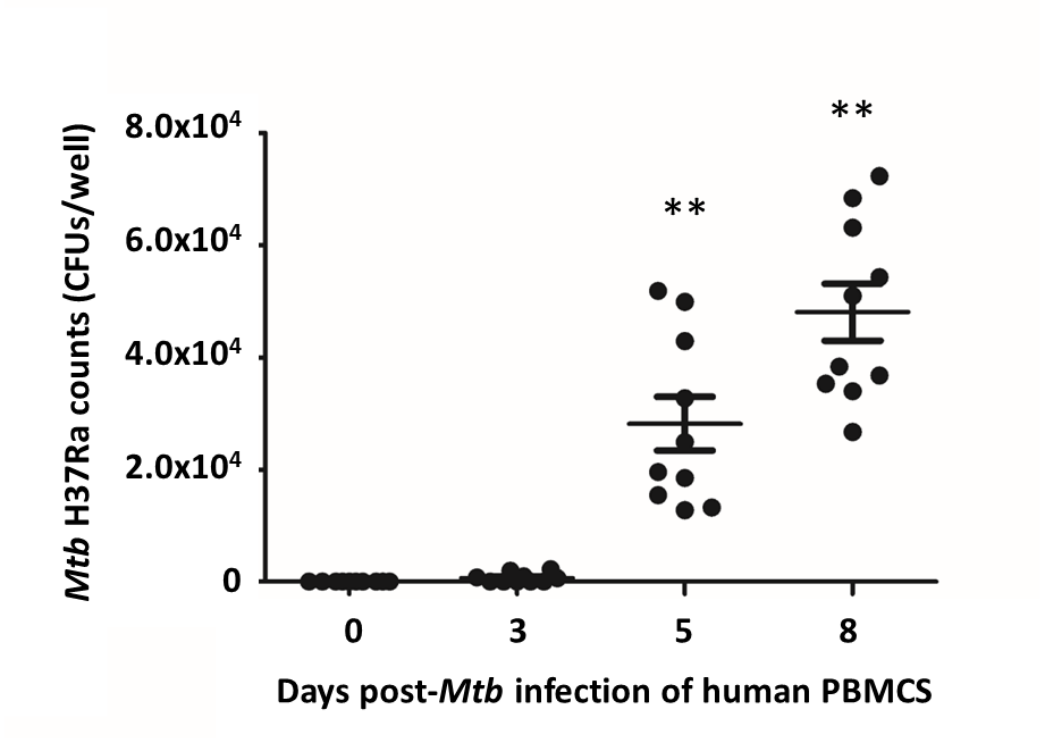

Figure2.4.Quantification of *Mtb* H37Ra post-infection on days 0, 3, 5 and 8. Bacterial counts on days 0, 3, 5 and 8 post-infection. Data were plotted as mean ± SD for 10 donors. Experiments were performed in triplicate per condition per donor. **p<0.01 compared to day 0 post-infection.

2.3.5 Changes in PBMC populations at day 8 post-*Mtb* infection

To evaluate the contribution of naive versus memory cells in response to *Mtb* infection, cell populations were characterized at day 8 post-infection. A comparison of PBMCs stimulated with ConA to unstimulated PBMCs (controls) was performed to validate that immune response could be measured in PBMCs following collagenase digestion of ECM. As expected, significant differences were observed in the percentage of total Th cells (Table 2.1) and the percentage of total Tc cells (Table 2.2) in infected versus uninfected cells. The percentages of activated naïve Th cells $CD3^{+}CD4^{+}CD45RA^{+}CCR7^{+}CD38^{+}HLADR^{+}$ and activated effector memory Th cells $CD3^{+}CD4^{+}CD45RA^{-}CCR7^{-}CD38^{+}HLADR^{+}$ were significantly higher ($p=0.032$) in infected cells (18.2±0.3) compared to uninfected cells (1.2±3.3) (Table 2.1). For Tc cells, the percentage of activated central memory cells $CD3^{+}CD8^{+}CD45RA^{-}CCR7^{+}CD38^{+}HLADR^{+}$ was significantly increased ($p=0.042$) in infected (15.3±1.2) versus uninfected cells (3.3±0.4) (Table2. 2).

Table2.1. $CD4^+$ T cell populations in PBMCs at 8 days post-infection with *Mtb* H37Ra

Th Cell Populations (Total, Subsets)		Th Cell Phenotype	Percentage of Th Cells Mean % of cells ± SD	
			Uninfected (n=10)	Infected (n=10)
Total Th cells [a]		$CD3^+CD4^+$	28.7±4.2	44.2±2.4*
Naïve		$CD3^+CD4^+$*CD45RA*$^+CCR7^+$	16.4±2.3	11.5±1.4
	Activated	$CD3^+CD4^+CD45RA^+CCR7^+CD38^+$HLA DR^+	1.2±3.3	18.2±0.3*
Central Memory		$CD3^+CD4^+CD45RA^-CCR7^+$	6.9±3.2	4.5±0.3
	Activated	$CD3^+CD4^+CD45RA^-CCR7^+CD38^+$HLA DR^+	2.7±0.4	1.5±0.5
Effector		$CD3^+CD4^+CD45RA^+CCR7^-$	11.2±0.3	16.2±4.7
	Activated	$CD3^+CD4^+CD45RA^+CCR7^-CD38^+$HLA DR^+	13.6±2.2	14.2±1.7
Effector Memory		$CD3^+CD4^+CD45RA^-CCR7^-$	32.4±3.2	56.0±5.2
	Activated	$CD3^+CD4^+CD45RA^-CCR7^-CD38^+$HLA DR^+	3.6±2.2	14.2±1.7*

[a] Percent of total gated lymphocyte population, Uninfected PBMCs, *Mtb*-infected PBMCs
* p <0.05, Infected compared to Uninfected

Table2.2. $CD8^+$ T cell populations in PBMCs at 8 days post-infection with *Mtb* H37Ra

Tc Cell Populations (Total, Subsets)		Tc Cell Phenotype	Percentage of Tc Cells Mean % of cells ± SD	
			Uninfected (n=10)	Infected (n=10)
Total Tc cells [a]		$CD3^+CD8^+$	9.7±7.0	25.8±3.5*
Naïve		$CD3^+CD8^+CD45RA^+CCR7^+$	3.5±0.5	5.6±1.2
	Activated	$CD3^+CD8^+CD45RA^+CCR7^+CD38^+$HLA DR^+	19.6±1.9	23.7±2.6
Central Memory		$CD3^+CD8^+CD45RA^-CCR7^+$	13.1±2.4	10.7±1.5
	Activated	$CD3^+CD8^+CD45RA^-CCR7^+CD38^+$HLA DR^+	3.3±0.4	15.3±1.2*
Effector		$CD3^+CD8^+CD45RA^+CCR7^-$	6.5±1.6	17.6±3.5
	Activated	$CD3^+CD8^+CD45RA^+CCR7^-CD38^+$HLA DR^+	3.6±0.5	6.4±1.0
Effector Memory		$CD3^+CD8^+CD45RA^-CCR7^-$	12.9±1.2	15.6±3.2
	Activated	$CD3^+CD8^+CD45RA^-CCR7^-CD38^+$HLA DR^+	1.8±0.6	1.1±0.2

[a]Percentage of total gated lymphocyte population, Uninfected PBMCs, *Mtb*-infected PBMCs
* p <0.05, Infected compared to Uninfected.

The percentage of $CD3^+CD4^+CXCR3^+CCR6^-$ Th1 cells (43.5±3.1) was significantly greater (p=0.024) in infected versus uninfected (22.8±2.8) cells. The percentage of $CD3^+CD4^+CD25^+$ activated T cells was significantly greater (p=0.026) in the infected (7.2±1.5) compared to the uninfected (3.6±2.1) cells, but no significant difference was observed in the percentage of $CD3^+CD4^+CD25^+CD127$lowCCR4$^+$ T reg cells in infected (6.2±1.5) and uninfected (5.9±1.2) groups. Generally, $CD4^+$CD25high T cells which coexpress FoxP3 are recognized as Tregs; however, FoxP3 is an intracellular marker. Studies of Treg were difficult due to the lack of a suitable cell surface marker apart from CD25. Liu et al. reported that the downregulation of the α-chain of the interleukin-7 receptor (CD127) on the majority of the

FoxP3 positive $CD4^+$ T cells that distinguishes Tregs from CD25 activated T cells. Since CD127 is a cell surface marker, the $CD4^+$CD25highCD127low phenotype allows for reliable identification of human Tregs (27).

2.3.6 *Mtb* H37Ra infection increased transient production of cytokines on day 3

The concentrations of cytokines in cell culture supernatants were measured on days 0, 3, 5 and 8 post-infection. The mean concentrations of Th1 cytokine IFN-γ was found to be significantly higher at day 3 (1659±553 pg/ml, p=0.007) and day 5 (434±182 pg/ml, p=0.009) post-infection compared to the respective uninfected group at day 3 (246±48 pg/ml) and day 5 (180±66 pg/ml) (Figure 2.5a). The mean concentrations of other Th1 cytokines were significantly increased only at day 3 in the infected group for TNF-α (1457±637 pg/ml, p=0.008) (Figure 2.5b) and IL-6 (1254±626 pg/ml, p=0.009) (Figure 2.5c) compared to the mean values observed for the respective uninfected group for TNF-α (246±48 pg/ml) and IL-6 (210±96 pg/ml).

IL-10 is referred to as a Th2 cytokine in this study but IL-10 is a pleiotropic cytokine characterized by its anti-inflammatory activities. The mean production level of Th2 cytokine IL-10 was also significantly higher (p=0.008) in the infected group on day 3 (1612±555 pg/ml) compared to the uninfected group (28±19 pg/ml) (Figure 2.5d). In contrast, the concentration of IL-4 did not vary between infected and uninfected groups (Figure 2.5e). The production of T cell-derived pro-inflammatory cytokine, IL-17 was significantly higher (p=0.007) in infected cells on day 3 (1170±333 pg/ml) compared to uninfected cells (216±77 pg/ml), and although infected cell levels fell over time, they remained significantly elevated throughout day eight of infection (Figure 2.5f).

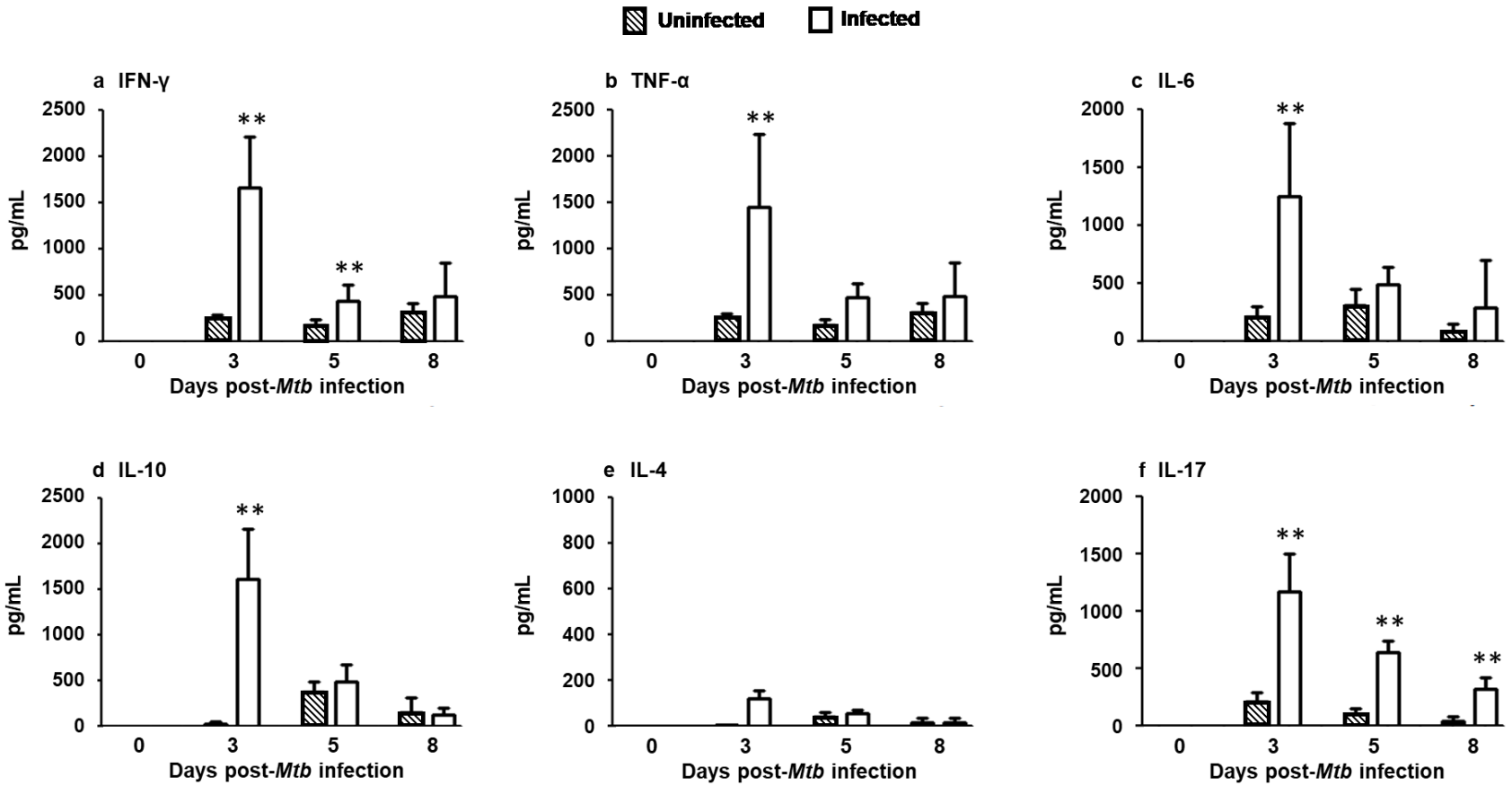

Figure2.5 Cytokine levels in cell culture supernatants of PBMCs ± *Mtb* H37Ra infection on days 0, 3, 5 and 8. Cytokine concentrations in cell culture supernatants were determined by ELISA for PBMCs infected with *Mtb* H37Ra, (open bars) uninfected PBMCs (hash bars). Data were plotted as mean ± SD and represent 10 samples per group. Experiments were performed in triplicate per condition per donor. **p<0.01 infected compared to uninfected cells.

2.3.7 Immune responses to *Mtb* H37Ra of PBMCs from BCG vaccinated and BCG unvaccinated donors differ

The impact of previous BCG vaccination on immune responses and bacterial growth was assessed by stratifying the 10 samples according to BCG vaccination history. Mycobacterial counts were observed to be significantly lower ($p<0.01$) in the BCG+ group on days 5 and 8 post-infection compared to the BCG- group, but no significant differences ($p=0.058$) were found in bacterial growth between the two groups on days 0 and 3 post-infection (Figure 2.6).

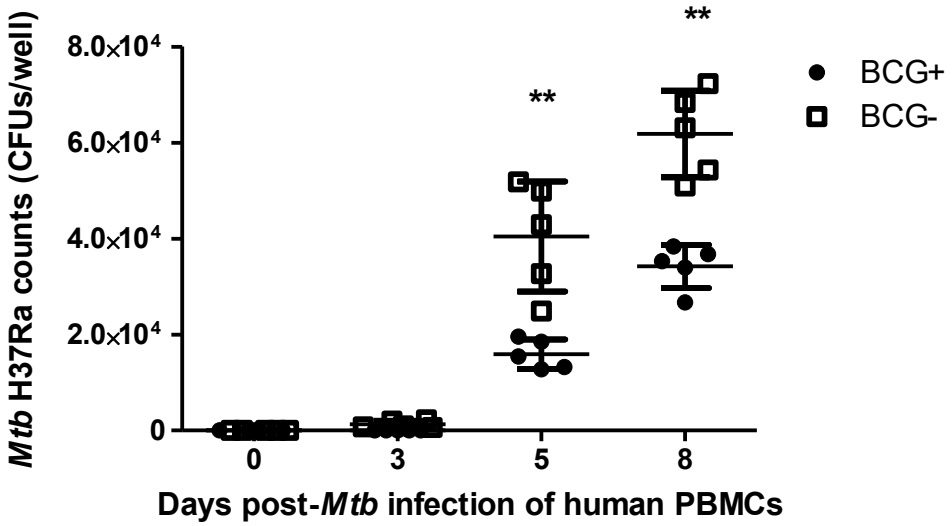

Figure2.6. Quantification of *Mtb* H37Ra post-infection in PBMCs from BCG+ and BCG- donors on days 0, 3, 5 and 8. Bacterial counts in PBMCs from BCG+ (solid bars) and BCG- (open bars) donors at days 0, 3, 5 and 8 post-infection with *Mtb* H37Ra. Data were plotted as mean ± SD of 5 samples per group. Experiments were performed in triplicate per condition per donor. **$p<0.01$, PBMCs from BCG+ donors compared to PBMCs from BCG- donors.

There was a difference however in the percentage of activated T cells between PBMCs infected with *Mtb* H37Ra from BCG+ and BCG- donors at day 8 post-infection. The percentage of activated naïve Th cells $CD3^+CD4^+CD45RA^+CCR7^+CD38^+HLADR^+$ (3.3±1.2) and activated

effector memory Th cells $CD3^+CD4^+CD45RA^-CCR7^-CD38^+HLADR^+$ (2.8±1.1) in infected PBMCs from the BCG+ donors were significantly lower (p=0.032) compared to the respective BCG- donors (15.1±2.2 and 13.0±1.6) (Fig 7). In contrast, the percentage of activated central memory Tc $CD3^+CD8^+CD45RA^-CCR7^+CD38^+HLADR^+$ cells was higher (p=0.045) in infected PBMCs from the BCG+ donors (7.4±1.4) compared to the BCG- donors (2.4±1.3) (Figure 2.7).

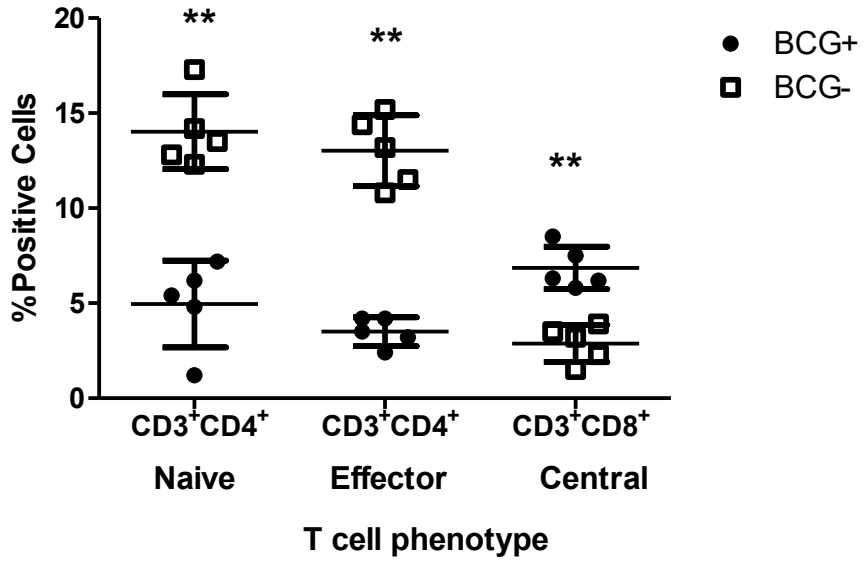

Figure2.7. Expression of cell markers on PBMCs from BCG+ and BCG- donors. Activated $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations of PBMCs from BCG+ donors (solid bars) and BCG- donors (open bars) infected with *Mtb* H37Ra were determined by flow cytometry at day 8 post-infection: activated naïve $CD3^+CD4^+$ T cells $CD3^+CD4^+CD45RA^+CCR7^+CD38^+HLADR^+$), activated effector $CD3^+CD4^+$ T cells ($CD3^+CD4^+CD45RA^-CCR7^-CD38^+HLADR^+$) and activated central memory $CD3^+CD8^+$ T cells ($CD3^+CD8^+CD45RA^-CCR7^+CD38^+HLADR^+$). Data were plotted as mean±SD and represent 5 samples per donor group, experiments were performed in triplicate per condition per donor. *p<0.05, PBMCs from BCG+ donors compared to PBMCs from BCG- donors.

The concentrations of cytokines detected in the cell culture supernatants also varied depending upon whether PBMCs were from BCG+ or BCG- donors. On day 3 following infection, the concentrations of Th1 cytokines were lower in cell culture supernatants from the BCG+ donors compared to the BCG- donors (1225±409 *vs* 2095±216 pg/ml) for IFNγ (p=0.042) (Fig 2.8a), TNF-α (1169±352 *vs* 2165±226 pg/ml, p=0.035) (Figure 2.8b), and IL-6 (718±148 *vs* 1790±377 pg/ml, p=0.029) (Fig 2.8c). The concentration of the Th2 cytokine IL-10 was significantly lower (1462±265 *vs* 1761±753 pg/ml, p=0.031) in cell culture supernatants from the BCG+ group compared to the BCG- group on day 3 post-infection (Figure 2.8d). At all-time points measured following infection, the concentrations of IL-4 (Figure 2.8e) and IL-17 (Figure 2.8f) in cell culture supernatants were not significantly different between BCG+ and BCG- groups.

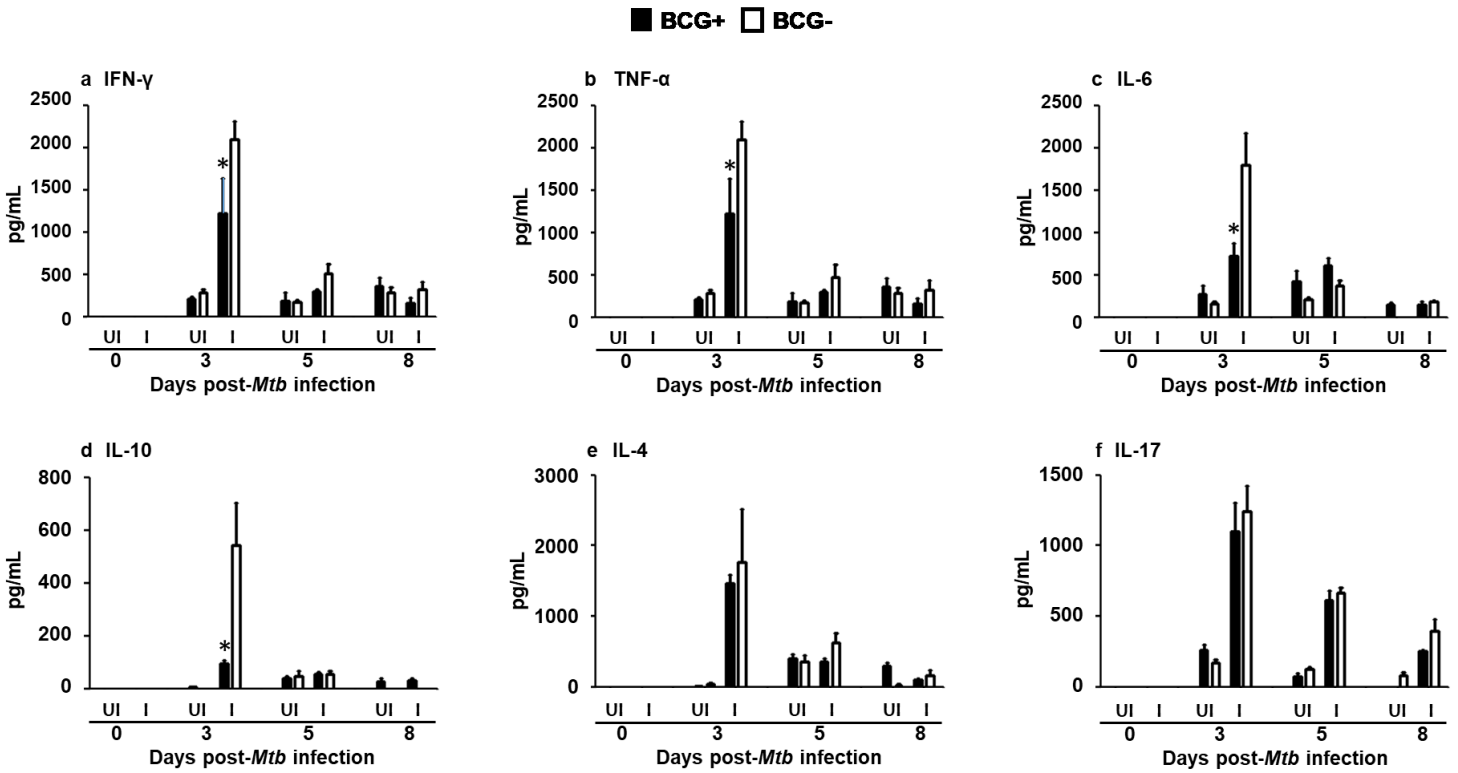

Figure2.8. Decreased Th1 and Th2 cytokine production by *Mtb* H37Ra infected PBMCs from BCG vaccinated versus BCG unvaccinated donors. Cytokine concentrations in cell culture supernatants were determined by ELISA at days 0, 3, 5 and 8 for uninfected PBMCs (UI) and PBMCs infected with *Mtb* H37Ra (I) stratified by BCG vaccination status. Data were plotted as mean ± SD and represent 5 samples per donor group. Experiments were performed in triplicate per condition per donor. *$p<0.05$, compared to BCG+ donors compared to BCG- donor**.**

2.4 Discussion

In this study, *Mtb* growth was the one infection outcome that was most consistently confounded by PBMC donor BCG vaccination status at multiple time points over the 8 days infection period. Cytokine expression may be another infection outcome to assess for perturbation by PBMC donor BCG vaccination status; however, in the present experiments, cytokine expression was generally more transient. In this study, a significant increase in the concentration of all three inflammatory cytokines (TNF-α, IFN-γ, IL-6) was observed in the supernatants of PBMCs infected with *Mtb* H37Ra compared to uninfected PBMCs at 3 days post-infection. These increases agree with previous studies where infections of human macrophages with both *Mtb* H37Rv and *Mtb* H37Ra led to TNF-α expression (28). In addition, TNF-α, IFN-γ, and IL-10 have all been shown to be induced in *Mtb* H37Ra infections of PBMCs from tuberculin-positive donors (29). Furthermore, IFN-γ production following infection of *Mtb* H37Rv was previously described by similar models involving either antigen stimulation or infection of PBMCs (15,16,23,30-32). However, differences in immune responses and mycobacterial growth rates have also been described between models involving infection with *Mtb* H37Rv and *Mtb* H37Ra. For example, H37Ra bacteriostasis and survival in murine macrophages has been previously associated with Th1 cytokine production. In contrast, *Mtb* H37Rv growth within murine macrophages was shown to be associated with the expression of Th2 cytokines (33). Therefore, cytokine expression following *Mtb* infection may be affected by the eukaryotic cell line as well as the *Mtb* strain used.

In the present study, the concentration of the Th2 cytokine IL-10 was higher in supernatants of 3-day infected versus uninfected PBMCs. Increases in Th2 cytokine (e.g. IL-10) concentration following infection may be a result of immune cells attempting to reduce the

potentially damaging effects of the Th1 cytokine-mediated inflammatory reactions without impairing the clearance of *Mtb* infection (34). IL-10 may be produced by a variety of cells identified in the PBMCs utilized in the present study including Th2 (e.g B cells), Treg cells (e.g $CD4^+CD25^+$ Tregs) (35,36) Th1 (e.g $CD8^+$ T cells) Th9, Th17 and $CD8^+$ T cells (37-40). The role of Tr1 cells was not discussed in this study due to the resource limitations. The role of Tr1 is discussed briefly in chapter 4. Other *in vitro* models have shown expression of both Th1 and Th2 cytokines following challenge with *Mtb* H37Ra. For example, $CD4^+$ T cells isolated from bronchoalveolar lavages of TB patients stimulated with heat-killed *Mtb* strain H37Ra produced both IFN-γ and IL-10 (41).

The concentration of IL-17 was significantly increased in the culture supernatants of infected versus uninfected PBMCs. Previous studies reported an increase in IL-17 production by T cells isolated from healthy tuberculin test-negative donors in response to *Mtb* antigens (32,42,43). It is known that IL-17 can be produced by Th17 cells, γδ T cells, and NKT cells (44,45). IL-17 most likely promotes the development of mature granulomas, since *Mtb*-infected IL-17-knockout mice failed to develop mature granulomas (46). However, Il-17 may not have a direct impact on mycobacterial load because in an IL-17 deficient mouse model, BCG infection, did not impact bacterial load (47).

In the present study, both the percentages of $CD4^+$ and $CD8^+$ T cells were significantly higher at day 8 in *Mtb*-infected versus uninfected PBMCs. An increase in both activated naïve $CD4^+$ T cells and activated effector memory $CD8^+$ T cells was observed compared to uninfected PBMCs. These patterns of proliferation require further investigation and may be related to the time frame used in with these experiments. Cell proliferation patterns may vary when longer time frames are assessed after infection. For example, in a clinical study, *Mtb* infection was

associated with CD4 T cell lymphocytopenia without a change in CD8 T cell population (48). A decrease in $CD4^+$ T cells was also demonstrated in an *in vitro* granuloma model using a virulent *Mtb* H37Rv strain (15).

In a previous study, bacteriostasis and survival of *Mtb* H37Ra within murine macrophages were associated with induction of Th1 cytokines (33). Similarly, an association of higher Th1 cytokine concentrations was observed with higher *Mtb* H37Ra CFUs and lower Th1 cytokine concentrations with lower *Mtb* H37Ra CFUs. However, these cytokines and mycobacterial responses were affected by PBMC donor BCG vaccination history but no differences in the cell aggregate formation was observed between BCG+ versus BCG- donor PBMCs. In the present study, lower concentrations of Th1 cytokines (IFN-γ, TNF-α, IL-6) on day 3 post-infection) and lower concentrations of one Th2 cytokine (IL-10) were observed in infections of BCG+ donor PBMCs compared to BCG- donor PBMCs (Fig 9). Similar trends may be seen in the literature with different models of infection. Cattle that were first vaccinated with BCG and then infected with *M. bovis* had a lower level of IFN-γ, TNF-α, and IL-2 than unvaccinated cattle (49). However, in our study, these changes in Th1 and Th2 cytokines are transient and may not be as effective markers for the assessment of confounding factors as the more stable trends in growth seen with *Mtb* CFUs.

T cell subset populations characterized at day 8 post-infection with *Mtb* H37Ra were associated with a decrease in the percentage of activated naïve and activated effector memory T helper cells in infected PBMCs from BCG+ versus BCG- donors. However, much of what is currently known about T cell subset populations is limited to studies in mouse models (50-52). While these studies have shown potentially important roles in the host response to *Mtb* infection, evidence of their protective roles remains unclear. We speculate that the higher levels of

activated naïve T helper cells in the PBMCs from BCG- donors may reflect an immune response to a primary *Mtb* exposure with significant levels of bacterial replication (53). It is possible that the increase in activated effector memory T cells (within day 8) in BCG- *vs* BCG+ PBMCs represents a shift from naïve T helper cells in an unvaccinated population (54). In contrast, an increase in the percentage of activated central memory cytotoxic T cells was observed in PBMCs from BCG+ donors infected with *Mtb* H37Ra compared to BCG-donors at day 8 post-infection. Central memory T cells are thought to provide long-term protection to mycobacterial infection and also generate effector memory and effector cells (55). A previous experiment by Li et al. identified the proliferation of both functional effector and central memory T cells following BCG infection of PBMCs from BCG- donors (56). Further, it has been demonstrated that BCG vaccinated cattle that were challenged by *M. bovis* produced expanded effector and central memory T cell populations (49). A recent review identified both central and effector memory T cell populations as being key targets of activation in *Mtb* vaccine studies (57). In CD4KO mice, it has been demonstrated that protective effector or memory $CD8^+$ T cells can be maintained by BCG vaccination without continuous boosting of the vaccine (58).

The series of experiments presented in this study have some limitations. Host immune responses in human PBMCs were assessed against attenuated *Mtb* H37Ra strain. Since the 1940s, both strains of *Mtb* H37Ra and *Mtb* H37Rv have been widely used for studying the pathogenesis of *Mtb* (59). Previous work has indicated that the choice of strains (e.g. virulent H37Rv *vs* attenuated H37Ra) can affect the cytokine responses and the growth or survival of mycobacteria in murine macrophage models of infection. In particular, bacteriostasis/survival (instead of death) of *Mtb* H37Ra was associated with induction of Th1 cytokines, while the growth of *Mtb* H37Rv was associated with the induction of Th2 cytokines (33). Although the use

of attenuated strains in infection models may not mirror infection with wild-type virulent strains, use of attenuated strains allowed assessment of the impact of potential confounders on experimental models using tools that are outside of a Biosafety Level 3 laboratory (22). Finally, no viability staining was done for flow cytometry analysis but to confirm the viability of the cells 0.4% Trypan Blue exclusion dye (Sigma-Aldrich, Oakville, Ontario) was used. The viability of the cells were above 90%. This may slightly bias the results.

BCG vaccination history was hypothesized to be a potential confounding variable in studies of the early host immune response to *Mtb* infection. In this study, BCG vaccination status was established by donor recall, which may be considered controversial but is commonly used in vaccine effectiveness studies for other pathogens (60). Misclassification of BCG vaccination status was further reduced by TB-expert physician evaluation of a declared immunization scar and a review of the BCG vaccination strategies in the country of birth in donors declaring a prior BCG vaccination. All of the BCG+ donors were immigrants from countries where BCG vaccination is commonly administered at the time of birth and this contributed to the limited number of BCG+ participants we were able to recruit for this study (61). In contrast, donors who declared no BCG vaccination history, all came from Canada where BCG is not routinely used. Although BCG vaccination does not always result in a scar and scars can wane with time, all BCG+ donors in our study exhibited an identifiable scar (62-64). It is important to note that this study focused on host responses early after infection using an attenuated strain of *Mtb*. *Mtb* H37Ra was used in these experiments as it can initiate early host immune responses and can be used in research laboratory settings with limited access to enhanced biosafety facilities. This study did not address possible variations in responses due to infections with wild-type strains of *Mtb* or an infection that occurs over a longer time period.

In conclusion, the findings in this study identified a potential confounding effect of prior BCG vaccination history on cytokine profiles and mycobacterial loads in infection models using human donor PBMCs. In particular, mycobacterial loads were clearly impacted by PBMC donor history of BCG vaccination very early following infection (Figure 2.9). In addition, the results revealed an impact of BCG vaccination history on T cell populations following PBMC infection with *Mtb* H37Ra, with higher levels of central memory T cells from BCG+ donors compared to BCG- donors. Central memory T cells have been identified to play a key role in the response to *Mtb* infections in mice previously vaccinated with a recombinant BCG strain (65). However, more work is required to determine the role of these T cells in controlling *Mtb* infection in a human PBMC model of *Mtb* infection. In particular, the present study demonstrates that use of the attenuated strain, *Mtb* H37Ra, allows for the assessment of potential confounding factors to infection models and can still act as a valuable tool to investigate and characterize key elements of the early human host response to *Mtb* infection in a less stringent biosafety environment prior to initiation of experiments with the more pathogenic wild-type strains in Biosafety Level 3 laboratories.

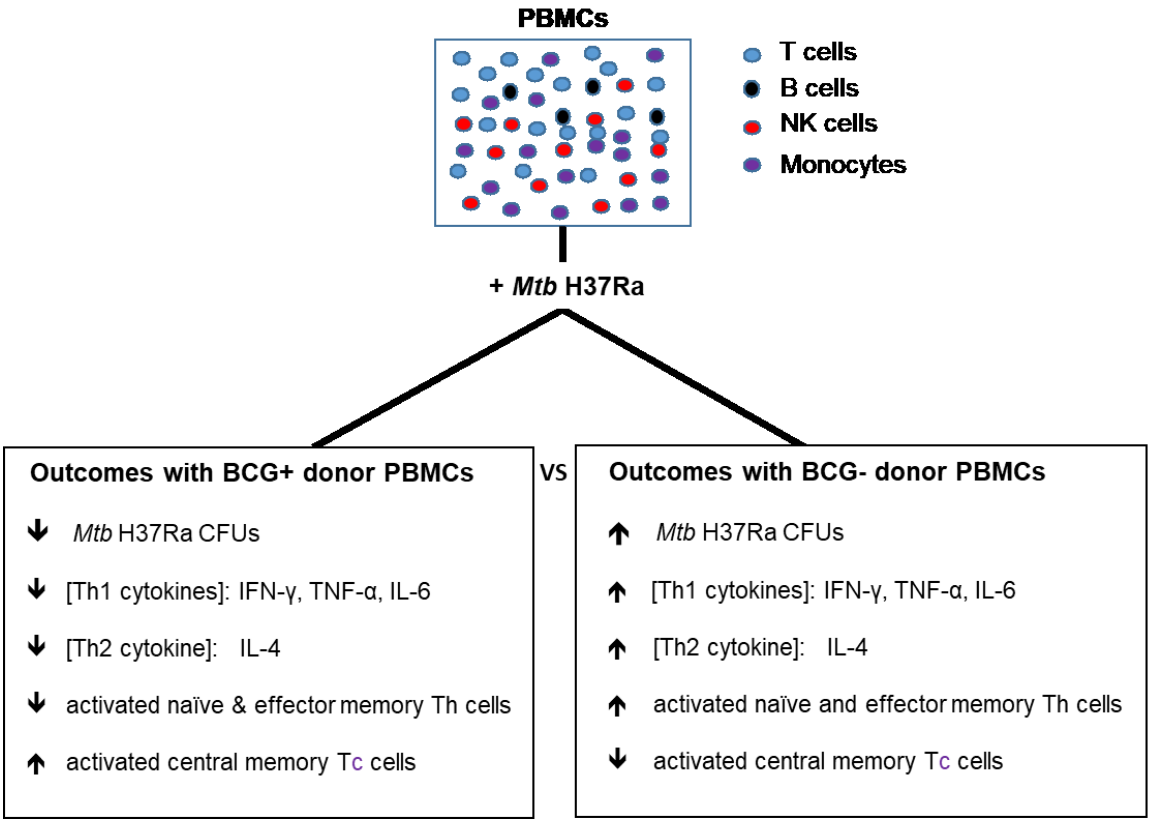

Figure2.9 Outcomes of *Mtb* H37Ra growth and cytokine expression in infections of PBMCs from BCG+ compared to BCG- donors. Infections of PBMCs from BCG+ donors were associated with lower *Mtb* H37Ra CFUs, lower concentrations of Th1 and Th2 cytokines, but higher proportions of activated central memory $CD8^+$ T cells compared to infections of BCG- donor PBMCs. Closed square brackets ([]) indicate concentration. IL-10 was not addressed in this figure because no significant difference was observed between BCCG+ and BCG- groups.

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Chapter 3: Evaluation of the effect of T regulatory cell depletion and donor BCG vaccination on *Mycobacterium tuberculosis* (*Mtb*) H37Ra infection using an *in vitro* model of human PBMC-*Mtb* infection

3.1 Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*), remains a major global health problem. It is estimated that 8.9–10.4 million incident cases and approximately 2 million deaths from TB were reported for the year 2015 (1). However, of those infected with *Mtb,* only 5-10% will develop the active disease over their lifetime and approximately 90% of the infected individuals will develop asymptomatic infection also known as latent infection (2-4).

Mtb are inhaled as droplets and infect the lung macrophages (2). The bacilli then undergo robust replication before the initiation of the adaptive immune responses (2). The expansion of effector T cells takes place in the lymph nodes (LN), where they are primed and then traffic back to the lungs to recognize the *Mtb* antigens presented by the macrophages and/or dendritic cells (DCs) (3). The adaptive immune response to *Mtb* develops much slower than in other infections such as *Salmonella enterica* (5), *Listeria monocytogenes* (6), *Francisella tularensis* (7), Influenza virus (8), or *Leishmania major* (9,10). In humans, the development of an adaptive immune response to *Mtb* occurs around five to six weeks after infection as indicated by tuberculin skin test conversion (11). In mice, a minimum of 12 days after aerosol infection is required for the initial arrival of *Mtb*-specific $CD4^+$ T cells in the lungs (12,13). The mechanisms that account for the delay in adaptive immune responses in *Mtb* infection remains poorly understood.

The current vaccine against *Mtb* is the live attenuated *M. bovis* Bacillus Calmette-Guérin (BCG) strain. Variability in the effectiveness of this BCG vaccine has been reported and may be a product of both the variability in BCG sub-strains (e.g. BCG Japanese, BCG Danish and Glax and BCG Connaught, Pasteur, and Tice) as well as human genetic factors (14,15). Other reports suggest that the limited efficiency of BCG vaccination may be due to the modulation of the *Mtb*-

specific immune responses by Treg cells (16-18). For example, Treg cells modulate the immune responses and promote enhanced *Mtb* growth by delaying the priming, expansion, and mobilization of both $CD4^+$ and $CD8^+$ effector T cells (19). Treg cells also counteract the action of Th17 cells, thus inhibiting the early recruitment of IFN-γ-producing $CD4^+$ T cells to the site of infection (20).

Treg cells are a subset of $CD4^+$ T lymphocytes, comprising ~1 to 5% of all circulating $CD4^+$ T cells (19,21). The important function of these cells is to prevent autoimmunity by maintaining self-tolerance and suppression of immune responses after eliminating invading organisms that have been cleared from the infection site (22,23). Treg cells were initially identified by their expression of $CD4^+$ $CD25^+$ cell surface markers but later studies further identified expression of the intracellular markers forkhead/winged helix transcription factor 3 (FoxP3) and low expression of IL-7 receptor α-chain (CD127) in humans (24-26).

Distinct effects of Treg cells have been demonstrated in response to different pathogens and in different stages of infection. Increases in adaptive immune responses have been reported with Treg cell depletion in chronic uncleared human infections, such as *Helicobacter pylori* (27), human immunodeficiency virus (HIV) (28,29), hepatitis C virus and (HCV) (30). In mice, infection models, such as *Leishmania major* (31), Herpes Simplex Virus-1 (32), and *Candida albicans* (33), depletion of Treg cells resulted in enhanced CD4 effector T cell function and rapid clearance of pathogen. In humans, the role of Treg cells in response to TB infection remains unclear (34-36). In this study, Treg cells are hypothesized to play a role in controlling early *Mtb* infections. Given the sparse literature in the field, this study was initiated with two aims. The first was to determine the impact of Treg cell depletion on immune cell populations from human PBMCs prior to infection with an attenuated *Mtb* H37Ra strain, as well as the growth of this

strain in an *in vitro* granuloma model of early human *Mtb* infection. The second aim was to determine the impact of BCG vaccination history of PBMC donors on the immune and bacterial responses in an *in vitro* granuloma model of early human *Mtb* infection depleted of Treg cells.

3.2 Materials and methods

Materials were obtained from Fisher Scientific, Ottawa, Ontario unless stated otherwise.

3.2.1 Ethics Statement

The study was approved by the ethics committee of the University of Alberta Health Research Ethics Board (Pro00057636). A questionnaire approved by the institutional ethics board was used to assess for the study participants.

3.2.2 Participant recruitment and selection

The inclusion criteria for study enrollment were healthy males and females between the ages of 18-54 years without the potential confounders such as: exposure to someone with active TB, had previously tested positive with a tuberculin skin test (TST)/interferon-gamma release assay (IGRA), recalled a history of latent TB infection, or had a recent gastrointestinal or respiratory illness, or were vaccinated with live or attenuated vaccine within the previous four weeks. Males and females under the age of 18 years, individuals who were vaccinated within the last 4 weeks with the live or attenuated vaccine, had a current known active infection or were taking any immunosuppressive drugs, and pregnant women were excluded from the study. Both arms of volunteer blood donors were inspected for scars consistent with BCG vaccination by DK, who has been a tuberculosis specialist for more than 25 years.

3.2.3 Blood collection and PBMCs isolation

Blood was drawn by a phlebotomist at the Alberta Diabetes Institute Clinical Research Unit of the University of Alberta. PBMCs were isolated from peripheral blood following our

standard protocol using density gradient centrifugation. Briefly, blood was diluted with an equal volume of saline and layered on Lympholyte® cell separation media (Cedarlane, Burlington, Ontario). Sample gradients were centrifuged at 870 x g, for 30 min at room temperature in a swinging-bucket rotor without the brake applied (IEC Centrifuge Model CRU-5000, O'Fallon, Missouri). The PBMCs interface was carefully removed by pipetting and washing twice in 50 mL phosphate-buffered saline (PBS) by centrifugation at 200 x g, for 5 min at 4°C (Beckman Coulter Allegra X-15R centrifuge, Mississauga, Ontario; used for all subsequent centrifugations). Following PBMCs isolation, Treg cells were depleted.

3.2.4 Depletion of $CD4^+CD25^+CD127^{low}$ Treg cells

PBMCs were subjected to the Treg cell depletion using a human $CD4^+CD25^+CD127^{low}$ regulatory T cell kit (Stemcell, Vancouver) according to the manufacturer's instructions. Briefly, PBMCs isolated following Lympholyte® gradient separation were stratified into two groups (PBMCs-Tregs and PBMCs+Tregs). The PBMCs-Tregs and PBMCs+Tregs were each transferred into a polystyrene round-bottom tube. To deplete Treg cell from PBMCs (PBMCs-Tregs), $CD25^+$ positive selection component A was added to the cells (10 µL per 10^7 cells), mixed and incubated at room temperature for 5 min. Following, incubation $CD25^+$ positive selection component B was added to the cells (10 µL per 10^7 cells), mixed and incubated at room temperature for 5 min. Following incubation, magnetic EasySep™ Releasable RapidSpheres™ (10 µL per 10^7 cells) (Stemcell, Vancouver) were added to the cells and vigorously pipetted up and down for more than 5 times, incubated at room temperature for 5 min. Next, the $CD4^+$ T cell enrichment cocktail (10 µL per 10^7 cells) was added to the sample, mixed and incubated at room temperature for 5 min. Following incubation, cells were resuspended in 2.5 mL of media (PBS containing 2% FBS and 1 mM EDTA) and the tube is

exposed to a magnetic field (EasySep™, Stemcell, Vancouver) without lid and incubated at room temperature for 10 min. Following incubation, the magnet along with the tube was inverted in one continues motion and the supernatant is collected into a new tube. The tube was then removed from the magnet and the cells that were bound by magnetic particles were resuspended in 2.5 mL of media, mixed gently 5 times by pipetting up and down and the tube was re-exposed to the magnetic field (EasySep™) without lid for 5 min at room temperature. The magnet along with the tube was inverted in one continues motion and the supernatant was discarded. The same procedure was repeated 5 times. The tube was then removed from the magnet and the cells that were bound by magnetic particles were resuspended in 2.5 mL of media and mixed gently by pipetting up and down for 5 times. Release buffer (20 µL per 10^7 cells) was added to the sample and vigorously mixed by pipetting, CD127high depletion cocktail (10 µL per 10^7 cells) was then added to the sample mixed and incubated at room temperature for 5 min. Then, Dextran RapidSpheres™ (2 µL per 10^7 cells) were added to the sample and incubated at room temperature for 5 min. After incubation, 2.5 mL of media is added to the sample mixed gently by pipetting up and down 3 times and the tube was then exposed to a magnetic field without lid for 5 min at room temperature. The magnet along with the tube was inverted in one continues motion and the supernatant was discarded. The tube was then removed from the magnet and the cells that were bound by magnetic particles were re-suspended in 2.5 mL of media, mixed gently 5 times by pipetting up and down and the cells were transferred into a Falcon™ round bottom polystyrene tube and placed on ice. The same wash procedure was repeated 5 times. After the final wash, the total number of live cells and cell viability were determined by counting the cells using Trypan Blue Exclusion dye (Sigma-Aldrich, Oakville, Ontario Aldrich. For PBMCs+Tregs, the cells were passed through the

column the same way as described above, except in this case the cells were not labeled with antibodies.

3.2.5 Growth of *Mtb* H37Ra

The bacterial strains *Mtb* H37Ra (ATCC 25177) was obtained from the American Type Culture Collection (ATCC) (Cedarlane). Bacteria were thawed rapidly in a 37°C water bath (Innova 3100 Water Bath Shaker, New Brunswick Scientific, Edison, New Jersey) and centrifuged at 3000 x g for 20 min at room temperature. Bacteria were cultured to log phase in 15 mL conical Pyrex tubes containing 5 mL Difco Middlebrook 7H9 broth supplemented with 10% albumin, dextrose, sodium chloride, catalase (ADC) enrichment and 0.05% Tween 80 (BD Biosciences, Mississauga, Ontario) at 37°C without CO_2 for 3-4 weeks. Every 5 days, optical density (OD600) was measured on 1 mL of bacterial suspension (Pharmacia Biotech Ultrospec 3000 UV/Visible Spectrophotometer, Scinteck Instruments, Manassas Park, Virginia) until it corresponded to the log phase (OD600 = 0.4-0.5). Bacteria were harvested during the log phase by centrifugation for 5 min at 1200 x g and then re-suspended in an equal volume of fresh Difco Middlebrook 7H9 media. The bacterial concentration (CFUs/mL) was determined by dividing the number of CFUs by the product of the dilution and the volume of the plated dilution. *Mtb* H37Ra was then centrifuged at 3000 x g for 20 min to pellet the bacteria. *Mtb* H37Ra cells were resuspended in PBS and cryopreserved in PBS.

3.2.6 Identification of Treg cells by Flow Cytometry

To assess the efficiency of Treg cell depletion, PBMCs isolated from the Treg cell depletion column were stained with a cocktail of antibodies (BD Biosciences, Mississauga, Ontario) CD3-FITC, CD4-BV510, CD25-eFluor, and CD127-PE by adding the appropriate volume of the antibody as per the manufacturer's protocol. Samples were incubated for 30 min in

the dark on ice. Following incubation, cells were washed 3 times by centrifugation at 525 x g for 5 min and resuspended in 400 µL of ice-cold fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 2% heat-inactivated FBS) and stored at 4°C in the refrigerator until analysis on the same day. Compensation tubes (BD Biosciences, Mississauga, Ontario) were used to distinguish each fluorochrome. Data was acquired by running the samples on the BD FACSCantoTM II (BD Biosciences, Mississauga, Ontario) with 10,000 events collected for each tube. The data were analyzed using the BD FACSDivaTM software (BD Biosciences, Mississauga, Ontario).

3.2.7 Infection of Human PBMCs

PBMCs were removed from the liquid nitrogen and thawed for 2 min in a 37°C water bath (Innova 3100 Water Bath Shaker). The cryovials were inverted twice to resuspend the cells, and cells were gently aspirated using a 2 mL pipette and transferred into a 15 mL Falcon tube containing Roswell Park Memorial Institute culture medium (RPMI, Sigma-Aldrich, Oakville, Ontario) supplemented with 1% Penicillin-Streptomycin and 10% FBS. To recover the remaining cells, the cryovials were rinsed by adding 1 mL of growth medium. To remove DMSO, the cell suspension was centrifuged for 5 min at 72 x g at 4°C. The cells were re-suspended in the growth medium with 20 µL volume of DNase solution (Sigma-Aldrich, Oakville, Ontario Aldrich) and incubated for 30 min at 37°C in a water bath (Innova 3100 Water Bath Shaker). The cells were then filtered through Falcon™ Cell Strainers to remove the cell debris. The cells were pelleted by centrifugation at 200 x g for 5 min at room temperature. The viability was determined by 0.4% Trypan Blue Exclusion dye (Sigma-Aldrich, Oakville, Ontario). The final concentrations of the cells were adjusted to 1 x 10^7/mL. To infect the cells, an extracellular matrix (ECM) was prepared as described by Kapoor et al. (37). Briefly, 1 mL of

ECM was prepared by mixing 0.95 mL Purecol® collagen solution (Cedarlane) with 50 µL 10×DPBS (Sigma-Aldrich, Oakville, Ontario Aldrich), 4 µL fibronectin (Sigma-Aldrich, Oakville, Ontario Aldrich) and 10 µL 1 N NaOH (Sigma-Aldrich, Oakville, Ontario Aldrich) and kept on ice (pH 7.0). PBMCs were mixed with ECM at a concentration of 5×10^5 PBMCs cells/50 µL/well at room temperature. PBMCs-ECM mixture samples were either infected with 5 x 10^6 *Mtb* H37Ra for an MOI of 0.1 (1:10) or left untreated. The MOI for infection is based on an estimate of 5% PBMCs are macrophages MOI relative to the macrophage population is 10 (10:1). Infected and uninfected samples were incubated at 37°C for 45 min, followed by an overlay with RPMI containing 20% human serum and incubated at 37°C for 8 days in a Forma Scientific CO_2 Water Jacketed Incubator (ThermoFisher Scientific, Mississauga, Ontario).

3.2.8 Screening for host immune cell aggregate structures using light microscopy

To detect the formation of cell aggregate structures, samples were visualized under the Zeiss Axio Scope A1 light microscope (Carl Zeiss Canada Ltd, Toronto). Images were acquired at 10X magnification using a Zeiss Axiocam camera (Carl Zeiss Canada Ltd).

3.2.9 Determination of *Mtb* H37Ra colony forming units in infected wells

Bacterial numbers were quantified on day 0, 3, 5 and 8 post-*Mtb* H37Ra infection. Briefly, culture supernatants were aspirated from each well, ECM was digested with collagenase (Sigma-Aldrich, Oakville, Ontario Aldrich) and cells were lysed in 0.1% Triton X-100 (Sigma-Aldrich, Oakville, Ontario Aldrich). Serial dilutions (ten-fold) of lysed samples were plated on Middlebrook 7H11 Selective Agar (BD Biosciences, Mississauga, Ontario) and incubated at 37°C for 3 to 4 weeks. Bacteria were counted and the number of CFUs was multiplied by the dilution factor, averaged and reported as CFUs/mL.

3.2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

Culture supernatants harvested on days 0, 3, 5 and 8 from triplicate wells per condition were stored at -70°C until use. Samples were then thawed on ice, cytokines IL-10, IL-17A, IL-4, IL-6, IFN-γ, TNF-α (BioLegend, San Diego, California), and TNF-β (R & D Systems, Minneapolis, Minnesota) were analyzed by ELISA using commercially available kits as per the manufacturer's instructions. Cytokine concentrations in the test samples were determined by diluting the test sample and tested in duplicate. The assays lower detection limits for IL-10, IL-17A and IL-4 were 3.9 pg/mL and for cytokines IFN-γ, TNF-α and IL-6 were 7.8 pg/mL, 31.25 pg/mL for TGF-β. Absorbance was measured at 450 nm using an EnVision™ multilabel plate reader model 2104 (PerkinElmer, Guelph, Ontario). Cytokine concentrations in the test samples were calculated based upon standard curves generated with known amounts of recombinant human cytokine. The standard deviation was calculated from the means and was not normalized.

3.2.11 Characterization of donor PBMCs

In order to characterize different subsets of cell populations (e.g. T cells, B cells, Treg cells, macrophages, dendritic cells (DCs) and Th1, Th2, and Th17) PBMCs were prepared for flow cytometry. Following thawing of PBMCs, 1 x 10^6 cells/mL were transferred to Falcon™ round bottom polystyrene tubes and re-suspended in ice-cold FACS buffer. Cells were washed twice in cold PBS and then centrifuged at 200 x g for 5 min and the supernatant was discarded. For characterization of cells post-infection, culture supernatants were aspirated from each well and ECM was digested with collagenase (Sigma-Aldrich, Oakville, Ontario Aldrich), cells were centrifuged at 200 x g for 5 min and the supernatant was discarded.

The cells were pelleted for 5 min at 1100 x g and resuspended in 100 µL of human BD Fc block (BD Biosciences, Mississauga, Ontario) (diluted 1:50 in FACS buffer) and incubated

for 30 min on ice to avoid any non-specific binding and background fluorescence. Each panel of cells was stained with a cocktail of monoclonal antibodies directly conjugated to fluorochromes (BD Biosciences, Mississauga, Ontario) by adding the appropriate volume of the antibody as per manufacturer's protocol (Supplemental Table 3). Cells were incubated for 30 min in the dark on ice. Following incubation, cells were washed 3 times by centrifugation at 525 x g for 5 min and re-suspended in 400 µL of ice-cold FACS buffer and stored at 4°C in the refrigerator until analysis on the same day. Compensation tubes (BD Biosciences, Mississauga, Ontario) were used to distinguish each fluorochrome and to avoid any spectral overlap.

3.2.12 Flow cytometry analysis and data acquisition

Data were acquired by running the samples on the BD FACSCantoTM II (BD Biosciences, Mississauga, Ontario) with 10,000 events collected for each tube. The data were analyzed using the BD FACSDivaTM software (BD Biosciences, Mississauga, Ontario). First, the cells were gated using the forward scatter (FSC) and side scatter (SSC) to find viable single cell events. Gating excluded events with low FSC and high SSC. Using a bivariate histogram, four different populations of cells are analyzed: double-positive, single positive for each antibody, and negative for both. The percent of each cell population queried was automatically generated by the software in each quadrant and was compared among the samples.

3.2.13 Statistics

All experiments were performed in triplicate for each donor. Data were analyzed using SPSS software version 13.0 (IBM Analytics, Armonk, New York). The normal distribution of the data was tested by the Shapiro–Wilks test. All values were reported as a mean ± standard deviation. Statistically significant differences among the groups were determined using two way

ANOVA, followed by a Bonferroni post hoc test. Values were considered significantly different when $p<0.05$.

3.3 Results

3.3.1 Selective Treg cell depletion from PBMCs

To differentiate the contribution of Treg cells from the immune responses by PBMCs to *Mtb* H37Ra infection, Treg cells were depleted from human donor PBMCs. Flow cytometry analysis confirmed that $CD4^+CD25^+$CD127low Treg cells were specifically depleted from PBMCs (PBMCs-Tregs, 0%, Figure 3.1a) when compared to PBMCs that were not depleted of Treg cells (PBMCs+Tregs, 28.8%±3.1 of $CD4^+CD25^+$ cells, Figure 3.1b). Generally, CD4+CD25high T cells which coexpress FoxP3 are recognized as Tregs; however, FoxP3 is an intracellular marker. Studies of Treg were difficult due to the lack of a suitable cell surface marker apart from CD25. Liu et al. reported that the downregulation of the α-chain of the interleukin-7 receptor (CD127) on the majority of the FoxP3 positive CD4+ T cells that distinguishes Tregs from CD25 activated T cells. Since CD127 is a cell surface marker, the CD4+CD25highCD127low phenotype allows for reliable identification of human Tregs (38).

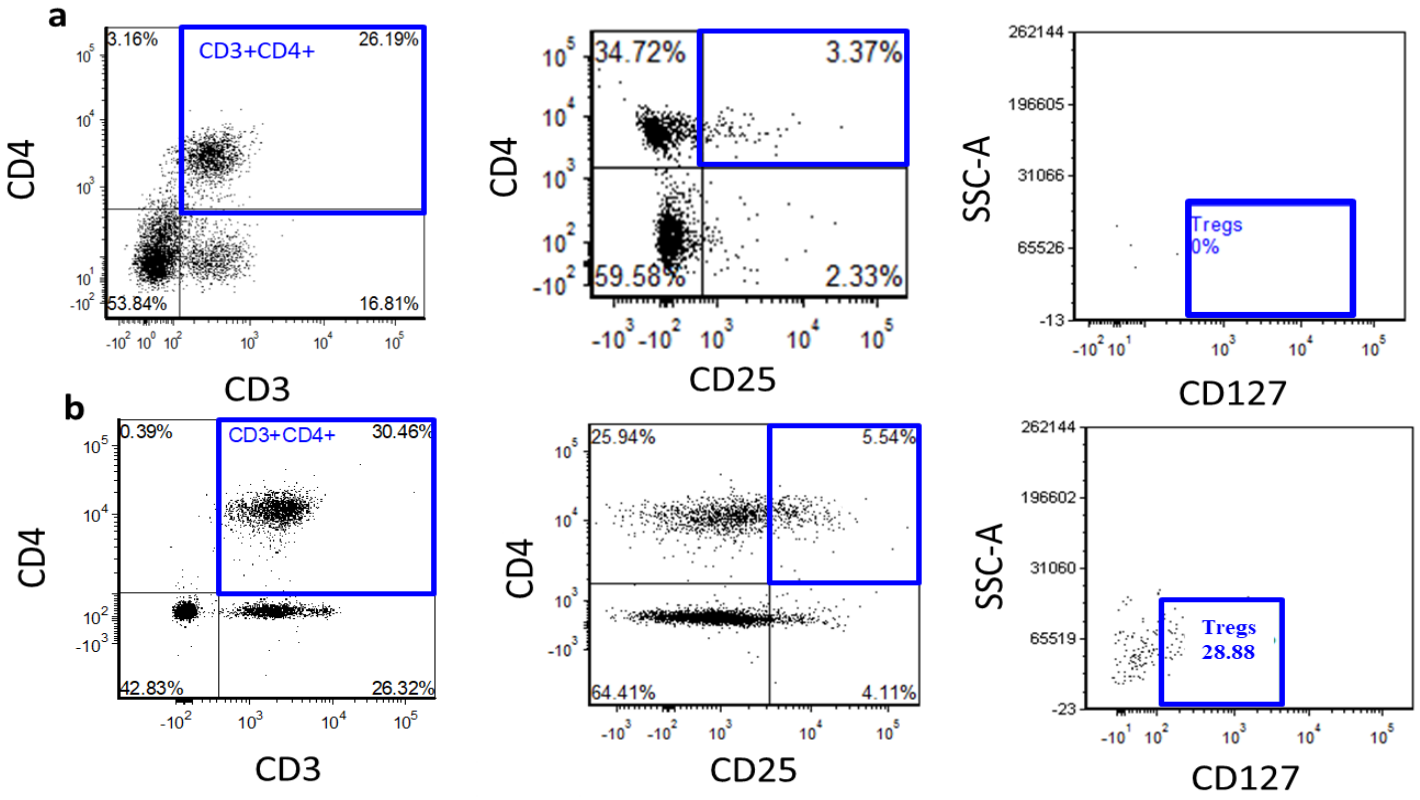


Figure3.1 Characterization of the Treg cell population of PBMCs with or without depletion. Lymphocytes were defined using flow cytometry based on their forward and side scatter. (a) T cells were first identified as $CD3^{+}CD4^{+}$T cells gated within this lymphocyte population, then further identified as $CD3^{+}CD4^{+}CD25^{+}$ and later identified based on $CD127^{+}$ population. Treg cells were depleted from PBMCs (PBMCs-Tregs) by magnetic separation. (b) Treg cell undepleted PBMCs (PBMCs+Tregs). One representative data set is shown per group. PBMCs from 10 donors were analyzed per group.The Tregs make up 28.88% of $CD3^{+}CD4^{+}$and$CD25^{+}$ cells.

In addition, flow cytometry analysis also confirmed that there were no other differences observed between the immune cell populations before and after Treg cell depletion (n=10/group). The populations of cells in both groups were; $CD3^+CD4^+$ (35.2%±4.2 *vs* 35.1%±3.5), $CD3^+CD8^+$T cells (18.9%±5.1 *vs* 15.8%±4.6), $CD3^+CD4^+CD25^+$ (2.1%±0.8 *vs* 1.9%±3.8), Th1 cells ($CD3^+CD4^+CXCR3^+CCR6^-$, 24.4%±5.3 *vs* 28.3±3.7), Th2 cells ($CD3^+CD4^+CXCR3^-CCR6^-$, 71.7%±2.6 *vs* 70.4%±4.1), B cells ($CD3^+CD19^+$, 7.2%±5.6 *vs* 6.6%±6.2) (Figure 3.2a), monocytes ($CD3^-CD19^-CD14^+$, 15.8%±3.1 *vs* 14.5%±6.3), DCs ($CD3^-CD19^-CD14^-CD20^-HLADR^+CD11C^+$, 2.5%±3.4 *vs* 1.5±2.9), and natural killer (NK) cells ($CD3^-CD19^-CD14^-CD20^-CD56^+CD16^+$, 14.3%±2.9 *vs* 13.1±5.2) (Figure 3.2b). No statistically significant differences were observed in these cell populations before or after Treg cell depletion ($p>0.05$).

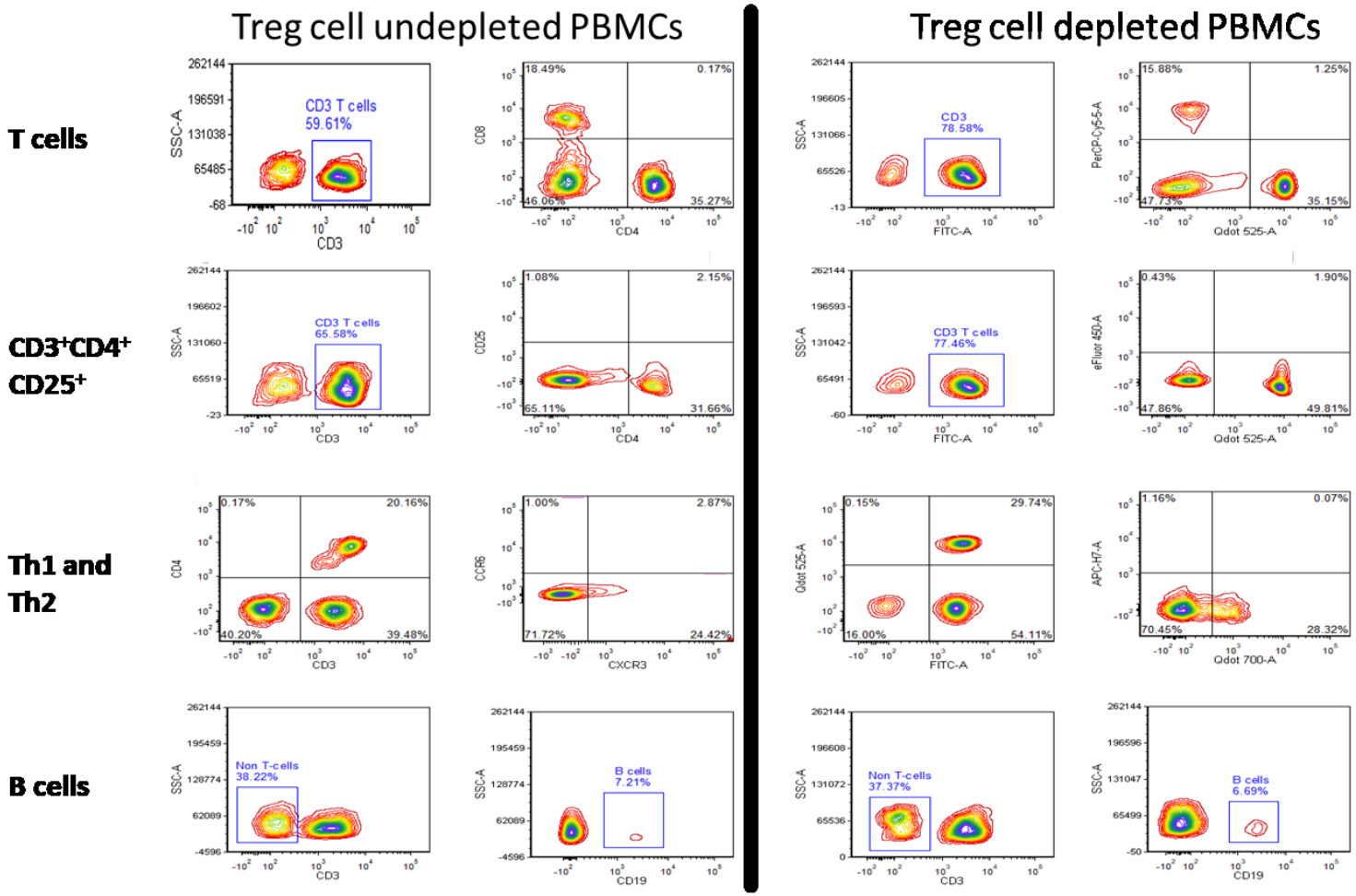


Figure3.2a: Human immune cell populations unaffected by Treg cell depletion. Lymphocytes were identified based on their forward and side scatter. Treg cells were depleted by magnetic separation. Cell populations evaluated were T cells, $CD3^{+}CD4^{+}CD25^{+}$, Th1 and Th2 cells, and B cells. One representative data set is shown. PBMCs from 10 donors were analyzed per group. T cells, $CD3^{+}CD4^{+}CD25^{+}$, Th1 and Th2, B cells are described horizontally.

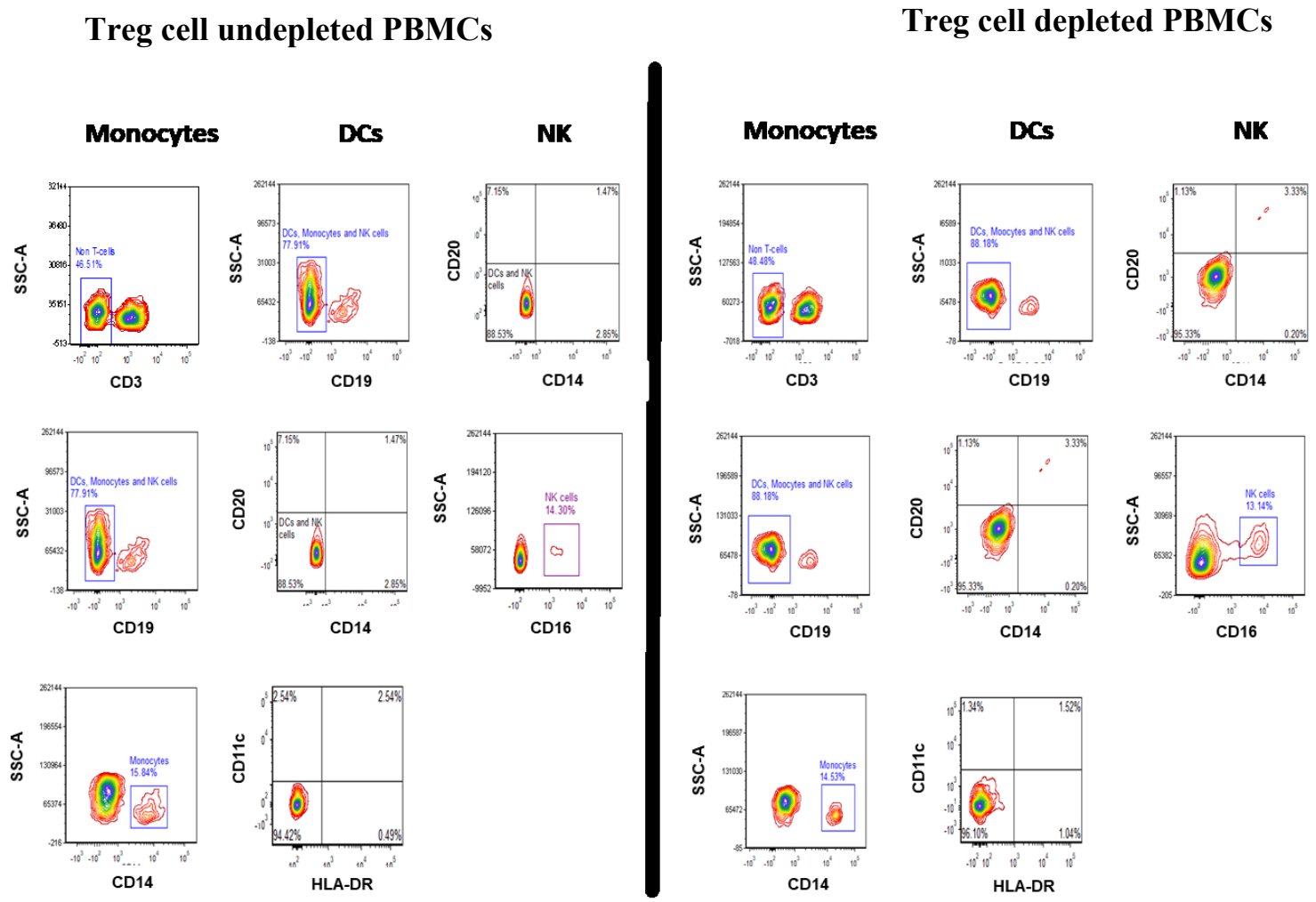


Figure3.2b: Human immune cell populations before and after Treg cell depletion. Cell populations assessed included monocytes, dendritic cells (DCs) and natural killer (NK) cells, identified based on their forward and side scatter. One representative data set presented. PBMCs from 10 donors analyzed per group. DCs are a rare population and should be theoretically described using dot plots. However, reanalysis of data using dot plot was not possible due to software upgrade. Monocytes, DCs and NK cells are described vertically.

3.3.2 Impaired cell aggregation in *Mtb*-infected Treg cell-depleted PBMCs

The impact of Treg cells depletion on the formation of cell aggregates was assessed by stratifying PBMCs from 10 donor samples into two groups: PBMCs-Tregs and PBMCs+Tregs prior to infection with *Mtb* H37Ra. Infection of the PBMCs-Tregs with *Mtb* H37Ra resulted in small cellular aggregates that appeared by day 5 and increased in number by day 8 post-infection without a marked difference in size. The PBMCs-Tregs aggregates reached a diameter of less than 50 μm by day 8 (Figure 3.3a). In contrast, larger aggregates were observed in PBMCs+Tregs by day 3 post-infection with *Mtb* H37Ra, which became even larger by day 5 and reached diameters of up to 100 μm by day 8 (Figure 3.3b). Donor-matched control PBMCS-Tregs (Figure 3a) or PBMCs+Tregs (Figure 3.3b) without *Mtb* H37Ra infection did not form cell aggregates when incubated with concanavalin A (ConA). These results indicate that the formation of large cellular aggregates (up to 100 μm) was specific to infection with *Mtb* H37Ra and that Treg cells were required for the formation of the large sphere-like cell aggregates.

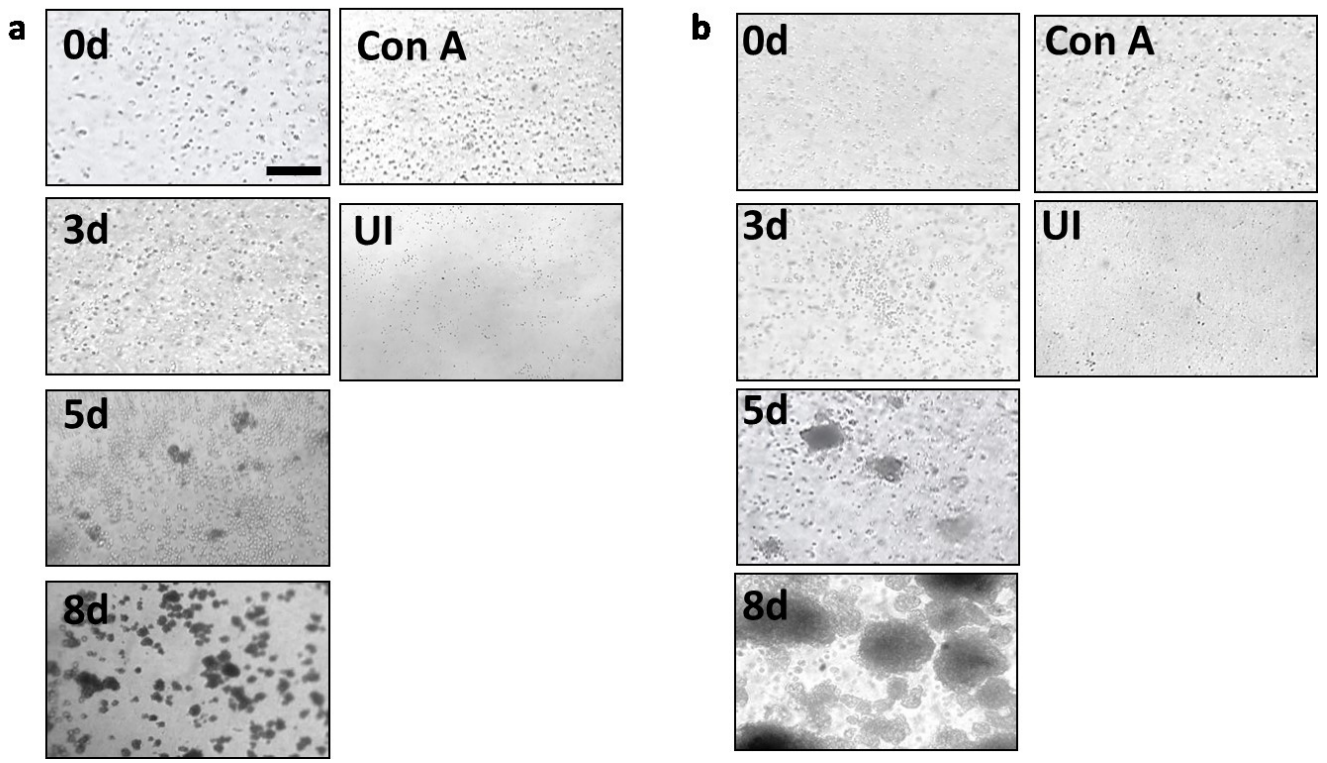
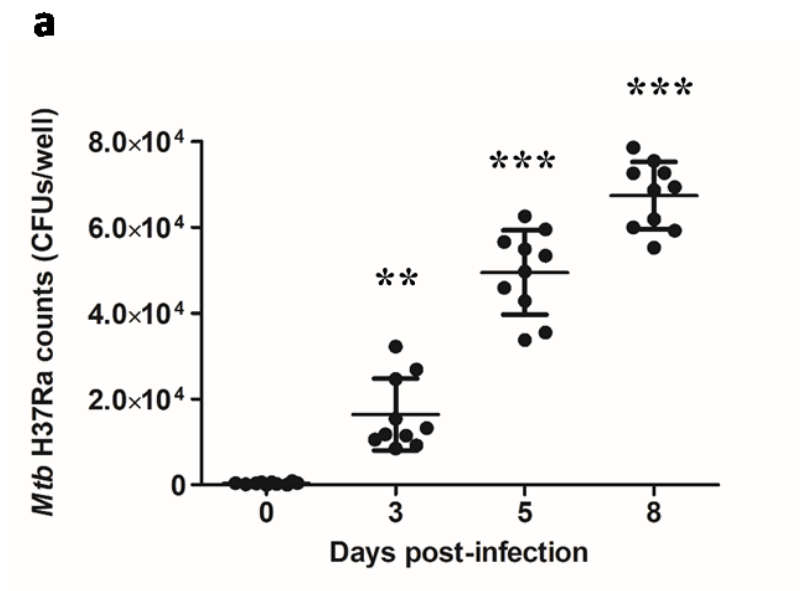
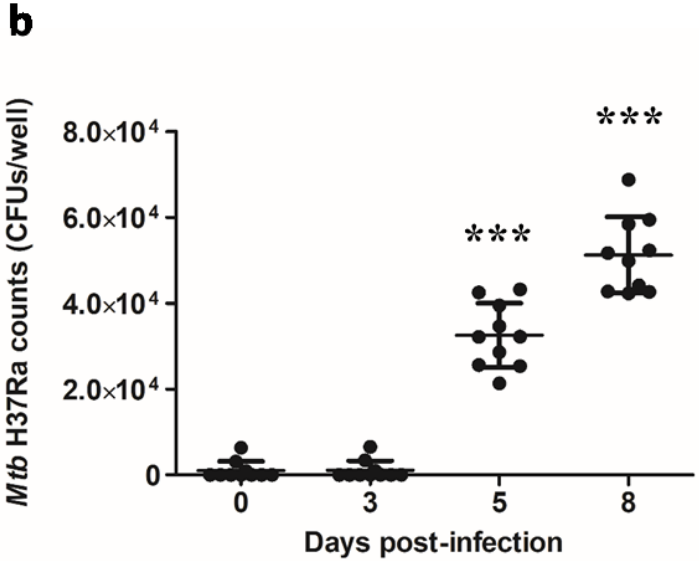


Figure3.3: Impaired formation of cellular aggregates following *Mtb* H37Ra infection in Treg cell-depleted PBMCs. Microscopic examination of *Mtb* H37Ra infected PBMCs. (a) PBMCs with Treg cell depletion (PBMCs-Tregs) and (b) PBMCs without Treg cell depletion (PBMCs+Tregs). Cells were examined at days 0, 3, 5 and 8 post-infection and included two control wells: one treated with ConA, and one uninfected (UI) PBMCs. One representative experiment is shown from experiments using PBMCs from 10 donors. Experiments were performed three times in triplicate per condition per donor. Images were obtained at 10X magnification (size bar = 50 μm).

3.3.3 Increased counts of *Mtb* H37Ra colony forming units (CFUs) in Treg cell depleted PBMCs

Mycobacterial growth (load) was quantified and expressed as CFUs/well at days 0, 3, 5 and 8 post-infection. Infection of PBMCs-Tregs with *Mtb* (n=10), resulted in an early increase in CFUs/well by day 3 (16,401±8,391) compared to day 0 (388±281, $p=0.007$) post-infection (Figure 3.4a). This increase in *Mtb* load continued at day 5 (49,458±9,808, $p<0.001$) and at day 8 (67,360±7814, $p<0.001$) post-infection. In contrast, PBMCs+Tregs (n=10) infected with *Mtb* did not increase in CFUs/well until day 5 (32,538±7,499, $p<0.001$) post-infection, but continued to increase at day 8 (51,246±8,853) compared to day 0 (1,055±2,124, $p<0.001$) post-infection (Figure 3.4b). *Mtb* loads were significantly higher in PBMCs-Tregs compared to PBMCs+Tregs at day 3 (16,401±8391 *vs* 1,111±2,200, $p<0.001$), day 5 (49,458±9,809 *vs* 32,538±7,499, $p<0.001$) and day 8 (67,360±7,814 *vs* 51,246±8,853, $p<0.001$) post-infection (Figure 3.4c). These results demonstrate that Treg cells were required to control early *Mtb* replication and were required for the formation of large size cell aggregates.


a
Mtb H37Ra counts (CFUs/well)
8.0×10⁴
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
**
***
***
0
3
5
8
Days post-infection


b
Mtb H37Ra counts (CFUs/well)
8.0×10⁴
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
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***
0
3
5
8
Days post-infection

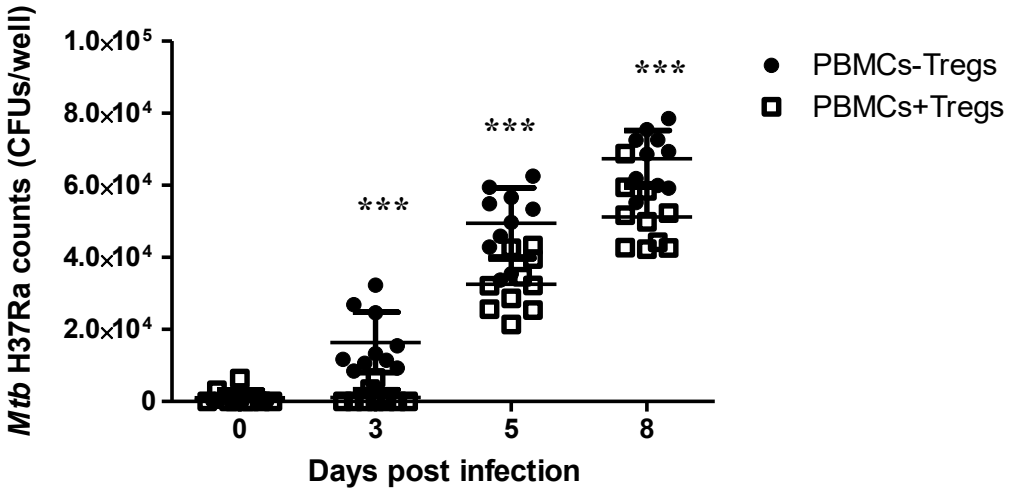

Figure3.4. Quantification of *Mtb* H37Ra in PBMCs with and without Treg cell depletion at 0-8 days post-infection. (a) Treg cell-depleted PBMCs infected with *Mtb* H37Ra, (b) Treg cell undepleted PBMCs infected with *Mtb* H37Ra (c) PBMCS-Tregs (Tregs depleted PBMCs) PBMCS+Tregs (Tregs undepleted PBMCs) infected with *Mtb* H37Ra. Data are plotted as mean ± SD and represent 10 samples per group (1 sample per donor, 10 donors per group). Experiments were performed in triplicate per condition and per donor. **p<0.01, compared to day 0 post-infection ***p<0.001, compared to day 0 post-infection.

3.3.4 Th1 and Th2 cytokine expression following *Mtb* H37Ra infection is affected by the presence or absence of Treg cells

The concentrations of cytokines in cell culture supernatants were measured on days 0, 3, 5 and 8 post-infection (Figure 6) and quantified based on the standard curve and the total cytokine concentrations were not normalized to cell number or protein concentration. The mean level of Th1 cytokine IFN-γ was significantly decreased in PBMCs-Tregs (n=10) compared to PBMCs+Tregs (n=10) by more than 7-fold at day 3 (1,710±489 *vs* 12,349±2784 pg/mL, $p<0.001$) and more than 11-fold at day 5 (451±95 *vs* 5,233±1,199 pg/mL, $p=0.036$) post-infection compared to PBMCs+Tregs respectively (Figure 3.5a). In addition, the mean concentrations of other Th1 cytokines were significantly lower only at day 3 post-infection in PBMCs-Tregs compared to PBMCs+Tregs, such as TNF-α, by more than 3-fold (362±150 *vs* 1,369±286 pg/mL, $p<0.001$) (Figure 3.5b) and IL-6 by more than 22-fold (379±157 *vs* 8,540±3,141 pg/mL, $p<0.001$) (Figure 3.5c). In contrast, the mean production of Th2 cytokines were significantly increased in PBMCs-Tregs at day 3 post-infection compared to PBMCs+Tregs by more than 2-fold for IL-10, (4,355±1,001 *vs* 1,796±479 pg/mL, $p=0.007$) (Figure 3.5d) and more than 9-fold for IL-4 (4,955±1,329 *vs* 506±170 pg/mL $p=0.006$) (Figure 3.5e). The T cell-derived pro-inflammatory cytokine, IL-17 was significantly lower in PBMCs-Tregs compared to PBMCs+Tregs by more than 2-fold on day 3 (295±104 *vs* 670±187 pg/mL, $p=0.032$) and more than 7-fold on day 5 (55±103 *vs* 401±101pg/mL, $p=0.041$) post-infection (Figure 3.5f).

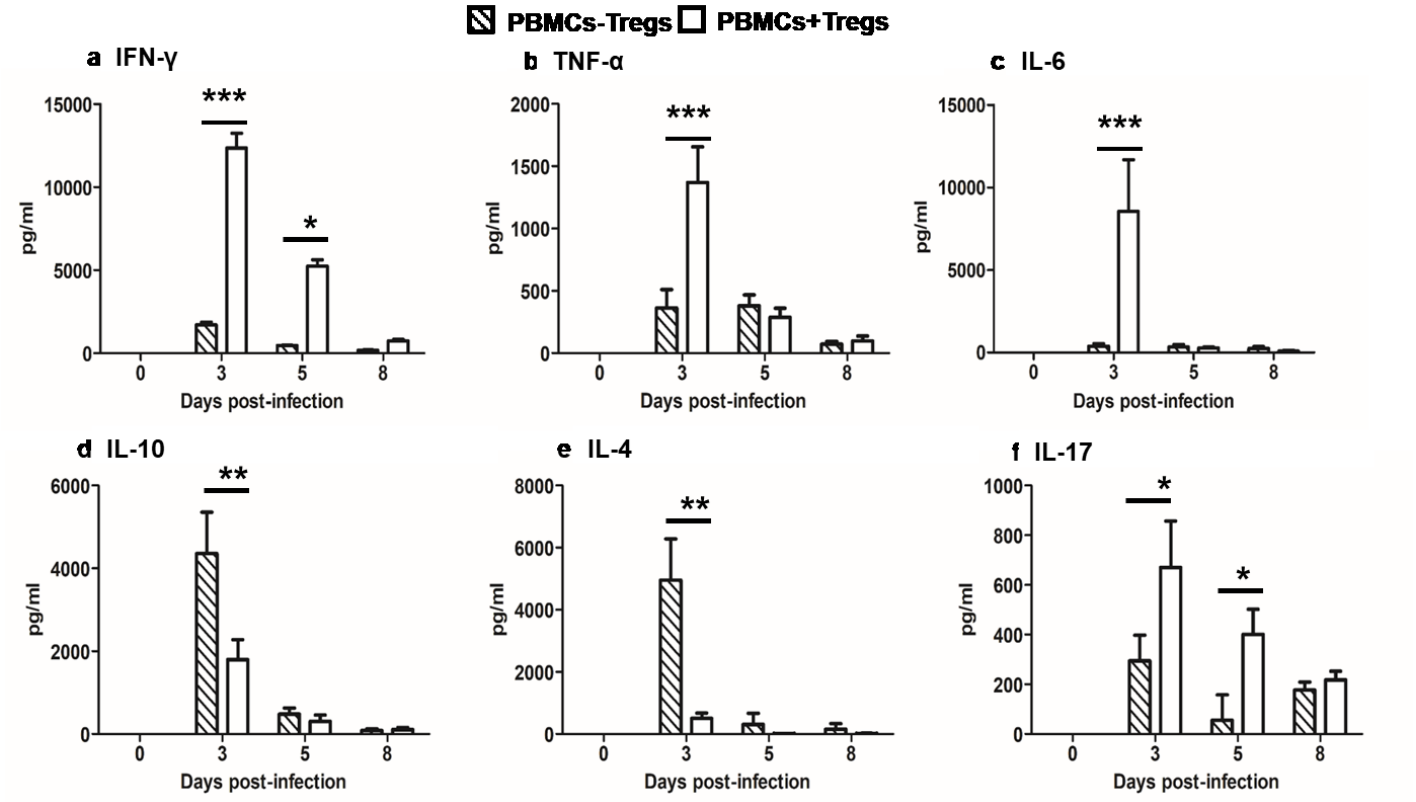

Figure3.5. Altered cytokine response to *Mtb* infection in Treg cell-depleted PBMCs. Cytokine concentrations in cell culture supernatants were determined by ELISA for PBMCs without Treg cells infected with *Mtb* H37Ra, (hash bars) and PBMCs with Treg cells (open bars). Data were plotted as mean ±SD and represent 10 samples per group. Experiments were performed in triplicate per condition per donor. *p<0.05, compared to day 0 PBMCs-Tregs **p<0.01, compared to day 0 PBMCs-Tregs ***p<0.001, compared to day 0 PBMCs-Tregs.

3.3.5 Infection of Treg cell-depleted PBMCs with *Mtb* H37Ra increased effector T cell populations at day 8 post-infection

Changes in the populations of PBMCs following collagenase digestion of matrices were observed at 8 days post-infection. Significant increases were observed in PBMCs-Tregs compared to PBMCs+Tregs in the percentages of $CD4^+$ (helper T cells; Th) by 2-fold (65.9±7.4 *vs* 32.7±4.8, $p<0.007$, Table 3.1) and $CD8^+$ (cytotoxic T cells; Tc) by 1.5-fold (35.9±6.3 *vs* 23.7±2.8, $p<0.004$, Table 3.2). Effector cells were approximately 2.5-fold higher in infections of PBMCs-Tregs compared to PBMCs+Tregs for both Th cells ($CD3^+CD4^+CD45RA^+CCR7^-$; 29.8±3.8 *vs* 11.6±2.5, $p<0.004$, Table 3.1) and Tc ($CD3^+CD8^+CD45RA^+CCR7^-$; 29.8±3.2 *vs* 12.6±4.3, $p<0.006$, Table 3.2). We found no significant differences in the percentage of B cells and memory B cell population; Treg cells; Th1, Th2, and Th17; NK cells; DCs and monocytes between infected PBMCs with Treg cells and without Treg cells.

Table3.1 $CD4^+$ T cell populations at 8 days post-infection with *Mtb* H37Ra

Th Cell Populations (Total, Subsets)		Th Cell Phenotype	Percentage of Th Cells Mean % of cells ± SD	
			-Treg (n=10)	+Tregs (n=10)
Total Th cells [a]		$CD3^+CD4^+$	65.9±7.4	32.7±4.8*
Naïve		$CD3^+CD4^+CD45RA^+CCR7^+$	14.4±6.3	18.3±5.6
	Activated	$CD3^+CD4^+CD45RA^+CCR7^+CD38^+$HLA DR^+	8.5±2.4	11.8±4.1
Central Memory		$CD3^+CD4^+CD45RA^-CCR7^+$	7.2±2.5	10.5±3.8
	Activated	$CD3^+CD4^+CD45RA^-CCR7^+CD38^+$HLA D	3.2±1.5	3.8±1.1
Effector		$CD3^+CD4^+CD45RA^+CCR7^-$	29.8±3.8*	11.6±2.5
	Activated	$CD3^+CD4^+CD45RA^+CCR7^-CD38^+$HLA DR^+	10.5±1.8	14.3±2.6
Effector Memory		$CD3^+CD4^+CD45RA^-CCR7^-$	43.8±5.1	49.5±4.3
	Activated	$CD3^+CD4^+CD45RA^-CCR7^-CD38^+$HLA DR^+	17.5±1.3	13.6±3.8

[a] Percent of total gated lymphocyte population, * p <0.05, -Tregs compared to +Tregs

Table3.2 $CD8^+$ T cell populations at 8 days post-infection with *Mtb* H37Ra

Tc Cell Populations (Total, Subsets)		Tc Cell Phenotype	Percentage of Tc Cells Mean % of cells ± SD	
			-Tregs (n=10)	+Tregs (n=10)
Total Tc cells [a]		$CD3^+CD8^+$	35.9±6.3	23.7±2.8*
Naïve		$CD3^+CD8^+CD45RA^+CCR7^+$	5.4±1.3	7.8±2.3
	Activated	$CD3^+CD8^+CD45RA^+CCR7^+CD38^+$HLA DR^+	22.3±2.4	28.3±4.1
Central Memory		$CD3^+CD8^+CD45RA^-CCR7^+$	14.2±3.6	13.8±2.2
	Activated	$CD3^+CD8^+CD45RA^-CCR7^+CD38^+$HLA DR^+	4.3±1.8	15.3±1.2
Effector		$CD3^+CD8^+CD45RA^+CCR7^-$	29.8±3.2*	12.6±4.3
	Activated	$CD3^+CD8^+CD45RA^+CCR7^-CD38^+$HLA DR^+	5.2±1.1	8.2±2.3
Effector Memory		$CD3^+CD8^+CD45RA^-CCR7^-$	16.3±3.1	18.9±4.2
	Activated	$CD3^+CD8^+CD45RA^-CCR7^-CD38^+$HLA DR^+	2.3±1.1	3.4±0.6

[a] Percent of total gated lymphocyte population, * p <0.05, -Tregs compared to +Tregs

3.3.6 Impact of Treg cell depletion on *Mtb* H37Ra infection of PBMCs from BCG vaccinated and BCG unvaccinated donors

The impact of previous BCG vaccination and Treg cells depletion on immune responses and bacterial growth was assessed for 10 donor samples according to BCG vaccination history. BCG+ donors (n=5) were from India and China, where BCG vaccination is typically administered at the time of birth. All donors who declared a BCG vaccination history also had an identifiable BCG scar upon examination by an experienced TB physician. All BCG- donors (n=5) were born in Canada where routine administration of BCG vaccine was stopped in the 1970's and is currently not available. PBMCs from 10 donors were stratified into four groups: +BCG-Tregs (n=5), +BCG+Tregs (n=5), -BCG-Tregs (n=5), and -BCG+Tregs (n=5). At day 0 of infection, no significant differences were observed in the bacterial load (CFUs/well) among the groups (data not shown). Bacterial loads were significantly increased in +BCG-Tregs compared to +BCG+Tregs on day 3 (14,807±7202 *vs* 2,193±2,822, $p<0.001$), day 5 (48,186±9,307 *vs* 35,657±5,806, $p<0.001$), and day 8 (71,059±7,434 *vs* 52,801±11,172, $p=0.006$) post-infection. In addition, bacterial loads were also significantly higher in the -BCG-Tregs compared to -BCG+Tregs on day 3 (17,994±10,010 *vs* 29±8, $p<0.001$), day 5 (50,731±11,217 *vs* 29,419±8,276, $p<0.001$), and day 8 (63,661±6,922 *vs* 49,691±6,746, $p=0.006$) post-infection (Figure 3.6). In contrast, there were no significant differences observed in CFUs between +BCG+Tregs *vs* -BCG+Tregs, or +BCG-Tregs *vs* -BCG-Tregs groups.

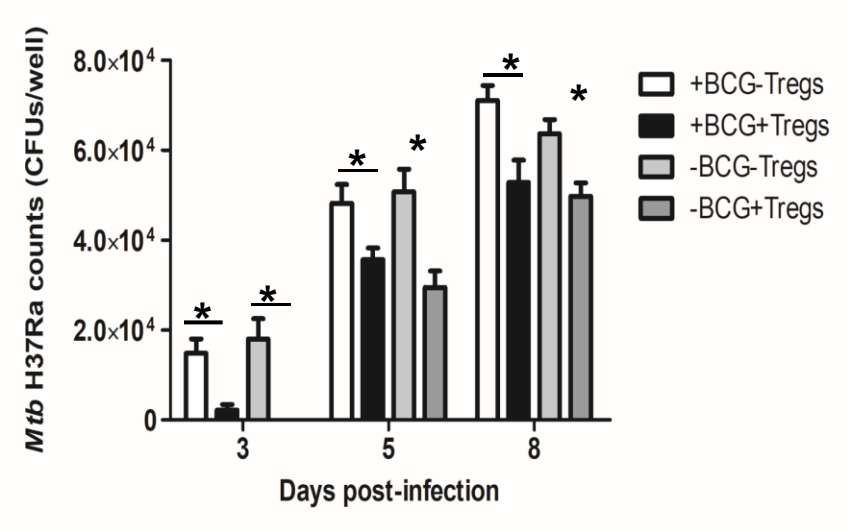

Figure3.6. Quantification of *Mtb* H37Ra post-infection in PBMCs from BCG+ and BCG- donors on days 3, 5 and 8. Bacterial counts at days 3, 5 and 8 post-infection. Data are plotted as mean ± SD and represent n=5 per group, experiments were performed in triplicate per condition per donor. **P<0.01 ***P<0.001

The differences in the percentages of T cells, Th1, Th2 and Th17 cells, B cells, DCs, monocytes and NK cells between PBMCs+Tregs and PBMCs-Tregs from BCG- donors and BCG+ donors post- infection were not significantly different.

The levels of cytokines varied between *Mtb*-infected PBMCs-Tregs and PBMCs+Tregs regardless of the BCG donor history. On day 3 following infection, the IFN-γ concentrations in the cell culture supernatants were lower in +BCG-Tregs (1,852±735 pg/mL) compared to +BCG+Tregs (12,813±2,875 pg/mL, p<0.001). Similarly, the levels of IFN-γ were significantly lower in the -BCG-Tregs (1,568±232 pg/mL) compared to -BCG+Tregs (11,885±3,315 pg/mL, p<0.001) (Figure 8a). At day 5 post-infection, the concentration of IFN-γ was also lower in the cell culture supernatants of +BCG-Tregs (429±88 pg/mL) compared to +BCG+Tregs (5,239± 1,599 pg/mL, p=0.006) and in -BCG-Tregs (473±80 pg/mL) compared to -BCG+Tregs (5,228±1,244 pg/mL, p=0.006) (Figure 3.7a).

The levels of TNF-α were significantly lower at day 3 post-infection in +BCG-Tregs compared to +BCG+Tregs (439±176 *vs* 1,369±310 pg/mL, p<0.001) and in the -BCG-Tregs (285±70 pg/mL) compared to -BCG+Tregs (1,368±330 pg/mL, p<0.001) (Figure 3.7b). Similarly, the concentrations of IL-6 were lower in +BCG-Tregs compared to +BCG+Tregs (319±161 *vs* 9,048±5,047 pg/mL, p<0.001); and in -BCG-Tregs (439±176 pg/mL) compared to -BCG+Tregs (11,232±2,065 pg/mL, p<0.001) at day 3 post-infection (Figure 3.7c).

In contrast, the concentrations of Th2 cytokines such as IL-10 at day 3 post-infection were significantly higher in +BCG-Tregs (4,654±1,197 pg/mL) compared to +BCG+Tregs (2,019±626 pg/mL, p<0.001) and in -BCG-Tregs (4,056±937 pg/mL) compared to -BCG+Tregs (1,573±268 pg/mL, p<0.001) (Figure 3.7d). Similarly, the levels of IL-4 at day 3 post-infection were higher in the +BCG-Tregs (5,736±698 pg/mL) compared to +BCG+Tregs (514±126 pg/mL); and in -BCG-Tregs (4,175±1,060 pg/mL) compared to -BCG+Tregs (499±213 pg/mL, p<0.001) (Figure 3.7e). The concentrations of T cell derived pro-inflammatory cytokine, IL-17 were significantly lower on day 3 in +BCG-Tregs (269±117 pg/mL) compared to +BCG+Tregs (802±151 pg/mL, p=0.028) and in -BCG-Tregs (320±90 pg/mL) compared to -BCG+Tregs (539±117 pg/mL, p=0.038). IL-17 concentrations were also significantly lower on day 5 following infection in +BCG-Tregs (96±9 pg/mL) compared to +BCG+Tregs (442±96 pg/mL, p=0.039) and in -BCG-Tregs (15±4 pg/mL) compared to -BCG+Tregs (361±123 pg/mL, p=0.032) (Figure 3.7f). There were no differences in cytokine expression patterns between infection of PBMCs-Tregs and PBMCs+Tregs at day 8 post-infection (data not shown). In this series of experiments, BCG vaccination history did not affect the cytokine expression patterns of PBMCs infected with *Mtb* H37Ra with or without Treg cells (Figure 3.7 a-f).

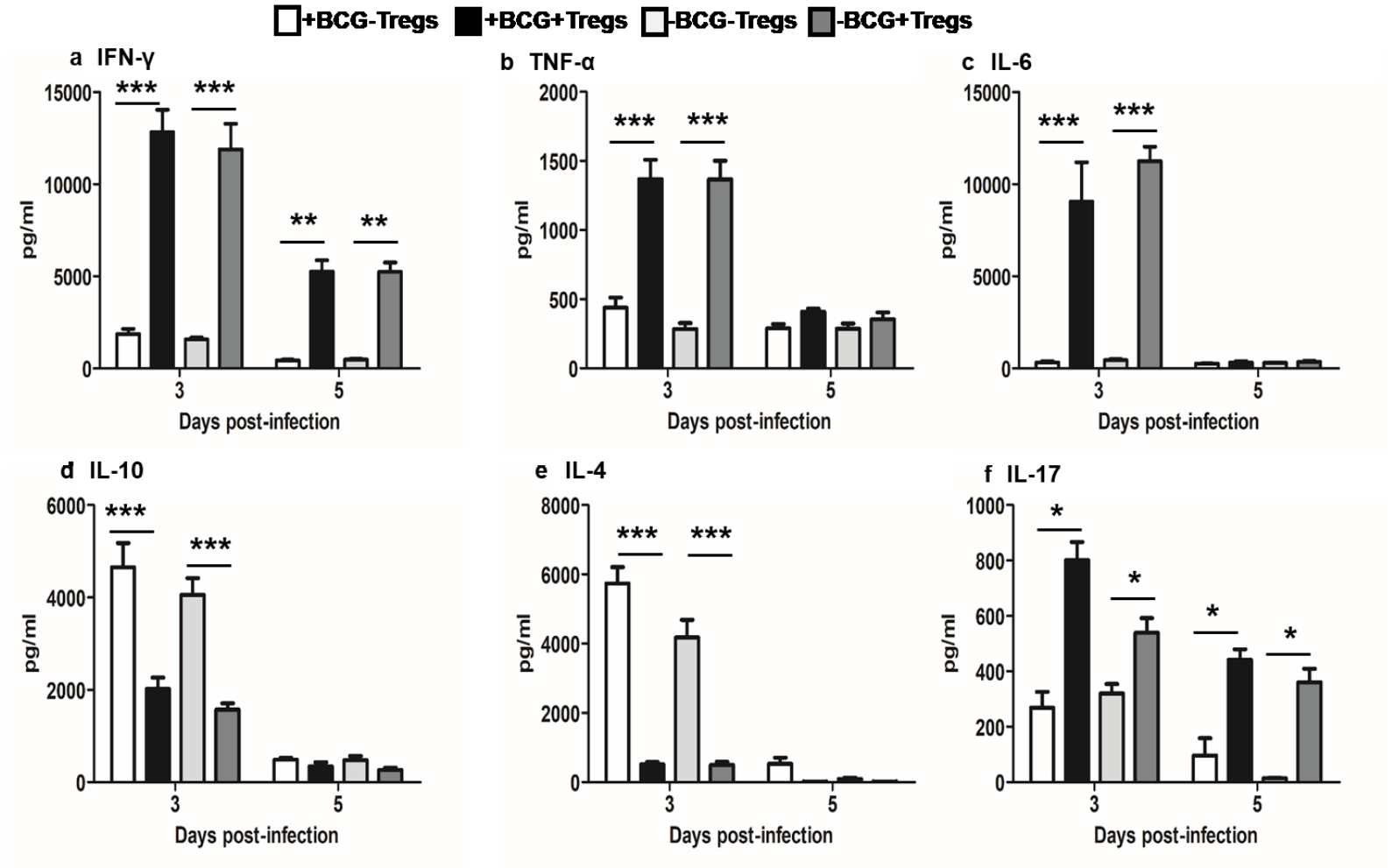

Figure 3.7 *Mtb* infection of PBMCs-Tregs produces a shift to a Th2 cytokine response. Cytokine levels in cell culture supernatants were determined by ELISA at days 0, 3, 5 and 8. Data are plotted as mean ± SD and represent 5 samples per group, experiments were performed in triplicate per condition per donor. *P<0.05 ***P<0.001

3.4 Discussion

In the present study, the effect of Treg cells depletion from PBMCs was examined in an early model of *Mtb* infection. Treg cells were depleted from donor PBMCs using a column selective against $CD4^+CD127lowCD25^+$ markers. Control PBMCs were passed through the columns without antibodies to cell surface markers. In general, previous studies have demonstrated that Treg cells suppress responses to self-antigens to prevent autoimmunity, and regulate immune responses to infections (39). Since the exact role of Treg cells during early human *Mtb* infection and following formation of granulomas is still unknown, an *in vitro* model of infection was used because it allowed the ability to control the conditions in an early infection of PBMCs by *Mtb*.

Mtb H37Ra was used to infect PBMCs in this study since the previous work Chapter 2 showed that *Mtb* H37Ra can be used as a model to understand the impact of the host or environmental factors such as BCG vaccination status and time from infection. The impact of Treg cells on early infections of PBMCs with *Mtb* H37Ra are summarized in Figure 3.9. Depletion of Treg cells from human PBMCs is associated with impaired immune cell aggregation during the 8 days of PBMCs infection with *Mtb* H37Ra. Variability in the size of cell aggregates was associated with the presence or absence of Treg cells. The cell aggregates in PBMCs+Tregs infected with *Mtb* H37Ra became denser in appearance and increased in size by day 8 of infection. However, in the PBMCs-Tregs, an increase in the size of immune cell aggregates was not observed. This observation was in agreement with previous findings by Quinn et al. where a $CD4^+CD25^+$ depleted murine model of *Mtb* infection displayed smaller granuloma structures compared to those infected mice that were not depleted of $CD4^+CD25^+$ T cells (40).

Increases in *Mtb* loads, measured as CFUs/mL, following *Mtb* infection were also associated with the depletion of Treg cells (summarized in Figure 3.8). At each time point over the 8 days post-infection, the burden of *Mtb* was higher in the PBMCs-Tregs infections than in PBMCs+Tregs. The prior literature is variable, with some studies showing similar, neutral or contradictory results (40-43). Depletion of Treg cells may have no effect on mycobacterial load in some *Mtb* infection models. For example, in an early primary mouse model of infection, depletion of Treg cells by treatment with anti-CD25 mAb did not have an impact on the mycobacterial load in the lungs (40). In another mouse model of infection, the adoptive transfer of $CD4^{+}CD25^{+}$ Treg cells into mice lacking T and B cells ($Rag1^{-/-}$ mice) did not control *Mtb* loads compared to wild-type C57BL/6 mice (41). Other studies contradict the present study and suggest that Treg cells are associated with poor control of mycobacterial loads and that depletion of Treg cells is associated with improved ability to control mycobacterial loads. PBMCs pre-primed with protein purified derivative for 6 days and co-cultured with infected monocyte-derived macrophages were able to reduce mycobacterial load, but co-addition of Treg cells increased the growth of *Mtb* (43). Infection of a chimera C57BL/6 mouse model (reconstituted with bone-marrow cells from Thy1.1 $FoxP3^{+/+}$ and Thy1.2 $FoxP3^{+/+}$ mice) with *Mtb* H37Rv was able to better control *Mtb* load when $FoxP3^{+}$ T cells were depleted by anti-Thy-1.1 mAb treatment (42). Differences in experimental conditions, timings of infection, mycobacterial strains used, or the nature of the host cell/organisms could explain these different observations between these studies. In the present study, an increase in the bacterial load after Treg cell depletion suggests that $CD4^{+}CD25^{+}CD127^{low}$ cells do not suppress immune responses that control *Mtb*. Nevertheless, the importance of the Treg cell response during tuberculosis remains an important question that needs to be addressed.

In the present study, an increased production of the Th1 cytokines (e.g. IFN-γ, TNF-α, and IL-6) and Th17 (e.g. IL-17) cytokine following infection of PBMCs was more likely to be associated with PBMCs+Tregs than PBMCs-Tregs. However, the increased production of Th2 cytokines (e.g. IL-10 and IL-4) following infection at day 3, was more likely to be associated with infection of PBMCs-Tregs than PBMC+Tregs. These findings agree with studies reporting an increased production of IFN-γ (40,41), TNF-α (40,43), and IL-6 (40,42) in mouse models where Treg cells were not depleted. However, in the present experiments, cytokine expression was generally more transient, which suggests that early suppression of *Mtb* growth is associated with the presence of Treg cells. In contrast, other work reported an association between *Mtb* progression and Treg cell population expansion, indicating that the expansion of Treg cells may be focused on different phases of infection (44) and may involve antigen-specific Treg cell responses (45). The present experiments did not assess the specificity of Treg cell populations to *Mtb* antigens. The role of Tr1 cells was not discussed in this study due to the resource limitations. The role of Tr1 is discussed briefly in chapter 4.

The present study did not show an impact of previous BCG vaccination on *Mtb* growth or cytokine responses in the presence or absence of Treg cells. At first glance, this finding appears to contradict the results from the Chapter 2 study where the growth of *Mtb* was affected by PBMC donor BCG vaccination. However, one of the possible reason would be variability between the results may be due to the differences in the manipulation of PBMCs between the studies. Other confounding reasons could be that the column used to deplete $CD25^{+}$ may have removed the activated T cells, some of which may be the memory T cells or the influence of BCG vaccination strain on the immune responses between the studies. In the Chapter 2 study, PBMCs were immediately cryopreserved following Lympholyte® gradient separation. In this

study, PBMCs were manipulated following Lympholyte® gradient separation prior to cryopreservation of PBMCs. PBMCs were antibody-labeled ($CD4^+CD127lowCD25^+$) for Treg cell depletion or unlabeled (Treg cells undepleted PBMCs). Both labeled and unlabeled cells were applied to a column exposed to a magnetic field. Magnetically labeled cells were retained, whereas unlabeled cells passed through the column were collected and cryopreserved. The complete process took an additional 5-6 hrs at a variety of temperatures and conditions as well as exposure to different mechanical stresses (e.g. columns, centrifugation, resuspension/pipetting). Therefore, it is clear that the PBMCs with Treg cells in this series of experiments would not be equivalent to PBMCs that were immediately frozen after processing.

The series of experiments presented in this study have some limitations. Although BCG vaccination history was established by donor recall, misclassification of BCG vaccination status was reduced by evaluation of a declared immunization scar and review of the BCG vaccination strategies in the country of birth in donors declaring a prior BCG vaccination. Note that this study focuses on host responses early after infection using an attenuated strain of *Mtb* and does not address possible variations in responses to infections with wild-type strains of *Mtb*. The study is focused on an early time, which may influence the role that Treg cells play in these experiments and did not address any impact of *Mtb*-specific Treg cells during this early infection (45).

In conclusion, this study showed that depletion of Treg cells in an early *in vitro* PBMC model of *Mtb* H37Ra infection was associated with an increased growth of *Mtb*. It also identified a decrease in Th1 responses (IFN-γ, TNF-α, and IL-6) and an increase of Th2 cytokines in the absence of Treg cells. BCG vaccination history was not a confounding factor in these series of

experiments. However, more work is required to determine the impact of these Treg cells in controlling *Mtb* infection in PBMC infection models.

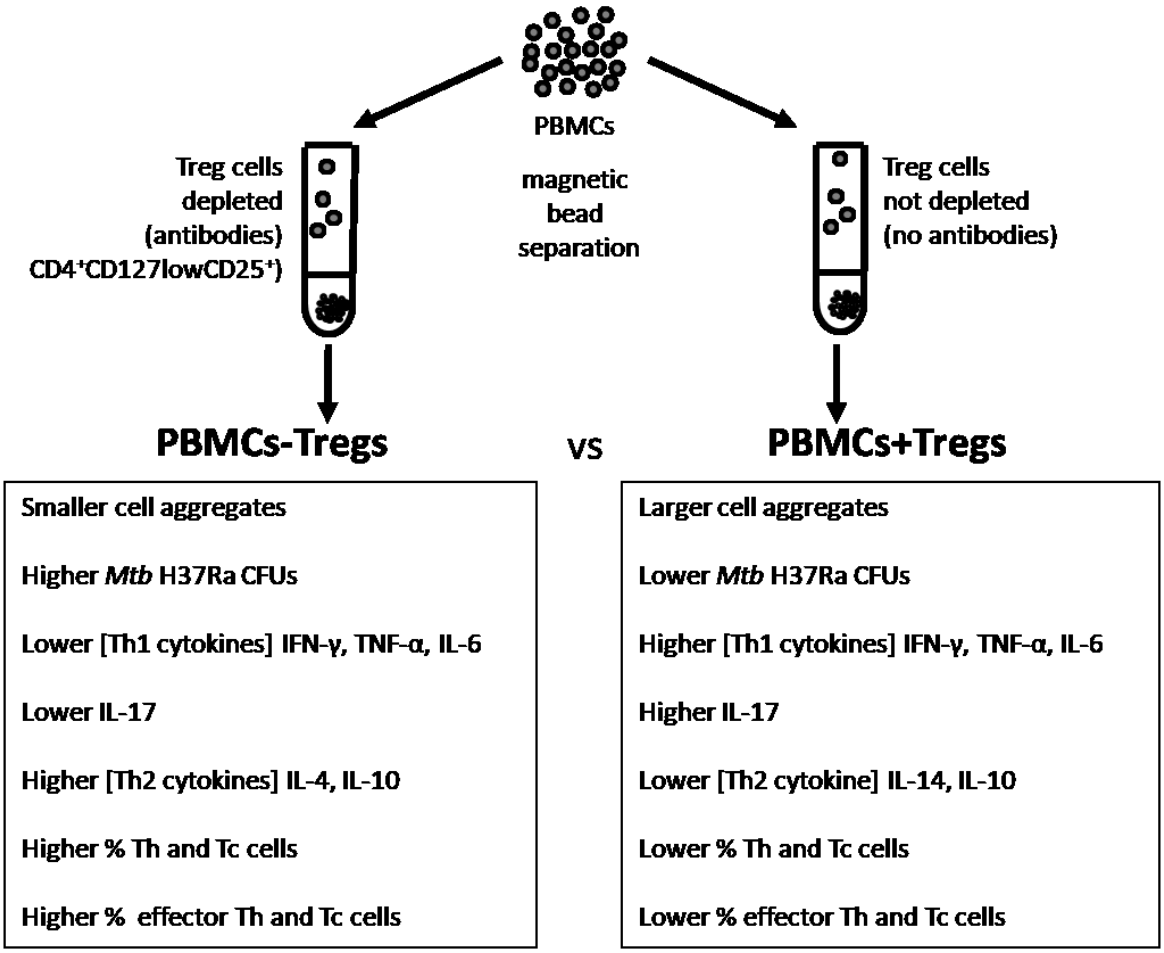

Figure3.8 *Mtb* H37Ra growth and cytokine expression in PBMCs±Tregs. Infections of PBMCs without Treg cells were associated with higher *Mtb* H37Ra CFUs, lower concentrations of Th1 cytokines, higher concentrations of Th2 cytokines; but higher proportions of total Th and Tc cells, and effector Th and Tc cells than infections of PBMCs with Treg cells. Closed square brackets ([]) indicate concentration.

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Chapter 4: Discussion and Future Directions

4.1 Discussion

In TB patients, *Mtb* bacilli survive within the TB granuloma (1). Most *Mtb*-infected individuals are able to control the mycobacteria within this granuloma during their lifetime without showing any clinical symptoms. In contrast, immunocompromised individuals are more likely to develop active disease accompanied by granuloma breakdown (2). The classically observed granuloma (present during the adaptive immune response) may represent a balance between host immune response and survival as well as growth of *Mtb*. A better understanding of early immune responses leading to the creation of TB granulomas would help us to identify new therapeutic targets that could limit long-term *Mtb* survival or enhance the immune response against *Mtb*. When using *in vivo* animal models of mycobacterial granulomas, several weeks of infection with bacilli are necessary for the granulomas to be large enough to enable their localization in the animal tissues (3-5). These time limitations prevent the analysis of the very early stages of the granulomatous reaction. Studying the earliest stages of the granuloma formation may aid in our understanding of key molecular effectors involved in the mycobacterial expansion and dissemination. Pioneering work by different researches has demonstrated *in vitro* models as an excellent model to understand various aspects of host-pathogen interactions in *Mtb* pathogenesis (7-13).

In the *in vitro* model presented in this thesis, I observed the infection of human PBMCs with *Mtb* H37Ra and the creation of early host cell aggregates composed of macrophages and T lymphocytes within 8 days of infection. The time frame of this study is consistent with another study, which observed the formation of human immune cell aggregates *in vitro* following infection with the virulent strain *Mtb* H37Rv (10). The early immune cell aggregates formed at day 8 of infection contain both macrophages and T cells, which are also found in granulomas formed in humans (14). This study also confirms that cells are continuously being recruited to the infection site as evaluated by the increase in the size of cell aggregates (Chapter 2, Figure 2.2). Together, both these studies demonstrate that although there are some differences between the attenuated strain of *Mtb* H37Ra and *Mtb* H37Rv, these two strains prompt the formation of early immune cell aggregates.

Host responses against *Mtb* infection mainly involve cell-mediated immunity (15). Cell-mediated immune responses include activation of phagocytosis, mycobacterial antigen presentation and antigen-specific cytotoxic and helper T cell responses (15). These responses stimulate host immune cells to secrete a variety of cytokines (e.g. IFN-γ, IL-12, and TNF-α) that induce migration and the close interaction of immune cells, leading to granuloma formation (16). In this study, cell-mediated responses were assessed on day 8 post-infection. The data from Chapter 2 Table 2.1 and Table 2.2 suggest that infection of human PBMCs with *Mtb* H37Ra induces two separate $CD4^+$ T cell populations; the first expresses an activated naïve phenotype. These naïve $CD4^+$ T cells are activated after interaction with antigen and differentiate into specific subtypes depending mainly on the cytokine secretion (17). For example, IL-12 and IFN-γ are key cytokines that initiate the downstream signaling cascade to activate Th1 cells (18,19). The second $CD4^+$ population expresses an activated effector memory T cell phenotype (17).

These effector memory T cells are a non-activated phenotype, but can very rapidly, and efficiently, expand into an effector population upon exposure to the challenge infection (17). An increase in $CD8^+$ memory T cells that express CCR7 was also observed, this phenotype of cells produced high amounts of IL-2 but low levels of other effector cytokines (e.g. IL-4, IL-5, and IFN-γ) (17).

A wide range of cytokines contributes to the immunological balance in *Mtb* infection. A Th1 response dominated by TNF-α and IFN-γ is the principal mediator of protective immunity in TB, while the Th2 cytokine IL-4 has the opposite effect (17). As described in Chapter 2 Figure 2.5, cytokine expression in this study occurred transiently with significant increases in pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IL-6) as well as the anti-inflammatory cytokine IL-10 at day 3 of post-infection. Studies have shown that granulomas that had a higher proportion of T cells producing IL-10 in combination with T cells producing pro-inflammatory cytokines IL-2, TNF or IL-17 were associated with sterilization of mycobacteria (20,21). Further, our data provide evidence for the co-existence of both pro- and anti-inflammatory cytokine responses in infection models during the creation of cell aggregates. This supports the idea that a balance of pro and anti-inflammatory cytokine responses may contribute to the ability of granulomas to kill *Mtb* and limit pathology.

In this study, IL-10 is referred as a Th2 cytokine but IL-10 is a pleiotropic cytokine characterized by its anti-inflammatory activities. IL-10 when bound to its receptor is produced by different cell including T cells, Bcells, and NK cells, DCs, macrophages, mast cells, neutrophils, and eosinophils. IL-10 activates the STAT3-mediated signaling which results in the inhibition of different target genes (22). IL-10 acts primarily on APCs and inhibit the upregulation of MHC class II and costimulatory molecules and the release of proinflammatory cytokines and

chemokines, there by limiting the antigen-presenting function. IL-10 can also directly inhibit T-cell function and cytokine production and proliferation (22). Recently, it has been been demonstrated that IL-10 is also involved in the suppressive function of a subset of adaptive regulatory T cells, CD4+ T regulatory type 1 (TR1) cells. TR1 cells secrete high levels of IL-10 and are known to play a key role in maintaining immune tolerance. However, due to limitation PBMCs I did not asses the role of TR1 cells in the production of IL-10 (22).

The *in vitro* early infection models presented here assessed the formation of cell aggregation following infection of PBMCs. This thesis distinguished between PBMCs isolated from BCG vaccinated donors and those from BCG unvaccinated donors over 8 days of infection. No differences in time relative to the formation of aggregates or the size of cell aggregates were observed in the infections of PBMCs between the BCG vaccinated and non-BCG vaccinated individuals. Although *Mtb* survival in a non-replicating form within granulomas is often described as a stationary balance between the host and pathogen (23), evidence points to a more dynamic host-pathogen balance. For this thesis, I predicted a decreased *Mtb* H37Ra load in PBMCs infections from BCG vaccinated individuals. As per Chapter 2, Figure 2.4, there was a decrease in *Mtb* CFU counts following the infection of the PBMCs from BCG vaccinated donors when compared to non-vaccinated donors. Therefore, *in vitro* infection models can be impacted by PBMC donor BCG vaccination history.

I then sought to determine the role of Tregs in affecting the outcome of infection in an early *in vitro* model of human PBMCs (Chapter 3). The literature focusing on *in vitro* infection models of human cells was sparse on this topic. However, the original thought based on the literature was that Tregs play an immunosuppressive role in *Mtb* infection. Therefore, this study was initiated with the hypothesis that the depletion of Treg cells from donor PBMCs, followed

by *Mtb* infection, would lead to the creation of smaller cell aggregates and decreased *Mtb* H37Ra loads.

As speculated, data from Chapter 3 Figure 3.3 supports the hypothesis that depletion of Tregs is associated with an impaired (smaller) immune cell aggregate formation. These results are in agreement with previously published data where a $CD4^+CD25^+$ depleted murine model of *Mtb* infection displayed smaller granuloma structures compared to those infected mice that were not depleted of $CD4^+CD25^+$ T cells (23). A previously published murine *Mtb* aerosol infection model demonstrated that Tregs delayed priming of $CD4^+$ and $CD8^+$ T cells in the lymph nodes (27).

In my study, percentages of $CD4^+$ and $CD8^+$ T cells increased in infections of PBMCs-Tregs compared to PBMCs+Tregs (Chapter 3 Table 3.1 and 3.2). Prior to this study, it was thought that Tregs in a granuloma (25) prevent the clearance of *Mtb* (24), suggesting that Tregs contribute to the delayed onset of adaptive immune responses. Surprisingly, the number of *Mtb* CFU counts (Chapter 3 Figure 3.5), was higher in the infected of PBMCs that were depleted of Tregs. This is the first such report and contrasts with previously published data, from animal experiments, which indicated that the depletion of $CD25^+$ cells early after *Mtb* infection was associated with the decreased bacterial load (23-26). This discrepancy may be due to a variety of factors. Other work has reported an association between *Mtb* progression and Treg cell population expansion, indicating that the expansion of Treg cells may be focused on different phases of infection (35) and may involve antigen-specific Treg cell responses (35). The experiments in this thesis did not assess the specificity of Treg cell populations to *Mtb* antigens. Prior animal studies used CD25 as a marker of $CD4^+$ Tregs and anti-CD25 antibodies to deplete the Treg population PBMCs, this approach may not completely eliminate Tregs. CD25 is

upregulated in effector T cells, and furthermore, CD25 is not expressed in all Treg cells. CD8+ Tregs are generally less studied compared to $CD4^+$ Tregs. In a previous study it has been demonstrated that $CD8^+$ Tregs suppressive activity among live BCG-stimulated PBMCs of *in vitro* PPD-responsive donors. This study also demonstrated that $CD8^+$ Treg cells isolated from live BCG-stimulated PBMCs enriched for expression of LAG-3 and CCL4, co-expressed CD25 and Foxp3, and inhibited Th1 cell proliferation (36). In this study we did not demonstrate the role of $CD8^+$ Tregs on the secretion of CCL4, which may have reduced the $Ca2^+$ influx early after TCR triggering. It could have been possible in my study that $CD8^+$ Tregs may be showing a memory recall response following infection and BCG activated $CD8^+$ Tregs could have inhibited Th1 responses, via CCL4 and via CD39 (36) Clearly, further research is needed to understand the impact of Tregs in early human *Mtb* infection and understand their impact on early *vs*. chronic infection.

4.2 Conclusion

Due to the limited access to human biopsy samples of granulomas, *in vitro* early infection models have been used to study factors that impact the structure and function of developing granulomas. In this study, I identified a potential confounding effect of prior BCG vaccination history on cytokine profiles and mycobacterial loads in infection models using human donor PBMCs. In particular, *Mtb* CFU counts were influenced by PBMC donor BCG vaccine history very early following infection. In addition, this study characterized the key elements of the early human immune response to *Mtb* infection and the impact of BCG vaccination history on T cell populations following PBMC infection with *Mtb* H37Ra. In particular, T cells with memory properties are higher in BCG vaccinated donor compared to BCG unvaccinated donors. Given the longevity of memory T cells, clearly this subset plays a decisive role in TB vaccines.

Although we still do not fully understand, what govern the development of Memory T cells, but these cells seems to control the mycobacterial load. In this study, I identified that Tregs play an important role in early host immune responses and *Mtb* growth. Collectively, the results presented here are novel because this is the first report that demonstrated that Tregs are important in controlling early *Mtb* infection and a delicate balance exists between Tregs and non-Tregs, and any changes to this equilibrium can alter the outcome of infection.

4.3 Future Directions

The BCG vaccine has been used since 1921 to prevent tuberculosis (TB) (37). The protective efficacy of BCG varies depends on the geographical location the BCG vaccine has been given and little is known about the variable protection against pulmonary TB (37). The genomic sequences of different strains of BCG vaccination compared with *Mtb* as a common reference is the deletion of RD1 that is present in *M. bovis*. The different strains of BCG vaccine such as, BCG-Moreau and BCG-Japan, have two copies of the insertion region (IS) 6110. BCG-Denmark, BCG-Tice and BCG-Glaxo, have only one copy of IS6110 and have lost RD2 in addition (37). Although, it is known that BCG vaccine strains induce different levels of protective immunity, currently, it is unknown the immune responses that are generated by these different strains of BCG vaccine to *Mtb* infection. The observations from chapter 2 suggest that host prior BCG vaccination may be one of the confounding factor in controlling early *Mtb* infection, and in chapter 3 this BCG vaccine effect was not observed. In order to understand if the BCG varies depends on the geographical location the next step would be is to recruit donors who had prior BCG vaccination from different geographical locations and asses the bacterial load and immune responses such as cytokines and immune cells populations in an early *Mtb* infection in vitro. Confirmation of host BCG vaccination with IGRA would strengthen the results.

The BCG vaccine remains important for the development of new TB vaccines, and as identified by many studies the benefits to administer BCG vaccination as a booster vaccine, it would be critical to evaluate the immune factors that affect how BCG works and the impact on new genetically modified BCG vaccines or other live mycobacterial vaccines.

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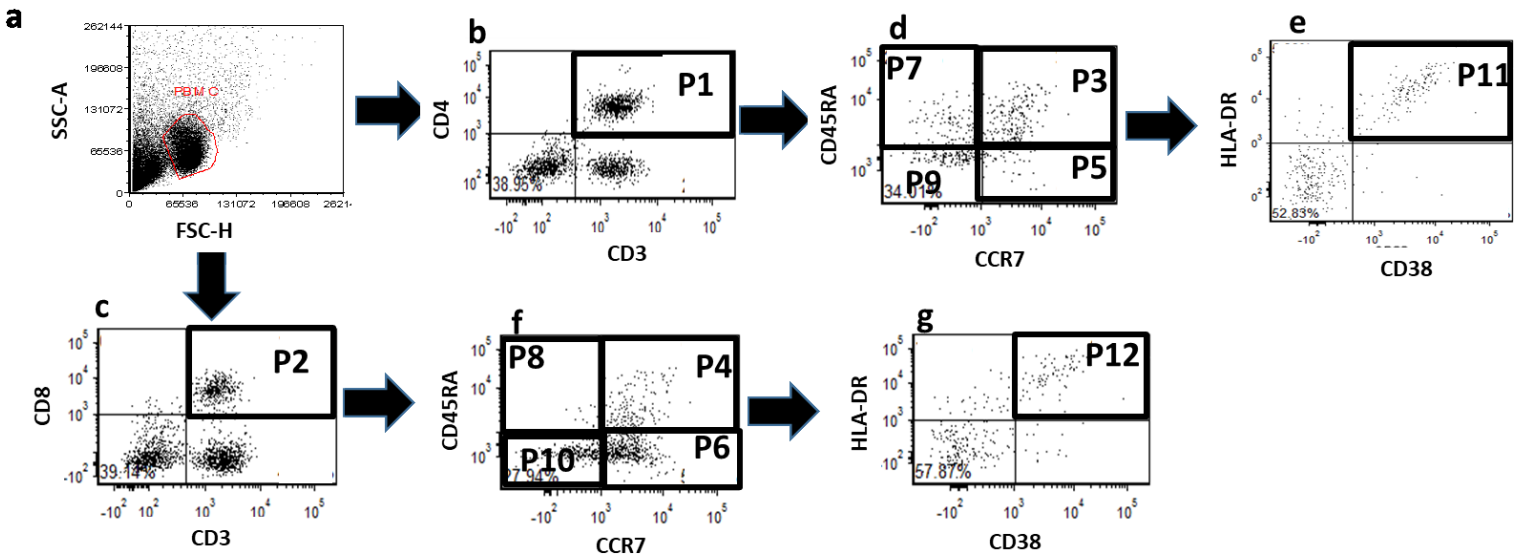
Appendix A

Antibody panels for PBMC immunophenotyping

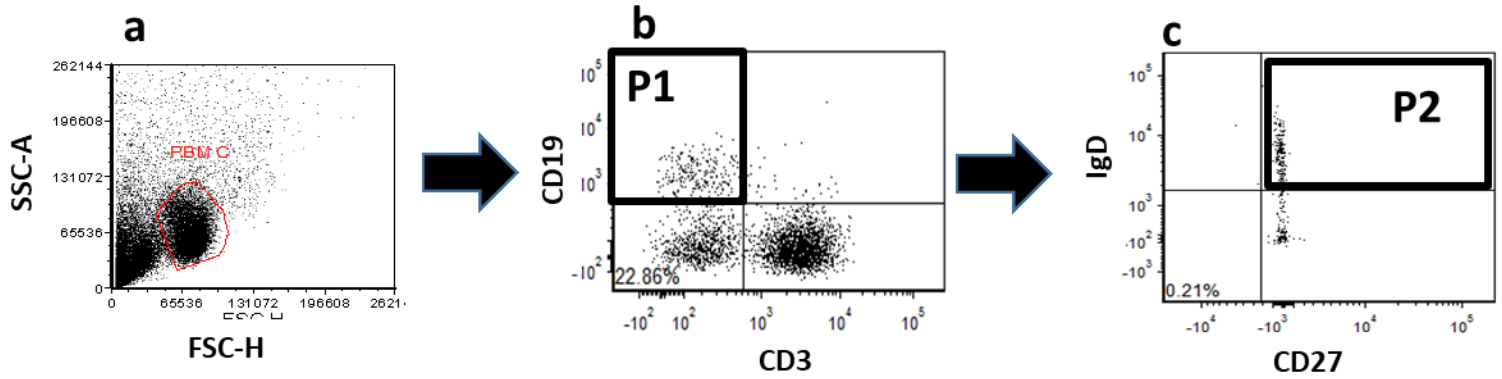
Fluorochrome	PBMC Immunophenotyping Markers				
	T cells	T Regulatory cells	Th1, Th2 and Th17 cells	B cells	Monocyte, DCs, and NK cells
PE	CCR7	CD127	CXCR3	CD24	CD16
V510	CD4	CD4	CD4	CD20	CD20
PE-Cy7	CD45RA	CCR4	CCR6	CD27	CD11c
V421	CD38	CD25	CD38	CD38	CD14
PerCP-Cy5.5	CD8	CD45RO	CD8	CD19	CD19
FITC	CD3	CD3	CD3	CD3	CD3
APC-H7	HLA-DR	HLA-DR	HLA-DR	IgD	HLA-DR

Appendix B



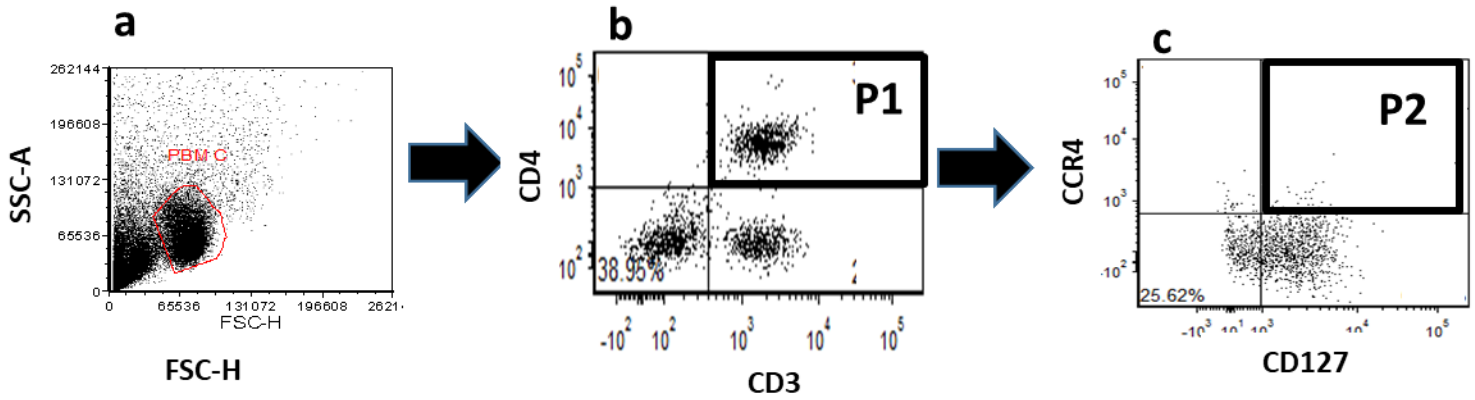
Characterization of T cell subpopulations.Lymphocytes were defined based on their forward and side scatter (a). T cells were defined as $CD3^+$ $CD4^+$T cells P1-gated within this lymphocyte gate (b), $CD3^+$ $CD8^+$ T cells P2-gated within this lymphocyte gate (c), naïve T helper cells P3 $CCR7^+CD45RA^+$ (d) naïve cytotoxic T cells P4 $CCR7^+CD45RA^+$ (f), central memory $CCR7^+CD45RA^-$ (P5 in d and P6 in f), effector $CCR7^-CD45RA^+$ (P7 in d and P8 in f), effector memory $CCR7^-CD45RA^-$ (P9 in d and P10 in f), activated population $CD38^+$HLA-DR^+ in the gated palpation (P11in d and P12 in f).

Appendix C



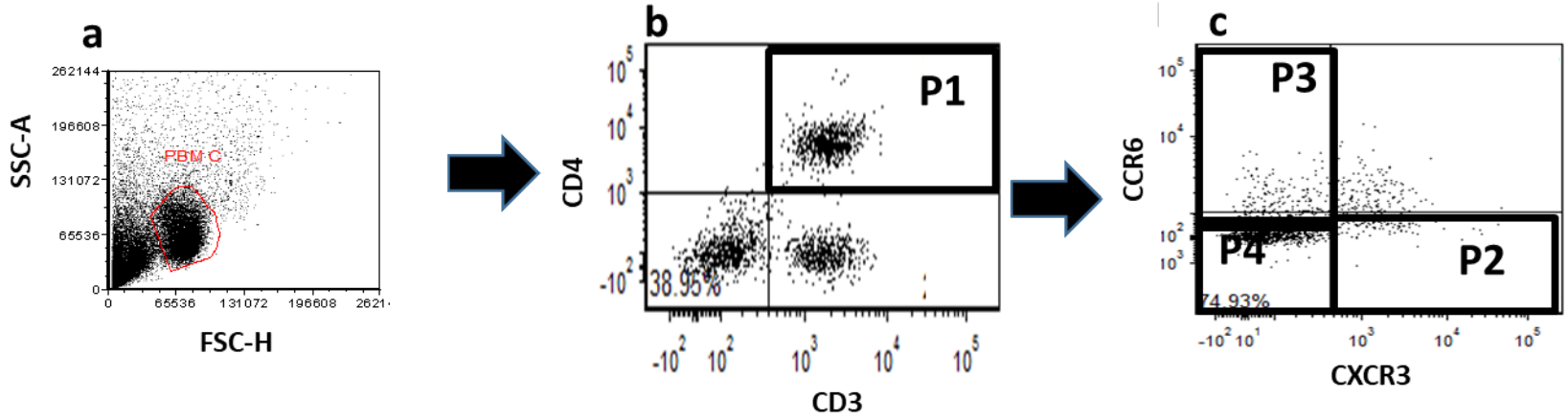
Characterization of B cell populations.Lymphocytes were defined based on their forward and side scatter (a) B cells were defined as $CD3^{-}CD19^{+}$ P1 gated within this lymphocyte gate (b), memory B cells $CD3^{-}CD19^{+}CD27^{+}IgD^{+}$ (P2 in c).

Appendix D



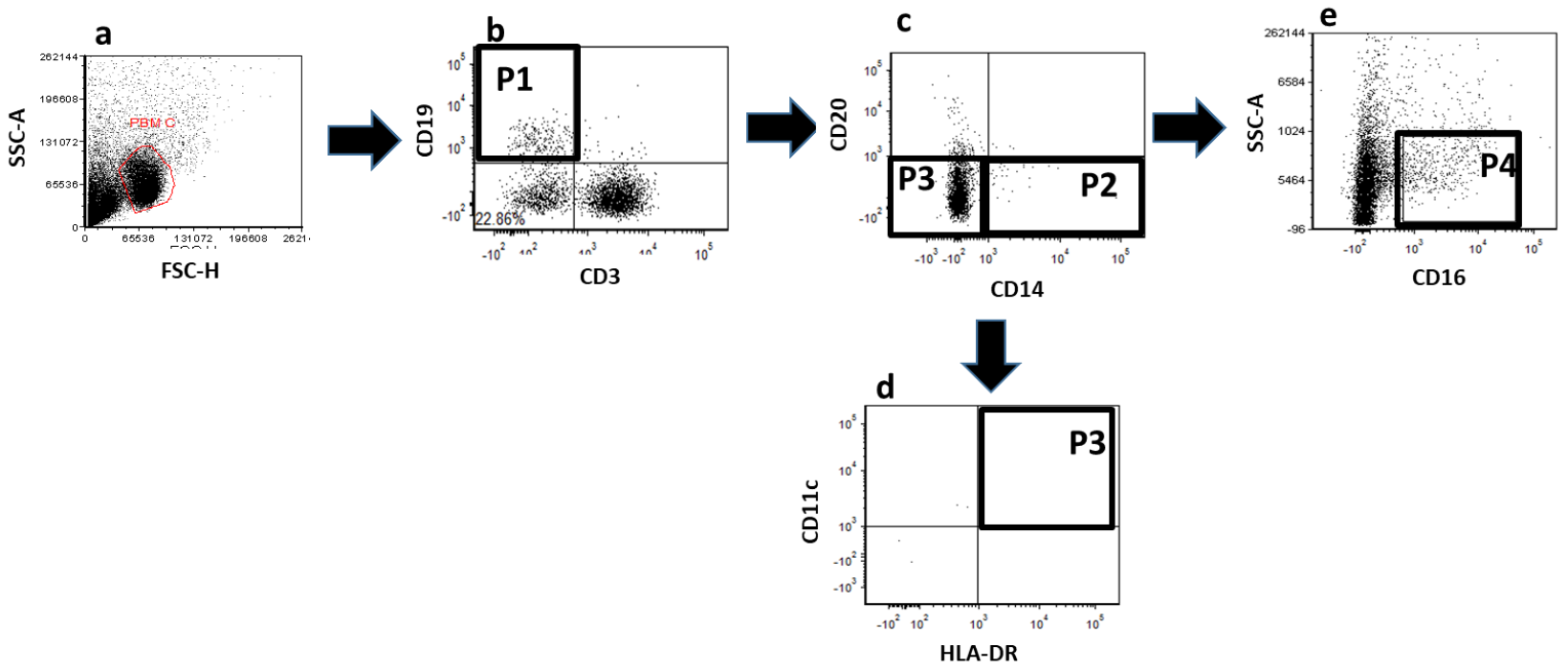
Characterization of T regulatory cell population.Lymphocytes were defined based on their forward and side scatter (a) T cells were defined as $CD3^+$ $CD4^+$T cells P1gated within this lymphocyte gate (b) T regs were defined as $CD3^+CD4^+CD25^+CD127^+CCR4^+$(P2 in c).

Appendix E



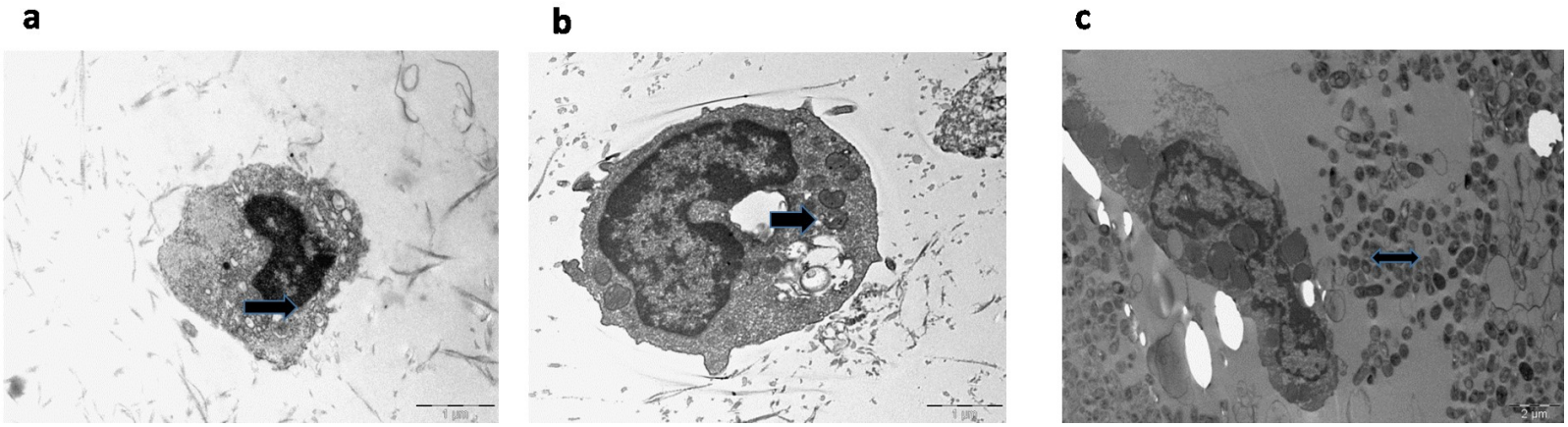
Characterization of Th1,Th2 and Th17 cell populations. Lymphocytes were defined based on their forward and side scatter (a) T cells were defined as $CD3^+$ $CD4^+$T cells P1gated within this lymphocyte gate (b) Th1 $CD3^+CD4^+CXCR3^+CCR6^-$ (P2 in c), Th2 $CD3^+CD4^+CXCR3^-CCR6^+$ (P3 in c).

Appendix F



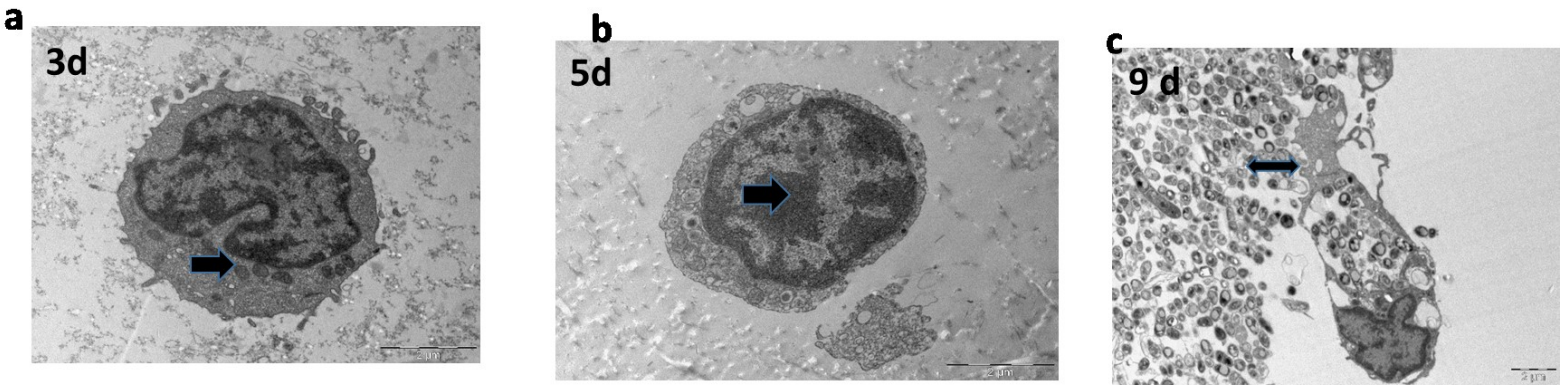
Charecterization of Monocytes, DCs and NK cells. Lymphocytes were defined based on their forward and side scatter (a) Non B cells CD3-CD19- gated within this lymphocyte (P1 in b), monocytes $CD3^{-}CD19^{-}$ $CD14^{+}$ (P3 in c), DCs and NKs $CD3^{-}$ $CD19^{-}$ $CD14^{-}CD20^{-}$ (P3in c), DCs $CD3^{-}CD19^{-}$ $CD14^{-}CD20^{-}HLA^{-}DR^{+}CD11c^{+}$ (P3in d), NK cells $CD3^{-}CD19^{-}$ $CD14^{-}CD20^{-}$ $CD56^{+}CD216^{+}$ (P4 in e).

Appendix G



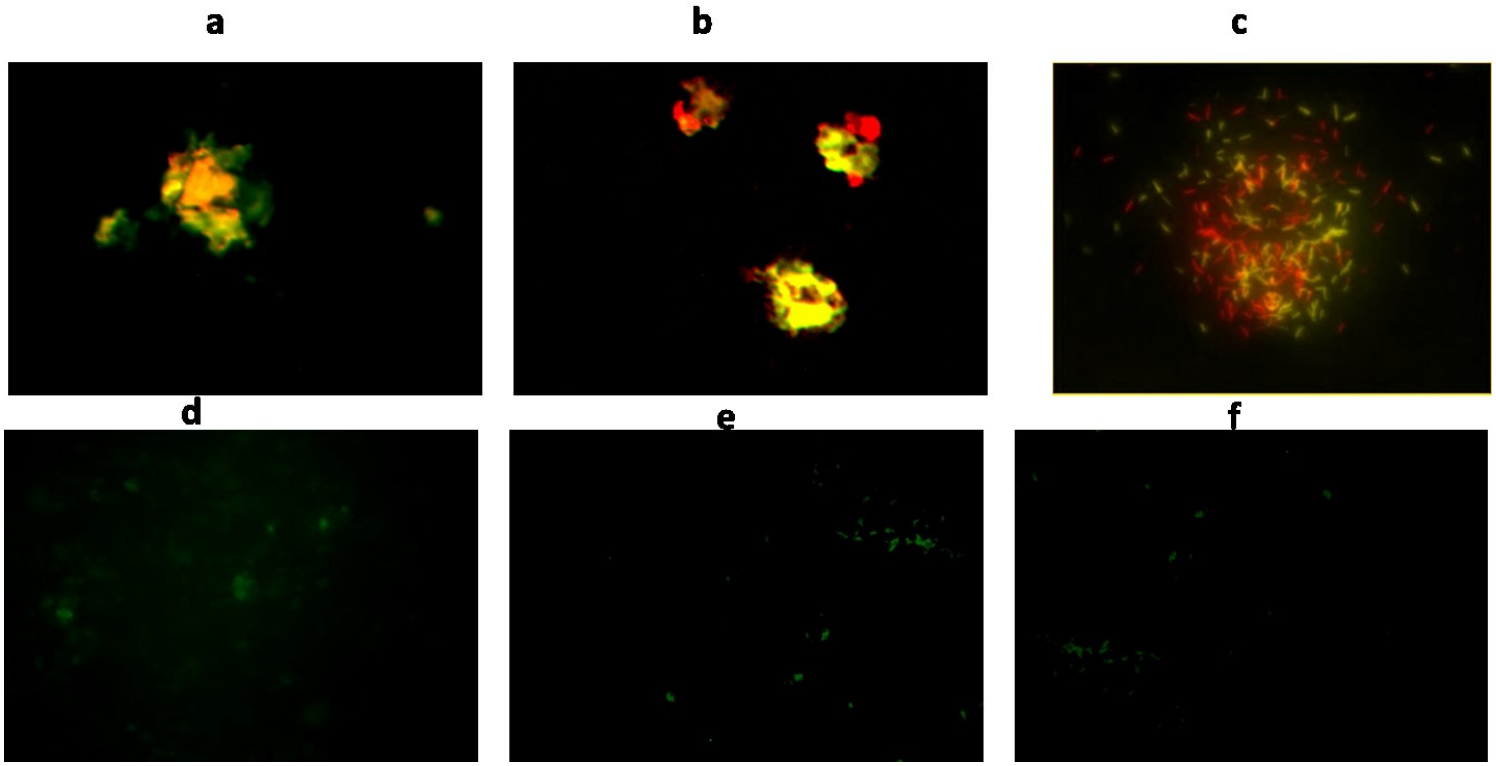
Transmission electron micrographs of internalization of *Mtb* H37Ra by macrophages. Intracellular infection is evident in the macrophage cell aggregates: (a) infection of macrophages by *Mtb* H37Ra at MOI **10:05,** bacteria indicated by arrow. (b) infection of macrophages by *Mtb* H37Ra at MOI **1:0.1**, bacteria indicated by arrow. Scale bar = 1 µm. (c) infection of macrophages by *Mtb* H37Ra at MOI **1:1** bacteria indicated by arrow.

Appendix H



Transmission electron micrographs show that intracellular infection is more evident in early infection (a) infection of macrophages by *Mtb* H37Ra on day 3 of infection, bacteria indicated by the arrow. (b) infection of macrophages by *Mtbs* H37Ra on day 5 of infection, bacteria indicated by arrow. (c) infection of macrophages by *M. tuberculosis* H37Ra on day 9 of infection, bacteria indicated by the arrow and a double arrow indicated lysis of the cell.

Appendix I



Co-localization of *M. tuberculosis* H37Ra to granuloma-like structures by Auramine-Rhodamine fluorescent staining.

(a) cell aggregates from BCG-unvaccinated donors (BCG-), (b) cell aggregates from BCG vaccinated (BCG+), (c) control (bacteria alone), (d) control ConA (PBMCs stimulated with ConA), (e) control (cells infected with *S.aureus*), (f) control (cells infected with *E.coli*). *Mtb* stained auramine (yellow) and rhodamine (red) stained actin.