IMPROVING THE EFFICACY OF BACILLUS CALMETTE GUERIN VACCINE BY CONCOMITANT INHIBITION OF T REGULATORY AND T HELPER 2 CELLS

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THESIS
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ABSTRACT

Tuberculosis (TB) remains a major threat to human population as currently *Mycobacterium tuberculosis* (MTB) infects nearly 33% of the global population. Annually, about one and a half million deaths are caused by tuberculosis (TB). Current reports suggest that approximately nine million new TB cases are reported every year. There is an available therapy for TB, however it is quite lengthily and consists of numerous antibiotics leading to treatment dropout. This treatment incompletion has been linked to the major cause for the appearance of drug-resistant species of MTB. Consequently, alternate therapies to treat TB are needed. Bacillus Calmette-Guerin (BCG) remains the only vaccine of choice since its inception in 1921. Although BCG mounts host protective T helper1 (Th1) cell activation, which plays a pivotal role in host protection against TB, its efficacy is inadequate, suggesting that additional methods to enhance protective immune responses are needed. We have also shown that simultaneous inhibition of Th2 cells and Tregs by using pharmacological inhibitors (suplatasttosylate and D4476, respectively) dramatically enhance MTB clearance and induces a superior Th1 response. Here we show that treatment with these two immuno-modulators during BCG vaccination dramatically improves vaccine efficacy. Furthermore, we demonstrate that these drugs induce a shift in T cell memory development, towards central memory T (Tcm) cell responses. Collectively, our findings provide evidence that concurrent inhibition of T helper cells type 2 and Tregs during BCG vaccination promotes vaccine efficacy.
PREFACE

This study represents original work by the candidate has not been submitted in the other form to any other University. All routine and experimental work (Bacterial Infections, Immunization of the mice and treatment with immunomodulators, Drug Treatment., Cell Staining and Flow Cytometry, $^{3}$H-Thymidine incorporation assay of splenocytes, Histology, T cell adoptive transfer, Statistical Analysis described in the thesis carried out by candidate in the Tuberculosis Aerosol Challenge Facility, International Centre of Genetic Engineering and Biotechnology (ICGEB), New Delhi, India and the Infection Prevention and Control, Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the joint supervision of Prof. Prashini Moodley and Prof. Gobardhan Das.

(Candidate)  
SANTOSH KUMAR

(Supervisor)  
Prof. Prashini Moodley
DECLARATION

I, SANTOSH KUMAR, declare that

(i) The research reported in this thesis, except where otherwise indicated, is my original research.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other person’s data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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</tr>
<tr>
<td>ADC</td>
<td>Albumin-Dextrose-Catalase</td>
<td></td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
<td></td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BAL</td>
<td>Broncho alveolar Lavage</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CD4(^+)</td>
<td>Cluster of Differentiation Antigen 4</td>
<td></td>
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<td>cDNA</td>
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<td>CFU</td>
<td>Colony Forming Unit</td>
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<td>CSA</td>
<td>Complete Soluble Antigen</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
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<tr>
<td>EDTA</td>
<td>Ethylene di amine tetra acetic acid</td>
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ELISA  Enzyme Linked Immunosorbent Assay
ESAT-6  Early Secreted Antigenic Target protein -6kDa
FACS  Fluorescence Activated Cell Sorter
FCS  Fetal Calf Serum
FITC  Fluorescein Isothiocyanate
GM-CSF  Granulocyte Macrophage Colony Stimulating Factor
Hr  Hour
HIV  Human Immunodeficiency Virus
IFN-α  Interferon-α
IFN-β  Interferon-β
IFN-γ  Interferon-γ
Ig  Immunoglobulin
IL-6  Interleukin-6
IL-10  Interleukin-10
IL-12  Interleukin-12
IRAK-1  Interleukin-1 receptor associated kinase-1
Kb  Kilobase
kDa  Kilodalton
KO  Knock out
LPS  Lipopolysaccharide
LT  Lymphotoxin
M  Molar
<table>
<thead>
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<td>MACS</td>
<td>Magnetic Cell Sorting</td>
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<tr>
<td>MDR TB</td>
<td>Multi-Drug Resistant Tuberculosis</td>
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<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
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<tr>
<td>Mg</td>
<td>Milligram</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>Min</td>
<td>Minute</td>
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<tr>
<td>ML</td>
<td>Millilitre</td>
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<td>MOI</td>
<td>Multiplicity of Infection</td>
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<tr>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation protein 88</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
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<tr>
<td>NK T cell</td>
<td>Natural Killer T cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Ng</td>
<td>Nanogram</td>
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<tr>
<td>OADC</td>
<td>Oleic acid-Albumin-Dextrose-Catalase</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Patterns</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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</table>
PE Phycoerythrin
Pg Pictogram
PPD Purified Protein Derivative
PRR Pattern Recognition Receptor
RBC Red Blood Corpuscles
RD1 Region of Difference1
STAT Signal Transducer and Activator of Transcription
SDS Sodium Dodecyl Sulphate
TB Tuberculosis
TCR T Cell Receptor
TGF-β Transforming Growth Factor-β
Th1 T helper cell type 1
Th2 T helper cell type 2
Th17 T helper cell type 17
TLR Toll Like Receptor
TNF-α Tumour necrosis factor-α
TRAF6 TNF receptor-associated factor -6
Treg T Regulatory Cells
WHO World Health Organization
XDR TB Extensively-Drug Resistant Tuberculosis
1. INTRODUCTION

Tuberculosis (TB) places a massive burden on global public health. Every year nearly nine million new TB cases appear and it causes approximately one and a half million deaths annually (WHO, 2012). MTB is mainly transmitted via the respiratory route and nearly 5-10 % of the individuals develop active tuberculosis after MTB infection. The host immunity is a key determinant of outcome to infection and disease. Current studies on TB patients and mice models have demonstrated the fundamental roles of CD8⁺ and CD4⁺T cells in clearing MTB (Chensue, S et al, 1994). However, HIV-infected individuals are reported to extremely susceptible to acquisition of new MTB infection along with reactivation of latent infection (Barnes et al 1991, Snider, D. E., Jr. et al 1992).

Altogether, these reports have taken us to the conclusion that the CD4⁺ T helper (Th1) cells play a decisive role in combating pulmonary TB. MTB overcomes host immunity by modulating the equilibrium of Th cell. Th1 cells play vital role in host protection from MTB infection. Consequently, individuals with alterations in their Th1 responses remain exceedingly susceptible to MTB infection (Flynn, J. L et al 2001, Lienhardt et al 2002). Nevertheless, activation of Th1 cell alone is not adequate to confer protection against MTB (Agger et al 2001, Demissie et al 2004). Activation of Th2 cells promotes TB disease development by antagonizing Th1-type immune responses (Lienhardt, C. et al 2002). IL-4 cytokine expression has been observed in human granulomas (Fenhalls, G et al 2000) and correlate with the immunopathology of tuberculosis (Ordway, D. J et al 2005, Seah, G. T et al 2000). Additionally, it has been reported that Bacillus Calmette Guérin (BCG)
vaccinated individuals mounted robust Th2 responses and failed to protect them from MTB infection (Dlugovitzky, D. et al 1999, Rook et al 2005). In the same way, it was observed that mice lacking IL-4 exhibited limited resistance to MTB infection (Hernandez-Pando et al 2004, Rook et al 1996). Collectively, this suggests that Th2 cells augment host susceptibility to MTB infection. However, other investigations further showed that MTB induces the proliferation of MTB specific FoxP3-expressing regulatory T cells (Tregs) (Scott-Browne et al 2007) in the draining lymph nodes (Shafiani, S et al 2007). These MTB specific Tregs cells hamper IFN-γ production by T cells and inhibit activation of protective immunity within the host (Nakamura, K. et al 2001, Randolph, D. A. 2006), thereby promoting disease progression.

As mentioned previously, the only TB vaccine presently available is the BCG, and it is estimated that three billion people have received it to date. BCG does not protect against MTB infection, but it has been shown to play a role in preventing dissemination of TB meningitis. Its efficacy against adult pulmonary tuberculosis was reported to vary greatly between 0-80% in different populations depending on the ethnicity and geographical locations (Colditz, G. A et al 1994). Recent studies have indicated that BCG vaccinated animals mainly developed antigen-specific CD4+ cells effector memory (Tem) cells. This failure of BCG to induce central memory cells (Tcm), may be the reason for its poor efficacy (Henao-Tamayo, M. I. 2011).

It is well documented that MTB survives, replicates and multiplies unhindered by modulating Th cell responses. Studies from patients and animal models indicate
that T cell activity is indispensable for anti-tuberculosis immunity. Previous studies suggest that, those that resist TB disease manifestation, mount tuberculosis antigen specific Th1 responses, as determined by the production of IFN-\(\gamma\), lymphotoxin (LT) and tumour necrosis factor-alpha (TNF-\(\alpha\)) (Goldsack, L et al 2007). Similarly, individuals with faulty genes for the production of IFN-\(\gamma\) or IFN-\(\gamma\) receptors are highly vulnerable to TB disease manifestation (Ottenhoff, T. H 1998). Recent reports support the well-established fact that, Th1 cells generated against MTB are indispensable for the expulsion of harbored tubercle bacilli from the host (Flynn, J. L.et al 1993). However, several studies indicated that only Th1 response is inadequate for the protection against TB (Bhattacharyya et al 1999). On the other hand, TB disease susceptible animals mount progressive Th2 responses, predominated by production of IL-4, IL-5 and IL-13 (Rook et al 2007). Thus, elevation of Th2 responses might be responsible for enhanced susceptibility to TB disease. This hypothesis was strengthened by the fact that IL-4 deficient mice show resistance to MTB infection (North R. J., 2007). Similarly, studies assessing the expression of cytokines in TB granulomas in patients with advanced TB, detected an enhanced IL-4 production (Fenhalls, G et al 2000). Interestingly, elevated Th2 responses has been noted in the patients who were not protected by BCG (Dlugovitzky D et al 1999). Based on the above-mentioned points, it is clear that Th2 cells alone are not the only factor contributing to host susceptibility to TB. Another T cell subset recently described, Treg cells, are phenotypically distinct, CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells and have been discovered to have vital role in immuno-regulations and control of autoimmune disorders
(Allan, S. E. et al 2008). It has been documented that TB induces a state of immuno-suppression in susceptible hosts. The progression of tuberculosis disease has been linked to the expansion of Treg cells (Scott-Browne, J. P. et al 2007). Antigen specific Treg cells increased within three weeks of infection, creating a conducive environment for proliferation of bacterial cells (Shafiani, S. et al 2011), and inhibiting effective Th1 responses. Cytokine requirements for the differentiation of Treg cells remain unclear. However, it has been established that FoxP3 transcription factor is necessary, which is induced by TGF-β signalling. Literature also suggest that combined inhibition of Th2 responses and Tregs are necessary for protective immune responses in the host and generation of Th1 cells responses is obligatory and adequate for controlling TB disease (Sweeney, K. A. et al 2011).

Thus, it is conceivable that mounting Th1 responses along with simultaneous inhibition of Th2 and Treg responses may be the answer to effective resistance against TB. To address this, we attempted to enhance the host protective immune response against MTB by inhibiting Th2 and Treg cells through small molecule inhibitors with BCG. Here, we simultaneously used both Th2 and Treg inhibitors to enhance the efficacy of BCG vaccination which generated higher Th1 responses. Furthermore, we observed a dramatic switch of Tem to Tcm. As Tcm plays the central role in host protection and determines vaccine efficacy, animals treated with these two inhibitors exhibited significantly improved vaccine efficacy when used along with BCG. Therefore, this strategy needs to be further evaluated for developing vaccine strategy.
2. REVIEW OF LITERATURE

2.1 Tuberculosis: The Global Epidemic

TB annually cause nearly 1.7 million deaths (WHO, 2012) and is the second leading cause of mortality from any single infectious disease globally, after HIV/AIDS (Corbett et al. 2003). Nearly 33% of the total world population carry latent MTB infection (WHO, 2004). On an average approximately 10 percent of MTB infected individuals develop active TB disease (Bloom and Small, 1998), and this percentage dramatically increases in the event of HIV infection (Selwyn et al., 1989; Girardi et al., 2000). HIV immunosuppression fuels the TB epidemic and both diseases have become global public health emergencies.

Figure 1 Estimated TB incidence rates, 2012 (Adapted from WHO Report 2013, Global Tuberculosis Control)
On an average, out of ten immuno-competent individuals infected with MTB, one can develop active TB in his whole life, but in co-infection with HIV, this rate is one out of ten in just a single year. In tuberculin test positive AIDS patients, one in two or three has a chance of mounting active TB disease (Corbett et al 2003). In industrialized countries, HIV makes up only a small number of TB cases but in developing countries, this situation is worst and remains as serious concern.

The worldwide spread of TB is generally associated with economic settings, maintaining its tightest grip on the populations of poor and under developed continents such as Africa, Asia, and Latin America (Frieden et al 2003; Corbett et al 2003). The rate of TB incidence largely ranges from >25/100,000 in North America to > 299/100,000 in western Russia and Asia to ~300/100,000 in Central and Southern Africa (Figure 1) (WHO, 2012). Furthermore, 95% of new TB cases and 99 % of deaths due to TB occur primarily in low income countries (Dye, 2006). Prior to the mid-1980s, a steady drop in TB cases was observed in developed countries, which then subsequently, began to see an increase in recent times. The re-emergence of TB is a severe global public health threat and this became more complex with the emergence of Drug-Resistant TB like MDR, XDR and TDR-MTB).
2.2 MTB

2.2.1 Historical Perspective

*Mycobacterium* genus was originated nearly 150 million years ago (Daniel, 2006). Presumably around 3 million years ago, MTB predecessor co-evolved with early hominids in East Africa. Reports suggest that nearly 15,000 - 35,000 years ago the more recent members of MTB complex were originated from a common progenitor (Gutierrez *et al*., 2005). The presence of MTB has been documented in the prehistoric humans remnants (4000 BC) and in Egyptian mummies (3000-2400 BC) (Zink *et al* 2003). In earlier times TB was known as “phthisis” - a Greek term that means ‘consumption’ indicating extensive weight loss due this disease. It was Hippocrates who first identified tuberculosis as a fatal disease around 460 BC and named it *phthisis*. J.L. Schoenlein gave the disease its present name “tuberculosis” in 1839.

MTB, the bacillus causing TB was first described in 1882 by Robert Koch. Albert Calmette and Camille Guerin introduced *Mycobacterium bovis* BCG vaccine in 1921, which remains the only vaccine against TB to date; however BCG efficacy against adult pulmonary TB remains questionable. In 1946, the antibiotic streptomycin was utilized as a treatment drug for TB (MRC, 1948). Notwithstanding, such advancements in the field of TB treatment, in 1980s and early 1990s multi-drug resistant tuberculosis (MDR-TB) and extensively-drug resistant tuberculosis (XDR TB) causing MTB strains emerged as a serious threat to TB control and management (CDC, 1990; CDC, 1993; CDC, 2007; Crawford, 1994; Dheda *et al* 2010; Neville *et al* 1994). The high prevalence of HIV/AIDS
has compounded the problem by fueling the drug susceptible and drug resistant TB epidemics.

2.2.2 Taxonomic Position of MTB

Superkingdom : Bacteria  
Phylum : Actinobacteria  
Class : Actinobacteria  
Subclass : Actinobacteridae  
Order : Actinomycetales  
Suborder : Corynebacterineae  
Family : Mycobacteriaceae  
Genus : Mycobacterium  
Species group : Mycobacterium tuberculosis complex  
Species : Mycobacterium tuberculosis

Major characteristics of *Mycobacterium tuberculosis* complex organisms are:

- MTB is an obligate aerobe and grows most efficiently in the lungs
- It is a facultative intracellular pathogen and typically infects mononuclear phagocytes such as monocytes, macrophages or dendritic cells
• It grows very slowly (generation time is 12 to 18 hours) compared to other bacteria (for example, the generation time of *Escherichia coli* is only about 20-30 minutes)

• Its cell wall is rich in lipids and extremely hydrophobic in nature. Therefore they are impervious to most of the regular stains like Gram’s stain

• Due to the presence of lipid-rich cell walls, these bacteria are comparatively impervious to a variety of basic dyes unless they are pooled with phenol. The stained MTB resists de-colorization from acidified organic solvents, hence called “acid-fast” bacilli.

### 2.2.3 Morphology of MTB

MTB bacteria appear as long thin rods, usually straight or slightly curved (*Figure 2*) that frequently show irregular beading due to vacuoles and polyphosphate granules. The bacillus is 1-10µm (usually 3-5µm) long and 0.2-0.6µm wide. This is a non-motile, non-spore forming and non-capsule forming bacterium. The lipid content in this bacteria is high, possibly the highest among all other bacteria. This waxy coat confers the acid fastness, extreme hydrophobicity, and little permeability to many known antibiotics (Chambers *et al.*, 1995; Bhatt *et al* 2007; Glickman and Jacobs, 2001). In the envelope structure, *Mycobacteria* contain mycolic acids and complex long-chain fatty acids that are found only in *Nocardia* and *Coryne bacterium*. Owing to the presence of N-glycolyl muramic acid in the place of N-acetyl muramic acid in the peptidoglycan cell wall, MTB is an acid-
fast bacterium. This characteristic is of a great practical importance since it is used to identify *Mycobacteria* in pathological specimens.

![Figure 2. Scanning Electron Micrograph of Mycobacterium tuberculosis (Image courtesy-Dr. Ray Butlar / CDC)](image)

### 2.3 Pathogenesis

This tubercle bacillus mainly infects hosts via the respiratory tract. The inhaled bacteria proceed towards distal sites in lungs and establish infection (Gupta and Chatterjee, 2005). MTB preferably infects lungs although it has capacity to infect in other tissues as well. Extra pulmonary TB develops in 10 to 25 percent of all the reported cases (Bloom and Small, 1998; Golden and Vikram, 2005; Mehta *et al* 1991; Yoon *et al* 2004). Patients suffering from TB expel MTB bacilli in the form of droplets while coughing and sneezing. Alveolar macrophages engulf these droplets and migrate towards draining lymph nodes where they help to
mount MTB specific T cells immune response and form granuloma in lungs to contain MTB on site of infection (Shoenfeld and Rose, 2005). MTB has the ability to replicate within the macrophages (Algood et al 2003; Algood et al 2005; Flynn and Chan, 2005; Ulrichs and Kaufmann, 2006). Macrophages and lymphocytes migrate to the site of infection and form a granuloma (Gonzalez-Juarrero et al 2001). Besides concentrating the immune response directly at the site of MTB infection, the other function of the granuloma is to avoid spread of MTB to other parts of the lung as well as different organs. The granuloma can develop into tuberculous lesions, most likely due to chronic stimulation of the immune cells at the site of infection (Flynn and Chan, 2001). Live bacilli have been isolated from the granulomas of patients with clinically inactive tuberculosis, which suggest that MTB can persist for long periods within the granulomatous lesions (Opie and Aronson., 1928; Robertson, 1933). Calcification in granuloma is observed if MTB infection is effectively contained at the site of its infection (Doherty and Andersen, 2005). However, granulomas enlarge in size and cellularity, if host immune response is not able to effectively control MTB replication. Ultimately, necrosis and cell death take place at hypoxic centre of the granuloma. In individuals with active TB disease, bacteria replicate and develop cavitary lesions. Subsequently, granuloma becomes caseous as a result of cellular degeneration and devastation (Dannenberg, 1993). During TB disease progression lipid rich material acts as a nutrient supply for the growth of MTB. Finally, dissemination of MTB takes place because of subsequent destruction and liquefaction of the granuloma. In some case cavitation is observed in TB patients,
as a result of lung tissue destruction and necrosis which ultimately develops into classical TB symptoms like blood in sputum and persistent cough (Abebe et al 2010; Doherty and Andersen, 2005). During this phase, patient is infectious and able to spread MTB via aerosol to healthy individuals. Patient with active TB disease can spread this disease and according to reports, can perpetuate this infection to about 10-15 people (Kaufmann, 2000).

Nearly 10 percent of the MTB infected people in their lifetimes will develop clinically active TB (Bloom and Small, 1998), but it becomes fatal if the disease is established and remain untreated (Tiemersma et al 2011). Disease outbreak is delayed because the progress of infection is very slow. In the adult, tuberculosis occurs most commonly as a result of the reactivation of the existing foci, rather than as a direct outcome of primary infection (Manabe and Bishai, 2000; Russell, 2007).

2.3.1 Granuloma

A granuloma is a mass of immune cells, comprised of a core of activated macrophages, enclosed by activated lymphocyte, which is formed to restrict or contain the foreign substance or pathogen that cannot be eliminated. The center of the granuloma often contains multinucleated giant cells, langhans cells, formed by the fusion of activated macrophages (Pritchard et al 2003; Lay et al 2007; Murphy et al 2008). These extremely large cells are generally enclosed by large modified macrophages which look like epithelial cells and referred to as epithelioid cells (Goldsby et al 2003).
The granuloma is the result of a vigorous cellular immune reaction to MTB components. The inhaled bacteria are internalized by alveolar macrophages and encouraged to infect the lung epithelium cells (Algood et al 2005; Flynn and Chan, 2005; Ulrichs and Kaufmann, 2006). The production of inflammatory chemokines and TNF-α by MTB infected macrophages results in the recruitment of different immune cells like natural killer NK T cells, neutrophils, CD8+ and CD4+ T cells, which subsequently amplify the cellular localization and remodeling of the MTB infection site (Algood et al 2003). Altogether, this inflammatory response is synchronized and superseded by the production of interferon IFN-γ. Subsequently, granuloma becomes stable, identifiable and emergence of the stratification takes place (Kaplan et al 2003; Ulrichs et al 2005). Furthermore neo-vascularization is observed in mature-phase granulomas (Kaplan et al 2003; Dheda et al 2005a; Ulrichs and Kaufmann, 2006).

In presence of a strong immune response, latent infection results in asymptomatic and non-transmissible state and finally granulomas may be reduced to small fibrous and calcified lesions (Doherty and Andersen, 2005). On the contrary, suppression of the immune status of host, the granuloma casemates, rips apart and leaks viable and infectious MTB into the airways, leading to the spread of infectious MTB bacilli (Figure 3).

The formation of granuloma has long been considered to be a necessary mechanism for the MTB containment, however a recent study suggested that granulomas might promote MTB infection (Davis and Ramakrishnan, 2009). This
finding indicates that granuloma formation cannot only be considered as an important process for host protective immunity against TB, but also leads to the establishment of latent TB infection, which makes it difficult for host immune system to resolve and clear MTB.

**Figure 3. The pathology of granuloma.** The granuloma comprises of a core of MTB infected macrophages encircled by foamy macrophages and other mononuclear phagocytes, with a layer of lymphocytes in association with a
fibrous cuff of collagen and other extracellular matrix components that delineates the periphery of the structure. (Adapted from Russel, 2007)

2.4 Innate immune response to MTB Infection

MTB infects, survives, and replicates within phagocytes (macrophages, monocytes, and immature dendritic cells) of susceptible hosts. These phagocytes provide a first line of defense, and in most cases, degrade the microorganisms shortly after engulfment. Disintegration of the ingested organisms is initiated after phagolysosome fusion. The acidic environment of the lysosomal contents and the hydrolases that are present in this compartment, destroy the microorganisms. However, MTB counters this host immune defense by several mechanisms such as mycobacterial sulfatides and lysosomotropic polyanionic glycolipids which have the ability to inhibit phagolysosome fusion (Goren et al 1976a; Goren et al 1976b; Middlebrook et al 1959). Furthermore, MTB produces copious amounts of ammonia, which effectively neutralizes the acidic environment of the phagolysosome, and successfully inhibits phagolysosome maturation (Gordon et al 1980).

Enzymatically, cleaved antigens intersect/interact/interfere with the cellular antigen processing machinery and are eventually presented on the cell surface via Major Histocompatibility Complex (MHC) molecules. The antigenic peptides are recognized by T cells, which lead to the generation of MTB specific T cell responses. In addition to the MHC-bound peptide, optimal activation and polarization of T cells require a microenvironment consisting of co-stimulatory
molecules on the same APCs appropriate cytokines and chemokines, and lipid mediators secreted at the inflammatory site. The microenvironment at the inflammatory site is predominantly generated by innate immune components upon recognition of Pathogen-Associated Molecular Patterns (PAMPs). These PAMPs are recognized by Pattern Recognition Receptors (PRRs) expressed by innate immune cells.

MTB produces several PAMPs, including lipoarabinomannan, phenolic glycolipids, phosphatidylinositol mannosidase and lipoproteins. These molecular patterns are recognized by innate receptors, called TLRs, on macrophages and DCs (Brightbill et al 1999). Interestingly, ligation of these PAMPs triggers both protective and pathogenic immune responses (Hawn et al 2006). However, it is not clear whether the balance by which these receptors are engaged during MTB infection contributes to susceptibility and resistance. It has been shown that signaling through TLR2 and TLR9 provides the strongest host resistance in TB (Bafica et al 2005). A range of microbial products can be recognized by TLR2 and heterodimered with other TLR like TLR1 or TLR6 (Takeda and Akira, 2005). TLR2 binds early secreted antigenic target protein 6 (ESAT-6) of MTB (Pathak et al 2007) which results in extensive modulation of host immune response. TLR2/1 recognizes mycobacterial phosphatidylinositol mannosidase and other lipoprotein, leading to pro-inflammatory cytokines production (Brightbill et al., 1999; Underhill et al 1999; Hawn et al 2006). On the contrary, recognition of lipoarabinomannan by TLR2 induces production of IL-10, which acts as an attenuator of protective immune responses (Quesniaux et al., 2004).
Unmethylated CpG motifs are recognized by TLR9 which results in the secretion of pro-inflammatory cytokines (Hemmi et al. 2000). There are two types of CpGs, namely A/D-type and B/K-type CpG, both of which are recognized by TLR9 (Hemmi et al. 2003). B/K-type CpG interaction leads to secretion of pro-inflammatory cytokines like IL–α, which IL-12 and TNF-α which facilitate Th1 responses (Takeda and Akira, 2005). Whereas, A/D-type CpG triggers interferon (IFN-γ)- (a type-I IFN) production by plasmacytoid DCs (Krug et al. 2001; Verthelyi et al. 2001). It has been shown that infection of macrophages by MTB induces production of type-I IFNs. Type-I IFNs also assist in mounting Th1 responses. Although some reports have showed the involvement of TLR-4 in mounting inflammatory responses, the role of this TLR in tuberculosis infections remains controversial. Upon recognition of ligands, TLRs form dimers, which then recruit the TIR domain-containing adapter molecule MyD88, this, in turn activates its downstream signals (Takeda and Akira, 2005) except for TLR3, all TLRs signal through MyD88. For instance, TLR3 and TLR4 activation signaling pathways induces type-I IFN, in contrast activation of TLR5 and TLR2 pathways does not induce this cytokine (Takeda and Akira, 2005). However, it is clear that the MyD88 pathway is essential for production of immune stimulatory and immune inhibitory cytokines as well as accessory molecules by APCs. MyD88-deficient mice are shown to be susceptible towards infection against MTB in comparison with wild type littermates (Ryffel et al. 2005).
2.5 Dendritic Cells and MTB

Dendritic cells (DC) are the most effective APCs and play a vital role in initiating immune responses in naïve animals (Steinman, 1991; Banchereau and Steinman, 1998). Current lines of research advocate that both myeloid and lymphoid precursors may lead to the generation of DCs. DCs are regarded as most potent APC because they have a unique ability to find antigen using numerous routes via macropinocytosis, clathrin-coated mediated endocytosis or phagocytosis which depends upon DC cell maturation state. Additionally, these cells express high levels of co-stimulatory molecules like CD80, CD86 and CD40 and MHCII which armor these cells to become the potent APCs (Banchereau and Steinman, 1998).

Interaction of MTB with DCs has been reported in number of studies. For instance, it has been shown that the MTB infection in DCs cause their activation (Henderson et al., 1997). Additionally, it has been reported that DCs infected with MTB produce increased levels of IL-12, IL-1 and TNF-α as inflammatory cytokines. Furthermore, it has been established that MTB could multiply in murine bone marrow derived dendritic cells (Bodnar et al. 2001). Activation and maturation of DCs can be induced by MTB lipo-peptides and proteins (Hertz et al. 2001). In addition, CD40 mediated stimulation of MTB infected DCs has enhanced potential to mount effective T cell responses by increasing MHC and other co-stimulatory molecules expression on DC cell surface (Demangel et al. 2001). DCs mediated induction of protective immunity was observed in a murine
model of MTB (Demangel et al 1999). These reports suggest that DCs are important in mounting and priming immune responses against MTB.

2.6 The Th1/Th2 Paradigm in TB

As mentioned previously, in the pre-AIDS era, approximately 10% MTB infected individuals, developed TB in their lifetime (Bloom and Small, 1998). The host had therefore evolved the resistance mechanism for controlling tuberculosis. Studies from patients and animal models indicate that T cells are an intrinsic part of anti-tuberculosis immunity. Historically, it is well-known that CD4+ T cells play the central function in the resistance to MTB infection (Caruso et al 1999; Mogues et al 2001; Scanga et al 2000). However, recently the role of CD8+ T cells in TB disease has been underscored. TB resistant individuals usually mount MTB antigen-specific Th1 responses, as determined by the production of IFN-γ, lymphotoxin (LT), and TNF-α by these cells (Kutlu et al 2007; Salgame, 2005). In contrast, susceptible animals mount progressive Th2 responses, predominated by the production of IL-5, IL-4 and IL-13 (Kutlu et al 2007; Rook, 2007; Rook et al 2004). Animal models of tuberculosis have confirmed that MTB specific Th1 cells are indispensable for expulsion of the harbored tubercule organisms (Flynn et al 1993; Cooper et al 1993). Similarly, individuals malfunctioning in the IFN-γ or the IFN-γ receptor encoding genes are reported to be highly susceptible to MTB (Ottenhoff et al 1998). However, several reports have indicated that only Th1 cell responses are not adequate for protection against TB (Bhattacharyya et al 1999; Elias et al 2005a; Leal et al 2001; Majlessi et al 2006). In fact, tuberculosis disease is often characterized by DTH induced by
PPD, which is the sign of IFN-γ mediated immune response. Therefore, elevation of Th2 responses might be responsible for enhanced susceptibility to TB. This hypothesis was strengthened in mice lacking IL-4, which show accelerated resistance towards MTB (North, 1998; Saunders et al 2000). Similarly, studies investigating the expression of cytokines in human granulomas in advanced TB disease have detected an enhanced IL-4 transcription (Fenhalls et al 2000). Several other studies have indicated that production of IL-4 and show a relationship with the immunopathology and disease progression in animal models and patients (Seah et al 2000; Dheda et al 2005b; Ordway et al 2005; van Crevel et al 2000). Furthermore, strong Th2 responses have been noted in patients who were not protected by BCG (Rook et al 2005; Dlugovitzky et al 1999). Nonetheless, it is apparent that Th2 cells are not the only cell type conferring disease susceptibility. Thus, it is very likely that promoting Th1 responses and simultaneously inhibiting Th2 responses holds the key to effective resistance against TB. This hypothesis is strengthened by the fact that latently infected individuals, in which MTB is effectively controlled, secrete a great amount of IL-4, an endogenous antagonist of IL-4 generated by alternative splicing from the primary IL-4 transcript (Rook, 2007).

2.7 Th17 cells in TB

The function of T cell responses in TB appears complex. Several other T cell subsets have been discovered previously, and the list continues to grow. The contribution of these cells in the outcome of tuberculosis pathogenesis has not
been studied extensively. Recently Th17 cells have been described as another subset of the Th cell lineage. These inflammatory phenotypic cells, are shown as a cause for the pathogenesis of several autoimmune disorders, and provide resistance to certain bacterial and fungal infections (Jin *et al* 2008). It appears that during MTB infection and TB disease progression, IL-17 does not have a defending role (Khader *et al* 2005; Cruz *et al* 2006). It has been shown that BCG-vaccinated IFN-γ-deficient mice develop enhanced numbers of cells producing IL-17, but the susceptibility of these animals to BCG remained unaltered (Cruz *et al* 2006). Another study indicated that IL-17 production during primary immune response inhibits the generation of an effective secondary immune response against TB (Romano *et al* 2006). In contrast, several other reports have established a protective function of IL-17-producing cells for the development of MTB specific secondary immune response (Khader *et al* 2007; Wozniak *et al* 2006). Additionally, studies documented that in IFN-γ deficiency, a strong IL-17-dependent memory response is produced in BCG-vaccinated animals. This memory response successfully protected animals upon subsequent challenge with MTB (Wozniak *et al* 2006).

Th17 cells differentiation requires simultaneous occurrence of TGF-β and IL-6 (Bettelli *et al* 2007). MTB infections in macrophages have been shown to produce significant amounts of both TGF-β and IL-6 (Toossi *et al* 1995; Van Heyningen *et al* 1997). Thus, it is expected that MTB infections facilitate Th17 differentiation. However, it is evident that both Th1 and Th2 subsets inhibit Th17 cells differentiation (Stockinger and Veldhoen, 2007). It is worth noting that the
Th1/Th2 paradigm in patients and animal models of TB has been well established. Furthermore, recent report suggest that for the molecular orchestration of Th17 differentiation TGF–β is not essential (Das et al 2009) instead, it strongly inhibits differentiation of both Th1 and Th2 cells. Consistent with this idea, IL-6-deficient mice showed a marginally increased bacterial burden in the initial phase of infection, suggesting a minor protective role of IL-6 confined within the innate immune response (Saunders et al 2000). Altogether, it suggests the protective role of both Th1 and Th17 cells, in contrast Th2 response have role in the progression of TB disease.

2.8 Treg cells in TB:
Treg cells are a phenotypically distinct T cell subset, characterized as CD4+CD25+FoxP3+ T cells, which play a decisive role in the control of autoimmune disorders and other immuno-regulatory mechanisms (Allan, S.E et al 2008). It is well known that TB induces immunosuppression in susceptible hosts. Recent report suggests that regulatory T cells become expanded during TB progression, and contribute to disease susceptibility (Scott-Browne, J.P., et al 2007). However, it is not clear whether these Tregs are natural Tregs or induced in the periphery in response to specific MTB antigens (adaptive Tregs). Treg cells exert suppressive functions by the production of TGF-β (Kretschmer, K., et al 2006; Lohr, J., et al 2006). Cytokine requirements for the differentiation of Treg cells remain unclear. However, it has been established that expression of FoxP3 transcription factor, can be induced by TGF-β (Liu, Y., et al 2008).
2.9 The BCG vaccine – Success, Failures and Reasons

BCG still remains the choice of vaccine against TB and it closely resembles the MTB with approximately 90% DNA homology. BCG was developed by two French scientist Albert Calmette and Camille Guerin of Pasteur institute during early 20th century using *M. Bovis* by growing it on culture medium and observing its reduced virulence in animals (Calmette and Plotz, 1929). For the first time in 1921 in France, this newly developed BCG vaccine was used for administration to infants, and it was a great success as it was able to significantly reduce infant mortality. Since its inception, BCG remains the only choice of TB vaccine (Lugosi, 1992). Reports suggests that annually over 0.1 billion doses of BCG are administered and in total approximately 3 billion individuals have been immunized with BCG (WHO, 2004). Although, BCG vaccine does not confer total and permanent immunity, it has been established that BCG induces a certain level of protection, mainly in children (Kaufmann, 2000). Large scale studies on the efficacy of BCG established that it shows greater protection in children, (Colditz *et al* 1995; Trunz *et al* 2006) but it has limited efficacy in adults (Fine, 1995).

Number of reports suggest that efficacy of BCG in imparting protection against TB varies from 0-80% (Colditz *et al* 1994; Brewer, 2000). In high income countries such as UK, North America and Denmark, clinical trials were conducted in 1940s and 1950 and it was established that the vaccine is highly efficient, in contrast recent trials conducted in Chingleput district of India which showed that BCG is unable to protect adults against pulmonary TB (ICMR, 1999), and some
studies performed in the US showed “negative” efficacy (Bannon, 1999; Fine, 1995). The reasons behind this variably in protection are largely unknown but several hypotheses have been stipulated, which includes genetic or nutritional dissimilarity in human populations, exposure of trial populations to environmental MTB, differences in trial methods, presence of diversity among the vaccine strains used in clinical trials (Behr, 2002; Brandt et al 2002; Comstock, 1994; Demangel et al 2005; Fine, 1995; Tsenova et al 2007). Studies using deletion analyses of different strains of BCG genome reviled that in several BCG strains, there has been loss few important genes required to mount protective immunity against TB. Sixteen deletions encoding 129 Open Reading Frames (ORFs) have been reported, which encodes a number of key T cell antigens such as the CFP-10, ESAT-6, CFP-21 and others (Mahairas et al 1996; Weldingh and Andersen, 1999; Skjot et al 2000). One hypothesis postulated that the absence of such immune-dominant antigens in BCG may be the reason why BCG is unable to prime a potent MTB protective immune response (Andersen, 2001; Behr et al 1999; Skjot et al 2000). Support for the significance of these antigenic proteins in protection comes from novel vaccine that performs better than BCG, which is BCG strain engineered to overproduce ESAT-6 protein (Pym et al 2003).

It is assumed that some of the important genes of BCG might have been lost during attenuation and/or at some stage in propagation (Behr et al 1999). Key antigenic proteins either not expressed or absent in a few BCG vaccines, whereas they are present in parental strain. However, seeing the experimental inconsistency in the efficacy of BCG vaccine, it is doubtful to conclude that
whether or not these strain variations may account for these ambiguities in findings (Oettinger et al 1999). Furthermore, reports have also suggested that, the presence of persistent helminth infections can hamper protective anti-TB responses induced by BCG. By shifting the host immune response towards a Th2 type, helminthes can drastically reduce the BCG protective efficacy (Elias et al 2005b; Malhotra et al 1999). It has been suggested that in developing countries failure of BCG vaccine is not due to low Th1 responses but rather because the vaccine is rendered ineffective and immuno-pathological in individuals exposed to the environmental immunological stimuli abundant in such countries (Rook et al 1981). All these details are not equally exclusive; rather all of these may give rise to the differences in vaccine efficacy. Besides variable efficacy, there are number of other limitations and major drawbacks of BCG. BCG boosters have been found to be ineffective (von Reyn and Vuola, 2002), possibly because the vaccine strain may not replicate in persons with previous immunization. PPD conversion after BCG vaccination has been considered a disadvantage in countries where determining MTB infection using the tuberculin skin test is used (von Reyn and Vuola, 2002). Severe and life-threatening complications may occur, including severe disseminated disease in immune compromised individuals including AIDS patients (Quinn, 1989).

Thus, in view of variable efficacy and various limitations of BCG, development of new, efficacious and safe vaccines appears to be the only option left. We believe that modification of BCG has the potential to be a future effective vaccine. Therefore, determination of the interaction of immune responses
modulated by H37Rv and the immune response mounted by BCG is a necessary first step.

2.10 RD1 region of MTB genome

A comparative evaluation of the genomes of the virulent MTB: H37Rv and *M. bovis* with *M. bovis* BCG revealed that many regions were missing in BCG when compared to the MTB and *M. bovis*. These were grouped into what is commonly known as Regions of Difference (RDs) (Mahairas et al 1996). It has been reported that during long term in vitro propagations of BCG, a large number of mutations have taken place and which have resulted in the deletion of many ORFs (Andersen, 2001; Behr et al., 1999; Kaufmann, 2001). Furthermore, 129 ORFs encoded by 16 deletions have been accounted for in the expression of several vital T cell antigens such as CFP-10, ESAT-6, CFP-21 etc (Mahairas et al 1996; Weldingh and Andersen, 1999; Skjot et al 2000) and 11 out of 16 deletions were exclusive to *M. bovis* while the other 5 deletions were unique to only BCG. One deletion which is designated as RD1, was deleted from BCG strains but present in the MTB complex (Harboe et al 1996). RD1 region is a genetic locus encoding 9 proteins from Rv3871 to Rv3879 (Cole et al 1998). Two open reading frames of RD1 region, Rv3874 and Rv3875 encode CFP-10 and ESAT-6 respectively (Cole et al 1998) and are co-transcribed (Renshaw et al 2002).

RD1 region encodes the T-cell antigen ESAT-6, which was originally isolated from a highly stimulatory low-molecular-mass fraction of MTB culture filtrate (Sorensen et al 1995). *In vitro* studies using human and mice models reported that
ESAT-6 is a possible diagnostic marker for active TB (Ulrichs et al 1998). Another MTB antigen CFP10 gene was similarly characterized like ESAT-6, which is co-localized in ESAT-6 gene operon (Berthet et al 1998). Using southern blotting approach it has been revealed both the *esat-6* and *cfp10* genes are present in MTB, virulent *M. bovis* and *M. africanum*, in contrast these two genes were not observed in the BCG vaccine strains as well as in most NTM (nontuberculous mycobacteria) (Harboe et al 1996; Skjot et al 2000).

Though none of the nine open reading frames (ORFs) that comprise RD1 have a biochemically assigned function, this region has been carefully scrutinized *in silico*. The predicted functions of several RD1 region genes suggest that they may have roles in protein translocation. Further, as CFP-10 and ESAT-6 lack clear secretion signals, they may require a novel secretion machinery for export (Braunstein and Belisle, 2000), and components of RD1 may form that machinery (Cole et al 1998; Gey Van Pittius et al. 2001; Lewis et al 2003; Pallen, 2002; Tekaia et al 1999). This hypothesis was proven true when genes (*Rv3870, Rv3871* and *Rv3877*) were deleted, which ultimately stopped the secretion of ESAT-6 and CFP-10. Furthermore, Cox and others also reported that disruption of individual RD1-region genes did not put off production of CFP-10 or ESAT-6. Nonetheless, an integrated RD1 region was required to secret these proteins (Pym et al 2003; Stanley et al 2003; Guinn et al 2004).

In an experiment when RD1 locus was reintroduced in BCG, it was observed that recombinant BCG was more virulent as well as additionally protective compared to the control BCG strain (Pym et al 2003). Additionally, immune response
similar to that of MTB was elicited by BCG:RD1, in particular, when BCG:RD1 was used for vaccination, it was found to induce migration of effector cells to the infection sites in lungs of subsequently MTB infected mice more efficiently than the mice infected with wild type BCG strain (Majlessi et al. 2005). These reports imply that RD1 antigens are lost during the attenuation of BCG and can comprehensively modulate BCG induced immune response.

RD1 region seems to be the most important determinant of MTB pathogenesis but the mechanism behind this is largely unknown. A number of research studies have suggested that the RD1 region modulates early events in MTB infection. Hence, RD1 region can be represented as an increasingly relevant genomic region of MTB, since it seems to be concurrently involved in better virulence in immuno-compromised hosts and increased protection in immuno-competent hosts and presents a challenge to researchers to identify the tactics employed by MTB to ensure its survival in the intracellular environment.

2.11 The Present Study
Numerous studies from literature established that Th1 cell responses are obligatory to combat TB. It is also known that BCG significantly induces Th1 cell response, however, it does not show adequate defense against TB in a lot of individuals. In the current study, we found that Stat-6^−/−CD4-TGF-βRII^DN mice which lack the capacity to mount Tregs and Th2 cell response are resistant to MTB infection. Moreover, concurrent inhibition of Tregs and Th2 cell subsets using chemical inhibitors noticeably reduced bacterial load in different organs of
mice. As these therapeutic agents are not directed to the harbored organisms, they should also avoid the risk of promoting the development of drug-resistant MTB. We have also shown that simultaneous inhibition of Th2 and Tregs by using pharmacological inhibitors D4476 and Suplatasttosylate dramatically enhance MTB clearance, and mounts an enhanced Th1 response. Additionally, we observed that administration of these two drugs during vaccination dramatically improves vaccine efficacy of BCG. Furthermore, we demonstrate that there is a shift in effector memory T (Tem) to central memory T (Tcm) cells. As a whole we provide evidence that inhibition of Th2 and Treg cells during BCG vaccination is the strategy for better efficacy of BCG vaccine.
3. RATIONALE OF THE STUDY AND OBJECTIVES

Currently BCG remains the only available TB vaccine, and it has been globally used since its inception in 1921. According to estimations about 3 billion people have received it worldwide. But, its efficacy in adult pulmonary tuberculosis is below par. Therefore; an effective vaccine is urgently needed. To design an effective TB vaccine, it is important to understand why the BCG is not working well. During the last two decades it was generally believed that MTB hinders host protective Th1 responses, but facilitates Th2 responses to survive within susceptible hosts. As these two subsets of Th cells are counter regulatory. Many studies were conducted to examine the therapeutic benefit of inhibiting Th2 responses during MTB infection. Indeed, inhibition of Th2 responses induces partial resistance to MTB infection. Thus, in addition to Th2 cells, additional immune components also participate in facilitating disease progression during tuberculosis. In fact, current reports have recommended that Treg cells play a decisive role in facilitating TB disease progression. However, in recent years, the members of Th cell family have been increasing at a rapid pace. Therefore, immunological parameters induced by BCG and the requirement of immunologic responses for optimal vaccine efficacy needs to be re-evaluated. Our first aim will be to identify T cell responses, in addition to those induced by BCG, for optimal vaccine efficacy. Formerly it has been observed that MTB infected animals that were cured with antibiotics show dramatically enhanced host protective immune responses as compared with animals vaccinated with BCG. However, T cell
responses induced in these animals have not been evaluated. We have shown that mice that are incapable in mounting both Th2 and Tregs cell responses (Stat-6-/-CD4-TGFβRIIDN) are wholly resistant to MTB infection. On the other hand, either the Th2 deficient (Stat-6-/-), or Treg deficient (TGF-βRIIDN) only animals were partially resistant to MTB infection. Surprisingly, each of these animals in T-bet-/- background were dramatically susceptible to MTB infection. These observations suggested that combined inhibition of Th2 responses and Treg cells has therapeutic benefit, whereas Th1 responses are indispensable for protective immune responses in the host. We further validated the data by applying small molecule directed immunotherapy using suplatasttosylate and D4476, which inhibit Th2 and Treg cells differentiation, respectively.

Therefore, it is highly likely that inhibition of IL-4 and TGF-β signaling will improve vaccine efficacy of BGC. Hence, our second aim is to test vaccine efficacy of BGC in the presence or absence of suplatasttosylate and/or D4476. Furthermore, to unfold the mechanism of such improved vaccine efficacy we will determine which subset of T cell is implicated in the enhancement of efficacy of BCG as our third aim.
4. MATERIALS AND METHODS

4.1 Ethics Statement:
The guidelines approved by the Institutional Animals Ethics Committee at ICGEB (ICGEB/IAEC/IMM-17/2008 and ICGEB/IAEC/IMM-22/2010), New Delhi, India were followed for every animal experiment. The *in vitro* experiments conducted in the School of Laboratory Medicine and Medical Sciences, were approved by the Biomedical Research Ethics Committee at the UKZN, Durban, South Africa (**BREC reference No: BCA 274/09**)

4.2 Bacterial Infections:
BCG and MTB H37Rv strains were grown and maintained in 0.02% Tween 80 containing Middlebrook 7H9 broth (BD Biosciences, Sparks, MD). For the determination of the number of viable MTB, oleic acid albumin dextrose catalase (OADC) enriched 7H11 agar plates were used. Mice infections were carried out via aerosol route. MTB burden in lung, spleen, and liver of mice was determined by using nutrient Middlebrook 7H11 agar plates. All MTB experiments were carried out in a either BSL II+ or BSL III facility at ICGEB, New Delhi, India and UKZN, South Africa following recommended guidelines.
4.3 Mice:
BALB/c mice, either Thy1.1<sup>+</sup> or Thy1.2<sup>+</sup> (Jackson Laboratory, USA) were bred and maintained in the ICGEB, New Delhi animal facility.

4.4 Immunization of the mice and treatment with immunomodulators:
Mice were immunized with BCG subcutaneously (1x10<sup>6</sup> bacteria) and the following day, treated with D4476 (TocrisBiosciences) and suplatasttosylate (Tocris Biosciences) at 16 nmol/g of body weight for a total of 10 days and subsequently kept in rest for 20 days. Mice were then infected with H37Rv MTB via aerosol route and the organs were harvested for determination of bacterial burden at consecutive days following infection.

4.5 Drug Treatment:
H37Rv infected mice were intraperitoneally treated with or without D4476 and/or suplatasttosylate (Tocris Biosciences) at 16nmol/g of mice body weight from day 1 till day 44 after MTB infection.

4.6 Solutions for Flow Cytometric Analysis:
Phosphate Buffer Saline (PBS, pH 7.4):13.7 mM sodium chloride (NaCl), 0.27 mM potassium chloride (KCl), 10 mM Di-Sodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>) and 0.2 mM Sodium di-hydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>)
Fixing Solution: 4% Paraformaldehyde solution in PBS
Permeabilization Buffer: 0.1% saponin, 1% Fetal calf serum (FCS) in PBS

RBC lysis buffer: 0.826 % ammonium chloride (NH₄Cl), 0.1 % potassium bicarbonate (KHCO₃) and 0.0037 % tetra sodium ethylene-diaminetetraacetic acid (EDTA) in double distilled water, pH adjusted to 7.2, filtered and autoclaved

### 4.7 Cell Staining and Flow Cytometry:

Intracellular cytokine staining was performed on cells which were first treated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml)(added during last 6 hr of culture) (Sigma-Aldrich or eBiosciences, USA). After washing the cells twice, they were permeabilized using Cytofix/Cytoperm kit (BD), and stained with the given antibodies: anti-mouse CD4 (clone GK1.5)-PE-Cy5, anti-mouse CD4 (clone GK1.5)-APC, anti-mouse CD4 (clone GK1.5)-FITC, anti-mouse CD8 (clone 53-6.7)-PE-Cy5, anti-mouse CD4 (clone GK1.5)-PE, anti-mouse CD62L (clone MPL-14)-APC, anti-mouse FoxP3 (clone FJK-16s)-APC, anti-mouse CD25 (clone PC61)-APC, anti-mouse IFN-γ (clone XMG1.2)-APC, anti-mouse IL-4 (clone 11B11)-PE, anti-mouse IL-17A (clone TC11-18H10.1)-PE, anti-mouse FoxP3 (clone MF-14)-PE, anti-mouse TNF-α (clone MP6-XT22)-PE, anti-mouse IL-6 (clone MP5-20F3)-PE, anti-mouse IL-12 (clone C15.6)-PE, and anti-mouse IL-10 (clone JES5-16E3)-PE (purchased from Biolegend, USA). The fluorescence intensity was measured by flow cytometry using (FACS CantoII or FACS Calibur from BD) and FlowJo software was used for data analysis (Tree star, USA).
4.8 $[^3]$H]-Thymidine incorporation assay of splenocytes:

Single cell suspensions of mice spleens were prepared by macerating them in complete RPMI 1640 (Gibco, Invitrogen, UK). RBC lysis buffer was used to lyse RBCs and washed with complete RPMI 1640. Splenocytes were plated in 96-well plate at a density of $0.1 \times 10^6$ cells/well and stimulated with different concentrations of MTB complete soluble antigen (CSA). After 48 hours, these cells were pulsed with tritiated thymidine (1.0 $\mu$Ci per well) (Amersham Biosciences UK). Cells were harvested using cell harvester (WallacTrilux) and $[^3]$H-TdR incorporation was measured using a liquid scintillation counter (Perkin Elmer, UK).

4.9 Cytokine assay:

The spleens of the adoptive transfer mice at 60 days post H37Rv challenge were isolated and macerated to single cell suspension and used in the estimation of antigen-specific cytokine responses. RBCs lysis was carried out using Tris/NH4Cl. After extensive washing, single cell suspensions were plated in 24 well plates at a density of $5 \times 10^6$ cells/well. These cells were stimulated using CSA of H37Rv (50 $\mu$g/ml), for 48 hours. The cytokine assay was performed using the culture supernatant by Luminex following manufacturer’s protocol (BioPlex, Bio-Rad).
4.10 Histology:
After 60 days of MTB infection, lungs were harvested from mice and processed as per guidelines. The dissected lung tissues were fixed in 4% paraformaldehyde and processed through alcohol grades, xylene and embedded in paraffin. Following paraffin embedding, the tissues were sliced, mounted on slides and dried. After drying, the slides were processed by treatment with xylene, alcohol and water. Using hematoxylin, slides were stained, rinsed and counter-stained with eosin prior to viewing under the microscope.

4.11 T cell adoptive transfer:
Thy1.1\(^+\) mice were gamma-irradiated (8 rads/sec for 100 seconds) and then rested for a day. Using lymph nodes of Thy1.2\(^+\) background mice, CD4\(^+\) T cells were isolated and then adoptively transferred into the irradiated recipient mice (2 x 10\(^6\) cells per mouse). After 10 days the recipient mice were challenged via aerosol route with H37Rv for further experiment.

4.12 Statistical Analysis:
SPSS10 software was used to carry out statistical analyses. All experiments were carried out for three times and significance in data was determined by Tukey’s multiple comparison tests and ANOVA (SPSS Software). \( p \) value >0.05 was considered as statistical significance.
For our genetically engineered mice data represent mean \( \pm \) S.D values of four mice/group/time point whereas experiments with small molecule-mediated immunotherapy represent value of six mice/group/time points.
5. RESULTS

5.1 Treatment with Th2 and Treg inhibitors increase the efficiency of BCG

To examine the effect of Th2 and Treg inhibitors on BCG vaccinated mice, we immunized C57BL/6 mice with BCG and concurrently treated them with D4476 and suplatasttosylate, for 10 days. These animals were rested for an additional 90 days, and re-infected with MTB H37Rv through the aerosol route. Organs were harvested and determined the bacterial burden at different time intervals (Figure 5A). We observed that co-treatment with these compounds drastically enhanced BCG vaccine efficacy, as determined by the significant reduction of bacterial loads in various organs (Figure 5A). In order to assess changes in the immune responses, we challenged spleen cells isolated from 60 days post re-infected animals with MTB derived complete soluble antigen (CSA) and measured the proliferative response. We noticed that animals that received immunomodulators along with BCG, exhibited superior proliferative responses (Figure 5B). Histological studies also revealed that animals treated with immunomodulators along with BCG shows dramatic reduction of granulomatous region (Figure 5C).
Figure 4. Schematic diagram of the immunization, treatment and infection of
BALB/C mice.
Figure 5. Curative treatment with immuno-modulators along with single BCG immunization reduces bacterial burden significantly. (A) After immunization BALB/c mice were treated with suplatasttosylate and/or D4476 at 16 nmol/g of body weight for a total of 10 days continuously and then kept the mice for another 20 days before the aerosol challenge with MTB (H37Rv). Bacterial loads (CFU) were measured in lungs and spleens at 60 days post infection. Data represent mean ± SD values from two independent experiments of four mice/group/time point. (B) Proliferation of spleenocytes in response to CSA was measured by $[^{3}H]$-thymidine incorporation assay. (C) Photomicrographs ($\times10$) of histological lung sections (6 μm) at 60 days after infection of the
indicated mice, stained with haematoxylin and eosin. Results shown here are representatives of at least three independent experiments.

5.2 Inhibition of Th2 and Treg by suplatasttosylate and D4476 in BCG immunized animals significantly enhanced CD4^+CD44^{hi}CD62L^{hi} T cell response

To provide insight into the T cell response induced by BCG immunized-immunomodulators treated MTB re-challenged animals we isolated T lymphocytes from spleens of different groups of animals. Phenotypic characterization revealed that CD4^+ T cells in suplatasttosyle and D4476 treated animals were increased significantly than that of BCG alone primed animals (Figure 6 A& B). Vaccine efficacy is mostly dependent on the pool of central memory cells. BCG primarily induces effector memory cells in lung, and possibly that’s why BCG is ineffective. As inhibition of Th2 and Tregs by the use of pharmacological inhibitors culminated in a higher resistance to MTB, we determined effector memory and central memory cells in immunized animals. Phenotypic analysis revealed that approximately half of CD4^+ cells are CD44^{hi}CD62^{hi}, which is significantly higher than any other group (Figure 7A & B). In contrary number of CD44^{hi}CD62^{lo} cells were dramatically decreased as low as 2.79% (Figure 7A & B).
Figure 6. CD4 expression after immunization and treatment of mice. (A & B) CD4 and CD8+ T cells count 60 days post infection of immunized and treated mice.
Figure 7. Multifunctional CD4 expression after immunization and treatment of mice. (A & B) Expression of different phenotypes (CD44^{hi}CD62L^{lo}, CD44^{hi}CD62L^{hi}, CD44^{lo}CD62L^{hi}) by multifunctional CD4^+ T cells. Results are representatives of three independent experiments.
5.3 T helper subset responses in immunomodulators-treated BCG immunized mice

To determine whether the treatment with suplatasttosylate and D4476 has an effect on the immune responses in BCG immunized animals, we next quantified the frequencies of cytokine producing T cells. Our experimental results demonstrated that IFN-γ producing CD4⁺T cells were dramatically increased whereas Th2 cytokine producing cells were decreased in drug treated BCG immunized animals, and an opposite trend observed in only BCG immunized animals (Figure 8 A & B). Interestingly, we did not detect any changes in Th17 cytokine producing cells in all groups (Figure 8 A, B&C). Although Th17 responses help in better vaccine efficacy (Chatterjee, S. et al 2011, Khader, S. A., et al 2007) it appears that it’s not absolutely necessary. Treg cells have a prominent role in establishing pathogenesis of TB, and hence we wanted to establish the status of Treg cells in these animals. We noticed an abrupt decrease of CD4⁺CD25⁺FoxP3⁺ T cells in drug treated BCG-vaccinated animals. We also determined other host protective cytokines, that is, IL-12 and IL-6. It was found that IL-12, IL-6 and TNF-α producing cells were considerably increased in drug treated BCG immunized (Figure 9 A & B). We examined IL-10 production in these conditions, and found there were no significant difference, which is in agreement with previous reports, that IL-10⁻/⁻ animals are as susceptible as wild type littermates (Figure 9 A & B).
**Figure 8. BCG immunization and immunomodulators treatment induces Th1 phenotype.** (A) Intracellular staining for IFN-γ, IL-4, IL17 from CD4⁺ T lymphocytes isolated from the spleens of different groups (infected, BCG immunized, BCG immunized and treated) mice. (B) FoxP3 (CD4⁺ CD25⁺) of CD4⁺ T lymphocytes isolated from the spleens of different groups (infected, BCG immunized, BCG immunized and treated) mice. (C) Bar diagram to show the percentage of Intracellular level of cytokines. Results are representatives of three independent experiments.
Figure 9. Intracellular Cytokine Profiling. (A & B) Intracellular cytokine staining for the IL-10, IL-6, IL-12 and TNF-α production from different groups (infected, BCG immunized, BCG immunized and treated) of mice. Results are representatives of three independent experiments.
5.4 Enhanced central memory T cells protect animals from MTB infection

To provide further evidence that central memory is generated by treatment with immuno-modulators, we immunized Thy1.2 congenic animals and treated the animals with suplatasttosylate and D4476. The isolated CD4$^+$ T cells were adoptively transferred into irradiated Thy1.1 congenic animals followed by infection with H37Rv (Figure 10). Fifteen, thirty and sixty days post infection, we measured the bacterial load in mice spleen and lungs, interestingly we observed that drug treated mice displayed significantly less bacterial burden than untreated mice (Figure 11). This observation pointed out that indeed central memory cells generated by the treatment of immuno-modulators are host protective.
Figure 10: Diagrammatic sketch of adaptive transfer of CD4$^+$ T cells from Thy1.2 to gamma-irradiated Thy1.1 mice.
Figure 11. Adoptive transfer of T lymphocytes from BCG immunized and treated mice confirm the reduction of bacterial burden. After immunization Thy1.1 mice were treated with D4476, and/or suplatasttosylate at 16 nmol/g of body weight for a total of 10 days continuously and then kept the mice for another 20 days then T lymphocytes were isolated from lymph node and injected intraperitonialy (5 x 10^6 cells) to gamma irradiated Thy1.2 before the aerosol challenge with low dose of M.tb (H37Rv) (~100 CFU). Bacterial burdens (CFU) were measured in lungs and spleens at 15, 30 and 60 days post infection. Data represent mean ± SD values of four mice per group per time point.
5.5 Th2 and Treg inhibitors induce a different distribution of cytokine-producing CD4+T cell subsets in BCG immunized mice.

It is well established that pro-inflammatory cytokines IL-6, TNF-α, and Th1 associated cytokines play central role in host protection. Therefore, we measured these cytokines in animals that received memory cells and protected from MTB infection (Figure 12 A). We observed that IFN-γ level was much higher in the treated mice than untreated, whereas IL-4 level was almost similar (Figure 12 A & B). We also determined the T cell polarizing cytokines IL-12, IL-6, TNF-α and IL-10. Interestingly we observed that IL-12, TNF-α and IL-6 level were higher in the treated mice (Figure 13 A & B).
Figure 12. Frequency of T cells and cytokine producing cells in post adoptively transferred mice. (A) Profile of CD4 and CD8⁺ T cells count 60 days post infection of adoptive transferred mice. (B & C) Intracellular staining for IFN-γ and IL-4 of CD4⁺ T lymphocytes isolated from the spleens of different groups adoptive transferred mice.
Figure 13: (A & B) Frequency of IL-6, IL-10, IL-12 and TNF-α intracellular cytokine producing cells of different groups of adoptive transferred mice. Results representative of three independent experiments.
6. DISCUSSION

The role of host Th cells balance is well established in MTB infection (Walzl, G., et al 2011). In susceptible hosts MTB induces Th2 and Treg cell responses and antagonize host protective Th1 cell immune responses. In this study we demonstrated that Th1 cells are indispensable for mounting effective immune response against MTB, which is in accordance with recently published studies (Sweeney, K. A., et al 2011). There is a dynamic balance between Th1 and Th2 cells and therefore, induction of Th2 decreases Th1 responses and compromises host protection. Indeed, literature suggests that Th2 responses deficient mice are moderately resistant towards MTB infection (Rook, G. A., et al 2004). Therefore, inhibition of Th1 responses in MTB infected host and induction of Th2 responses symbolizes a strategy of MTB to evade host immunity.

Recently, reports have shown that MTB induces strong Treg cell responses as well as facilitates Th2 responses. Treg cells hamper host protective immunity (Boussiotis, V. A., et al 2000). Additional studies suggest that Treg cell differentiation needs TGF-β and it is secreted by the MTB infected cells as well as by infiltrated mesenchymal stem cells (Raghuvanshi, S., et al 2010, Toossi, Z., et al 1998, Shafiani, S., et al 2013), our data also provides proof of intense Treg cells expansion in MTB infection. One report also suggests that TGF-β is required for Tregs cell development, maintenance and expansion. Indeed, previous data also pointed out that TGF-β signaling deficient mice are partly resistant towards MTB infection (Jayaswal, S., et al 2010). Recent data also suggest that individual inhibition of Tregs or Th2 cells can confer fractional resistance towards MTB.
Consequently, to understand whether or not concurrent suppression of Tregs or Th2 cells does have a complementary effect; we created Stat-6\textsuperscript{-/-} CD4-TGF-βRIIDN mice. We found that these mice demonstrated increased resistance towards MTB infection in comparison with the only Stat-6 and only TGF-β deficient transgenic strains. Consequently, our data advocate that concurrent inhibition of Tregs and Th2 cells may have better therapeutic advantage in comparison to the inhibition of only one subset at a time. Nevertheless, the possibility of some additional influences by Stat-6 and TGF-β deficiency on MTB infection cannot be excluded. It is also worth considering that inhibition of Th2 and Tregs cells differentiation may also be achieved by using IL-4 and TGF-β antibodies. But, these antibodies are quite costly and encompass a few disadvantages like antigenicity. We chose to scrutinize small molecule inhibitors that could possibly inhibit Tregs and Th2 cells. In this view we found that suplatasttosylate has the ability to interrupt IL-4 and other cytokines signaling with no effect on IFN-β production (Oda, N., et al 1999). The molecular mechanism behind the mode of action of this compound has not been completely elucidated, but it has been shown that suplatasttosylate inhibits production of IL-4 cytokine by directly affecting T cells (Hennekes, H., and Asadullah, K., 2002). D4476 works as an ALK5 inhibitor and down regulates Smad3 activation and thereby inhibits expression of TGF-β receptor I (Callahan, J. F., et al 2002).
Treatment of mice with MTB infection on currently with these two immunomodulators considerably lowered MTB load in diverse organs of mice. This immunotherapy failed in mice lacking T-bet, signifying that therapy is largely reliant on Th1 cell responses activation. Although the possibility remains that these reagents have some direct effect on MTB beside their natural effect on Treg and Th2 cell. Nonetheless, our observations reveal that immunotherapy using small molecules is effective for TB treatment. The immunomodulatory agents used in this study were found not to act directly against harbored MTB; therefore this therapy should not discriminate between different drug resistant MTB variants. Despite the fact that this therapy against TB treatment is novel, it cannot be used for an extended time. It is well acknowledged that TGF-β has an indispensable role in host immune homeostasis but dominant negative TGF-β RII mice suffered from multi organ inflammation and ultimately death (Kulkarni, A. B., et al 1993; Gorelik, L., and Flavell, R. A. 2000). In the similar view, Th2 inhibition may also results in vulnerability of host toward parasitic infections (van der Werf, N., et al 2013). Therefore, we suggest the use of these modulators in human TB patients for short treatment periods. It is possible that antibiotics can also be used in combination with these immunomodulators which canpotentially help in reducing treatment duration and prevents the generation of drug-resistant TB strains. Our research revealed that, using small molecule inhibitors; concurrent inhibition of Tregs and Th2 cells may have significant potential in TB treatment. Additionally, these chemical agents are cheaper than antibodies and
offer very little complications associated with recombinant immunotherapeutic agents.

The Th1 and Th17 cells have important role in host TB protection (Chatterjee, S. et al 2011). It is worth highlighting here, that BCG induces sufficient Th1 responses, however not protective. Therefore, there are two possibilities that explain the lack of protection of BCG. Firstly, either BCG does not mount an immune response that is critical for optimum host protection, supported by reports indicating that BCG is unable to mount Th17 responses, which may play additional role in host protection (Chatterjee, S., et al 2011, Wareham, A. S., et al, 2011, Samuchiwal, S. K., et al 2014). However, in these reports it has been shown that mounting additional Th17 responses indeed provides better albeit, incomplete protection (Chatterjee, S., et al 2011, Wareham, A. S., et al 2011, Samuchiwal, S. K., et al 2014). Secondly, BCG may produce additional immune responses, which may hinder host protective immune responses. We have shown that inhibition of Th2 and Treg cells by small molecules, significantly enhanced host protective immunity. BCG also induces Th2 and Treg cells, and thus may hinder host protective Th1 and/or Th17 cells. Our results demonstrated that inhibition of Th2 and Treg cells by small molecules enhanced BCG induced vaccine efficacy. Our observation that simultaneous inhibition of Th2 and Treg culminates enhanced host protective immunity however was not sufficient to completely eradicate harbored MTB. Therefore, it appears that a recombinant BCG that can mount both Th1 and Th17 responses, and along with inhibition of Th2 and Treg cells is required to clear off MTB bug.
Although BCG is not effective in adult pulmonary TB, but its effective in children and approximately 4 billion of doses have been administered, a strategy that will support booster BCG, that selectively enhances Th1 and Th17 responses is highly desired. In this respect, a recombinant BCG along with inhibition of Th2 and Tregs may be the strategy that has potential.
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8. PUBLICATION EMANATING FROM THIS THESIS

9. APPENDICES

List of antibodies used in the study

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