INDUCTION OF STRONGER AND LONG LASTING VACCINE IMMUNITY TO TUBERCULOSIS

Pearl Bakhru

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INDUCTION OF STRONGER AND LONG LASTING VACCINE IMMUNITY TO TUBERCULOSIS [DISSERTATION]

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INDUCTION OF STRONGER AND LONG LASTING VACCINE IMMUNITY TO
TUBERCULOSIS

A
DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
And
The University of Texas
M.D Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Pearl Bakhru, B.S., M.S.
Houston, Texas
December, 2012
Dedication

To my parents whose unconditional love and support has encouraged me to stay strong during thick and thin. Also, to my uncle and aunt, who were very instrumental in helping me during the toughest times and made me feel at ease throughout this journey. I love you all immensely.
ACKNOWLEDGEMENTS

With a deep sense of gratitude and respect, I would like to acknowledge my mentor Dr. Chinnaswamy Jagannath for providing me the opportunity to work under his esteemed guidance and being a source of immense inspiration and support during the course of my project. He has been like a father figure to me and his patience and trust in my scientific ability has helped me during the tough times of my PhD.

I would also extend my utmost appreciation to my advisory, exam as well as my supervisory committee members: Dr Diane Bick, Dr Bradley McIntyre, Dr Rick Wetsel, Dr John Klein, Dr Keri Smith, Dr Audrey Wanger, Dr Jagannadha Sastry, Dr Jeffrey Actor and Dr Joseph Alcorn. Their critical reviews and analysis has changed my approach to a scientific problem and their guidance has helped me be a better researcher.

I would like to acknowledge Dr Robert Hunter for his financial support, Dr Dorothy Lewis and her lab members for their help in flow cytometry analysis and NIH vaccine center at Emory University for providing us with the tetramers. I would also want to thank Dr Steve Norris and all the other faculty members involved with Molecular Basis of Infectious Diseases (MBID) program for the financial support as well guidance involved with scientific writing.

My utmost gratitude to all my lab members who are also my dear friends- Dr Arshad Khan, Dr Emily Soudani, Dr Eulogia Roman, Dr Chris Singh, Dr Jaymie Estrella, Dr
Shen-An Huang, Karie Herdtner, Daniel Yuan, Smriti Malasi, and Shruti Malasi for helping me with many techniques and providing me with tremendous assistance during heavy work load. Also, I would like to thank Natalie Sirisaengtaksin and Seema Mukherjee for their help during certain projects.

A huge hug and thanks goes to all my friends for being there for me during the most insane times in my project where I was suffering with data and results and whenever I was feeling low. Their humor, love and support made this journey easy for me and instilled confidence and kept me going. You all are like my family.

Most importantly my sincere appreciation goes to the Graduate School of Biomedical Sciences (GSBS) for allowing me to be a part of this institute and providing me with an amazing opportunity to work with the best people in research.
Abstract

Induction of stronger and long lasting vaccine immunity to tuberculosis

Publication No------------------

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Tuberculosis is a major cause of death due to an infection in mankind. BCG vaccine protects against childhood tuberculosis although, it fails to protect against adult tuberculosis. BCG vaccine localizes to immature phagosomes of macrophages, and avoids lysosomal fusion, which decreases peptide antigen production. Peptides are essential for macrophage-mediated priming of CD4 and CD8 T cells respectively through MHC-II and MHC-I pathways. Furthermore, BCG reduces the expression of MHC-II in macrophages of mice after infection, through Toll-like receptor-1/2 (TLR-1/2) mediated signaling. In my first aim, I hypothesized that BCG-induced reduction of MHC-II levels in macrophages can decrease CD4 T cell function, while activation of other surface Toll-like receptors (TLR) can enhance CD4 T cell function. An in vitro antigen presentation model was used where, TLR activated macrophages presented an epitope of Ag85B, a major immunogen of BCG to CD4 T cells, and T cell derived IL-2 was quantitated as a measure of antigen presentation. Macrophages with BCG were poor presenters of Ag85B while, TLR-7/9/5/4 and 1/2 activation led to an enhanced antigen presentation. Furthermore, TLR-7/9 activation was found to down-regulate the degradation of MHC-II through ubiquitin ligase MARCH1, and also stimulate MHC-II expression through activation of AP-1 and CREB transcription elements via p38 and ERK1/2 MAP kinases. I conclude from vi
Aim-I studies that TLR-7/9 ligands can be used as more effective ‘adjuvants’ for BCG vaccine. In Aim-II, I evaluated the poor CD8 T cell function in BCG vaccinated mice thought to be due to a decreased leak of antigens into cytosol from immature phagosomes, which reduces the MHC-I mediated activation of CD8 T cells. I hypothesized that rapamycin co-treatment could boost CD8 T cell function since it was known to sort BCG vaccine into lysosomes increasing peptide generation, and it also enhanced the longevity of CD8 T cells. Since CD8 T cell function is a dynamic event better measurable in vivo, mice were given BCG vaccine with or without rapamycin injections and challenged with virulent Mycobacterium tuberculosis. Organs were analysed for tetramer or surface marker stained CD8 T cells using flow cytometry, and bacterial counts of organisms for evaluation of BCG-induced protection. Co-administration of rapamycin with BCG significantly increased the numbers of CD8 T cells in mice which developed into both short living effector-SLEC type of CD8 T cells, and memory precursor effector-MPEC type of longer-living CD8 T cells. Increased levels of tetramer specific-CD8 T cells correlated with a better protection against tuberculosis in rapamycin-BCG group compared to BCG vaccinated mice. When rapamycin-BCG mice were rested and re-challenged with M.tuberculosis, MPECs underwent stronger recall expansion and protected better against re-infection than mice vaccinated with BCG alone. Since BCG induced immunity wanes with time in humans, we made two novel observations in this study that adjuvant activation of BCG vaccine and rapamycin co-treatment both lead to a stronger and longer vaccine-mediated immunity to tuberculosis.
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CHAPTER 1

INTRODUCTION
Tuberculosis (TB) is a disease caused by the rod shaped tubercle bacillus Mycobacterium tuberculosis (Mtb) which mainly infects the lungs and brain. In 2007, there were an estimated 13.7 million chronic active cases globally, while in 2010 there were an estimated 8.8 million new cases and 1.5 million associated deaths, mostly occurring in developing countries. There is an increased incidence of TB in Asia and Africa whereas, only about 5-10% of population in USA and Europe are affected (61). The main reason behind this disparity is the prevalence of AIDS in the developing countries where TB co-infection occurs as a consequence of HIV-infection induced reduction in CD4 T cell mediated immunity (105).

Symptoms of TB are chronic cough with blood tinged sputum, weight loss, fever and night sweats. If TB spreads to other organs, the symptoms are widely distributed (57). To diagnose active TB, the most important method is either the detection of mycobacteria in sputum or the X-ray of lungs which detects granuloma, which is a typical histological presentation of TB. For diagnosis of latent TB, physicians rely on tuberculin/PPD skin test or an Interferon-γ release (IGRA) test performed on blood samples. Treatment of TB is intensive and requires a long time to completely clear the pathogen. Health care workers recommend directly observed therapy (DOTS) for comprehensive treatment of TB (40). It is emphasized in case of patients suffering from multi-drug resistant (MDR) TB, patients with HIV-1 and co-infection with TB (82). Prevention in developing countries relies on administering the vaccine BCG soon after birth.
Pathogenesis: In advanced stages TB can also spread to other parts of the body. Transmission of TB usually occurs through aerosol route; when an infected person sneezes or coughs, the TB droplets are released into the air and the latter can infect a healthy individual (33). Most of the infections are latent or asymptomatic and only about 10 % of infected humans develop active disease, which if not treated can have serious consequences (112).

Bacillus Calmette-Guérin (or Bacille Calmette-Guérin, BCG) is a vaccine which is an attenuated form of Mtb derived from *Mycobacterium bovis*, which usually infects the cattle and other animals. BCG is less virulent because it has been subcultured for 230 passages in an artificial medium for over 13 years (65). BCG still holds its strong immunogenicity and thought to be an effective and safe vaccine in children. It is the most widely used and distributed vaccine by WHO and is the first vaccine given to children immediately after birth (87). However, BCG has lost some gene segments (e.g., RD1- immunodominant region present in both *M.bovis* and Mtb) during its long term-passage *in vitro*, and it has been thought that this is the reason why it protects children only up to 5-10 years.

Variable Efficacy of BCG vaccine. The first large scale trial evaluating the efficacy of BCG was conducted from 1956 to 1963, and involved 54,239 school children who received BCG at the age of 14 or 15; this study showed an efficacy of 84% up to 5 years after immunization. However, a US Public Health Service trial of BCG in Georgia and Alabama published in 1966, showed an efficacy of only 14% and did
much to convince the US, that it did not want to implement mass immunization with BCG. A further trial conducted in South-India and published in 1979 (the "Chingleput trial"), also showed no protective effect due to BCG. The duration of efficacy is not exactly known for BCG vaccine. However, meta-analysis of data from different trials indicate that protection level of 80 % decreases after 15 years of age and by the time the individual is 20, the protection is almost close to zero (4). The most important effect of BCG is preventing miliary TB and TB meningitis in different parts of the world. This is one of the most important reasons why BCG is still administered in developing countries and is an essential part of childhood immunization (24).

**Reasons for variable efficacy:** Many reasons have been cited for the variable efficacy of BCG; although most remain to be explained.

a) *Genetic variations in BCG strains:* Because BCG has been passaged for a number of times, there have been genetic modifications (deletions) in the strains. A major example is the deletion of RD1 operon that encodes for the secreted immune-dominant antigens ESAT6 and CFP10 conserved in both *M. bovis* (parent of BCG) and in wild type Mtb (94).

1) *Genetic variations in human population:* The genetic makeup of human populations in different countries and their diet and lifestyle may have a strong influence on the vaccine efficacy of BCG strain (46). However, interesting data emerged from the Birmingham BCG trial published in 1988. The trial was based in Birmingham, United Kingdom, and examined children born to families who originated from the Indian subcontinent (where vaccine
efficacy had previously been shown to be zero). The BCG trial showed a 64% protective effect, which is very similar to the figure derived from other UK trials, arguing against the human genetic variation hypothesis.

2) *Interference by non-tuberculous mycobacteria*: Humans are also exposed to environmental (*M. vaccae*) or non-tuberculous mycobacteria (*M. Kansasi*) (76). This can generate a non-specific immune response to the mycobacteria and administering BCG may become less effective. There is clinical evidence from parallel studies performed on adolescent school children in the UK and Malawi. In this study, the UK school children had a low baseline cellular immunity to mycobacteria which was increased by BCG; in contrast, the Malawi school children had a high baseline cellular immunity to mycobacteria which was not significantly increased by BCG. This was thought to be due to the induction of suppressive immunity by environmental mycobacteria. An alternative explanation was suggested by mouse studies: immunity against mycobacteria prevents BCG from replicating and preventing from its efficient induction of immune responses. This is called as the blocking hypothesis.

3) *Interference by concurrent parasitic infection in humans*: Simultaneous infection with parasites may change the immune response to BCG, making it less effective. A T-helper 1 (Th1) response is required for an effective immunity to tuberculosis; parasites induce a counter-productive Th2-response which blunts the Th1-inducing effect of BCG (89).

4) *Exposure to ultraviolet light*: Concentration of ultraviolet light (particularly UV-blue) from the Sun may have some effect on efficacy of the BCG vaccine.
UVB light has been demonstrated to reduce efficacy of BCG vaccine in guinea pigs. The concentration gradient of UVB light increases geographically closer to the Earth's equator. It is possible, though currently unproven that this effect may occur as a result of sunlight-dependent Vitamin-D production in humans (50).

5) **Immunological sequestration of BCG in antigen presenting cells.** BCG vaccine has a mechanism to evade the host immune responses by sequestering within phagosomes that do not fuse with lysosomes (97). Lysosome mediated degradation of BCG antigens is a pre-requisite for activation of CD4 T cells through MHC-II and this has been thought to affect the efficacy of Th1 immunity induced by BCG. It is also known that BCG phagosome membrane is not permeable enough for antigens to leak across into cytosol preventing cross-presentation (41). These aspects are further discussed below under the context of peptide processing mechanisms.

**Immune responses against Tuberculosis.** For the optimal protection against TB, T lymphocytes are the sentinel cells used for defense mechanism. Macrophages and dendritic cells (DCs) (antigen presenting cells; APCs) present antigens from Mtb or BCG vaccine to T cells through the surface MHC-I and MHC-II molecules. T lymphocytes defend against pathogens through 3 distinct mechanisms; a) CD4$^+$ T cells recognize peptide antigens via Class II Major Histocompatibility complex (MHC-II) (10). b) CD8$^+$T cells recognize peptide antigens via Class I Major Histocompatibility complex (MHC-I) (17) and c) in addition, gamma delta (γδ) T cells
and CD1 restricted T cells may play a significant role at mucosal surfaces (17). B-cell mediated responses and antibody responses are evident in tuberculosis but have not been shown to be associated with protection (114). Thus, current vaccine improvement strategies are focused on improving T lymphocyte activation against Mtb.
Figure 1: Major immune mechanisms involved in immunity to tuberculosis and relevance of immune cells for vaccine development. *Mycobacterium tuberculosis* (Mtb) and BCG vaccine persist within the phagosomes of infected macrophages or dendritic cells (DCs). Peptides from these organisms are routed through MHC-II and MHC-I pathways to activate respectively CD4 and CD8 T cells. The latter are effector T cells that secrete active cytokines and through feedback activation drive macrophages to kill intracellular mycobacteria. CD8 T cells are thought to persist as long living memory T cells and can kill macrophages with Mtb or BCG through cytolytic granules. Better understanding of antigen processing in tuberculosis, and the role of CD4 and CD 8 T cells in immunity will facilitate future vaccine design against tuberculosis (9).
The peptide processing mechanisms that leads to Th1 immunity. The MHC-II pathway of peptide presentation depends upon the lysosomal degradation of microbial antigens to peptides. The peptides are then transported to the MHC-II containing compartments (MIIC) in APCs (99). They are assembled into MHC-II and sorted to plasma membrane. Some pathogens like Mtb and BCG vaccine have an atypical pathway and macrophages can assemble a limited number of peptide epitopes in their phagosomes, since the latter contain MHC-II as well as peptide cleaving enzymes. However, both BCG and Mtb sequester in phagosomes that do not fuse with lysosomes and this seriously limits the levels of peptides that can be produced from degradation of the total proteins of the organisms (97). BCG also suppresses the MHC-II expression by macrophages. The mechanisms of intervention that can bypass this block in production of peptides are a major aim of this thesis.

The MHC-I pathway usually presents self-peptides on the surface of APCs. The de novo synthesized antigens in the cytosol undergo proteolysis by proteasomes and the peptides generated get transported to the endoplasmic reticulum via a ‘transporter associated with antigen processing molecule’ (TAP). TAP then sorts the peptides to MHC-I loading sites, and the MHC-I-peptide complex is exported to plasma membrane (102). A Cross-presentation mechanism allows processing of exogenous antigens from phagocytosed microbes via MHC-I. These are at least three cross-presentation mechanisms through which M. tuberculosis or BCG vaccine antigens are loaded into MHC-I (52) (42). First, the antigens leak from phagosomes into lysosomes of Mtb or BCG infected cell for direct cross
presentation. Secondly, Mtb or BCG infected cells produce exosomes, that can be taken up by bystander dendritic cells or macrophages, which then present antigens via MHC-I. In the third mechanism, the Mtb or BCG infected cells can undergo apoptosis and release the apoptotic bodies containing antigens, which can be taken up by bystander APCs (15). The relative efficacy of these three mechanisms is unclear. However, in the context of this proposal, BCG phagosomes are not leaky enough that peptides do not get access to cytosol. *A mechanism to enhance peptide production and activate CD8 T cells for a better function is the second major aim of this proposal.*

**Role of Toll-like receptors (TLRs) in immunity and pathogenesis.** TLRs are similar to the toll-gene products of *Drosophila*, and the ten mammalian TLRs can recognize specific molecular patterns of a microbe, and generate protective immune responses (59). The TLRs utilize the adaptor protein MyD88 and downstream signals affect specific gene targets and transcription factors. Thus, MyD88 can activate NF-κB and trigger production of cytokines (7). MyD88 plays an important role in combating tuberculosis infection and MyD88 deficient mice are markedly susceptible to tuberculosis, dying within 4 weeks after infection (22). TLRs are known to link both innate and adaptive immunity. Thus, MyD88 can induce the ‘inducible nitric oxide synthase’ (iNOS) mediating nitric oxide secretion, and MyD88 deficiency affects the production of Th1 cytokines like TNF-α, IL-6 and IL-12p40; major mediators of Th1 immunity. When the lungs of MyD88 deficient mice were examined, a massive infiltration of mononuclear cells and neutrophils occurred.
during tuberculosis, but interestingly, there was no difference in the recruitment and numbers of CD4 and CD8 T cells (38). Thus, MyD88 may selectively affect immune responses.

While TLRs are emerging as the major mediators of protective immunity, it has also been recognized that certain lipids of mycobacteria can in fact induce immune-suppression in macrophages and DCs. The lipid components of the cell wall of both Mtb and BCG are known to reduce macrophage function and down-regulate MHC-II expression (80) (37). Thus, it appears that pathogens such as Mtb have evolved to modulate phagocyte function to survive within. Likewise, due to its cell wall lipids, BCG vaccine also has the same suppressive influence (20). In our proposal we propose to examine whether we can activate selective TLR-pathways to positively regulate the function of macrophages and DCs.

Relative role of CD4 and CD8 T cells during Th1 immunity. It is known that CD8 T cell depleted mice or mice which have a defective β2-microglobulin or TAP1 gene (making it MHC-I deficient) do not effectively control tuberculosis compared to control wild type mice (39). In striking contrast, depletion of CD4 T cells in mice rapidly leads to death due to tuberculosis. In humans as well, depletion of CD4 T cells during HIV-1 infection rapidly leads to active tuberculosis and accelerated death due to infection (78). Thus, CD4 T cells seem to control acute infection. In humans perhaps it is the CD8 T cell that controls infection over long term. This function is consistent with the ability of CD8 T cells to develop into long living memory T cells (54). Whether long living CD4 cells occur during tuberculosis of
mice or humans remains unclear. CD8 T cells also generate cytotoxic T lymphocytes which induce perforin and granzyme-B mediated killing of Mtb or BCG infected macrophages and DCs (67). Whether long living CD8 T cells play a role in the control of tuberculosis remains unclear and we propose to investigate this aspect in one of our aims.

The mouse is a good tuberculosis model to dissect the relative importance of CD4 and CD8 T cells during short term protection against tuberculosis. However, the phenotype of T cells mediating effector and memory functions still remains unclear in tuberculosis. One of the reasons is that antigen specific, tetramer positive CD8 T cells were found infrequently in BCG vaccinated mice. Research has indicated a limited set of antigen specific CD8 T cells that could be associated with protection in mice. When the CD8 T cell epitopes were analyzed to check for the most immune-dominant regions of TB, the homologous proteins of the early secretory antigenic target-6 kDa (ESAT-6) family: TB10.3 and TB10.4 were discovered (31). TB10.43-11- specific CD8 T cells seem to be important during the infection because they are recruited to the lungs, and express the Th1 cytokines TNF-α and IFN-γ, and they up-regulate the expression of FasL and LAMP-1/2 (CD107A/B) upon activation (110). Such cells are also present in secondary lymphoid organs like spleen. However, the expansion and contraction of CD8 memory T cells and their specific recall expansion after re-infection are relatively unknown. In this proposal, we will investigate these events associated with BCG vaccination to better understand vaccine mediated immunity.
Rapamycin as an immune-regulator. The role of memory T cells in protecting against infection is better understood in viral infections, several of which induce specific CD8 T cell responses in mice. Rafi Ahmad et al. have used the Lymphocytic-choriomeningitis virus infection (LCMV) of mice to demonstrate the memory CD8 T cell responses (2). These studies revealed an interesting observation that low doses of Rapamycin can have an immunomodulatory effect. Rapamycin is a well-known immunosuppressive drug used in anti-cancer therapy and in transplant patients. Rapamycin effects are dose-dependent and it induces FoxP3 regulatory T cells, inhibits type I interferon production by plasmacytoid dendritic cells and modulates T cell trafficking (91). It binds to the FKBP12 protein and forms an inhibitory complex which suppresses the mTORC1 pathway (23).

During LCMV infection of mice, Rapamycin treatment increased CD8 T cells compared to untreated but virus infected mice. Memory CD8 T cells expressing markers CD127, CD62L and Bcl2 were increased while KLRG-1 marker was reduced (2). These data suggested that inhibition of mTOR pathway using Rapamycin not only increased the magnitude of the virus specific CD8 T cell response, but also improved the functional quality of the memory CD8 T cells since memory cells with the phenotype (CD127$^{\text{High}}$CD62L$^{\text{High}}$Bcl-2$^{\text{High}}$ and KLRG-1$^{\text{Low}}$) are associated with long-lived protective immunity. *These studies now provide a model for investigating the emergence and maintenance of memory T cells during BCG vaccination in this proposal.*
Potential approaches for improving BCG vaccine:

Aim-I: Investigate whether BCG induced suppression of MHC-II leads to reduced antigen presentation in macrophages and this process can be enhanced through Toll-like receptor activation.

Hypothesis: Since BCG suppresses MHC-II, we generated a hypothesis that TLR-activation in macrophages increases MHC-II expression and enhances antigen presentation to CD4 T cells. This would enhance the efficacy of the vaccine.

Results: We show that BCG suppresses antigen presentation to CD4 T cells and this is due to the lipids of its cell wall. Using an *in vitro* antigen presentation where BCG derived-Ag85B is presented by macrophages and DCs to CD4 T cells, we then show that activation of TLRs increases antigen presentation. We report that TLR-ligands (7/9) were the most effective to enhance MHC-II levels in BCG. They reduce the degradation of MHC-II, through down-regulation of ubiquitination by MARCH1 enzyme. They increase surface expression of MHC-II through a process dependent upon p38/ERK-1/2, AP-1 and CREB activation. TLR-7/9 ligands can be therefore used adjuvants with BCG vaccine.

Aim-II: Investigate whether MHC-I induced activation of CD8 T cells in BCG infected mice can be enhanced using Rapamycin co-treatment.

Hypothesis: Rapamycin enhances the function of APCs and also affects the longevity of CD8 T cells. We therefore developed a hypothesis that rapamycin
enhances autophagic delivery of BCG vaccine into lysosomes increasing peptide generation and routing of peptides into MHC-I for activation of CD8 T cells. We further proposed that Rapamycin would enhance the function of CD8 T cells and prolong the vaccine-induced protection against tuberculosis.

**Results:** We found that Rapamycin has a dual effect in modulating APCs as well as CD8 T cells. Rapamycin was found to increase the numbers of antigen specific CD8 T cells both during natural tuberculosis infection of mice and after vaccination with BCG. Rapamycin enhanced the efficacy of BCG vaccine through increasing the memory precursors as well as effectors, affecting both CD4 and CD8 T cells. Finally, Rapamycin enhanced the longevity of CD8 T cells by expanding central memory T cells which persisted and showed a robust recall protection of mice against re-challenge with tuberculosis. Thus rapamycin enhanced the short term and long term efficacy of BCG vaccine and helped to identify Eomes as biomarker for long living memory T cells.
CHAPTER-2.1

ACTIVATION OF TOLL-LIKE RECEPTORS (TLRs)
ENHANCE EXPRESSION OF MHC-II IN BCG INFECTED
MACROPHAGES
**Background**

Efficacy of BCG vaccine in adult pulmonary tuberculosis is variable, and it ranges anywhere between 0-80% in protecting adults from developing an acute infection. There are many reasons explaining the variability like antigenic drift, nutritional discrepancies amongst humans, difference in ethnic groups and exposure of individuals to other kinds of mycobacteria. It is known that BCG vaccine does not protect for a long period of time and strikingly, booster doses with BCG also do not boost the vaccine efficacy. This strongly indicates that immune memory mechanisms are defective or do not last longer following BCG vaccination. Interestingly, Rook et al. have proposed that BCG induced Th1 response switches to Th2 type, particularly in tropical regions where parasitic infestations are frequent (90). BCG also fails more often to protect children in this region. Whether progressive Th2 responses in humans interfere with memory development is not clear.

**Figure 2:** Antigen presenting cells (APCs) process peptides from BCG vaccine to activate T cells via MHC-I and MHC-II pathways.

Research over the past decade has identified that BCG is immunologically sequestered in phagocytes like macrophages and dendritic cells (Fig.2). This event may affect the
ability of phagocytes to cross-talk with T cells, which mediate Th1 immunity to tuberculosis. It is known that, antigen presenting cells like macrophages and dendritic cells (APCs) present antigenic peptides of BCG to CD4 or CD8 T cells via MHC-II and MHC-I, respectively. The bacterial peptides are usually generated by the proteolytic breakdown of BCG derived antigens in the lysosomes, which needs BCG phagosomes to fuse with lysosomes. However, there are two major flaws in this antigen processing mechanism. First, BCG sequesters within immature phagosomes of macrophages and does not fuse with lysosomes. This affects peptide generation before loading into MHC-II (97). Secondly, the phagosome membrane of BCG is not permeable to peptides as much, and this prevents leakage of peptides into cytosol, where they are normally processed by proteasomes to generate peptides for further loading into MHC-I (12, 64). This reduces efficacy of MHC-I pathway in BCG infected APCs.

Figure 3: Processing and presentation of peptides from BCG vaccine is defective in antigen presenting cells (APCs) which reduces activation of T cells.

A third defect became apparent when BCG was found to inhibit the expression of MHC-II in macrophages and DCs (92). Lipids of BCG were found to suppress MHC-II on macrophages via the TLR
pathway (20) (Fig.3). Since MHC-II molecules load peptides and present to CD4 T cells to trigger the major component of Th1 immunity, we hypothesized that lack of lysosomal fusion of BCG in APCs coupled with reduced MHC-II expression may seriously affect the ability of APC to activate T cells. In phagocytes, MHC-II is constitutively synthesized but its expression can be up-regulated in response to infection or treatment with IFN-γ. Unloaded MHC-II, misfolded MHC-II or membrane internalized MHC-II can all be labeled with ubiquitin and targeted to proteasomes and lysosomes for breakdown (32). This process is mediated by a group of ubiquitin labeling enzymes. Membrane-associated RING-CH (MARCH) proteins like MARCH1 represent a family of E3 ubiquitin ligases, which contain a variant catalytic RING-finger domain (RING-CH domain, C4HC3) located at the N-terminus (30). Earlier studies in mouse DCs proposed that ubiquitination of lysine-225 in the cytoplasmic tail of MHC-II β-chain through MARCH1 is a determinant for the targeting and accumulation of the mature form of MHC-II in the lysosomes of DCs (111). Loss of MHC-II ubiquitination was accompanied by the down-regulation of MARCH1 expression; therefore, MARCH1 is thought to be an important regulatory molecule of MHC-II expression (30).

**Specific Hypothesis:** Mycobacteria like BCG contain lipoproteins (LP) like the 19 kDa LP and lipo-arabinomannan (LAM), which are ligands for Toll-like receptor, TLR-1/2 (37). We hypothesized that these lipids suppress MHC-II expression in macrophages, and thereby suppress antigen presentation to CD4 T cells. Since the lipids are contained in BCG, we anticipate that BCG by itself will cause down-
regulation of MHC-II through up-regulation of MARCH1 enzyme in macrophages. Finally, we propose that alternate activation of TLRs other than TLR-1/2 can bypass BCG mediated suppression of MHC-II.

**Methods:** Even though live BCG does not undergo lysosomal fusion, limited peptide antigen processing occurs in BCG infected macrophages. This has been thought to be due to sorting of soluble Ag85B to lysosomes, sorting of dead BCG to lysosomes or limited antigen presentation within phagosome itself (13). The BB7-CD4 T cell hybridoma cell line is specific for p25 epitope of Ag85B, a major secreted immune-dominant antigen of BCG. When these T cells are overlaid on macrophages containing BCG, IL-2 is secreted, which is dependent upon their specific recognition of p25 epitope from Ag85B bound to MHC-II on macrophages. This process does not occur when Ag85B negative strain of BCG or *M.tuberculosis* is used to infect macrophages (97). We therefore used this well established, *in vitro* system to investigate whether, BCG infected macrophages present Ag85B epitope to CD4 T cells (BB7 T cells), and study the effect of lipids and other activators on macrophages (Fig.4).

**Figure 4:** An *in vitro* antigen presentation to CD4 T cell assay using macrophages and DCs infected with BCG vaccine.
Results

Lipids of BCG decrease in vitro antigen presentation. Macrophages were infected with BCG alone or were treated with increasing doses of lipids (19 kDa LP and LAM) as indicated. (Fig.5). Macrophages were overlaid with BB7 T cells and supernatants analyzed for IL-2 using ELISA. Fig.5 shows that both 19 kDa lipoprotein and LAM suppressed antigen presentation in a dose-dependent manner. Since LP and LAM are integral components of cell wall in BCG and are shed from growing bacteria, it follows that BCG by itself suppresses antigen presentation.

Since BCG can decrease MHC-II levels, but may also exert additional non-specific effects, we sought to determine whether lipids could interfere with the processing of soluble Ag85B. Macrophages were therefore treated with soluble Ag85B, followed two hour later by T cell overlay. Macrophages processed Ag85B and activated T cells to secrete IL-2 (Fig.6). Prior treatment of macrophages with lipids however, suppressed antigen presentation.

Macrophage surface MHC-II levels determine peptide presentation while, intracellular MARCH1 enzyme determines degradation of MHC-II. Thus, BCG infected macrophages were analyzed using flow-cytometry. BCG induced a down-regulation of surface MHC-II while, concurrently, intracellular MARCH1 was up-regulated (Fig.7).
Figure-5: Macrophages (MΦs) infected with *Mycobacterium bovis* BCG vaccine present Ag85B epitope to CD4 T cells (BB7) and induce IL-2 secretion. Lipids of BCG suppress IL-2 responses. C57Bl/6 derived primary macrophages (MΦs) were infected with BCG, and tested alone or were added with lipids as indicated for 4 hrs. Washed macrophages were overlaid with BB7 T cells and supernatants analyzed for IL-2 using ELISA. Lipids (19 kDa lipoprotein and lipoarabinomannan, LAM) suppress IL-2 elicitation. Data from one of three separate similar experiments are shown. Triplicate wells of macrophages per dose per reagent in each experiment (mean values of IL-2 expressed ± SD; p values by t test).
Figure-6: Lipids of BCG suppress presentation of soluble Antigen-85B of BCG vaccine from macrophages (MΦs) to CD4 T cells. MΦs were treated with 100 ng/mL of recombinant Ag85B (from BEI, NIH) and after 2 hrs, washed and overlaid with BB7 T cells for 4 hrs. Supernatants were tested for IL-2 as in Fig.5. MΦs were tested untreated or treated with 19 kDa lipoprotein and LAM as in Fig.5, prior to the addition of Ag85B. MΦs treated with lipids alone without antigen, and MΦs with BCG alone are shown as controls. Data from one of two separate similar experiments are shown. Triplicate wells of macrophages per reagent in each experiment (mean values of IL-2 expressed ± SD; ** p< 0.009, t test, 2 experiments).
Figure-7: BCG infected macrophages show suppression of MHC-II surface expression which correlates with an enhanced expression of intracellular MARCH-1 ubiquitin ligase. C57Bl/6 derived BMA.A4 MΦs were infected with BCG and were tested 4 hrs later for surface expression of receptors using specific antibodies, isotypes and flow cytometry. BCG infection down-regulates MHC-II but up-regulates ubiquitin ligase MARCH-1 in MΦs. One histogram is shown of three similar experiments.
**TLR-ligands increase in vitro antigen presentation.** Macrophages were infected with BCG with or without prior activation using various TLR ligands. Fig.8 shows that prior activation with multiple TLR-ligands increased antigen presentation by macrophages. Ligands for TLR-9, TLR-7 and TLR-5 were better activators than ligands for TLR-4 or TLR-1/2. Ligands for TLR-3 and TLR-6 had no beneficial effect.

**TLR-ligands down-regulate MARCH-1 in BCG infected macrophages and increase MHC-II expression.** Since the levels of MHC-II and MARCH1 in macrophages were inversely correlated during antigen presentation following BCG infection, the effect of various TLR-ligands on MHC-II and MARCH1 in macrophages was evaluated using flow-cytometry. Fig.9 illustrates that macrophages activated with various TLR-ligands show two profiles. Some like TLR-9/7 ligands induced a strong up-regulation of MHC-II while decreasing MARCH1. Others like TLR-1/2 ligand induced a down-regulation of MARCH1 but had no affect the overall levels of MHC-II. Yet others had no effect on either MHC-II or MARCH1. None of the ligands affected the levels of MHC-I, indicating that TLR-activation affected only MHC-II expression.

**TLR-ligands affect CD86 and intracellular IL-10 in BCG infected macrophages.** The co-stimulatory molecules CD80 and CD86 participate during MHC-II mediated peptide presentation to CD4 T cells. Fig.10 demonstrates that TLR-9/7 ligands up-regulate the expression of CD86 but not CD80 in macrophages. Furthermore, IL-10, a suppressive cytokine has been found to affect the levels of intracellular MARCH1.
Figure-8: Alternate activation of Toll-like receptors in macrophages infected with *Mycobacterium bovis* (BCG vaccine) enhances antigen presentation in *vitro*. C57Bl/6 derived primary macrophages (BMs; MΦs) were left untreated or activated with various TLR-ligands as indicated (1 µg/mL each) followed by infection with BCG vaccine (MOI=1) for 4 hr. Washed macrophages were overlaid with Antigen-85B specific BB7 T cells (1:20) and IL-2 in supernatant collected 4 hrs later was measured using sandwich ELISA. Prior TLR activation enhances antigen presentation. Triplicate wells of macrophages per reagent in each experiment (mean values of IL-2 expressed ± SD; p values shown above bars vs. BCG alone; t test). One of 3 similar experiments is shown.
**Figure-9:** TLR-activation of macrophages infected with BCG vaccine enhances the expression of surface MHC-II and induces down-regulation of MARCH1. C57Bl/6 macrophage derived BMA.A4 macrophage cell line (MΦs) were activated with TLR-ligands as in Fig.8, followed by infection with BCG for 4 hrs, and stained for surface receptors or intracellular MARCH1. Cells were analyzed by flow cytometry and representative histograms are shown. Data in Table shows up- or down-regulation of MHC-II and MARCH1 scored as log increase or log decrease averaged from three separate experiments. TLR-agonists enhance MHC-II expression but down-regulate ubiquitin ligase MARCH1, but have no effect on MHC-I expression by MΦs.
Figure-10: TLR-activation of macrophages infected with BCG vaccine enhances CD86 and down-regulates intracellular IL-10. Macrophages were activated and infected as in Fig.8, and stained for surface receptors or intracellular IL-10. Cells were analyzed by flow cytometry and representative histograms are shown. Data in Table shows up-or down regulation of CD80, CD86 and IL-10 scored as log increase or log decrease averaged from three separate experiments.
Ligands for TLR-1/2, TLR-4, TLR-7 and TLR-9 induced a down-regulation of IL-10. To determine if TLR ligands could act in synergy, a combination of TLR-ligands was tested on BCG infected macrophages. Again, only a combination of TLR-9 and TLR-7 ligands induced elevated MHC-II and reduced MARCH1 in macrophages (Fig.11). Generally therefore, TLR activation favored upregulation of MHC-II.

**siRNA knock-down of MARCH affects MHC-II levels in macrophages.** Since MARCH1 is known to ubiquitinate MHC-II and facilitate its degradation, macrophages were subjected to knock-down using siRNA vs. MARCH1 (Santa Cruz Biotechnology) or scrambled control sequence for 18 hrs. Rested macrophages were infected with BCG for 4hrs and surface expression of MHC-II was measured using flow cytometry. Fig.12 shows that knock-down of MARCH1 increased surface expression of MHC-II, even in BCG infected macrophages.
Figure-11: Combined TLR-activation of macrophages infected with BCG vaccine enhances the surface MHC-II and down-regulates MARCH1. Macrophages were activated (1 μg/mL of ligands) and infected as in Fig.8, and stained for surface receptors or intracellular MARCH1. Cells were analyzed by flow cytometry and representative histograms are shown from two separate experiments.
Figure-12: siRNA knockdown of MARCH1 in macrophages increases surface expression of MHC-II. Macrophages were treated with siRNA vs. MARCH1 (Santa Cruz Biotechnology) or scrambled control siRNA for 18 hr, rested for 18 hr, and infected with BCG for 4 hr. Surface expression of MHC-II was measured using flow cytometry, and data were expressed as log positive cells. Knock-down of MARCH1 increases surface expression of MHC-II, even in BCG infected macrophages. Data averaged for two separate experiments (p value, t test; ± SD of 2 experiments).
TLR-ligands decrease ubiquitination of MHC-II in BCG infected macrophages: MARCH1 is known to ubiquitinate MHC-II and ubiquitinated MHC-II is routed through proteasome for degradation. To validate that TLR-activation led to a decrease in ubiquitinated MHC-II, macrophages were activated with TLR-ligands, infected with BCG and their lysates were immune-precipitated (IP) with an antibody to MHC-II. The IP was then labeled with an antibody to polyubiquitin. Fig.13 indicates that TLR-ligands (7>5>9>3>1/2) reduced the levels of ubiquitinated MHC-II relative to MHC-II from BCG infected macrophages.
Figure-13: TLR-activation decreases the ubiquitination of MHC-II in BCG infected macrophages. MΦs of duplicate wells were activated with TLR-ligands (1 μg/mL for 18 hr), infected with BCG (MOI=1) for 4 hr, lysed and pooled. An antibody to MHC-II antibody was used to capture MHC-II from lysates at 4°C overnight and the immune-precipitate was subjected to western blot using another antibody to polyubiquitin. MΦ-activation with TLR-agonists for 9/7/5 decrease the levels of ubiquitinated MHC-II. One of three similar experiments is shown. MΦ lysate used for fractionation was loaded for actin control and the IgG control used for IP is also shown.
Discussion

Mouse infection and vaccination experiments have shown that BCG induces largely a CD4 T cell type of response with less strong CD8 T cell response [90]. Yet in the mice, BCG is barely able to control infection with Mtb. Several studies propose that the sequestration of BCG in immature phagosomes of APCs and the ability of live BCG to inhibit MHC-II is one main reason why APCs do not efficiently cross-talk to T cells. Such T cells are therefore poorly effective against Mtb in mice. We therefore investigated the hypothesis that moieties contained in BCG inhibit MHC-II. We used an *in vitro* antigen presentation that confirmed that lipids of BCG inhibited antigen presentation and since these lipids are integral in the cell wall of BCG, it seemed to have an inherent ability to suppress antigen presentation.

Macrophages sense pathogens and bacteria through specific Toll like receptors (TLRs) and LP and LAM lipids of BCG are known to mediate their suppressive effects through TLR-1/2. However, we hypothesized that alternate activation of TLRs may help bypass the suppressive effect of BCG on APCs. Toll like receptors (TLRs) are single membrane spanning non-catalytic receptor proteins and are similar to the toll gene products of Drosophila. They are present in the membranes of both vertebrates and non-vertebrates. They recognize and distinguish between special microbial patterns and hence allow the cell to distinguish between self and non-self (16). TLRs link innate immunity to adaptive response and most human tissues contain at least one type of TLR while, APCs express multiple TLRs (75). While TLR-1/2 induced a negative effect through BCG, we sought to activate other
TLRs. In this experiment, interestingly, TLR-9/7/5/4 &1/2 ligands enhanced antigen presentation indicating that, BCG induced suppressive effect on antigen presentation can be bypassed. This was not surprising since TLRs mediate their effects on signaling and cytokine secretion in APCs using multiple pathways, and it was anticipated that TLR-1/2 induced suppressive effects on antigen presentation could be neutralized. During MHC-II mediated priming of CD4 T cells, additional stimulation is required by co-stimulatory molecules like CD80 and CD86 for optimum activation (107) (27). For example, peptide presentation without co-stimulation can induce tolerance. It was therefore interesting to note that TLR-9 ligand boosted the CD86 response in addition to MHC-II in macrophages.

We also found that, intracellular MARCH1 levels were inversely correlated with surface expression of MHC-II confirming the regulatory role of MARCH1 on recycling of MHC-II. Many TLR ligands enhanced MHC-II through a down-regulation of MARCH1. They also induced a down-regulation of IL-10, a cytokine known to induce MARCH1 and thus negatively affect MHC-II levels (36). We noted however that there was some variability in the ability of TLR ligands to down-regulate MARCH1, enhance MHC-II, and increase antigen presentation. Only TLR-9/7 ligands performed all three functions. TLR-1/2 ligand down-regulated MARCH1 but did not significantly enhance MHC-II. Since intracellular loading of peptides into MHC-II, MHC-II recycling, and sorting of peptide loaded MHC-II to surface membrane together determine the eventual expression of MHC-II on plasma membrane, we propose that TLR-ligands perhaps influence different stages of the
MHC-II mediated peptide presentation. It is however clear that, at least TLR-9/7 ligands can be used to enhance the immunogenicity of BCG vaccine \textit{in vitro} and in animal models. Our data are novel in that; previous studies have only used TLR-4 or TLR-1/2 ligands to enhance the efficacy of subunit vaccines to tuberculosis. This study has identified more potent TLR-7/9 ligands as adjuvants to improve vaccines against tuberculosis.
CHAPTER-2.2

TOLL-LIKE RECEPTOR (TLR) LIGANDS ENHANCE MHC-II IN MACROPHAGES THROUGH THE ACTIVATION OF MAP-KINASES AND AP-1 OR CREB TRANSCRIPTION FACTORS
Background

MHC-II is required for activation of the CD4 T cells, which are obviously important to control multiple infections. In humans, depletion of CD4 T cells through AIDS results in extensive cross infection with tuberculosis and accelerated death. MHC-II is a crucial immune determinant and its abnormal expression or decreased expression can have strong pathological effects (47). Mitogen activated protein kinases (MAPKs) are highly conserved in eukaryotes and are important signaling components that regulate cytokine responses and transcription of cellular factors in multiple cell types. There are three major groups of MAPK in mammalian cells: the extracellular signal-regulated kinase (ERK), the p38 MAPK and the c-jun NH2-terminal kinase (JNK) (25, 113). When the cell receives an extracellular stimulus, MAPKs are phosphorylated and they mediate signaling for cellular transcription and induce cytokine secretion. MAPKs also activate transcription factors (TFs) like the cyclic AMP response element binding protein (CREB) and Activator protein (AP-1), which bind the Class II transactivator (CIITA), a master regulator of MHC-II synthesis (5) (66). The signaling pathways triggered by TLR ligands are illustrated in Fig. 14 and Fig.15.

Specific Hypothesis

Our recent studies (Chapter 2.1) showed that multiple TLR ligands induced an up-regulation of MHC-II in macrophages. We hypothesized that TLR-ligands regulated MHC-II through MAPK cascade and AP-1 or CREB transcription factors. We proposed that differences in the levels of MHC-II induced by TLR ligands could be attributed to specific signaling pathways.
Figure-14: TLR-4 induced MAPK activation: All TLRs (TLR4 shown here as an example) mediate signaling through MyD88 to IRAK1/2/4-TRAF6 complex (83). TLR-4 induced pathway of activation of AP1 and CREB are shown to illustrate that individual TLR-ligands may signal through multiple overlapping mechanisms. MAPKs thus occupy a pivotal role (42).
Figure-15: TLR-activations converge at TRAF-6 and MAPKs (p38 MAP, ERK1/2, JNK), which lead to activation of transcription factors AP-1 or CREB. The latter two bind the Class II transactivator (CIITA), a master regulator of MHC-II synthesis. Alternate mechanisms of CIITA activation of MHC-II via NF-kB may also exist.
Results:

TLR-activation induced MHC-II expression in macrophages is dependent upon activation of p38 MAPK, ERK1/2 and AP-1 or CREB mediated activation of MHC-II. MΦs were incubated with inhibitors of MAPKs (p38, ERK1/2 and JNK), AP-1 or CREB (all tested at 1 μM) for 2 hrs and were tested for surface MHC-II expression or were further activated with TLR-ligands for 2 hrs and BCG infection for 2 hrs before MHC-II analysis. Trypan blue vital dye was used to ensure that macrophages were > 95% viable. Fig.16 illustrates that MAPKs inhibitors alone did not affect MHC-II expression in macrophages. TLR-7 and TLR-9 ligands both induced an up-regulation of MHC-II in macrophages (Fig.17). Interestingly, MHC-II induced by TLR-7 activation was inhibited by blockade of p38-ERK1/2 and AP-1 while, TLR-9 induced MHC-II was affected by blockade of p38-ERK1/2 and CREB. Signaling induced by TLR-9/7 ligands was therefore subtly different. In striking contrast, TLR-2 and TLR-4 ligands induced only a modest up-regulation of MHC-II which was not affected significantly by inhibitors of MAPKs or AP-1/CREB (Fig.18).

TLR-activation increases MHC-II expression in dendritic cells dependent upon MAPKs and AP-1/CREB. DCs are another type of APCs and their role of MHC-II in priming CD4 T cells is of prime importance. When vaccines are initially given, DCs are the cells that process vaccine to prime ‘naïve’ T cells. In BCG infected DCs as well, TLR-9/7 ligands significantly shifted the MHC-II expression and this process was dependent upon MAPKs and both AP-1 and CREB (Fig.19). In addition, multiple TLR-ligands enhanced antigen presentation by DCs to CD4 T cells (Fig.19).
Figure-16: MAPK or AP-1/CREB inhibitors in macrophages do not affect MHC-II expression in macrophages. MΦs were incubated with inhibitors of MAPKs (p38, ERK1/2 and JNK), AP-1 or CREB (all tested at 1 μM), for 2 hr. Ligands for TLR-7 or TLR-9 was used as a control without infection of macrophages. Trypan blue vital dye was used to ensure that macrophages were > 95% viable. Macrophages were then fixed and stained for MHC-II using an antibody followed by flow cytometry analysis (2 separate experiments with duplicate samples in each). A 0.5 log-shift in fluorescence compared to TLR-ligand activated macrophages was considered significant. Inhibitors alone had no significant effect on the MHC-II expression of MΦs.
Figure 17: TLR-7 and TLR-9 activation enhances MHC-II expression in BCG infected macrophages and inhibitors of MAPK cascade and AP1/CREB transcription factors decrease surface MHC-II. C57Bl/6 derived BMA MΦs were treated with AP-1, CREB or MAPK inhibitors (p38, ERK and JNK) for 2 hrs, washed and activated with TLR-7 and TLR-9 ligands for 2 hrs, infected with BCG for 2 hrs and stained for surface MHC-II. Histogram from one of two similar experiments is shown. Log decrease in MFI shown for each activation parameter. BCG infection up-regulates MHC-II in MΦs (>0.6log vs.isotype) but prior TLR activation further enhances MHC-II (>1log10 vs.isotype). TLR-7 induced MHC-II is reduced significantly by inhibitors of p38/ERK MAPK and CREB transcription factor. In contrast, TLR-9 induced MHC-II is reduced significantly by inhibitors of all three MAPKs and AP-1 transcription factor.
**Figure-18: Activation of TLR-2 and TLR-4 has moderate effects on the levels of MHC-II in BCG infected macrophages.** C57Bl/6 derived BMA MΦs were treated with AP-1, CREB or MAPK inhibitors (p38, ERK and JNK) for 2 hrs, washed and activated with TLR-1/2 and TLR-4 ligands for 2 hrs, infected with BCG for 2 hrs and stained for surface MHC-II. Histogram from one of two similar experiments is shown. Log decrease in MFI shown for each activation parameter. BCG infection up-regulates MHC-II in MΦs (>0.6log vs.isotype) but prior TLR activation do not further enhance MHC-II. Neither MAPK nor AP-1/CREB inhibitors have a significant effect on MHC-II expression.
Figure-19: TLR-activation increases MHC-II expression and antigen presentation by dendritic cells, which is dependent upon activation of p38 MAPK, ERK1/2 and AP-1 or CREB transcription factors (TFs). Upper panels: C57Bl/6 mouse derived DC.2.4 dendritic cell line was activated and infected as for MΦs in Fig.17. TLR-9 and TLR-7 agonists enhanced MHC-II and the effect was inhibited by blockade of MAPKs and TFs. Table: DCs were activated, infected and tested for antigen presentation as in Fig. 8. Data indicate that prior TLR-activation enhances antigen presentation and TLR-9>TLR-7>TLR-4>TLR-1/2 ligands were most effective (pg/mL of IL-2 averaged from triplicate wells of two experiments ±; p values vs. untreated DCs (shown as none); t test ).
Overall Discussion- Up-regulation of MHC-II using TLR activation

BCG is the most frequently used vaccine in the world today with over a billion doses administered to date. Although, BCG is the safest vaccine used, it is less effective in generating stronger and longer lasting T cell responses. When vaccines are internalized through receptor mediated phagocytosis by APCs, they are enclosed within a plasma membrane bound phagosome, which is subsequently sorted to lysosomes through a series of fusion events. Lysosome breakdown of BCG vaccine generates peptides which are then routed into MHC-II loading compartments. MHC-II then primes CD4 T cells to generate a specific Th1 immunity. It has become clear now that BCG activates TLR-1/2 and TLR-4 when it is phagocytosed into APCs, leading to immunosuppression. Specifically, BCG down-regulates MHC-II in APCs when given to mice and has the same effect on APCs in vitro. Secondly, BCG stays put in immature phagosomes preventing lysosome fusion. We made some novel observations in this study that help to bypass these immune-suppressive events.

First, we confirmed that lipids of BCG suppressed in vitro antigen presentation, presumably through TLR-1/2 and TLR-4 (Fig.5-8). Alternate TLR-activation however boosted the ability of APCs to present antigen in vitro (Fig.8). TLR-ligands seemed to activate MHC-II through down-regulation of MARCH1 (Fig.5-9). In a previous study, TLR-1/2, 3 and 4 ligands were found to similarly enhance MHC-II levels through down-regulation of MARCH1 in naïve DCs. This report was different from our study in using ‘naïve’ DCs (108). Furthermore, our model was more relevant to vaccine development since, only live attenuated BCG vaccine suppressed MHC-II
in APCs. Interestingly, in our model, we showed that only TLR-9/7 ligands up-regulated MHC-II and down-regulated expression of MARCH1. In contrast, TLR-1/2, TLR-3 and TLR-4 ligands had a moderate effect in enhancing MHC-II mediated responses from BCG infected macrophages. It should be noted that BCG vaccine already has TLR-1/2 and TLR-4 binding lipids like LP and LAM. We therefore propose that adding similar ligands may not significantly help in boosting the vaccine. We further noted that the ligands for TLR-7 and TLR-9, single strand RNA and DNA are present in BCG vaccine, but due to its sequestration within phagosomes, these ligands may not be able to activate the TLRs inside macrophages. In addition to macrophages, we also found that TLR-ligands boosted the antigen presentation by DCs. Again, only TLR-9/7 ligands seemed to boost surface MHC-II levels as evaluated using flow-cytometry.

A review of the signaling mechanisms indicated that pathways induced by multiple TLR-ligands converge at the level of MYD88 and TRAF6 (Fig.14). These molecules in turn, trigger phosphorylation of the MAPKs prior to the activation of AP-1 and CREB transcription factors. Subtle differences in AP-1 and CREB signaling may account for differences in the effects of TLRs. Overall, our study identified a new rationale for the use of TLR-7/9 ligands as novel adjuvants to boost the efficacy of BCG vaccine. These studies are clinically relevant since a TLR-4 ligand MPL has been proposed as an adjuvant for subunit protein tuberculosis vaccine in humans. We show that TLR-9/7 ligands are stronger adjuvants and propose that adjuvants for TLR-7/9 can be used with BCG vaccine in humans to boost CD4 T cell responses.
CHAPTER-3

EFFECTS OF RAPAMYCIN ON T CELL FUNCTION DURING EXPERIMENTAL TUBERCULOSIS
Background

*Mycobacterium bovis* BCG is the approved vaccine for humans to prevent tuberculosis caused by *M. tuberculosis* (Mt). BCG vaccine protects against childhood tuberculosis but not adult disease. Thus, it lacks the ability to induce long term memory. BCG infected antigen presenting cells (APCs) like macrophages and dendritic cells prime effector CD4 and CD8 T cells to control tuberculosis, but only CD8 T cells are thought to persist as long term memory cells (68). (108)Since BCG induced Th1 response was sub-optimal in the mouse model (97), we proposed that, inherent defects of BCG in APCs may lead to less efficient expansion of CD8 T cells, and the latter do not transition into long living memory CD8 T cells.

The MHC-I dependent presentation of peptides to CD8 T cells (Fig.20) depends on the two pathways. In the first mechanism, BCG phagosome fuses with lysosomes and peptides generated are sorted to MHC-I. This process is defective since BCG does not fuse with lysosome (97). In the second mechanism, secreted antigens from BCG phagosomes escape into cytosol, where they are cleaved by proteasome into peptides, which are in turn, loaded into MHC-I. However, BCG has limited phagosome membrane pore size, because of which, leakage of antigens into cytosol is reduced (41). For example, cloning of pore forming toxin listeriolyisin (LLO) into BCG enables it to induce better CD8 T cells in mice (22). It is noted here that the wild type Mt also sequesters like BCG vaccine in macrophages, although it secretes pore forming antigens, unlike BCG.
Rapamycin is an anti-neoplastic drug which is shown to repress the mammalian target mTOR. Rapamycin has an effect on a wide variety of biological pathways; one of which is the autophagy. Autophagy is a process in which a double membrane auto-phagosome vesicle engulfs the cytosol and organelle. They are shuttled to lysosome to form an auto-phagolysosome, where degradation occurs. Various cellular components are recycled using autophagy, and the latter can be triggered by starvation. We recently found that autophagy can be induced by rapamycin in BCG infected macrophages which led to the delivery of BCG to lysosome, increased peptide production and enhanced efficacy for BCG vaccine (94). Since BCG and Mtb avoid lysosome fusion, in this study, we sought to determine if rapamycin treatment of mice would enhance delivery of BCG or Mtb to lysosome compartments, increased peptide antigen production and thereby enhance immune responses. More recent studies show that rapamycin also has multiple effects on T cells and these effects are discussed below in other chapters. Fig.20 outlines the current state of knowledge on the mechanisms of rapamycin as it pertains to Mtb or BCG infection of APCs.

**Overall Hypothesis:** Rapamycin has dual effects by enabling BCG vaccine to fuse with lysosomes and it prolongs the longevity of CD8 T cells. *Thus, I developed a hypothesis that rapamycin activation of APCs may enhance their ability to better process BCG vaccine, and concurrently induce more effective and long-lasting CD8 T cell responses by modulating mTOR of T cells.*
Figure-20. Dual effects of Rapamycin on APCs and T cells. In macrophages and DCs (APCs), BCG is arrested within immature phagosomes. Prior activation of APCs with rapamycin is known to trigger autophagy and deliver BCG into autophago-lysosomes (49). This facilitates degradation of BCG, generation of peptides for MHC-II pathway and priming of CD4 T cells (red arrows). Lysosome degradation can also route peptides into MHC-I pathway (blue arrow). The traditional route of antigens leaked from BCG phagosomes into cytosol and their processing by proteasome prior to MHC-I mediated presentation is also shown (blue arrow). The second emerging mechanism is the direct effect of rapamycin on the mammalian target of rapamycin (mTOR) of the CD8 T cells.
Methods

Since the expansion of CD8 T cells, elaboration of their effectors like perforin and granzyme-B and correlation of their function to protection against tuberculosis, can only be observed using *in vivo* models, I used mouse models to investigate specific questions. The C57Bl/6 mice are Th1 biased mice suitable to study immunity against tuberculosis. The BALB/c mice have a Th2 bias and are thought to be alternate models to study pathogenesis.

The tuberculosis mouse infection model (Fig. 21). The standard (NIH approved) mouse model of aerosol infection with tuberculosis consists of infecting C57Bl/6 mice with a low aerosol dose (approx. 100 CFU per mouse) of *M. tuberculosis* (*Mtb*) and observing infection for 4 weeks. Mice are sacrificed at 4 week, and the lungs and spleens are homogenized and plated on 7H11 agar plates for CFU counts. *Mtb* grows within lungs of mice rapidly to yield $6 \log_{10} (1\times10^6$ CFU) organisms per lungs, over the 4 weeks. Concurrent with growth in lungs and spleens, CD4 and CD8 T cells are induced and are measured through flow cytometric analysis of lung, spleen and lymph node derived suspension cells. IFN-$\gamma$ secreting CD4 and CD8 T cells are important in defense against tuberculosis, and when they reach to a significant number, the growth of *Mtb* is arrested and bacterial numbers start decreasing around 4 weeks. Recent reports indicate that even multi-functional, multiple cytokine secreting T cells can be induced during tuberculosis infection of mice although; their numbers are small during natural aerosol infection.
CHAPTER-3.1

RAPAMYCIN INCREASES CD8 T CELL FUNCTION DURING

EXPERIMENTAL TUBERCULOSIS
**Specific Hypothesis**

It is well known that aerosol induced infection of tuberculosis in mice is exponential up to 4 weeks after which it levels off. It has been shown that mice produce moderate CD8 T cell responses during experimental tuberculosis (35). Thus, the levels of antigen-specific CD8 T cells which can be demonstrated during infection of mice are lower. In this section, we hypothesized that rapamycin can enhance macrophage mediated-processing of wild type Mtb, induce better CD8 T cell responses and increase their protective function.

**Methods:** Mice were aerosol infected with Mtb and from day one, treated with i.p. doses of rapamycin given at 75 μg/kg dose, once a day for 30 days. Two days after the last dose, mice were sacrificed and lungs and spleens were processed for bacterial counts. The T cells of lungs, lymph nodes and spleens from sacrificed mice were also analyzed using surface markers (CD4, CD8 and tetramers) and flow cytometry. CD8 T cells were analysed using 2 different tetramers which were specific for Mtb antigens, TB10.4 and ESAT6 (PE-labeled tetramers were synthesized by the NIH Tetramer facility, Emory University). During Th1 immunity the transcription factors (TFs) T-box expressed in T cells (T-bet) and Eomesodermin (Eomes) seem to determine the effector and memory T cell lineage, respectively (86). T-bet is a major determinant of effector T cell immunity against many infections. To determine if rapamycin affected the expression of these TFs, CD8 T cells were also typed for intracellular levels of TFs.
Results:

**Rapamycin treatment enhances protection against tuberculosis in mice** (Fig. 21). Rapamycin treated macrophages can kill Mtb or BCG through autophagy [94]. However, in this experiment, the primary goal was not to determine the killing effect of rapamycin, but to evaluate the effects of low doses of rapamycin on T cell function. Fig 21 shows that rapamycin did not affect the lung counts of Mtb but had an inhibitory effect on Mtb counts of spleens. Since the low dose rapamycin was not sufficient in enhancing APC mediated killing of Mtb *in vitro*, we speculated that the anti-tuberculosis effect of rapamycin was likely due to an increased activation of T cells in the spleens. Activated T cells are known to enhance ability of macrophages to kill intracellular Mtb.

**Rapamycin increases expression of T-bet in CD4 T cells** (Fig. 22). Rapamycin is known to affect CD8 T cells positively but not CD4 T cells (41). Furthermore, rapamycin is also known to differentially affect the expression of T-bet and Eomes which are major regulators of the effector Th1 immunity and longevity, respectively (86). Interestingly, during tuberculosis of mice, rapamycin was only able to increase T-bet in CD4 but not in CD8 T cells. It did not affect T cells of spleen or lymph nodes, where T cell expansion and contraction usually occur following infection.

**Rapamycin increases the numbers of antigen specific-CD8 T cells** (Fig. 23). To determine whether cytotoxic CD8 T cells mediated protection in rapamycin treated mouse spleens; antigen specific, tetramer positive CD8 T cells were enumerated
using flow cytometry. A typical histogram using TB10.4-specific tetramer is shown in Fig.23. Rapamycin significantly enhanced the expansion of CD8 T cells specific for TB10.4 and ESAT-6. We speculate that a dramatically decreased level of TB10.4 specific CD8 T cells in spleens at 8 weeks could be due to cytolytic degranulation, and enhanced killing of splenic macrophages, which in turn led to decreased Mtb counts.
Figure-21. Effects of Rapamycin on experimental tuberculosis of C57Bl/6 mice. Four to 6 week old female mice were aerosol infected with 100 CFU of *M. tuberculosis* (Mtb) and injected i.p. with rapamycin as indicated (75 µg/kg daily for 30 days). Mice (n=5 per time point per group) were sacrificed on day 32 and day 60, and organ counts of Mtb determined by plating organ homogenates on 7H11 agar. Colony forming units (CFU) indicate bacterial burden. Rapamycin reduced bacterial numbers in spleens (p< 0.01; 2-way ANOVA vs. untreated).
Figure-22: Rapamycin treatment during tuberculosis enhances T-bet expression within CD4 T cells of lungs: (Left) T cells from lungs of mice (Fig.21) collected at day 60 after infection were stained for intracellular transcription factors T-bet and Eomes besides CD4 or CD8 surface phenotype using antibodies and isotype controls before analysis using a Beckman Coulter flow cytometer with Flow-Jo software. Representative histogram of 3 lungs is shown. Rapamycin did not affect the levels of TFs in either spleens or lymph nodes (right).
Figure-23: Rapamycin enhances levels of antigen (tetramer) specific CD8 T cells during tuberculosis. Lungs, lymph nodes and spleens of Mtb infected replicate mice (Fig.21) were tested for tetramer (PE-TB10.4/PE-ESAT6) positive T cells using flow cytometry at 32 (left vertical panel) and 60 (right vertical panel) days post infection. Rapamycin enhances the levels of TB10.4 specific CD8 T cells (* p < 0.05; *** < 0.01, t test; n=3 mice per time point per group). A typical dot plot is illustrated.
Discussion

A striking feature of human tuberculosis is that people recovering from tuberculosis do not become immune to infection. Furthermore, a significant proportion of TB patients treated with drugs also undergo relapse indicating that the process of immune memory is not well developed (101, 106). The purpose of this study was to determine if T cell function during tuberculosis of mice could be altered using rapamycin, paving the way for vaccination mechanisms to increase T cell responses and memory. Rapamycin treatment given for 30 days enhanced bacterial clearance only from spleens of mice but not lungs. Our *in vitro* studies showed that 5 μg/mL of rapamycin is required to activate 10^6 macrophages to kill *Mtb* *in vitro* (unpublished observations). Since the mice were treated with a low dose of rapamycin at 75 μg/kg dose, < 200 ng of rapamycin per mL of blood was available per mouse weighing 25 gm. We therefore propose that the rapamycin could only activate T cells of spleens. These in turn, may have activated macrophages locally to kill *Mtb* within macrophages. In support of these observations, rapamycin treatment increased the function of T cells.

The transcription factors, T-bet and Eomes are major determinants Th1 immunity respectively regulating, short term effector immunity and long term memory in T cells (6, 8). Surprisingly, rapamycin activated T-bet in CD4 T cells of lungs, but not T-bet or Eomes in T cells of spleens or lymph nodes. Earlier studies using Mtb infected mice show that, aerosol implanted Mtb drain from lungs into hilar lymph nodes, and then to spleen over the course of infection. Local priming of T cells
occurs and such primed T cells home back to lungs, either during primary infection or after re-infection of lungs. Migration to and from lung target organ to lymphoid organs is regulated by pro-inflammatory chemokine receptors on T cells and T-bet plays a major role in inducing their expression (6, 8). Enhanced expression of T-bet in CD4 T cells of lungs but not spleen or lymph nodes by rapamycin therefore indicates that the drug was able to enhance effector CD4 T cell function in the lungs. We also propose that these T cells were in the process of migration into lymphoid organs since, generally in the aerosol infected mice, bacterial numbers decline only after 4-8 weeks and homing of T cells into lymphoid organs occurs to facilitate contraction and memory development.

In order to further address this question, we analyzed antigen specific CD8 T cells. In our initial validation studies, we considered using four types of tetramer specific CD8 T cells to determine T cell activation. The antigens Mtb32a, PstS3 and TB10.4 are found in both wild type Mtb strain as well as BCG and could be used to detect CD8 T cell responses both during BCG vaccination and after Mtb challenge (11, 88). The antigen ESAT6 is an immune-dominant region of Mtb but absent in BCG (58). In our model, the numbers of CD8 T cells specific for Mtb32a, PstS3 were too low. Both ESAT6 and TB10.4 specific CD8 T cells were however found in significant numbers in the organs of mice with tuberculosis. Interestingly, rapamycin treatment enhanced the numbers of TB10.4 and ESAT6 specific CD8 T cells in all three organs (55). However, there were certain differences in their association with protection against tuberculosis in rapamycin treated mice. ESAT6 specific CD8 T
cells were not associated with protection since lung CFUs remained unchanged even in the presence of their abundant numbers. In contrast, TB10.4 numbers were significantly low in spleens when Mtb numbers declined (day 60). We propose that these CD8 T cells lysed, accounting for their reduced numbers coinciding with decreased CFU of Mtb. This observation is consistent with a previous report wherein, TB10.4_{20-28}-loaded H-2K^{d} specific CD8^{+} T cells were found at high frequencies in draining and non-draining lymph nodes, spleen, bone marrow, and blood following \textit{M. tuberculosis} infection (55). TB10.4_{20-28}-specific CD8^{+} T cells are known to be enriched in the lung and comprise up to 40% of the CD8^{+} T cells in the lung tissue and in the broncho-alveolar compartment. Finally, TB10.4 specific CD8 T cells express granzyme-B, an important cytotoxin mediating target cell lysis [66]. TB10.4 specific CD8 T cells therefore appear to be a biomarker indicating protection against tuberculosis of mice and could be used to monitor the expansion and contraction of protective CD8 T cells. We therefore conclude that rapamycin seems to exert a moderate protective function against tuberculosis of mice at low doses and we propose that perhaps, higher doses would be more effective.
CHAPTER-3.2

RAPAMYCIN INCREASES BCG VACCINE INDUCED PROTECTION AND CD4 AND CD8 T CELL FUNCTION AGAINST EXPERIMENTAL TUBERCULOSIS IN MICE
**Specific Hypothesis:** During natural infection with Mtb, low dose rapamycin was able to marginally enhance CD8 T cell function, which resulted in significantly increased clearance of Mtb from spleens. Since Mtb has been found to exert suppressive effects on the expression of MHC-II in mice (60, 70), decrease sensitivity of macrophages to IFN-γ and affect various cellular processes, we speculated that virulent Mtb could be invasive and not that susceptible to the action of low dose rapamycin. As is the practice in many infectious diseases, a vaccine has been used to prevent tuberculosis in humans. The *M.bovis*- derived BCG vaccine is the only vaccine approved for use against human tuberculosis. It protects children variably against tuberculosis but not adults. BCG has been thought to induce immunity for 5-10 years since around 10 years of age, children again become susceptible to tuberculosis despite childhood vaccination with BCG. We found earlier that, when dendritic cells containing BCG vaccine were pre-activated with rapamycin and injected to mice, an enhanced protection occurred against subsequent challenge with virulent Mtb (48). *We therefore hypothesized that rapamycin enhances the efficacy of BCG vaccine through its combined action on DCs and CD8 T cells* (Fig.24).

**Tuberculosis mouse vaccine evaluation model** (Fig. 24) The standard (NIH approved) mouse model of vaccine evaluation consists of mice which are first vaccinated with BCG vaccine (one s.c. dose per mouse given at 1X10^6 CFU per mouse) and rested for 4 weeks to develop immunity. They are then aerosol infected with *M.tuberculosis* (Mtb; 100 CFU per mouse) and observing infection for the next 4 weeks. Mice are sacrificed at 8 weeks after vaccination and 4 weeks after
challenge infection and lungs and spleens are homogenized and plated on 7H11 agar plates for CFU counts. Protection generated by BCG vaccine is measured by a \( \log_{10} \) decrease in Mtb counts of lungs and spleens. All experimental vaccines and vaccine protocols are compared against the BCG induced \( 1 \times \log_{10} \) reduction in bacterial numbers of lungs and spleens. T cell functions are evaluated using flow-cytometry using cells of lungs, spleens and lymph nodes collected from mice sacrificed after Mtb challenge (4 weeks and 8 weeks).

**Interpretation of vaccine model:** The BCG vaccine evaluation model has been routinely used by us and others to validate newer, emerging vaccines and vaccine-adjuvant strategies against tuberculosis (26, 49). It has become apparent that BCG vaccine by itself induces only a moderate protection against tuberculosis, reducing growth of virulent Mtb by \( 1 \times \log_{10} \) in the lungs. BCG induces moderately strong CD4 T cell expansion but a poor CD8 T cell response after vaccination. It is also a poor inducer of *multifunctional cytokine secreting T cells* (MFCs), thought to mediate better protection against tuberculosis. Against this background, using rapamycin to treat BCG vaccinated mice we asked the questions illustrated in Fig.24.
Figure-24. The NIH- mouse tuberculosis vaccine evaluation model used to determine the immunological basis of protection. BCG vaccine has been known to yield moderate protection against challenge with virulent *M. tuberculosis* in this model, which is mediated by the expansion of CD4 T cells after vaccination. A poor CD8 T cell response is evident after vaccination. Although, CD4 T cells again expand after challenge with Mtb, it is unclear whether it is fully effective, since Mtb declines by 1-log$_{10}$ at 4 weeks p.c., but persists at significant numbers (3-4 log$_{10}$ per lungs) for over 300 days in the lungs. Mice then begin to die of age or increased replication of Mtb. The NIH model is restricted to a 4 week post challenge window to determine if, other candidate vaccines induce better protection than BCG, which is associated with a significant change in T cell responses.
Results:

**Rapamycin enhances efficacy of BCG vaccine in mice.** Fig.25 shows that rapamycin treatment of mice after BCG vaccination led to a significant decrease in Mtb counts of both lungs and spleens compared to mice vaccinated with BCG alone (p< 0.02, 2-way ANOVA, 5 mice per group). Rapamycin alone had no significant effect on the growth of challenged Mtb organisms.

**Rapamycin enhances levels of antigen specific tetramer positive CD8 T cells.**

The organs of vaccinated mice were tested for antigen-specific CD8 T cells specific for TB10.4 & ESAT6-tetramers at 4 (left vertical panel) and 8 (right vertical panel) weeks post challenge (Fig.26). Data show that rapamycin treatment with BCG generally expands antigen-specific CD8 T cells. However, ESAT6 specific T cells tend to remain elevated in lungs while, TB10.4 specific CD8 T cells showed a decline over time, which may indicate degranulation and cytolytic effects associated with this population of CD8 T cells.
Figure-25. **Rapamycin enhances efficacy of BCG vaccine against tuberculosis**: Mice were vaccinated with BCG or BCG in combination with rapamycin (BCG+R) (given i.p. daily doses at 75 µg/kg for 30 days) followed by aerosol infection with 100 CFU of *M. tuberculosis*. Organs were collected at 4 weeks post challenge, homogenized and plated for CFU counts on 7H11 agar, which were expressed as log₁₀ CFU per organ. Rapamycin cotreatment following BCG vaccine induced better clearance of Mtb from both the organs (p values determined using 2-way ANOVA vs. BCG alone group; n=5 mice). Rapamycin alone has no bactericidal effect on the organ counts of Mtb.
Figure-26. Rapamycin enhances the levels of antigen specific tetramer positive CD8 T cells. CD8 T cells of organs of mice shown in Fig.25 were collected on week 4 and 8 post-challenge, stained for tetramers specific for ESAT6 and TB10.4 antigens of Mtb, and analysed using flow-cytometry. Percent positive T cells from 3 individual mice per group per time point were averaged (±SD). A representative dot plot is shown to left corner. Rapamycin cotreatment of mice enhances the levels of antigen specific CD8-T cells (** p < 0.05; *** < 0.01; t test).
Rapamycin enhances memory precursor T cells in mice vaccinated with BCG.

In mice, CD8 T cells are further divisible into short lived effector T cells (SLECs) and memory precursor effector T cells (MPECs), depending upon the expression of two transcription factors, T-bet and Eomes. The latter are known to respectively regulate short term effector function and memory in CD8 T cells. It remains unclear whether T-bet and Eomes expression in CD4 T cells translates into memory function, although some report that Eomes expression in CD4 T cells can ascribe them with a cytolytic function (34). SLECs are T-bet\textsuperscript{high} and Eomes\textsuperscript{low} while, MPECs are reverse. BCG is known to induce mostly SLECs in mice, although these cells in mice were typed on the basis of CD62L expression, where CD62L\textsuperscript{low} correspond to SLECs and MPECs are CD62L\textsuperscript{high} (90). SLECs are important for short term control of infection while MPECs transition into long term memory population. We therefore sought to determine whether rapamycin could positively affect these two cell populations. CD4 and CD8 T cells were stained for intracellular T-bet and Eomes and analysed using flow-cytometry. Fig. 27 shows that, rapamycin cotreatment with BCG helped to maintain elevated levels of T-bet\textsuperscript{+} CD8 SLECs until 8 weeks post challenge. Interestingly, rapamycin enhanced Eomes\textsuperscript{+} CD8 MPECs at 4 weeks in the target lung organ and these persisted at significant levels in the spleens at 8 weeks. This suggested that rapamycin enhanced the homing of CD8 memory T cells from lungs into the splenic lymphoid compartment. The role of T-bet and Eomes in the regulation of CD4 T cell function remain unclear. Even then Fig. 28
shows that rapamycin cotreatment increased the levels of T-bet\(^+\) and Eomes\(^+\) CD4 SLECs in the organs of mice.
Figure-27: **Rapamycin enhances the levels of T-bet$^+$ short living effector CD8 T cells (SLEC) and Eomes$^+$ MPECs memory precursor effector CD8 T cells (MPEC) after vaccination.** CD8 T cells of organs of mice shown in Fig.25 were collected on week 4 and 8 post-challenge, and stained for intracellular T-bet and Eomes prior to analysis using flow cytometry. Three individual mice were analysed per time point per group and percent positive T cells averaged. Rapamycin cotreatment with BCG increased T-bet$^+$ Eomes$^+$ CD8 SLECs in lymph nodes and spleen at 4 and 8 weeks post-challenge. Rapamycin cotreatment enhanced the Eomes$^+$ MPECs in lungs at 4 weeks and spleens at 8 weeks, post challenge (* p < 0.05; *** < 0.01; t test).
Figure-28: Rapamycin affects the levels of T-bet+ short-living effector CD4 T cells (SLEC) and Eomes+ MPECs memory precursor effector CD4 T cells (MPEC) after vaccination. CD4 T cells of organs of mice shown in Fig.25 were collected on week 4 and 8 post-challenge, and stained for intracellular T-bet and Eomes prior to analysis using flow cytometry (legend as in Fig.27). Three individual mice were analysed per time point per group and per cent positive T cells averaged. Rapamycin cotreatment with BCG increased T-bet+ Eomes+ CD4 SLECs in lymph nodes and spleens at 4 and 8 weeks post-challenge (p *** < 0.01, t test).
Discussion

In our previous study, during experimental tuberculosis, rapamycin was partially able to enhance clearance of virulent Mtb in the spleens of mice, although it did enhance the levels of antigen specific CD8 T cells in both lungs and spleens (Chapter 3.1). Since Mtb is a virulent organism capable of modulating immune responses, we sought to determine the immune responses elicited by the BCG vaccine in mice against tuberculosis. BCG is an attenuated mutant of *M. bovis* similar to *M. tuberculosis* and thought to be less immune-suppressive than virulent Mtb. Data presented here show that rapamycin was able to significantly boost the efficacy of BCG vaccine to reduce the numbers of Mtb in both lungs and spleens of mice. This correlated well with the expansion of antigen specific, tetramer positive CD8 T cells in lungs and spleens. Significantly, when lung counts of Mtb CFUs declined, the numbers of TB10.4 specific CD8 T cells declined from 10% to 4% but those of ESAT6 specific CD8 T cells remained unchanged. Furthermore, TB10.4 specific CD8 T cells increased dramatically in the lymphoid organs (spleens and lymph nodes). This is consistent with a pattern of homing of CD8 effectors from the target lung organs to the latter for transition into memory cells.

Recent studies indicate that transition of effector T cells into memory precursor T cells occurs under defined conditions which are probably dictated both by pathogen elimination and by intracellular transcription factors T-bet and Eomesodermin (Eomes) (86). First, infection is largely controlled by effectors, which removes or reduces the continuous priming signaling from APCs towards CD8 T cells. Secondly, perhaps due to cytokine signaling, a switch occurs from effector into
memory phenotype regulated by T-bet and Eomes. In support of this observation, CD8⁺ T cells lacking T-bet show enhanced differentiation as long lived memory cells. In addition, mice deficient in Eomes have a limited capability to compete for antigen specific memory compartment in CD8 T cells (6). Furthermore, CD8 T cells from Eomes deficient mice lack long term persistence and could not fight the secondary rechallenge infection since they did not produce a strong response of effector T cells (34). These observations led us to examine the numbers of T-bet⁺ and Eomes⁺ T cells operationally defined as short living effector cells (SLECs) and memory precursor effector T cells (MPECs) (1, 53). Interestingly, we found that rapamycin enhanced the accumulation of Eomes⁺CD8⁺ MPECs early after infection in the lungs (4 weeks) which persisted later in the spleens (Fig.23). At the same time, it maintained a strong expansion of SLECs. A similar profile was also observed for Eomes⁺CD4⁺ T cells (Fig.24). Although the function of Eomes in CD4 T cells remains unclear, some studies indicate that Eomes⁺CD4⁺ are cytotoxic. The major observation of this study was that rapamycin cotreatment not only enhanced the levels of T-bet⁺CD8⁺ SLECs but also Eomes⁺CD8⁺ MPECs. We therefore suggest that the novel, dual action of rapamycin in enhancing the CD8 and CD4 T cells enabled the BCG and rapamycin co-treated mice to control tuberculosis better.
CHAPTER-3.3

RAPAMYCIN AFTER BCG VACCINATION SHIFTS THE PHENOTYPE OF IMMUNE MEMORY CELL POPULATIONS THROUGH INHIBITION OF MTOR FACILITATING EXPANSION OF CENTRAL MEMORY T CELLS
Background:
In the previous chapter, we used rapamycin to boost the efficacy of BCG vaccine and found that it increased bacterial clearance Mtb from organs, induced enhanced levels of antigen specific CD8 T cells and boosted the levels of T-bet$^{+}$CD8$^{+}$ SLECs and Eomes$^{+}$CD8$^{+}$ MPECs (Figs.25-28). These observations were exciting but also raised additional questions. Emerging studies using virus infection models have revealed that the phenotypes of T cells in vaccinated mice are quite diverse and require the use of multiple surface markers to define effector and memory T cells. Some of these studies have used rapamycin based on the exciting observation that rapamycin represses mTOR to alter T cell function (3). Studies show that long living memory precursor T cells express distinct surface markers in addition to Eomes and undergo considerable flux (18, 104). Furthermore, there are divergent opinions on how effector and memory T cells arise after presentation of antigens from APCs, and the rate at which effector T cells undergo transition into memory cells (72). Finally, an effective transition from effector to memory phenotype seems to require a significant elimination of the pathogen in mice. Paradoxically, mice really do not eliminate Mtb organisms which persist for months. These considerations led us to re-examine the phenotype of memory CD8 T cells using rapamycin treated mice and re-evaluate their significance and correlation to protection.

Memory T cell phenotypes: A sustained T cell immune response is divided into two distinct phases. The first phase after infection is the activation event, where antigen specific T cells or effector T cells undergo an exponential increase (14, 62).
Expanded effector cells ensure enough T cells to attack and combat and contain the infection (Fig.29). As soon as the infection is controlled, the T cells undergo a controlled contraction phase where only 5-10% of cells remain after resolution of the infection (54, 103). These cells present during the contraction phase are referred to as the memory pool. The second phase of the immune response occurs when there is a ‘reinfection’ and a second exposure of the antigen to the resting T cell memory pool. When the memory T cells encounter the antigen again, they re-expand and divide much more vigorously than the initial effector T cell population. They produce cytokines at a much higher rate and have increased specificity against the antigen (81). Memory T cells are distinct compared to naïve T cells and possesses properties which are different and unique. They express high levels of IL-7 (CD127) receptor and also the anti-apoptotic protein Bcl2. These markers along with other co-receptors allow for the constant division and self-renewal properties of the memory T cells (85, 109).

Role of rapamycin in the regulation of memory phenotype in T cells: The mammalian target of rapamycin (mTOR), is emerging as a major regulator of T cell function. First, activated T cells down-regulate the receptors CD62L and CCR7, which are homing receptors usually present on naïve T cells (45). Since mTOR inhibits both CCR7 and CD62L, it has been proposed that repression of mTOR by rapamycin may facilitate the expression of chemokine receptors and enable homing of memory T cell populations (95) (73). Fig.30 illustrates another mechanism in which, rapamycin can affect T cells through repression of mTOR which reduces TRAF-6 mediated signaling. TRAF-6 activation has multiple consequences, one of
them being the reduction of IFN-αβ. It has been reported that TRAF-6 (a downstream signaling molecule inhibited by mTOR) deficient T cells could develop into effector T cells, but failed to differentiate into memory precursors (79). Furthermore, TRAF-6 deficient T cells exhibited lower levels of AMP-kinase, which is an inhibitor for mTOR. Interestingly, metformin, an AMPK agonist could reverse the effect, and restore the formation of memory T cells in TRAF-6 deficient mice (84). Since IFN-αβ enhances effector function of CD8 T cells in combination with IL-12 (see below), we propose that rapamycin inhibits mTOR and TRAF-6 thereby reducing IFN-αβ and this enables effectors to change into memory cells.

We initially used rapamycin to boost the efficacy of BCG vaccine due to its ability to increase macrophage mediated processing of BCG vaccine (49). Recent studies indicate that inhibition of mTOR in T cells through rapamycin could be another mechanism to enhance vaccine efficacy and memory cell formation in mice (86). Data show that mTOR plays a significant role in the formation of a functional memory pool. Araki et al. reported that low dose-rapamycin has an immune-modulatory role on the phenotype of the CD8 T cells during virus infections and can enhance different stages of the memory formation and development of the T cells (3).

While rapamycin affects expression of homing receptors by acting on mTOR, either prolonged or timed exposure can also suppress them (Fig.31). Furthermore, rapamycin has varied effects on the production of memory T cells depending on when it is given. Rapamycin given during the expansion of T cells improves the
number of memory T cells, but generates a similar number of effector CD8 T cells during the infection compared to mice untreated with rapamycin. However, the rate at which rapamycin treated CD8 T cells undergo apoptotic death is reduced when they enter the contraction phase. Rapamycin given during the contraction phase changes the phenotype of the effector T cells to a memory phenotype. Finally, rapamycin yields the best results when it is given during both expansion and contraction phase (2).

**Role of IL-12 and T-bet during memory development:** IL-12, a key cytokine that is secreted from BCG or Mtb infected APCs also plays a significant role in skewing effector vs. memory T cells. IL-12 activates T-bet in OT-I CD8 T cells leading to a burst in effector T cell population [75]. Paradoxically, rapamycin reduced T-bet and IL-12 expression but enhanced Eomes, thereby facilitating memory cells. The role of IL-12 in enhancing effectors is understandable since it is the first innate cytokine to be secreted after macrophages or DCs engulf mycobacteria, and the initial burst of effectors is required to control infection (83, 86). However, IL-12 must decline in order for memory cell expansion to continue. Since both BCG and Mtb persist in APCs for prolonged periods, an intriguing possibility is that, the continuous secretion of IL-12 by infected APCs may in fact interfere with the overall process of memory cell expansion.

**Synergy of IL-12 with IFN-αβ:** The other key cytokines which seem to play a role in the regulation of effector vs. memory are the IFN-α and IFN-β. Mescher et al. reported that both IL-12 and IFN-αβ can act as a ‘third signal’ to promote both IFN-γ
secretion and expression of perforin and granzyme in murine CD8⁺ cells (29). The latter are the effector cells that can potentially kill APCs containing mycobacteria. Paradoxically, they are also the cells which transition into long living MPECs in mice and when re-infection occurs, undergo recall expansion into effectors which contain re-infection. It is thus obvious that IL-12 and IFN-αβ are required to sustain both initial strong effector response, and recall expansion of memory cells into cytotoxic effectors. Interestingly, Fig.30 illustrates that IFN-αβ can be induced both through TLR-activation as well as through rapamycin mediated repression of mTOR.

**Specific Hypothesis**

*Cumulatively, these observations lead to our hypothesis that CD8 effector vs. memory switch following BCG vaccination may be defective due to both host and vaccine (BCG) dependent factors. BCG may hide in APCs not inducing enough IL-12 or IFN-αβ. On the other hand peptide antigens from BCG are also probably not enough to induce CD8 T cells. We propose that rapamycin treatment of mice during BCG vaccination may induce ‘functionally better CD8 T cells’ due to its dual effect on APCs and T cells.*
Figure-29: Memory CD8\(^+\) T-cell differentiation. Left: Antigen-specific CD8\(^+\) T-cell responses after an acute infection. In the expansion phase, naive CD8\(^+\) T cells proliferate to become effector cells. After clearance of infection, they enter contraction phase where 5-10% cells become memory population. Progressive memory CD8\(^+\) T-cell differentiation occurs and the subsets of effector and memory CD8\(^+\) T cells emerge. Key cell surface and intracellular markers for memory CD8\(^+\) T-cell differentiation are listed (Nature: (2))
**Figure-30: Rapamycin an immune-modulator affects mTOR and down-stream events regulating IFN-αβ.** The latter plays a role in regulation of effector T cells. Rapamycin mediated repression of mTOR can inhibit TRAF-6 mediated induction of IFN-αβ, which may in turn affect effector vs. memory cell development. TLR-7/9 activation may also influence the memory process through the induction of IFN-αβ. *(Nat.Immunology 2008 (28)).*
**Figure-31: Rapamycin improves the quantity and quality of memory CD8⁺ T cells.** (A) Without rapamycin treatment. (B) Rapamycin treatment during T-cell expansion phase increases memory precursor effector cells that survive during the contraction phase. A higher quantity of memory T cells is formed compared with control. (C) Rapamycin treatment during T-cell contraction phase accelerates effector to memory T-cell formation, and improves their quality. (D) Rapamycin treatment during both the expansion and contraction phase improves both quality and quantity of memory CD8⁺ T cells (*Nature*: (2)).
Methods

Evaluation of effector vs. central memory in mice: In our previous chapter, we used a single low dose of rapamycin to determine if it could increase effector and memory T cells and enhance efficacy of BCG vaccine. However, investigations in other laboratories have indicated that the dosing and timing of rapamycin can impact the development of effector and memory cells (Fig.31). Therefore, we sought to determine in this study, the effects of a low dose rapamycin (long term) vs. high dose (short term) on the course of tuberculosis and the expansion of effector and memory T cells. The mouse experiment is illustrated in Fig.32. Earlier, we identified SLECs and MPECs on the basis of T-bet and Eomes, which are transcription factors. More recent studies show that they also show a differential expression of CD62L, CD44, CD127 and CCR7, depending on whether they are SLECs and MPECs. For example MPECs are CD62L⁺, CD44⁺, CD127⁺ and CCR7⁺. In contrast the SLECs are CD62L⁻, CD44⁻, CD127⁻ and CCR7⁻. Organs of three mice per time point per group were individually analyzed and averaged for graphs and p value determined using graphpad prism. Typical dot blots are illustrated in Figs.33 and 34.
**Figure-32. Effect of low and high dose rapamycin on BCG induced protection and memory populations.** BCG induces $1 \log_{10}$ protection against tuberculosis using the standard NIH model of vaccine evaluation (C57Bl/6 mice). We sought to determine the effect of either a low (LR; 75 µg/kg; 30 days) or high dose (HR; 750 µg/kg; 10 days) rapamycin as indicated, on the efficacy of BCG vaccine. Mice were sacrificed 4 and 8 weeks after challenge and 5 mice analyzed for CFU counts and 3 mice for T cell functions at each time point.
Figure-33. Evaluation of memory population by gating on CD4 T cells: The phenotype of memory cells was optimized using antibodies to CD62L, CD44 and CD127 markers on CD3+CD4+ T cells using 3 individual mice per group sacrificed 4 and 8 weeks after challenge with *M.tuberculosis* (Fig.32). BCG vaccinated mice were compared with mice receiving BCG+ low dose or BCG+ high dose rapamycin besides, untreated but Mtb infected mice. Beckman Coulter flow cytometer and Kaluza software were used for analysis of T cells.
Figure-34: Assessing memory population on CD8 T cells: The phenotype of memory cells was optimized using antibodies to CD62L, CD44 and CD127 markers on CD3⁺CD8⁺ T cells using 3 individual mice per group sacrificed 4 and 8 weeks after challenge with *M.tuberculosis* (Fig.32). BCG vaccinated mice were compared with mice receiving BCG+ low dose or BCG+ high dose rapamycin besides, untreated but Mtb infected mice. Beckman Coulter flow cytometer and Kaluza software were used for analysis of T cells.
Results:

Cotreatment of rapamycin with BCG vaccine enhances the ability of vaccine to clear Mtb from organs. Fig. 35 shows that in both lungs and spleen of mice, rapamycin enhanced bacterial clearance well above the levels induced by BCG vaccine alone. Significantly, both low-dose and high-dose rapamycin boosted the efficacy of BCG vaccine in mice against tuberculosis. The high dose rapamycin was marginally better than low dose rapamycin although, the differences were not statistically significant. As anticipated, BCG alone protected against tuberculosis by decreasing organ CFUs of untreated mice by 1-log10.

Effect of Rapamycin on effector memory (MPEC) and effector T cell (SLEC) phenotype: Spleen, lymph nodes and lungs were analysed 4 week and 8 week post challenge to determine changes in MPEC vs. SLEC population (Fig. 32). CD4+ MPECs and CD8+ MPECs (CD62L+CCR7+) were again typed on the basis of CD44 and CD127. Thus, there were two groups of MPECs, CD62L+CCR7+CD127+ and CD62L+CCR7+. CD44+ distributed in the target organ lung and the lymphoid organs; spleen and lymph nodes. Rapamycin plus BCG treatment generally enhanced CD8+ MPECs compared to BCG alone group in the lungs at 4 weeks post challenge, when Mtb counts had declined (Fig. 36a-b). At 8 weeks again, only rapamycin plus BCG had accumulated higher numbers of CD8+ MPECs in the lungs (Fig. 36b). In contrast, Figs 37a-b shows the profiles of CD4+ and CD8+ SLECs. In general, rapamycin treatment did not markedly affect the distribution of SLECs.
which were present already in significant numbers. However, rapamycin tended to increase the numbers of CD8\(^+\) SLECs in lungs and spleens while increasing those of CD8\(^+\) SLECs in the lymph nodes.
Figure 35: Both low-dose long term and high-dose short term rapamycin dosing boost the efficacy of BCG vaccine in mice against tuberculosis. Mice were vaccinated with BCG and treated with rapamycin as indicated in Fig.32. After challenge, CFU counts of organs were determined by plating organ homogenates on 7H11 agar 4 weeks post challenge (CFUs did not change 8 weeks p.c; not shown). As anticipated, BCG alone protects by decreasing organ CFUs of untreated mice by 1-log_{10} (p < 0.009 for BCG vs. untreated; 2-way ANOVA). Data show that both doses of rapamycin significantly enhance the ability of mice to clear bacterial burden (p values determined using 2-way ANOVA; 5 mice per group analyzed; 4 weeks post challenge with Mtb).
Figure-36a: Rapamycin induces a better expansion of memory precursor effector (MPEC) T cells (CD62L⁺ CCR7⁺ CD44⁺ T cells). Mice sacrificed at 4 and 8 weeks post challenge were analyzed using flow cytometry (* p<0.02; **,*** < 0.01; t test; n= 3 individual mice per group per time point). Mice were vaccinated with BCG and treated with rapamycin as indicated in Fig.32.
Figure-36b: Rapamycin induces a better expansion of memory precursor effector (MPEC) T cells in lungs and spleens (CD62L⁺CCR7⁺ CD127⁺ T cells).

Mice sacrificed at 4 and 8 weeks post challenge were analyzed using flow cytometry (* p<0.02; **,*** < 0.01; t test; n= 3 individual mice per group per time point). Mice were vaccinated with BCG and treated with rapamycin as indicated in Fig.32.
Figure-37a: Rapamycin induces a better expansion of short living effector (SLEC) T cells lungs and spleen between 4-6 weeks and lymph nodes at 8 weeks post challenge with Mtb (CD62L^−CCR7^− CD44^+^ T cells). Mice sacrificed at 4 and 8 weeks post challenge were analyzed using flow cytometry (* p<0.02; **, ***< 0.01; t test; n= 3 individual mice per group per time point). Mice were vaccinated with BCG and treated with rapamycin as indicated in Fig.32.
Figure 37b: Rapamycin has only a moderate effect on the expansion of short living effector (SLEC) CD8 T cells in the spleens (CD62L-CCR7-CD127+ T cells). Mice sacrificed at 4 and 8 weeks post challenge were analyzed using flow cytometry (* p<0.02; **,*** < 0.01; t test; n= 3 individual mice per group per time point). Mice were vaccinated with BCG and treated with rapamycin as indicated in Fig.32.
Effect of Rapamycin on antigen specific memory T cells. CD8^+ SLECs and CD8^+ MPECs possibly contained T cells specific for different antigens. In order to determine if a distinct antigen specificity is associated with a memory phenotype, CD8 T cells of the organs were analysed in mice after infection was cleared using INH drug treatment (Fig. 38, inset). T cells of mice harvested at the 12\textsuperscript{th} week (1 week post drug treatment) were stained using TB10.4 tetramers and either CD62L or CD44. It has been known from previous studies that CD62L^+ or CD44^+ T cells represent central memory phenotype. Fig. 38 shows that rapamycin treatment significantly enhanced the numbers of antigen specific, central memory T cells specific for TB10.4, particularly in the spleens and lymph nodes.
**Figure-38:** Rapamycin induced antigen specific tetramer+ CD8 T cells are CD44+ and CD62L+ memory cells. To determine if CD8 T cells remaining in the from organs of mice were antigen–specific memory T cells, mice shown in Fig.32 were treated with isoniazid drug for 3 weeks and rested. Mice were then sacrificed at 12 weeks post challenge (1 weeks post drug treatment), and stained for both memory markers and tetramers. Both low and high dose rapamycin expand antigen specific CD8 T memory cells (*** < 0.01, p values shown for groups compared, t test; n= 3 individual mice per group). Note massive increase of memory T cells in spleens.
Discussion

Since BCG does not induce protection against adult tuberculosis, it has been hypothesized that BCG lacks the ability to induce central memory T cells. The central memory T cells are by definition CD8 or CD4 positive T cells that survive for prolonged periods and undergo recall expansion into effectors when rechallenge with infection occurs. Mechanisms of effector to memory switch are however debated.

Obar and Lefrancois proposed two concepts on the fate of effector and memory cells. In the ‘Transition hypothesis’ effector T cells or $T_{EM}$ are induced by a vaccine or infection and need to transition into memory T cells or $T_{CM}$ under the regulatory influence of distinct cytokines, including but not limited to IFN-γ, IL-2, TNF-α, IL-15 and IL-7 (63). In the ‘Induction of central memory’ hypothesis, memory T cells or $T_{CM}$ (=MPECs) are sufficiently primed at the initial administration of vaccine, and are present in low numbers. They are present at higher numbers only due to prolonged priming from APCs to CD8 T cells (72).

Since LLO expressing BCG leaks more antigens into cytosol and enhances CD8 T cell response in mice, it now appears that the short term protection provided by BCG vaccine is likely due to insufficient stimulation of CD8 T cells (41). BCG thus induces low levels of central memory T cells in mice (45).
To determine if BCG-induced levels of MPECs can be enhanced and such MPECs are functional, in this study, we used the mTOR suppressive action of rapamycin. The goal was to determine if, the phenotype of BCG induced CD8 T cells can be skewed from effector to memory phenotype using well defined surface markers including tetramers. We found that rapamycin co-treatment after BCG vaccination in mice indeed enhanced the levels of MPECs (Fig.36-37). This confirmed that BCG by itself induced low levels of MPECs but their numbers could be increased using mTOR inhibition. Most significantly, after clearance of infection, only rapamycin treated BCG vaccinated mice had elevated levels of central memory MPECS in lymphoid organs (Fig.38). Interestingly, rapamycin also affected CD4 T cells both in the MPECs and SLEC compartment, although the changes were distinct in organ compartments (Fig.36-37). Since it is well known that rapamycin does not affect CD4 T cells positively, we offer a novel explanation that rapamycin enhances the function of BCG and Mtb infected APCs in mice, which in turn enhance CD4 T cell numbers and quality.

We therefore conclude that BCG induces low levels of central memory T cells perhaps because of a defect in host-vaccine interaction or absence of key cytokines but immune-modulation can enhance vaccine induced central memory responses (98). We found that rapamycin induced both MPECS and SLECs in response to vaccination by BCG and this had a markedly beneficial action on the clearance of pathogenic Mtb in mice. We propose that rapamycin can enhance long term immunity engendered by BCG vaccine.
CHAPTER-3.4

EVALUATION OF THE PROTECTIVE FUNCTION OF MEMORY PRECURSOR EFFECTOR CD8 T CELLS (MPECs) INDUCED BY RAPAMYCIN AFTER RECHALLENGE OF BCG VACCINATED MICE
Background

In most developing and some developed countries, primary BCG vaccination is practiced for children soon after birth. Protection from BCG vaccination varies in different geographic regions. Nearly in all regions though, protection wanes by the age of 10-15 years and adults are susceptible to tuberculosis. Interestingly, tuberculosis infection does not lead to significant cross protection or ‘infection-induced’ immunity. This has been a problem for the natural control of the spread of tuberculosis. The take home lesson is that BCG does not protect against adult tuberculosis most probably due to the loss of long living memory T cells (central memory) that can persist in the human body.

To determine the importance of persisting memory T cells, however, there is a need for appropriate animal models (51). In previous studies along this direction, mice have been vaccinated with BCG or infected with wild type Mtb and memory cells have been examined over prolonged periods (45). In other studies, mice were infected with wild type Mb and re-infected to study persistence of memory T cells (55). Neither of these studies recapitulate the human situation, where BCG vaccination is followed by infection with wild type Mtb and then, T cells shift in their phenotype and function or lost. Since Vaccination induced central memory T cells undergo contraction and are present in small numbers for prolonged periods, their functional significance can only be ascertained by the recall expansion into effector T cells. In the previous chapter, we demonstrated that rapamycin cotreatment with BCG enhanced the numbers of central memory T cells. In this study, we examined
the hypothesis that rapamycin-induced central memory T cells are fully functional in mice and protect against tuberculosis after rechallenge.

**Methods:**

*Evaluation of protection against rechallenge tuberculosis in mice:* In our previous chapter, we demonstrated marked expansion of both SLECs and MPECs (Fig.32-38). To determine whether the TB10.4 specific MPECs are the cells that persist and undergo recall expansion, mouse vaccine experiments were performed as before. However, at 8 weeks (Fig.39), mice were treated with the isoniazid (INH) drug for 3 weeks. INH is lethal for both Mtb and BCG and clears the vaccine and Mtb from the organs reducing the load of bacteria and removing priming stimuli from APCs. Mice were rested and on week 12, challenged again with wild type Mtb. Re-infection or protection against re-infection was allowed to take place for the next four weeks and mice were sacrificed. Mouse organs were then plated for CFU counts of Mtb and T cells were phenotyped for effector T cell function (perforin and granzyme positive) using TB10.4 tetramer as a marker. The recall expanding T cells were therefore TB10.4 antigen specific T cells.
Results:

Prior rapamycin treatment of BCG vaccinated mice enables a better protection against tuberculosis in the mouse rechallenge model. Mice were vaccinated with BCG and treated with rapamycin as in Fig.39. Rechallenged mice were sacrificed on week 16 and CFU counts of organs determined. Fig.40 demonstrates that both low and high dose rapamycin given after BCG vaccination were able to enhance the ability of mice to reduce the bacterial burden of lungs and spleens better than those given with BCG vaccine alone. BCG alone was not protective against rechallenge in spleen and induced only a modest protection in lungs.

Effect of Rapamycin on the recall expansion of functional CD8 T cells. In the previous chapter we found that after drug induced bacterial clearance in BCG-rapamycin treated mice, significant numbers of TB10.4+ MPECs persisted in the spleens and lymph nodes (Fig.38). To determine if the protection generated during rechallenge among such mice was due to MPECs (Fig.40), the T cells of organs were stained for TB10.4 tetramer/IFN-γ and TB10.4 tetramer/perforin/granzyme and analysed using flow cytometry. Fig.41 shows that rapamycin treatment enhanced the numbers of TB10.4/IFN-γ+ T cells in the lungs. Furthermore, the proportion of TB10.4+ CD8 T cells expressing perforin and granzyme-B were also high in lungs (Fig.42). Finally, the numbers of functional CD8 T cells correlated well with those expressing both T-bet and Eomes (Fig.42). Thus, rapamycin strongly enhanced the recall expansion of functional CD8 T cells in the target lung organ which was
exposed to reinfection. Rapamycin had less significant effects on the recall expansion of CD8 T cells in the spleens and lymph nodes (Fig. 43-44, 45), despite reducing the numbers of Mtb in the spleens. Since lymphoid organs harbour resting MPECs, it is possible that after Mtb rechallenge, these cells underwent recall expansion and homed more rapidly to the target lung organ, accounting for their lesser numbers in spleens.
Figure-39: The mouse tuberculosis rechallenge model to determine the protective effect of memory precursor effector T cells (MPECs). Mice are vaccinated and treated with rapamycin as in Fig.32. However, at 8 weeks post challenge, mice were treated with isoniazid (25 mg/kg oral dose given daily for 3 weeks) drug to clear the organs of live Mtb and BCG vaccine. Mice were rested for 1 week, and then re-challenged with an aerosol dose of live Mtb (week 12; 100 CFU per mouse). Four weeks later (week 16) mice were sacrificed to determine protection mediated by persisting MPECs. Lungs and spleens were plated for CFU counts (5 mice per group) and T cells harvested from mice evaluated for CD8 T cell function (3 mice per group) (TB10.4 tetramer, granzyme-B and perforin) using flow cytometry.
Figure-40: Rapamycin treatment of BCG vaccinated mice enables a better protection against tuberculosis in the mouse rechallenge model indicating a protective function for memory precursor effector T cells (MPECs). Mice were vaccinated with BCG and treated with rapamycin as in Fig.39. Rechallenged mice were sacrificed on week 16 and CFU counts of organs determined. Both low and high dose rapamycin given after BCG vaccination are able to enhance the ability of mice to reduce the bacterial burden of lungs and spleens better than those mice vaccinated with BCG alone (p calculated using 2 way ANOVA; 5 mice per group). BCG alone gave modest protection against rechallenge in lungs and no protection in spleens. The log CFU numbers shown immediately above the bars, represent log$_{10}$ decrease in Mtb counts compared to untreated.
Figure-41: Rapamycin treatment following BCG vaccination enables mice to mount a stronger recall expansion of antigen specific tetramer+ CD8 T cells in the lungs upon rechallenge with virulent *M.tuberculosis*. **Left:** Representative dot plot of tetramer-PE and IFN-γ FITC stained CD8 T cells (one of three individual mice). Flow analysis using Beckman Coulter flow cytometer using Flo-jo software. **Right:** Lungs of rechallenged mice (week 16, Fig.39) show biomarkers of antigen specific CD8 T cells. Rapamycin enhanced the levels of TB10.4 specific CD8 T cells, (**p< 0.01 using t test; ± SD, 3 individual mice).
Figure 42: Rapamycin treatment following BCG vaccination enables mice to mount a stronger recall expansion of functional CD8 T cells in the lungs upon rechallenge with virulent *M. tuberculosis*. Lungs of rechallenged mice (week 16, Fig.39) have fully functional CD8 T cells which express granzyme-B and perforin biomarkers (** p< 0.01 using t test; ± SD, 3 individual mice).
Figure-43: Rapamycin treatment following BCG vaccination has no major effect on the numbers of antigen specific tetramer+ CD8 T cells in the spleens upon rechallenge with virulent *M.tuberculosis*. Left: Representative dot plot of tetramer-PE and IFN-γ FITC stained CD8 T cells. Flow analysis using Beckman Coulter flow cytometer using Flo-jo software (3 individual mice analyzed). Right: Spleens of rechallenged mice (week 16, Fig.39) show biomarkers of antigen specific CD8 T cells but there is no increase in numbers (± SD, 3 individual mice analyzed).
Figure-44: Rapamycin treatment following BCG vaccination has no significant effect on the expansion of CD8 T cells in the spleens upon rechallenge with virulent *M. tuberculosis*. Spleens of rechallenged mice (week 16, Fig.39) have functional CD8 T cells which express granzyme and perforin biomarkers but there is no increase in numbers ($\pm$ SD, 3 individual mice analyzed).
Figure-45: Rapamycin treatment following BCG vaccination has no significant effect on the expansion of CD8 T cells in the lymph nodes upon rechallenge with virulent *M.tuberculosis*. Lymph nodes of rechallenged mice (week 16, Fig.39) have functional CD8 T cells which express granzyme-B and perforin biomarkers but there is no increase in numbers (± SD, 3 individual mice analyzed).
Discussion

Two problems are evident in the control of human tuberculosis. First, primary childhood vaccination with BCG vaccine does not protect against adult tuberculosis occurring after 10 years of age. Second, tuberculosis does not generate a robust infection-induced immunity in adults (74). Thus, BCG induced long living memory T cells are not robust. Not surprisingly, booster vaccination with BCG is also not effective. This means that the central memory pool (MPECs) in adults is so small that it cannot be rejuvenated with booster BCG vaccine or through natural infection with tuberculosis. Thus, the first goal of our study was to determine if rapamycin in combination with BCG vaccine could be used to create a pool of MPECs which was different in quality. In other words, we proposed that the memory pool induced by BCG vaccine alone would be different than the one created through rapamycin-induced mTOR modulation during BCG vaccination.

Results of this study confirm that rapamycin induced MPECs were much better than BCG induced MPECs in protecting against rechallenge of tuberculosis in mice (Fig.40-44). As expected, BCG vaccine alone generated no protection against tuberculosis of the spleens and borderline protection against tuberculosis of the lungs. The protection data were interesting also because the recall population consisted of TB10.4 specific CD8 T cells expressing markers of CTL activity like perforin and granzyme. Our observations are novel because there are no previous reports which show CD8 T cell mediated protection of lungs in mice against rechallenge tuberculosis. It is thus clear that rapamycin treatment during BCG vaccination affects the quality and quantity of MPECs that can protect long term.
CHAPTER-3.5

*IN VITRO STUDIES ON THE EFFECT OF RAPAMYCIN ON DENDRITIC CELL- T CELL COCULTURES*
Background

Previous chapters show the dramatic result that rapamycin treatment induces a remarkable shift in the quality and quantity of antigen specific CD8 T cells which mediate protection against tuberculosis. In these studies, a complex mouse model was used to analyze the emergence of various subsets of T cells. Since rapamycin can affect both the APCs and T cells, it is possible that the mouse permits multiple interactions to take place between rapamycin, APCs and T cells. We therefore sought to determine if DCs treated with rapamycin in vitro could directly affect the signaling within T cells and allow them to change their phenotype. This would help to differentiate between the dual effects of rapamycin.

Various types of in vitro antigen presentation models are available to investigate the mechanism of T cell activation (21). We illustrated the use of APCs overlaid with BB7 T cells as models to investigate the MHC-II dependent presentation of peptides to CD4 T cells (Chapter 2). Typically, macrophages or DC’s are pulsed with soluble or bacterial antigens, which are processed by the DCs and epitopes presented in the context of either MHC-II or MHC-I on the plasma membrane (56). The DCs are then overlaid with either CD4 or CD8 T cell lines, which specifically recognize the peptide epitope (96). Overlaid T cells then respond by either secreting cytokines or another biomarker that indicates antigen recognition or binding [103]. In this study, we tested the ability of DCs to present a peptide to CD8 T cells and analyzed the expression of transcription factors that could affect the lineage and CTL function of the T cells.
Methods

A DC cell line DC2.4, obtained from bone marrow derived DCs of C57Bl/6 mice was used as the antigen processing cell in the *in vitro* assay. DCs were first activated with rapamycin overnight or left untreated. They were infected with a recombinant BCG strain which expressed an ovalbumin (Ova) derived peptide (H2Kb<sup>257-264</sup> peptide, SIINFEKL) and washed. DCs were overlaid with B3Z, a CD8 T cell hybridoma that is specific for Ova. B3Z CD8 specific T cells were gently scraped off after overnight incubation and flow cytometry analysis was performed to determine levels of T-bet, Eomes, perforin and granzyme-B using antibodies and using Flow-Jo software. It is noted here that, DCs were used as APCs although macrophages can also present antigen to T cells.
Results

DCs activated with rapamycin and infected with BCG were found to enhance the expression of T-bet and Eomes in CD8 T cells incubated overnight (Fig.46). Likewise, the coculture enhanced the expression of both perforin and granzyme in CD8 T cells (Fig.47). When a CD4 T cell line BB7 was overlaid on the same DCs, there was no significant change in the expression of T-bet and Eomes (Fig.48).
Figure 46: Rapamycin treated dendritic cells enhance the expression of T-bet and Eomes transcription factors (TFs) in CD8 B3Z-T cells early after antigen presentation. DCs (DC 2.4 cell line) activated with rapamycin (dose as indicated), overnight were infected with BCG vaccine expressing ova-SINFEKL for 4 hrs, washed and overlaid with CD8 T cells (B3Z T cells) specific for SINFEKL peptide. After 18 hrs, T cells were stained for intracellular TFs and analyzed using Beckman Coulter flow cytometer and Flow-jo software. Representative histogram of T-bet and Eomes expression from one of two similar 3 experiments is shown. Untreated DCs overlaid with B3Z T cells are controls.
Figure-47: Rapamycin treated dendritic cells enhance the expression of granzyme-B and perforin in B3Z CD8 T cells early after antigen presentation. CD8 T cells were overlaid on rapamycin activated and BCG-ova infected DCs as in Fig. 46. T cells were stained for granzyme-B and perforin and analyzed using flow cytometry. One representative histogram is shown from two similar experiments.
Figure-48: Rapamycin treatment of BCG infected DCs does not affect the intracellular expression of T-bet and Eomes in CD4 T cells. Rapamycin activated and BCG-ova infected DCs were overlaid with BB7 CD4 T cells as in Fig.46. BB7 T cells recognize the Ag85B epitope from BCG and therefore specific antigen presentation occurs between T-bet or Eomes stained BB7 CD4 T cells and DCs. T cells were analyzed using Beckman Coulter flow cytometer and Flow-jo software.
Discussion

In a previous study, we found that rapamycin activated DCs of mice enhance the intracellular processing of BCG vaccine and allow enhanced presentation of peptides to CD4 T cells \textit{in vitro} (49). This has been found to be due to the autophagy inducing effect of rapamycin, where BCG is sorted to into autophago-lysosomes resulting in enhanced peptide production. Although, these CD4 T cells recognize peptide in the context of MHC-II, it remained unclear if the process activated other intracellular events in T cells.

Recent research has shown that when DCs cross talk to T cells via TCR, a number of events are triggered within T cells that lead to their differentiation into Th1 immunity mediating effectors (43, 69). This process is of course dependent upon both MHC-TCR ligation and costimulation. Interestingly, CD4 and CD8 T cells seem to have several intracellular transcription factors that determine the eventual fate of T cells as effectors and memory cells. Thus, T-bet regulates the differentiation of Th1 T cells into effectors, while Eomes regulates the memory lineage of T cells. It should also be noted that T-bet and Eomes can have subtly different effects on subsets of T cells. Furthermore, T-bet also affects DCs (93). Coincident with the activation of these TFs, T cells may independently activate programs to secrete IFN-\(\gamma\), IL-2 and TNF-\(\alpha\) by CD4 T cells or enable perforin and granzyme synthesis among CD8 T cells to mediate cytolytic function (19, 110). The major finding of this study was that DCs activated with rapamycin induced a better expression of T-bet and Eomes in CD8 T cells along with enhanced perforin/granzyme-B expression.
upon antigen presentation. This did not occur during a similar treatment window *in vitro* with CD4 T cells. Thus, rapamycin preferentially and rapidly activated TFs in CD8 T cells which are consistent with its known positive effect on CD8 T cells *in vivo*. It should be noted that neither the CD8 T cells nor CD4 T cells were exposed to rapamycin directly *in vitro* in this study. However, when mice are given rapamycin *in vivo*, it seems possible that rapamycin could affect T cells separately. This has been shown in our *in vivo* studies where, rapamycin, in addition to affecting CD8 T cells also enhanced the function of CD4 T cells. Furthermore, rapamycin was given for longer periods in mice when it can have different effects and *in vitro* models do not recapitulate this event. We therefore tentatively conclude that rapamycin treated DCs have a rapid enhancing effect on the function of CD8 T cells through the induction of T-bet or Eomes. Additional studies seem necessary to determine if rapamycin treatment affects CD4 T cells when they are interacting with DCs.
CHAPTER-4

CONCLUSIONS
Tuberculosis is the leading cause of death due to infections in mankind with 2 million people dying of the disease. BCG is the only approved vaccine for humans and is being used in many parts of the world. Despite being the most frequently used vaccine, BCG generates partial immunity against childhood tuberculosis and does not protect against adult tuberculosis. Humans above the age of 10-15 years are not protected either by childhood vaccination or by infection with tuberculosis, which is expected to generate infection-induced immunity. Therefore, it has been proposed that the T cell responses induced by BCG are not strong and do not last longer. Experimental infections with *Mycobacterium tuberculosis* (Mtb) and vaccination with BCG are feasible using mice, which offer a model to evaluate vaccine induced T cell responses. These studies indicate that BCG induces moderately strong CD4 T cell responses in mice that are able to control tuberculosis to a certain extent. However, certain inherent properties of BCG vaccine seem to limit its potential as a vaccine. The overall goal of this proposal was therefore to investigate why BCG fails to induce robust CD4 and CD8 T cell responses, and identify newer strategies to strengthen the BCG vaccine.

Initial observations from our laboratory showed that antigen presenting cells (APCs) infected with BCG vaccine do not adequately present soluble mycobacterial antigen(s) to CD4 T cells via the MHC-II pathway (97). We therefore developed the first hypothesis under Aim-I that, lipids of BCG vaccine may bind the surface Toll-like receptors (TLRs) and inhibit expression of MHC-II and other cellular processes. Using *in vitro* assays for antigen presentation with BCG infected macrophages; we
confirmed that the 19 kDa lipoprotein and lipo-arabinomannan lipids, which are integral structures of cell wall in BCG, inhibited antigen presentation (Chapter 2). We then demonstrated that, BCG infection of APCs reduced the expression of MHC-II in macrophages. This observation explained why BCG vaccine was less than optimally effective as a vaccine. In mice this defect is apparent as a modest protection (decline in bacterial counts by 1-log_{10}) against aerosol induced tuberculosis. In humans, the protection induced by BCG vaccine is lost over the first 10 years of age. Investigation into the strategies to boost the efficacy of BCG vaccine then led us to the mechanisms of activation in APCs.

APCs like macrophages and dendritic cells use multiple TLRs to recognize microbial ligands. The latter are known to regulate cytokine expression by APCs and alter the micro-environment in which APCs cross-talk to T cells. However, TLRs also stimulate a wide variety of intracellular mechanisms in APCs and we sought to clarify them in the context of MHC-II mediated activation of T cells. First, we examined whether BCG induced down-regulation of MHC-II could be enhanced by alternate activation of TLRs. We discovered that BCG down regulated MHC-II through the up-regulation of the ubiquitinating enzyme MARCH1 and alternate activation of many TLRs, particularly TLR-7/9 strongly enhanced MHC-II in macrophages and DCs. Elsewhere, we reported that TLR-7/9 ligands are indeed the best adjuvants to boost the efficacy of BCG vaccine against experimental tuberculosis of mice (unpublished observations). Our studies were novel and thought provoking. First, the efficacy of BCG vaccine has been increased using
genetic manipulations but to date it has not been possible to counteract the suppressive effect of BCG vaccine for APCs. TLR-4 activating LPS derived MPL has been used as adjuvant with subunit protein vaccines against tuberculosis. This study shows that TLR-7/9 ligands are much more powerful than TLR-4 activating LPS, and they show an unique ability to enhance MHC-II unlike the less effective LPS. Since cpgDNA, a TLR-9 ligand has been approved for use as adjuvant against certain cancers in humans; we anticipate that similar ligands of TLR-7/9 will be used as adjuvants with BCG vaccine for humans.

Our investigations into the TLR mediated activation of APCs also identified several avenues for future research. Since the major lipids of BCG vaccine suppressed MHC-II, it seems worthwhile to evaluate lipid-deficient BCG vaccine mutants as possible vaccine candidates. In support of this observation, the 19 kDa lipoprotein knockout strain of BCG vaccine was reported more immunogenic but not tested as a vaccine (44, 100). Second, TLR-ligands were found to use subtly different activation pathways to trigger MHC-II via AP-1 and CREB before they bind the CIITA. It is possible that genetically engineering a protein inducer of AP-1 or CREB into BCG may yield a more immunogenic BCG vaccine. Finally, a combination of TLR-adjuvants may boost the efficacy of BCG vaccine than a single adjuvant. Overall, Aim-I studies of this proposal have unraveled newer novel TLR- dependent mechanisms to improve BCG vaccine.
Since the Aim-I studies established a mechanism to render the CD4 T cell response more robust, we next sought to prolong the ‘longevity of protection’ generated by BCG vaccine.

While CD4 T cells protect against acute infection, CD8 T cells have been proposed to be able to persist in the human body for years and undergo recall expansion to protect against infection and re-infection. However, BCG induces a poor CD8 T cell response in mice. Part of the problem is that the membrane structure of BCG phagosome within APCs prevents leakage of antigens into cytosol for proteasome processing prior to loading of peptides into MHC-I. One way to bypass this problem is to enhance the sorting of BCG to lysosomes, where peptides are produced and get routed to MHC-I. The second way is to enhance the secretion of antigens from BCG into the cytosol and this requires additional genetic engineering of the vaccine. Grode et al., cloned LLO into BCG improving the leakage of antigens from the phagosome into cytosol and better induction of CD8 T cell responses in mice (41).

Our previous studies indicated that rapamycin, an immune-modulator enhanced the sorting of BCG vaccine to lysosomes (49). Others reported that rapamycin increased the longevity of CD8 T cells by repressing mTOR. Thus, for Aim-II studies, we developed a new hypothesis that the dual effects of rapamycin may enhance CD8 T cell response to BCG vaccine if the drug were to be given to mice. We used three models of infection and vaccination followed by infection to determine the effect of rapamycin on CD4 and CD8 T cells during tuberculosis.
Since APCs prime and expand antigen specific T cells, one measure of the efficacy of APC mediated priming is the number of tetramer specific T cells. In an aerosol infection model, rapamycin was able to stimulate the expansion of tetramer specific-CD8 T cells but this correlated only with a limited control of tuberculosis. We next examined the ability of rapamycin to enhance the efficacy of BCG vaccine by administering low and high doses of rapamycin with BCG vaccine, and found several significant effects. First, rapamycin dramatically enhanced the efficacy of BCG vaccine to clear virulent Mtb from the organs of mice. Second, rapamycin enhanced the magnitude of antigen specific CD8 T cells specific for TB10.4 antigen. Third, rapamycin enhanced the magnitude of memory precursor effector T cells (MPECs), thought to persist in mice for prolonged periods. Finally, we found that the rapamycin-BCG induced functional MPECs persist in mice, and undergo robust recall expansion into effector CD8 T cells expressing perforin and granzyme, and protect against rechallenge of tuberculosis. Overall, these studies demonstrated that rapamycin combination with BCG could enhance short term effector responses as well as prolong long term memory.

The ability of rapamycin to enhance short term protection was obviously due to a strong effector T cell response (CD62L\text{low} population) although surprisingly, rapamycin enhanced CD4 cell responses in addition to CD8 T cells. We suggest that under \textit{in vivo} conditions, rapamycin boosted the function of APCs to sort BCG to lysosomes which enabled a better activation of CD4 T cells. It is well established that rapamycin does not affect CD4 T cells positively at the doses we used in mice.
However, other than our own previous study (49), the long term effects of low dose rapamycin has not been explored in the context of the ability to APCs to prime CD4 T cells. The dual enhancing effect of rapamycin is therefore a novel observation that suggests that mTOR repression is likely to markedly boost the efficacy of BCG vaccine. Since BCG is immunologically sequestered (49, 77), the use of mTOR repressors like rapamycin in humans to enhance processing of BCG antigens and ensure long lasting T cell responses remains an intriguing approach. Low doses rapamycin are also not likely to deleteriously affect the immune system in humans.

The beneficial effects of rapamycin on CD8 T cells in this study was anticipated but still brought out surprising observations. Soon after we described that rapamycin enhances lysosome mediated processing of BCG enhancing its efficacy against tuberculosis in mice, others reported that rapamycin enhanced the longevity of CD8 T cells in mice against viral infections.

There are two schools of thought on how long term memory develops against pathogens (63, 71, 73). The ‘transition hypothesis’ suggests that effector T cells switch to memory T cells through selective activation or deactivation of intracellular signaling mechanisms including mTOR, Eomes and T-bet and perhaps others. Under the ‘induction hypothesis’, memory T cells are always present at low numbers following APC mediated priming of CD8 T cells, but their numbers become robust after prolonged priming from APCs. Whether one or both mechanisms operate in the development of long term memory has been thought to depend upon
the pathogen. Data from our studies (Chapter 3) suggest that during BCG vaccination the beneficial effect of rapamycin likely involved both mechanisms. First, BCG was not processed efficiently by naïve APCs, but presumably rapamycin enhanced its processing. We propose that this led to enhanced activation and prolonged contact with T cells during the 30 days treatment of mice with rapamycin following BCG vaccination. In evidence of this observation, both CD4 and CD8 T cells increased in numbers in this mouse model. Second, rapamycin induced a robust tetramer specific CD8 T cell response which suggested that these antigen specific CD8 T cells had a better chance to transition into memory T cells. In Fig.38 for example, after mice have been treated with drugs and infection was cleared, there was a marked increase in the numbers of TB10.4 specific CD62L⁺ memory CD8 T cells in the spleens.

The final intriguing discovery of this study was that TB10.4 CD8 T cells that persisted in drug cured mice, underwent a strong recall expansion after rechallenge of tuberculosis and protected against infection. Since BCG vaccine generally fails to show strong protection against re-infection of tuberculosis, these studies strongly argue that rapamycin treatment of BCG vaccine qualitatively alters memory response enabling long term immunity. These data now suggest future studies which are of translational value. To boost the long term efficacy of BCG vaccine we speculate that infants could be given low dose rapamycin following BCG vaccine, although there are likely to be impediments in administering rapamycin to infants. The discovery of Eomes as a biomarker of long living CD8 T cells however
suggests that, cloning of an Eomes inducing cytokine like IL-15 into BCG can prolong its ability to confer long term memory. We conclude that Aim-I and II together have generated novel observations on boosting the ability of BCG vaccine and the data are also likely to be helpful for developing vaccines for other intracellular pathogens.
CHAPTER-5

MATERIALS AND METHODS
CHAPTER 2:

Macrophages: Primary bone marrow derived macrophages (MΦs) from C57Bl/6 mice (4-8 week old M/F, Harlan or Jackson animal providers, USA) were grown in Iscove’s medium with 10% FBS (IDM) and 10 ng/mL GM-CSF and CD11c beads (Miltenyi INc, USA; 130-052-001) were used to deplete DCs from bone marrow cells cultured for 7 days. The CD11b⁺ CD11c⁻ MΦs were plated in GM-CSF free medium and used for activation and infection. MΦ-derived BMA.A1 cell line (kind gift of Dr. Kenneth L. Rock, U. Massachusetts, USA), were similarly cultured in IDM and used for many experiments. MΦs were activated with TLR-ligands (1-5 μg/mL; dissolved in sterile endotoxin-free water, Invivogen, USA) and incubated overnight at 37°C. They were then infected with *Mycobacterium bovis* BCG (Pasteur strain, ATCC35734; MOI = 1), prepared as a single cell suspension, for 4 hr on a shaker at 37°C, washed and collected for further analysis. Viability of MΦs was > 90% using trypan blue.

*In vitro antigen presentation assay:* This has been described in detail earlier (97). Briefly, MΦs were treated or not with either mycobacterial lipids (19 kDa lipoprotein and lipoarabinomannan), inhibitors of TLR-signaling or activated with TLR-ligands, followed by infection with BCG (MOI = 1). Washed monolayers were overlaid with BB7 T cell hybridoma (kind gift of Dr. Cliff Harding, CWRU) (1:20 ratio) and supernatants collected either 4 hr or 18 hr after overlay and tested for IL-2 using sandwich ELISA.
CD4 T cell hybridoma: The Ag85 epitope-specific (241-256) T cell hybridoma (BB7) a gift from Dr. Clifford Harding (Case Western Reserve University, Cleveland, OH), was maintained in DMEM medium (Sigma, USA) supplemented with 10% heat-inactivated FBS (HyClone Laboratories), 50 µM 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES buffer, nonessential amino acids, 100 µg/ml penicillin, and 100 µg/ml gentamicin.

Inhibition of TLR-signaling: Various inhibitors of MAPK and API/CREB were used to block signaling due to TLRs. The inhibitors were from Calbiochem and used at doses recommended by the manufacturer. MΦs were treated with inhibitors of MAPKs and API/CREB for 2 hr, activated with TLR-ligands for 2 hr and infected with BCG for 2 hr prior to fixation and assay of surface MHC-II. This rapid procedure was adapted to ensure >90% viability of MΦs.

siRNA down-regulation of MARCH1: MΦs were treated with siRNA and scrambled siRNA directed against MARCH1 (sc-106199; sense/antisense and scrambled, SantaCruz Biotechnology, USA;) as recommended by the manufacturer and rested for 14 hr. They were tested naïve, or infected with BCG for 4 hr followed by surface expression of MHC-II evaluated using flow cytometry.

Immunofluorescent staining of cells: MΦs, BMA.A1 or DC.2.4 cell lines were stained using antibodies against MHC-II, MHC-I, CD80, CD86. After incubation for 30 mins on ice with antibodies, cells were washed with PBS and fixed with...
Cytofix/Cytoperm buffer (BD biosciences, #555028). Intracellular staining was using antibodies for MARCH-1 and IL-10. Cells were incubated for 30 mins and washed with PBS. MARCH1 assay did not require brefeldin-A as it is not secreted (MHC-II,#12-5322; CD80,#12-0801; CD86, #12-0862;IL-10, #11-7101; MHC-I, #17-5985, all from Ebiosciences USA) (MARCH1, #D16-sc104369; Santacruz Biotechnology, USA).

**Flow cytometry:** The fixed cells were acquired and analyzed for surface or intracellular markers using Gallios flow cytometer (Beckman Coulter) and the data analysis was carried out using Flow-Jo software (Treestar, Ashland). The graphical analysis was performed using the Prism Software.

**Immunoprecipitation and Western Blot analysis of ubiquinated MHC-II: MΦs in 6 well plates were treated with TLR-ligands at a concentration of 1 μg/mL or none overnight at 37°C. They were then infected with BCG for 4 hr (MOI=1:1) on a shaker at 37°C. Cells were centrifuged for 10 min (3,000 x g, 4°C) and the supernatant was removed. Cells were resuspended in 30 μL of the lysis buffer (mammalian Protein Extraction Reagent (M-PER, Pierce) with 5 μM MG132, a proteasome inhibitor, and a protease inhibitor cocktail (112 μM PMSF, 3 μM aprotinin, 112 μM leupeptin, 17 μM pepstatin). Samples were incubated with shaking at 4°C for 1 hr, centrifuged for 20 min (10,000 rpm 4°C) and nuclei free supernatants were collected and estimated for protein and normalized. One μg of anti-mouse MHC-II antibody (Ebioscience, #14-5321) was added per sample, which
were gently mixed overnight at 4°C. Fifteen μL of Protein A/G PLUS-Agarose beads (Santacruz Biotechnology, USA) were added to each sample and gently mixed for 4 hr at 4°C. Supernatants were removed through centrifugation (2000 rpm, 15 min) and beads were washed three times with 1x PBS. Sample buffer was added to each sample, and each tube was boiled for 5 minutes to elute proteins from beads. Samples were loaded onto a 12% SDS-PAGE gel and electroblotted onto a PVDF membrane. Western blotting was performed using an antibody directed towards polyubiquitin (Ebioscience, #14-6078).

**Statistical analysis:** Statistical analyses were done using GraphPad software. A p value ≤0.05 was considered statistically significant for these analyses. Student’s t-test and standard one-way ANOVA followed by Dunnett’s Multiple Comparison Test were used to determine statistical significance. Experiments were repeated a minimum of three times and in each, at least triplicate wells were used. Data are presented using the mean ± SD of results for 1 or 3 similar experiments or are averaged from 2-3 experiments as indicated.

**CHAPTER 3:**

**BCG & Rapamycin:** *M. bovis* BCG (ATCC-35734) was grown in Dubos broth and log phase organisms were washed thrice with PBS, sonicated CFU counts determined by plating before infections. Rapamycin was purchased from Santacruz Biotechnology.
Biotechnologies (USA). It was dissolved at a final concentration of 1 mg/ml in sterile PBS (phosphate buffer saline).

**Mouse immunization, challenge and lung CFU determination:** Female C57BL/6 mice of age 8–12 wk were used for most experiments. C57BL/6 were purchased from Jackson ImmunoResearch Laboratories. They were housed within a BSL-3 vivarium and fed food and water and chow. For most experiments, mice were immunized with 75 µg/kg of rapamycin (low dose) and 750 µg/kg (high dose) of mice intraperitoneally, with or without BCG administered subcutaneously. For most experiments, mice were immunized once. After 4 weeks of vaccination, mice were infected with a log phase, highly viable single-cell suspension of *M. tuberculosis* Erdman strain via aerosol using a Glas-Col aerosol apparatus, calibrated to deliver 100–200 CFU per mouse lungs over 30 min of nebulization. For Rechallenge experiments; after 8 weeks post challenge, mice were treated with the isoniazid (INH) drug for 3 weeks and were rested for a week followed by a rechallenge on week 12 with Mtb via aerosol using a Glas-Col aerosol apparatus, calibrated to deliver 100–200 CFU per mouse lungs over 30 min of nebulization. Protocols for the animal experiments described in this study were approved by Institutional Animal Care and Use Committee. The amount of bacteria delivered into the lung was confirmed 24 h after exposure by plating neat lung homogenates on 7H11 agar from three mice.
**Colony forming units (CFU) determination:** Protection was determined post-challenge and post rechallenge by harvesting the lungs and spleens from the infected mice, homogenizing the tissues in 0.05% PBS–Tween 80, and plating ten-fold serial dilutions on 7H11 agar plates for bacterial growth. Bacterial colonies were counted 4 weeks after incubation at 37°C. Values ranging between 10 and 100 colonies per plate per dilution were used to calculate the bacterial burden per organ and were expressed as: mean log$_{10}$ CFU saline/untreated - Mean log$_{10}$ CFU vaccine.

**P value for CFU counts vaccine vs. untreated control, 5 mice per time point:** Mice were left untreated (5 per group) or vaccinated with BCG followed 4 wks later with aerosol challenge with *M.tuberculosis*. Mice were sacrificed and colony counts of *M. tuberculosis* measured in the lungs. Data are plotted as log$_{10}$ CFU per organ per mouse. The example to the right shows that such early CFU data is highly discriminative and predictive of survival, and importantly the statistical power of these data is superior to survival data. Allowing for a statistical power of 0.8–0.9, and a usual variance of 0.2–0.3 log$_{10}$ CFU, a reduction in the mean CFU values between saline controls and test groups of about 0.7 log$_{10}$CFU is usually significant, when n= 5 animals are used (2-way ANOVA used for p values).
**In vitro Antigen presentation assay using CD8 T cells (Antigen processing).**

DC2.4-C57BL/6 specific cell line was grown in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% heat-inactivated FBS (HyClone Laboratories), 50 µM 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES buffer, nonessential amino acids, 100 µg/ml penicillin, and 100 µg/ml gentamicin. DC2.4 was plated in 6 well plates and used as naive cells or activated for 18 hours with different doses of rapamycin-5 µg or 10 µg. They were infected with BCG at MOI=1. After a 4-hr infection, monolayers were washed and were used directly to overlay B3Z T cells at a ratio of 1:20. The cells were incubated at 37°C overnight and was scraped the next day and analyzed for flow cytometry.

**CD8 T cell hybridoma:** B3Z cells, a CD8+ T-cell hybridoma has been engineered to secrete β-galactosidase when its T-cell receptor engages an Ova257-264:Kb complex (24, 25). It was kind gift of Dr. Nilabh Shastri (University of California, Berkeley) and were maintained in a medium (RP-10) consisting of RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum, L-glutamine, HEPES, 50 µM 2-mercaptoethanol, 50 U/mL penicillin, and 50 µg/mL streptomycin.

**Flow cytometry:** Lungs were obtained from C57Bl/6 mice at 4 weeks post aerosol challenge and 4 weeks post rechallenge with Mtb and processed with 1mg/ml collagenase and 1mg/ml elastase (Sigma Biologicals, USA) to break down the fibrous tissue material. It was then passed through cell strainers to achieve a cell
suspension devoid of tissue pieces. Spleen and lymph nodes were mashed using frosted slides till a suspension of cells was obtained. The tissues were further treated with ACK lysis buffer (BioWhittaker, USA) which removes RBCs followed by incubation. In some experiments were restimulated T cells with Phorbol 12-Myristate 13-Acetate, Ionomycin and Brefeldin-A (Sigma Biologicals, USA) to increase further stimulation and expansion of lymphocytes. Abs (CD4-FITC, CD8-FITC, IFNγ-APC, Perforin-APC, Granzyme-B-PE, Tbet-PE, Eomes-APC, PD1-APC, KLRG1-FITC, CD44-FITC, CD127-PE, CD62L-APC, CD3-APCCy7) were purchased from Ebioscience, USA and tetramers were obtained from NIH facility, Emory University. Flow staining was performed as per BD Biosciences protocol. Cell events were collected using Beckman-Coulter-Gallios cytometer and the cytokine profile was analyzed using FlowJo software (Tree Star, Ashland, OR). The graphs were plotted and analyzed using graph pad prism software.

**Flow cytometry for in vitro studies:** CD8 T cells, B3Z T cells and BB7 T cells were incubated with Phorbol 12-Myristate 13-Acetate, Ionomycin and Brefeldin-A (Sigma Biologicals, USA) for 4 hr to increase further stimulation and intracellular staining of markers. The T cells were collected, washed with PBS and stained with CD8-FITC, CD4-FITC, Tbet-PE, Eomes-APC, Perforin-APC and Granzyme-B-PE (Ebioscience, USA) according to BD biosciences protocol. Cell events were collected using Beckman-Coulter-Gallios cytometer and the cytokine profile was analyzed using FlowJo software (Tree Star, Ashland, OR). The graphs were plotted and analyzed using graph pad prism software.
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M. Torres. 2012. Human phagosome processing of Mycobacterium tuberculosis antigens is modulated by IFN-gamma and IL-10. Immunology.


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GRE.

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Research Positions

2003 Training at P.H.Medical Centre in Pathology, Hematology, Microbiology and Biochemistry, Mumbai, India
2004 Attended Plant Tissue Culture Workshop at Padmashri Research Institute at Bangalore, India
2005 Summer Training in Biotechnology at Haffkine Research Institute, Mumbai, India
2006 Training at Serum Institute of India in Bacterial Vaccine Production Department, Pune, India
2007 One Month Clinical Internship at K.E.M Hospital, Pune, India

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Posters and Presentations:
- Presentations include Somatic Embryogenesis in Plant Tissue Culture, High Performance Liquid Chromatography in Bachelors
- Neutraceuticals as Antivirals, Dengue and Hemorrhagic Fevers in Masters
- Associated with National Service Scheme (N.S.S) in 2005 and 2006.
- One of the student organizer for Autophagy Mini-symposium held on June 26, 2010 at UT Medical School
- Poster Presented at the American Society for Microbiology Conference (ASM), San Diego- May 2010: ‘TLR-adjuvanted BCG induces stronger Th1 responses in mice leading to increased vaccine efficacy.’
- Poster Presented at the the American Association of Immunologists Conference (AAI), San Francisco- May 2011. ‘Toll like receptor agonists stabilize MHC-II expression in macrophages thereby enhancing peptide presentation by BCG infected macrophages’
- 3rd Prize: Poster competition at the MBID retreat in May 2011: TLR-adjuvanted BCG induces stronger Th1 responses in mice leading to increased vaccine efficacy
- Oral Presentation at Molecular Basis of Infectious Diseases Convention (MBID), Houston- March 2012: Toll like receptor (TLR) agonists stabilize MHC-II expression in BCG infected macrophages and enhance peptide presentation
- Poster Presented at the American Association of Immunologists Conference (AAI), Boston-May 2012: ‘Enhancement of the long term efficacy of BCG vaccine against experimental tuberculosis in mice through rapamycin induced modulation of CD4 and CD8 T cell functions’

STUDENT MEMBER in COMMITTEE:
- Student member of Institutional Biosafety committee (IBC)- UT Houston from Feb 2011-present
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MANUSCRIPTS:

List of Publications
3. Arshad Khan, Jaymie Estrella, Eulogia Roman, Pearl Bakhru, Christopher R. Singh, Subramanian Dhandayuthapani, Dekai Zhang, Jeffrey D.Cirillo,
N.Tony Eissa, Robert L. Hunter & Chinnaswamy Jagannath. Toll-like receptor- ligands boost the efficacy of BCG vaccine against tuberculosis in mice, through increased processing of BCG antigens in macrophages and dendritic cells (Journal of Immunology JI: 12-00680-FL, pending revision)

MANUSCRIPTS in progress:


5. Pearl Bakhru, Emily Soudani, Arshad Khan, Dorothy Lewis, Robert L. Hunter and Chinnaswamy Jagannath. Rapamycin cotreatment with BCG vaccine enhances antigen-specific CD8 T cell responses and central memory in mice associated with increased protection against tuberculosis (under preparation) (*presented as a poster in Amer. Asso. Immunologists General Meeting, Boston 2012)

Area Course Requirements fulfilled:
• Basic Concepts of Tumor Biology – UT Smithville (Systems Requirement)- B
• Cancer Cell Signaling (Cellular Requirement)- B
• Current Methods in Molecular Research-I (Quantitative Requirement)- A
• Current Methods in Molecular Research-II (Quantitative Requirement)- A
• Principles in Pathology (Program in Molecular Pathology Requirement)- B
• Ethics (General Course Requirement)- P
• Eukaryotic Gene Expression (Molecular Requirement)- B
• Current topics in Translational Research (MBID requirement)- P
• Molecular basis of Pathogenesis (MBID requirement) - A

Seminars:
• Seminars in Molecular Carcinogenesis
• Seminars in Experimental Pathology

Overall GPA: 3.3/4