Novel Strategies to Prevent and/or Treat Tuberculosis

by

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Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), continues to be a leading infectious cause of death worldwide with approximately 10 million new cases and 2 million deaths attributed annually. There has been resurgence in active TB infections since 1980s and the World Health organization (WHO) has declared TB to be a "Global emergency". The lack of an effective vaccine, the lengthy treatment regimens with multiple chemotherapeutic agents that have serious side effects, the prevalence of co-infection with HIV and the increasing number of cases of multi-, extensively-, and totally-drug resistant tuberculosis (MDR, XDR, TDR-TB), necessitate the development of novel vaccine and therapeutic regimens to treat and control the global spread of tuberculosis. The immune correlates of protection from infection and control of an ongoing infection are not clear yet, but intensive studies suggest the important role of innate immunity and multi-specific, polyfunctional adaptive cellular immunity.

In this study, I have designed a novel subunit vaccine by conjugating a palmitoyl-lysine residue to peptides of the ESAT-6 antigen of *Mtb*, which corresponds to dominant human T cell epitopes. I sought to evaluate their ability to induce protective cellular immunity against *Mtb* (H37Ra) by subcutaneous (s.c.) and intranasal (i.n.) immunizations in a mouse model of *Mtb* infection. I have also evaluated how an adjuvant [Poly I:C, MPL, Gardiquimod (GDQ) and heat-killed *Caulobacter crescentus* (HKCC)] contributes to enhancing the induced responses and resulting protective efficacy of lipopeptides. My results demonstrated that single C-terminal palmitoyl-lysine modified lipopeptides of ESAT-6 elicited significant antigen specific $CD4^+$ and $CD8^+$ T cell responses upon subcutaneous immunizations. Intriguingly, a combination of immunogenic lipopeptides of ESAT-6 antigen exhibited local (pulmonary) and systemic immune responses along with efficient protective efficacy when administered intranasally or subcutaneously. Surprisingly, immunization with ESAT-6 derived lipopeptides with MPL and HKCC enhanced protection, whereas PolyI:C and GDQ led to reduce protection associated with specific local and systemic immune modulation. My studies demonstrate the potential of ESAT-6 derived lipopeptides as a promising vaccine candidate against *Mtb*, and emphasize that selection of the adjuvant is critical for the success of vaccines.

In addition, I have investigated the ability of the newly discovered immunomodulator HKCC to induce immune responses capable of controlling mycobacterial growth. I examined the effect of HKCC *in vitro* on various human immune cells present in human peripheral blood mononuclear cells (PBMCs). I also evaluated the host-mediated anti-mycobacterial effects of HKCC in human macrophages infected with *Mtb* (H37Ra) and *M. avium,* and

in a mouse model of *Mtb* infection. My results demonstrated that HKCC stimulate innate immune cells such as antigen-presenting cells (APCs), NK and NKT cells, and also lead to induction of multiple cytokines upon 24 h and 96 h culture with human PBMCs. Intriguingly, treatment with HKCC stimulated PBMCs supernatant led to significant reduction of *Mtb* and *M. avium* replication within human macrophages. Further, immunotherapy with HKCC alone and combination with isoniazid therapy significantly controlled mycobacterial growth in lungs, liver and spleen of *Mtb* infected mice with the induction of local and systemic protective immune responses. These findings reveal the promise of HKCC as a novel immunotherapy to treat mycobacterial infections.

Overall, a promising vaccine candidate and a novel immunotherapeutic approach have emerged from my studies, which may be developed to prevent and/or treat TB infections.

Preface

This thesis is an original work by Nancy Gupta. The animal experiments/procedures (Protocol# AUP 279, until July 15, 2016) were approved and conducted in accordance with the University Animal Care and Use Committee (ACUC) for Health Sciences. The collection of human blood was approved (Protocol# 3983 until Nov 9, 2016) by University of Alberta Human Research Ethics Board.

Chapter 2 of this thesis has been published as N. Gupta, S. Vedi, D.Y. Kunimoto, B. Agrawal and R. Kumar, "Novel lipopeptides of ESAT-6 induce strong protective immunity against *Mycobacterium tuberculosis*: Routes of immunization and TLR agonists critically impact vaccine's efficacy", *Vaccine*, Vol. 34, 5677-5688. I was responsible for the experiment design, execution, data collection and analysis, and manuscript writing. Dr. S. Vedi assisted with the collection and processing of BALs. Dr. B. Agrawal contributed to concept formation, manuscript edits and composition. Dr. R. Kumar and Dr. Kunimoto were the supervisory authors and were involved with concept formation, data analysis and manuscript composition.

Dedication

To

My Granny

With love and Honor

Acknowledgments

I thank my supervisor Dr. Rakesh Kumar for his encouragement, consistent support and guidance all along my Ph.D. work. His direct, straightforward and passionate approach to life and science is always inspiring me. I am deeply grateful to him for being a constant source of inspiration and for always sustaining me in pursuing my own ideas.

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AA	: Amino Acid
α-GalCer	: Alpha Galactosylceramide
ADCC	: Antibody-Dependent -Cell -Mediated Cytotoxicity
Ag	: Antigen
AIDS	: Acquired Immune Deficiency Syndrome
ANOVA	: Analysis of Variance
APC	: Allo-phycocyanin
APCs	: Antigen-Presenting Cells
ATCC	: American Type Culture Collection
BC	: Before Chirst
CCAC	: Canadian Council of Animal Care
CCL22	: C-C Motif Chemokine 22
CD	: Cluster of Differentiation
cDNA	: Complementary DNA
CFA	: Complete Freund's Adjuvant
CLRs	: C-type Lectin Receptors
CFSE	: 5(6)-Carboxy Fluorescein N-hydroxy Succinimidyl Ester
ConA	: Concanavalin A
CPM	: Counts Per Minute
CpG	: Cytosine-phosphate-Guanine
CTL	: Cytotoxic T Lymphocyte
CTLA-4	: Cytotoxic T-lymphocyte-Associated Antigen 4
CXCR6	: Chemokine (C-X-C Motif) Receptor 6
GrB	: Granzyme B
DAMPs	: Danger Associated Molecular Patterns
DCs	: Dendritic Cells
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylene Diamine Tetraacetic Acid
ELISA	: Enzyme-linked Immuno Sorbent Assay
ER	: Endoplasmic Reticulum
FACS	: Fluorescent-activated Cell Sorting
FBS	: Fetal Bovine Serum
FITC	: Fluorescein Isothiocyanate
FoxP3	: Forkhead Box P3
GM-CSF	: Granulocyte-macrophage Colony Stimulating Factor
GDQ	: Gardiquimod
HDL	: High Density Lipoprotein
HIV	: Human Immunodeficiency Virus
HKCC	: Heat-killed *Caulobacter crescentus*
HSLAS	: Health Sciences Laboratory Animals Services
ICAM-I	: Intercellular Adhesion Molecule-1
IFN-γ	: Interferon-gamma
IL	: Interleukin

IN or i.m.	: Intranasal
ISGs	: Interferon Stimulatory Genes
JAK	: Janus Kinase
kDa	: Kilo Dalton
LPS	: Lipopolysaccharide
mAb	: Monoclonal Antibody
mDCs	: Myeloid Dendritic Cells
mg	: Milligram
MHC	: Major Histocompatibility Complex
min	: Minute
MIP-1α	: Macrophage Inflammatory Protein-1 Alpha
MCP-1	: Monocyte Chemoattractant Protein-1
ml	: Milliliter
NK cells	: Natural Killer Cells
NF-κB	: Nuclear Factor-Kappa B
OD	: Optical Density
ORF	: Open Reading Frame
PAMPS	: Pathogen-associated Molecular Patterns
PBS	: Phosphate Buffered Saline
PBMCs	: Peripheral Blood Mononuclear Cells
PD-1	: Programmed Cell Death Protein 1
PDL-1	: Programmed Cell Death Protein Ligand-1
pDCs	: Plasmacytoid Dendritic Cells
PE	: Phycoerythrin
pg	: Picogram
PHA	: Phytohaemagglutinin
Poly I:C	: Polyinosinic-polycytidylic Acid
PPAR-γ	: Peroxisome Proliferation Activated Receptor Gamma
PRRs	: Pattern Recognition Receptor
RBCs	: Red Blood Cells
RIG-1	: Retinoic Acid Inducible Gene-I
RPMI	: Roswell Park Memorial Institute
RNA	: Ribonucleic Acid
rpm	: Revolution Per Minute
s.c.	: Subcutaneous
STAT	: Signal Transducer And Activator Of Transcription
TCR	: T Cell Receptor
TGF-β	: Transforming Growth Factor-beta
Th	: T Helper
TLR	: Toll Like Receptor
VLDL	: Very Low Density Lipoprotien
µg	: Microgram
µl	: Microliter

Chapter 1

Introduction to Tuberculosis

1.1 Tuberculosis: Ancient Enemy, Present Threats

Tuberculosis (TB) is one of the oldest known human diseases. TB is still one of the major causes of high mortality among infectious diseases. TB is thought to have originated more than 150 million years ago during the Jurassic period [1]. The causative agent of TB, *Mycobacterium tuberculosis* (*Mtb*) originated from soil and over the time some species of mycobacteria evolved to live in mammals. Literature reports have shown that early ancestors may have been infected with *Mtb* as far back as 3 million years ago in East Africa [2]. However, prevailing strains of *Mtb* are thought to have originated about 15,000-20,000 years ago. *Mtb* DNA was isolated from Egyptian mummies with bone disease more than 5,000 years ago. *Mtb* infection was also found in Israel (9000 year/ago), Peru (1000 year/ago), India (3300 year/ago) and China (2300 year/ago). TB reached Western Europe and North America during the 18^{th} and the first half of the 19^{th} centuries [3, 4].

TB has been known by many names over its long history in humans. Hippocrates (460–370 B.C.), the ancient Greek physician, identified TB as phthisis, the most widespread, and invariably fatal disease of his time. During the first half of the 20th century, TB was called "consumption" or "White Plague" [5-8].

Until 1882, the cause of TB was unknown. On March 24, 1882, however, the history of this disease changed when Robert Heinrich Hermann Koch identified tubercle bacteria that he presented as "Die Aetiologie der Tuberculose". Robert Koch isolated the bacteria from an infected host, grew it in culture and demonstrated that it is the causative agent of TB [9, 10]. In 1909, pediatrician Clemens Freiherr von Pirquet observed positive tuberculin reactions upon subcutaneous administration of *Mtb*-derived protein in children without any symptoms of disease and introduced the term "latent tuberculosis" [11]. In 1921, Albert Calmette and Camille Guérin

developed an attenuated strain of *Mycobacterium bovis (M. bovis)* as a preventive vaccine for TB, Bacille Calmette-Guérin (BCG) [12].

The 1950s brought about the great advances in treating TB with the development of several anti-tuberculosis drugs and their different combinations. In the 1970s, TB was starting to be considered as a disease of the past-era. However, decades of widespread use of antibiotics resulted in the new emergence of drug-resistant strains of mycobacteria. Unfortunately, in the 1980s, TB returned as one of the most dangerous infectious diseases, and presently it continues to spread in every corner of the globe [13-15]. The resurgence in the TB cases worldwide has been due to its linkages with the HIV/AIDS epidemic, increased mobility and immigration of people, increased poverty and homelessness, poor compliance of drug regimens and their side effects, and/or emergence of drug-resistant variants that are not susceptible to almost all of the available drugs [16-21]. Human immunodeficiency virus (HIV) is the most important determinant of the widely observed increases in TB in both developing and industrialized countries and is associated with a high TB attack rate, rapid disease progression and high mortality [22-24]. In addition, other immunocompromised individuals such as cancer patients, organ transplantation recipients and elderly people are also highly susceptible to TB infection, reactivation and spread [25]. The TB epidemic has exponentially worsened because of lack of effective treatment or vaccine.

In 1993, the World Health Organization (WHO) declared TB a "Global Emergency" due to increasing numbers of TB cases and a rise in multi-drug resistant cases [26]. In 1995, WHO introduced DOTS (directly observed treatment, short-course) to control the disease and prevent development of drug-resistance [27]. It has been estimated that 225 million new infections will occur and about 79 million people worldwide will die of TB between the years 1998-2030 [28]. In 2006, WHO launched the Global Plan to stop TB in line with the Millennium Development Goals, with

an aim to reduce TB prevalence and deaths by 2015 by 50% compared to a baseline of 1990, and eliminate TB as a public health problem by 2050 [29].

However, despite these global efforts, TB still represents an enormous challenge to health services and has a significant socioeconomic impact on communities. Further, in 2009, identification of total drug-resistant TB strains (TDR-TB), an incurable form of TB, suggests that the current efforts to curb TB disease are not sufficient and more research efforts are needed.

1.2 *Mycobacterium* Complexes

In the bacteria kingdom, Mycobacteria belong to the Phylum Actinobacteria, Order Actinomycetales, Family Mycobacteriacea and Genus *Mycobacterium*. Mycobacteria are classified as gram-positive bacteria due to their genetic similarities with other gram-positive bacteria [30, 31]. Mycobacterial species are further classified into different complexes based on DNA sequencing as follows:

The *Mycobacterium tuberculosis* complex includes *M. tuberculosis, M. canettii, M. africanum, M. microti, M. bovis, M. caprae and M. pinnipedii*, which are pathogenic to humans and animals. There is > 99.9% similarity in DNA sequences among the members of this complex [32-34].

The *Mycobacterium avium* complex (MAC) includes *M. avium* and *M. intracellulare*. These are atypical environmental bacteria causing infections in humans. They lead to pulmonary infection and disseminated TB disease in HIV/AIDS and other immunocompromised individuals. Pulmonary infections with MAC rarely occur in immune-competent hosts. MAC also comprises *M. avium* subsp. Paratuberculosis, the etiologic agent of Johne's disease or paratuberculosis [35, 36].

The *Mycobacterium fortuitum* complex includes *M. fortuitum, M. peregrinum, M. abcessus and M. chelonae,* which are frequently responsible for abscess formation in local infections and surgical wounds. These mycobacterial species, especially *M. abcessus*, can also lead to pulmonary disease, particularly in immunosuppressed individuals [37- 40].

1.3 Characteristics of Mycobacteria

Mycobacteria are aerobic, non-motile, non-spore forming, rod-shaped organisms (2-4 μm in length and 0.2-0.5 μm in width). The high density of lipids in the cell wall prevents gram staining, but they can be stained by Ziehl-Neelson acid-fast dyes, and are thus termed as acid-fast bacilli. After staining, they appear as slightly curved or straight and small red or pink rods [41-44]. Mycobacteria are facultative intracellular parasites, usually of macrophages. They are obligate aerobic bacteria since they require high levels of oxygen for growth. Mycobacteria are always found in the well-aerated upper lobes of the lungs in TB patients [45]. Mycobacteria are slow growing bacteria. They have a generation time of 15-20 hours to undergo one cycle of replication, which is extremely slow compared to *Escherichia coli* that can divide every 20 minutes. Besides its strictly aerobic intracellular nature and slow growth, the other unique features of the bacillus include dormancy, complex waxy cell wall and genetic homogeneity [46].

1.4 Transmission and Epidemiology

Mycobacteria are transmitted through the air when a person with active pulmonary tuberculosis coughs, sneezes or spits [47]. TB bacilli can infect people of all age groups. Approximately one third (2 billion) of the world's population is latently infected with TB bacilli; 10% of latently infected individuals develop active TB, while the remaining 90% harbor their latent bacilli throughout their lifetime. According to a WHO report worldwide TB accounted for 9.6 million new cases and 1.4 million deaths in the year 2014 alone [48]. Although most TB

infections and deaths were found to occur among men, the burden of disease is also high among women and children. In 2014, 480,000 women died from TB (300,000 among HIV-negative women and 180,000 among HIV-positive women) with an incidence of 3.2 million new cases. In the same year, approximately 140,000 HIV-negative children died from TB and 1.0 million new TB cases appeared in children. The estimates for HIV-positive children are not yet available [49-50].

In 2014, multidrug-resistant TB (MDR-TB) accounted for 190,000 deaths from 480,000 newly diagnosed MDR-TB cases. MDR-TB related deaths were over 10% of all TB-related deaths. More than half of these cases were in India, China and the Russian Federation. Unfortunately, about 9.0% of MDR-TB cases had extensively drug-resistant TB (XDR-TB).

People living with HIV are up to 37 times more likely to develop TB disease than people who are HIV negative [51]. In 2014, 12% of individuals diagnosed with TB were also co-infected with HIV, leading to 390,000 deaths due to TB. Most of the TB-HIV cases occurred in South-East Asia (56%), Africa (29%), the Eastern Mediterranean (8%), Europe (4%) and the America (3%).

There are 17 countries with a high burden of TB; Bangladesh, China, Democratic Republics of Congo and Korea, Ethiopia, India, Indonesia, Mozambique, Myanmar, Nigeria, Pakistan, Philippines, Russian Federation, South Africa, United Republic of Tanzania, Thailand and Vietnam. These nations account for 82% of all estimated cases of TB worldwide. The incidence rate of TB in these countries is reported as 100 cases per 100,000 populations or more. The countries that rank first to sixth in terms of the number of TB incidents in the year 2014 are India (2-2.3 million), Indonesia (0.75-1.4 million), China (0.9-1.1 million), Nigeria (340,000-880,000), Pakistan (370,000-650,000), and South Africa (410,000-520,000). India and China alone account for 24% and 11% of global cases of TB, respectively [52, 53].

1.5 Genome

The genome of the virulent strain of *Mtb*, H37Rv, was completely sequenced in 1998 by Cole *et al.*, providing greater insights about the biochemistry, physiology, genetics and immunology of this bacterium. The genome of *Mtb* comprises 4,411,529 base pairs and represents the second-largest bacterial genome sequence currently available (after that of *E. coli*). Bioinformatics analysis has led to the identification of approximately 4000 genes in the mycobacterial genome of which > 91% of the genes have potential coding capacity. Overall, 58% of the *Mtb* genes can be attributed a function, 27% show similarity to putative proteins and the remaining 15% are unrelated to known genes or proteins in the databank. It has been estimated that approximately 51% of the genes present in the *Mtb* genome have arisen because of gene duplication and domain shuffling [54, 55].

Interestingly, 6% of the total genome of *Mtb* is putatively devoted to 227 genes, which encode for enzymes involved in fatty acid metabolism. These enzymes allow the synthesis and degradation of several lipids from simple fatty acids to complex molecules such as mycolic acids. Among these, about100 genes are involved in β-oxidation of fatty acids, while in *E. coli* approximately50 enzymes are involved in fatty acid degradation [54, 56].

Approximately 4% of the *Mtb* genome consists of two unrelated families of acidic and glycine-rich proteins, named as PE and PPE families. These names are derived from the presence of conserved proline-glutamate (PE) or proline-proline-glutamate (PPE) residues near the N-terminus of 110 and 180 amino acids long proteins, respectively. The 104 PE genes can be further sub-divided into three classes containing 29 proteins within the PE region alone, 8 proteins in which the PE region is followed by unrelated C-terminal sequences and 67 proteins that form the PE-PGS family. These proteins are restricted to pathogenic bacteria; however, non-pathogenic mycobacteria also contain some similar genes. The functions of most of the members

of this large family of proteins are still not known, but size variation has been observed between different strains [54, 57, 58].

Genome analysis of the *Mycobacterium* revealed an efficient DNA repair system with nearly 45 genes related to DNA repair mechanisms and code for 13 putative sigma factors and more than 100 regulatory proteins. The presence of a single ribosomal ribonucleic acid (rRNA) operon (rrn), contrary to most eubacteria that have more than one rrn operon, has been noted to be a factor contributing to the slow growth of *Mtb* [59-60].

The genome of *Mtb* has a very high guanine + cytosine (G+C) content (65.5%). Though the G+C content in the genome is relatively uniform, the amino acid content of the proteome is non-random. The proteins are rich in glycine, alanine, proline, and arginine which are all encoded by G+C rich codons at the expense of amino acids such as lysine and asparagine encoded by adenine + thymine (A+T) rich codons [54].

The members of the *Mtb* complex have ≥ 99% 16S rRNA gene sequence homology [61, 62]. Genome analysis of the attenuated strain of *Mtb*, H37Ra, showed high similarity to that of its virulent parent strain H37Rv with respect to gene content and order as demonstrated by Zheng *et al* [63]. However, the genome size of H37Ra is 8,445 bp larger as a result of 53 insertions and 21 deletions in H37Ra compare to H37Rv. Further, H37Ra has several distinguishing characteristics, such as loss of cord function, impaired ability to arrest phagosome maturation and mutations in the PhoPR transcriptional regulator that results in a loss of virulence function [64].

1.6 Cell Wall

Mycobacteria have a unique and intricate cell wall among all gram-positive bacteria, which is crucial for its survival and virulence. The presence of long chain fatty acids is characteristic of the mycobacterial cell wall that accounts for 60% of the dry weight of the cell

wall. The cell envelope of *Mtb* consists of three main structural components a) the long chain mycolic acids, b) a highly branched arabinogalactan (AG) polysaccharide and c) a cross-linked network of peptidoglycan, which is surrounded by a non-covalently linked outer capsule of protein and polysaccharides. Therefore, the complexity and hydrophobicity of the cell wall are responsible for its intrinsic resistance to many antibiotics and host-microbicidal pathways. The mycobacterial cell wall does not contain lipoteichoic acid and lipopolysaccharides, typical of gram-positive and gram-negative bacteria, respectively [65-67].

Mycolic acids (MA) are the major lipid component of the mycobacterial cell wall and comprise 40–60% of cell wall dry weight. The structural component, long-chain α-alkyl-β-hydroxyl fatty acids (C_{70}-C_{90}) of mycolic acid, is highly conserved among mycobacterial species and contributes to the fluidity and permeability of the cell wall. They are three distinct types: α-MA found in more abundance and existing in a cis-cyclopropane configuration, whereas methoxy and keto mycolic acids exist either as cis- or trans configuration and are present in less abundance [71, 72]. MA are covalently attached to the cell wall or non-covalently associated with glycolipid trehalose 6, 6'-dimycolate (TDM) or cord-factor [73].

TDM is also one of the major structural components of the hydrophobic cell wall, which prevents phagosome-lysosome fusion and contributes to the survival of bacteria within granulomas. In addition, the cord factor is responsible for the serpentine or slender arrangement of mycobacterial cells [74-77].

The peptidoglycan layer surrounds the plasma membrane and is responsible for bacterial shape and mechanical strength. It comprises a carbohydrate backbone of repeating disaccharide N-acetyl glucosamine-N-acetyl muramic acid (NAG-NAM) residues cross-linked to peptides. Up to 80% of mycobacterial peptidoglycan contains 3-3 peptide cross-links compared to other bacteria that contain 4-3 cross-linking [68, 69]. Glycolylated NAM polymers of peptidoglycan

are recognized by innate receptor nucleotide oligomerization domain 2 (NOD2) and induce proinflammatory cytokines in *Mtb*-infected macrophages [70].

The arabinogalactan (AG) is the major polysaccharide of mycobacterial cell wall, consisting of arabinose and galactose sugars in a furanose configuration. They contribute to the mycobacteria survival inside the macrophages.

The outer surface of the mycobacterial cell wall also contains other complex lipids including lipoarabinomannan (LAM), glycopeptidolipids, sulpholipids, phthiocerol dimycocerosate and phenolic glycolipids [78-80]. LAM is one of the complex glycolipids that contains a phosphatidyl-myo-inositol anchor, a D-mannan polymer attached to the inositol ring, D-arabinose chains, and capping motifs at the end of the arabinose residues [81, 82]. LAM acts as a virulence factor and inhibits phagosomal maturation and interferes with host cell signaling, and as a result down-regulates the host immune responses [83]. Virulent, slow-growing mycobacteria like *Mtb* harbor mannose-capped LAM (ManLAM) in their cell wall, while rapidly growing non-virulent species of mycobacteria such as *M. smegmatis* harbor non-capped (AraLAM) or phospho-myo-inositol-capped LAM (PILAM) [84]. Thus, the type of capping is important among mycobacterial species for their virulence. The cell wall of mycobacteria also contains a 19-kDa lipoprotein of unknown function that has been implicated in virulence through a role in host cell death or apoptosis, and manipulation of bactericidal mechanisms [85].

The cell wall of mycobacteria also encompasses porin proteins, which have similar properties to those found in the outer membrane of other gram-negative bacteria, providing a pathway for low molecular weight hydrophilic nutrients [86, 87]. The lipid rich complex composition of *Mtb* cell walls provides a permeability barrier against hydrophilic drugs and host defense mechanisms [88, 89].

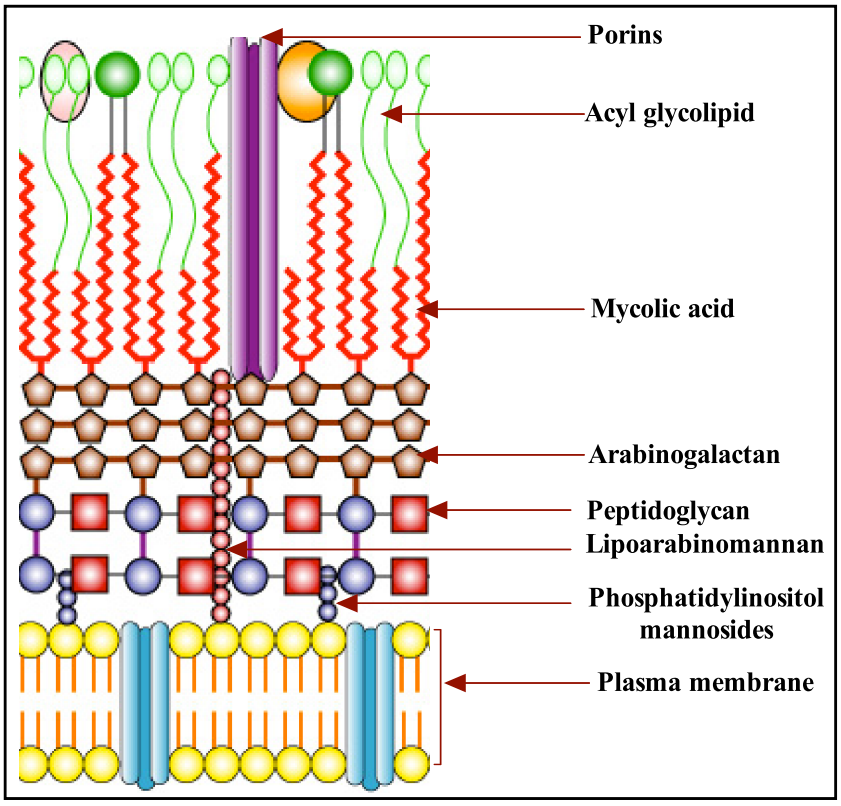

Fig 1.1: The basic structure of the *Mycobacterium tuberculosis* cell wall. [Figure has been modified from reference 232 with permission].

1.7 The Outcome of Mycobacterial Infection and Disease Pathogenesis

Mtb enters the human body after inhalation of aerosol droplets containing exhaled tubercle bacilli from an individual with active TB disease. Each aerosol droplet can contain 1 to 400 bacilli, and the infectious dose for a person is between 1 to 200 bacilli, making transmission through aerosol highly efficient [90]. Upon inhalation, mycobacteria travel to the lungs and end up in the alveolar space, where they are recognized as foreign and engulfed by professional phagocytic cells (macrophages) as part of normal host defense mechanism. However, the mycobacteria employs several mechanisms to counteract the anti-microbial activity of macrophages including phagosomal acidification, activation of proteolytic enzymes in the acidified phagosomes, and production of antimicrobial peptides as well as reactive oxygen and

nitrogen metabolites. Thus, mycobacteria overcome the early innate immune defenses, which allows survival and initial residence within macrophages [91-97].

Once alveolar macrophages phagocytose the bacteria, they either kill the ingested bacteria or bacteria keep multiplying until the primary host cell bursts. Mycobacteria also promote necrosis of macrophages, which facilitates bacterial dissemination and delays the onset of adaptive T cell immune responses. The inflammation triggered by dying or apoptotic macrophages leads to further recruitment of monocytes/macrophages. These macrophages are stimulated by ligation of various pathogen recognition receptors (PRRs), and trigger innate immune signaling pathways leading to the production of chemokines and cytokines that promote further recruitment of immune cells to the site of infection. Epithelial cells and neutrophils also secrete chemokines and cytokines in response to bacterial products and begin to organize early granuloma [99-101].

In addition, dendritic cells (DCs) can internalize the mycobacteria, apoptotic macrophages or mycobacterial products, and migrate to the local draining lymph nodes to present mycobacterial antigens to lymphocytes [102-105]. As a result, *Mtb* specific T and B cells are stimulated and migrate to the site of infection. Eventually, a well-organized granuloma forms that consists of central infected macrophages, surrounded by macrophages, foam cells, multinucleated giant cells, with peripheral recruited lymphocytes and a fibrous capsule. This granuloma formation is the hallmark of TB disease, which represents a fine balance between a potentially dangerous pathogen and host immune system [106, 107].

An infection with *Mtb* can have several possible outcomes depending on host immune status and the pathogen. In some of the cases, the initial infection subsides, and the collagen capsule contracts to form a scar or the calcified granuloma. However, in small percentage of individuals, the center of the granuloma undergo necrosis resulting in the formation of caseous

necrotic granulomas, and bacteria are released from caseating granulomas and cause active TB [108]. Approximately 5-10% of infected individuals develop severe, life-threating primary progressive disease at this stage. Despite wide dissemination of *Mtb* during primary infection, the majority of infected individuals contain infection without becoming symptomatic for years or decades in the form of solid granulomas known as latent TB [109-112] (**Fig. 1.2**). Later, any time in life, the onset of immunodeficient conditions, such as viral (HIV) infection, cancer, organ transplantation, immunosuppressive medications, age or malnutrition, can lead to reactivation of dormant bacteria, breakdown of the granulomas and progression to active disease. Five to ten percent of latently infected individuals develop reactivated or secondary disease. However, the actual cause of reactivation remains to be resolved. [113,114].

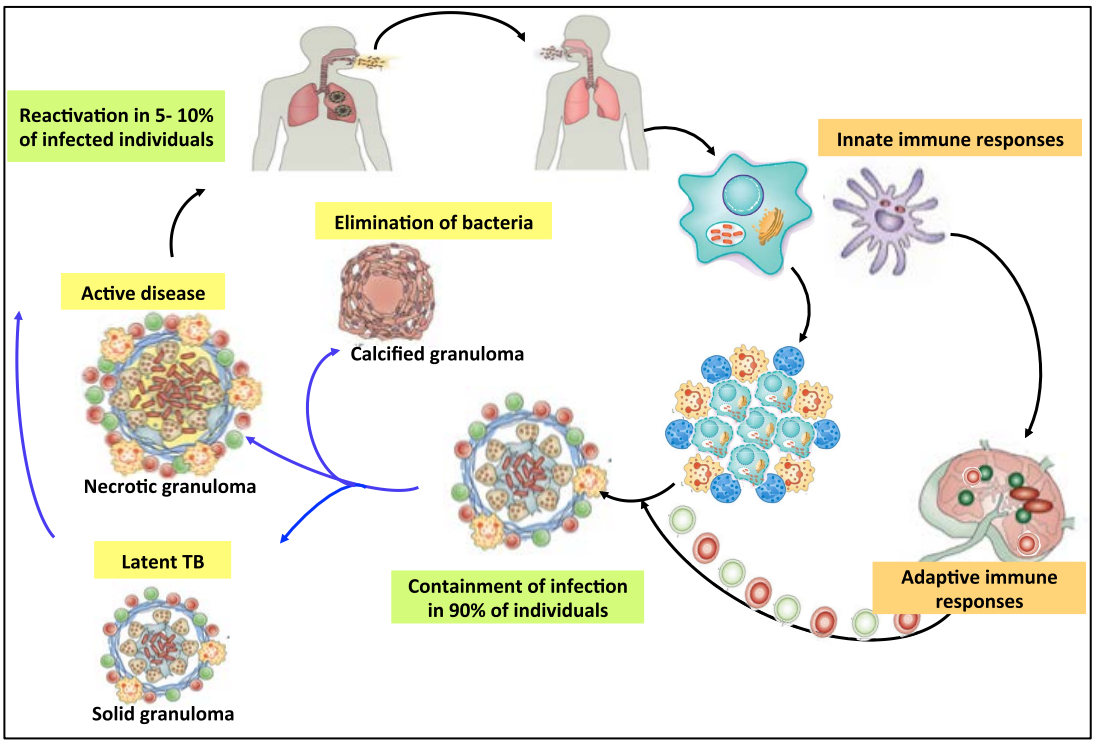

Fig 1.2: Different outcomes of *Mtb* infection and TB Pathogenesis. [Figure has been modified from reference 230 with permission].

1.8 Tuberculosis Disease and Infection

Mycobacteria mainly cause infections in lungs and lead to pulmonary TB, which can either progress to active or chronic latent infection.

1.8.1 Active Tuberculosis

In the case of active TB, mycobacteria keep multiplying in the body and spread efficiently from one person to another by sneezing, coughing, talking or laughing. Active TB is a fatal, symptomatic and contagious form of disease [115]. Only 5-10% of the initially infected people develop active TB. Symptoms of active TB in the lungs begin gradually and develop over a period of weeks or months. Clinical manifestations of active TB infection include a bad cough lasting three weeks or more, chest pain, weight loss, weakness or fatigue, loss of appetite, bloody sputum, chills, fever, and night sweats [116]. Besides the lungs, TB can infect other organs such as the kidney, brain, liver, bones etc.; the signs and symptoms vary according to the infected organs. Active TB is diagnosed with chest radiography, sputum smear microscopy, mycobacterial culture, and nucleic acid amplification tests [117].

1.8.2 Latent Tuberculosis

In latent TB infection, mycobacteria remain in the body in a dormant or non-replicating stage life-long and cannot be transmitted from one host to another. Latent TB is an asymptomatic and non-contagious form of disease. Approximately 90% of newly infected individuals develop latent TB infection. Latently infected people have a positive tuberculin skin test or TB blood test. However, in certain immune compromised conditions (5-10%), latent TB can be reactivated and cause active TB disease [118].

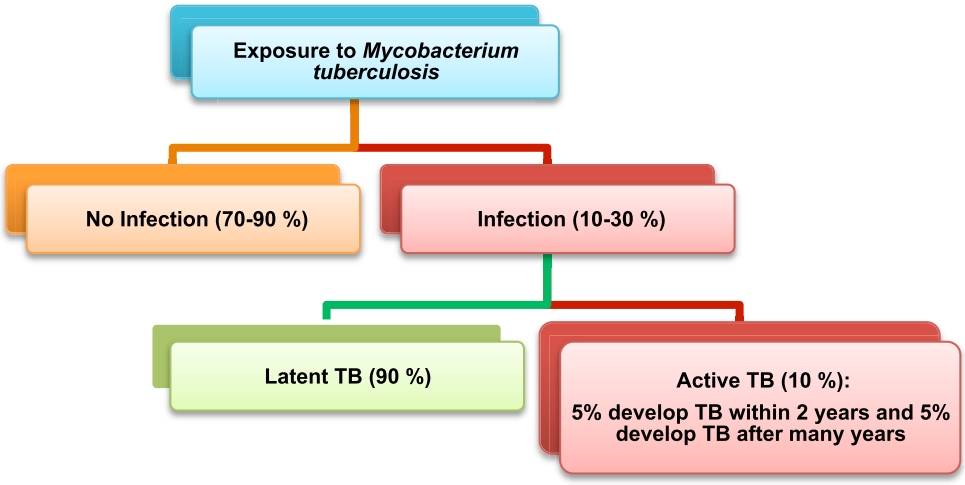

Fig 1.3: The natural history of TB infection.

1.9 Immune Responses against Mycobacteria

Two distinct arms of the immune system coordinate and protect a host from invading pathogens. Innate immunity is the most critical arm of the immune system that provides the first line of defense against infection. Adaptive immunity starts later and relies on the efficient coordination of innate immune cells. The interaction of microbial pathogen with innate immune cells influences the development of subsequent adaptive immunity [119]. The functional variability between the two arms of the immune system is mediated through the cells associated with each component. Macrophages, dendritic cells (DCs), neutrophils, gamma-delta- (γδ) T, natural killer (NK) and natural killer T (NKT) cells make up the innate immune responses, while T and B cells mediate adaptive immunity. Recent literature suggests that NK and NKT cells also have some features of adaptive immunity [120-122].

1.9.1 Innate Immune Responses

Innate immunity acts immediately after encountering a pathogen and therefore plays a crucial role in the early recognition and subsequent triggering of broadly specific immune responses to all pathogens. A growing body of new evidence suggests that the innate immune system can also produce pathogen-specific responses and mount resistance to secondary infections through 'innate immune memory' or 'trained immunity' [123].

Innate immune cells recognize pathogens through pathogen recognition receptors (PRRs), which bind to highly conserve broad microbial structures known as pathogen-associated molecular patterns (PAMPs). In addition, they recognize damage-associated molecular patterns (DAMPs) of host molecules released by infected cells. During *Mtb* infection, mycobacterial components are recognized by a variety of different PRRs such as Toll-like receptors (TLRs), complement receptors (CRs), scavenger receptors, nucleotide binding-oligomerization–domain (NOD)- like receptors (NLRs), dectin-1, surfactant protein A (sp-A) receptors, mannose receptors (MRs) and the dendritic cell specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN). These PRRs facilitate entry of the mycobacteria into host cells and, also send an alarm signal to initiate innate immune responses [124-127].

Broadly, upon mycobacterial PAMPs' engagement, PRRs trigger intracellular signaling cascades, which lead to the activation of nuclear factor kappa B (NK-κB), resulting in the production of cytokines, chemokines and antimicrobial effector molecules from infected cells [128-130]. These secreted cytokines or chemokines bind to the membrane bound receptors expressed on innate immune cells in autocrine and/or paracrine manner, and initiate the early host response to mycobacterial infection [131].

Therefore, the complex interaction of mycobacterial PAMPs with their specific PRRs present on host innate immune cells suggests the key role of innate immunity in controlling *Mtb*

infection. However, accumulating evidence suggests that the host has evolved multifaceted innate immune mechanisms to sense mycobacteria and elicit defense responses, while the mycobacteria has developed elaborated strategies to circumvent host defense mechanisms.

Macrophages (MΦ)

Macrophages, the first immunological barrier against mycobacterial infections, play a critical role in the evolution of *Mtb* infection. In addition to the recognition and immediate elimination of bacteria through phagocytosis and secretion of microbial products, macrophages are extremely important in orchestrating the immune response and shaping the adaptive immunity [132].

In response to *Mtb* infection, macrophages up-regulate or trigger antimicrobial effector molecules to both prevent mycobacterial replication and recruit other immune cells into the lungs. Macrophages upon recognition or uptake of mycobacterial glycolipids, lipoproteins, and carbohydrate components through PRRs, initiate the downstream signaling pathways, resulting in the activation of transcription factor NK–κB and PPARγ, which lead to the production of cytokines (TNF–α, IL-1β, IL-12, IL-8), chemokines (MIP-1α, CCL22), antimicrobial peptides (LL-37) reactive nitrogen (iNOS) and oxygen (iROS) species [133-138]. Cumulatively, these antimicrobial effector molecules induce autophagy to control mycobacterial replication inside the macrophages as well as activate neutrophils, DCs and T cells.

However, *Mtb* interferes with effector and signaling pathways of macrophages to reside unharmed in infected macrophages of the host for a long period of time. *Mtb* also down-regulates MHC II expression on macrophages and attenuates the T cell recognition of infected macrophages.

Neutrophils

Neutrophils are among the first immune cells to penetrate the physical barriers of the human body and play a crucial role in the development of innate and acute inflammatory responses against bacterial infections. During *Mtb* infection, neutrophils produce and secrete antimicrobial enzymes (α-defensins, matrix metalloproteases, lactoferin and lipocalin), to restrict the growth of mycobacteria within macrophages, and also promote apoptosis of infected macrophages, thereby limiting *Mtb* survival within the host. Upon stimulation with *Mtb*, they also secrete chemokines (IP-10, MCP-1, MIP-1α/β) and pro-inflammatory cytokines (IFN-γ and TNF-α) to recruit and activate other immune cells [139, 140]. However, these effector molecules also mediate lung tissue damage and sustained, hyper-activated inflammatory response.

Neutrophils are the second most abundant cells after lymphocytes found bronchoalveolar (BAL) and sputum samples of active pulmonary TB patients [141-143]. Further, neutrophils have been reported to highly express programmed death ligand-1 (PDL-1) and type I IFN-inducible genes in the blood of active TB patients [144, 145]. Whether the increased expression of PDL-1 on neutrophils is associated with suppression of protective immunity or in the resolution of inflammation remains to be elucidated.

Dendritic Cells (DCs)

DCs are the primary antigen-presenting cells and play a central role in bridging innate and adaptive immunity through their significant role in internalizing, processing and presenting antigens to T cells.

DCs can internalize mycobacteria or mycobacterial components through different PRRs. Several studies have suggested that *Mtb* is capable of replicating within DCs, but further studies are needed to substantiate these observations. Upon recognition, the fate of DC maturation is

dictated to a large extent by the interaction of DCs with *Mtb.* A number of studies have suggested that after interaction, DCs mature and migrate to lymphoid organs where they prime naïve T cells and secrete immunoregulatory cytokine IL-12, and therefore, strengthen the cellular immune responses against infection. In contrast, others reported that *Mtb* impairs DC maturation and manipulates its functions in order to prevent optimal induction of host adaptive immunity [146, 147]. However, some studies indicated that the outcome depends on the interaction of *Mtb* or its components with receptors present on DCs. Recognition of *Mtb* through TLRs leads to DC activation and IL-12 secretion during early infection, whereas interaction with DC-SIGN prevents DC maturation that results in high IL-10 secretion and inhibition of antigen-specific T cell proliferation [148,149]. Moreover, engagement of TLRs and DC-SIGN down-regulates an ubiquitin ligase RING-CH-1 protein (MARCH 1) critical for recycling the MHC-II complex on the cell surface of DCs, and interferes with their antigen presenting ability [150]. Therefore, the outcome of *Mtb* and DC interaction is complex and not fully understood.

Collectively, DCs are at the forefront in priming *Mtb* specific immune responses but their differential interaction with *Mtb* components influence the delayed onset of adaptive immunity during mycobacterial infection.

Natural Killer (NK) Cells

NK cells are prominent components of innate immunity, which recognize and kill infected cells, and thus play an important role in controlling intracellular pathogens. NK cells mediate their function through cellular cytotoxicity and production of a range of cytokines or immunoregulatory mediators.

During early infection, NK cells become activated by type I IFNs and IL-12 produced from the macrophages and DCs. These factors in turn can recruit more NK cells locally at the site

of infection [151]. NK cells produce several cytokines such as IFN-γ, TNF-α, IL-10, IL-4, IL-17 and IL-22, which can activate macrophages, modulate DCs functions, and downstream adaptive immune responses [152]. NK cells themselves are regulated by the expression of various activation and inhibitory receptors [153].

In acute mycobacterial infection, NK cells have been shown to possess increased cytotoxic activity, IFN-γ and TNF-α production, and up-regulation of activation marker NKG2D/NKp46. They have been also shown to lyse infected monocytes and alveolar macrophages through NKp46 and NKG2D receptors [154]. NK cells not only have direct anti-mycobacterial activity, they can also regulate other immune cells' functions to control *Mtb* infection. The cytokines produced by NK cells further activate DCs, favor Th1 adaptive immune responses, and restrict the expansion of regulatory T cells. NK cells were found to lyse *Mtb* expanded T regulatory cells, induce γδ T cell proliferation and promote IFN-γ production from $CD8^+$ T cells [155, 156]. In addition, NK cells can directly recognize mycobacterial the cell wall component mycolic acid and peptidoglycan through NKp44 and TLR-2 receptor, respectively [157, 158].

However, NK cells have been reported with reduced cytotoxicity, depressed IFN-γ production, and lowered expression of NKp30 and NKp46 activating receptors in patients with active TB [159].

Natural Killer T (NKT) Cells

NKT cells have both NK and T cell markers, and possess both effector and regulatory functions. NKT cells are mainly classified as two types: Type I or invariant NKT (iNKT) cells are characterized by their restricted expression of an invariant Vα24-Jα18 T cell receptor (TCR) paired with Vβ11 chain in humans and Vα14-Jα18 paired with Vβ2, Vβ7 and Vβ8.2 chains in

mice, whereas heterogeneous Type II NKT cells are characterized by their less restrictive TCR repertoire. Once NKT cells mature in the thymus, they migrate to the periphery, where they can be activated through recognition of endogenous and exogenous lipids presented by CD1d [160, 161]. They can also be activated in a CD1d-independent manner [162]. Upon stimulation, NKT cells produce immune-stimulatory cytokines that can alter the strength and quality of adaptive immune responses through cross talk with DCs, and by shifting cytokine response to Th1, TH2 or TH17 cell type profiles [163].

Growing evidence suggests that murine and human NKT cells mediate protection against *Mtb* [164]. In mice, administration of α-GalCer (a known iNKT agonist) both alone and in combination with anti-TB drugs, improved the clinical outcomes of *Mtb* infection [165]. Incorporation of α-GalCer in BCG vaccine has been shown to enhance the host immune responses [166]. Also, patients with active TB were found to have dysfunctional NKT cells with increased expression of PD-1 inhibitory molecule [167]. Recently, it has also been shown that NKT cells isolated from pleural fluid of TB patients produce IFN-γ, TNF-α, IL17, IL-2 and IL-21 upon *ex-vivo* antigen stimulation [168, 169]. Therefore, NKT cells become activated during the early response to pulmonary TB and actively participate to resolve *Mtb* infection. Whether NKT cells are associated with early innate resistance to mycobacterial infection is not clear yet.

Gamma-delta (γδ) T cells:

γδ T cells carry a T cell receptor encoded by Vγ and Vδ gene segments. They recognize unprocessed, non-peptide phosphate antigens in a non-MHC restricted manner [170]. During the early phase of *Mtb* infection, γδ T cells expressing IFN-γ and IL-17 along with cytotoxic effector function are recruited in the lungs. It has been shown that γδ T cells are the primary source of IL-17 in early infection and they appear with increased frequency in patients with active TB [171].

Mice and human studies suggest that γδ T cells expanded after BCG vaccination are capable of restricting mycobacterial growth in perforin and granulysin dependent manner. γδ T cells also elicit protective immune responses upon interaction with NK, DCs and $CD8^+$ T cells [172, 173]. Thus, γδ T cells represent an early defense against pulmonary TB and link between innate and adaptive immunity.

1.9.2 Adaptive Immune Responses

Activation of innate signaling pathways and immune cells of the innate system set the stage for downstream adaptive immune responses. Inflammatory mediators such as cytokines or chemokines, secreted from APCs or various innate immune cells stimulate T lymphocytes, which in turn aid B cells and orchestrate adaptive immunity. Adaptive immunity encompasses both humoral and cellular immunity mediated by B and T cells, respectively.

1.9.2.1 Humoral Immune Responses

Humoral immune responses are represented by B cells and antibodies produced by them. Antibodies play a pivotal role in defending a host against extracellular pathogens. Antibodies neutralize and disarm invading pathogens with effector mechanisms including neutralization, complement activation, opsonization, and antibody-dependent cell-mediated cytotoxicity (ADCC) [174].

Protection against intracellular pathogens such as mycobacteria is believed to be exclusively T cell mediated, with B cells and antibodies playing a limited role. The contribution of humoral immunity to protect against *Mtb* infection has been controversial for more than a century, because animal and human studies have provided inconsistent and sometime contradictory results. The evidence for the role of humoral immunity in defense against *Mtb* is inconsistent. However, a substantial number of studies conducted with serum therapies, mouse

polyclonal antibodies, human polyclonal antibodies, secretory human IgA and monoclonal antibodies to purified mycobacterial antigens ranging from surface proteins to polysaccharides, suggest a protective role of antibodies against *Mtb* [175]. The complexity and heterogeneity of antibody responses during *Mtb* infection suggest that both protective and non-protective antibodies exist and that could be one of the reasons for inconsistent results in animal and clinical studies. Furthermore, several studies have demonstrated that BCG vaccination also elicits IgG and IgM responses against several mycobacterial antigens, which enhance both innate and cell-mediated effect against *Mtb*. Antibody titers in individuals with active and latent TB are highly variable. Interestingly, it has been shown that people with active TB have antibody responses with low avidity and low IgG/IgM ratio for surface antigens, and that they fail to produce high-avidity IgG against surface antigens of the mycobacteria [176, 178]. In addition, patients with active TB have antibody responses with increased avidity to inactivated intracellular antigens of *Mtb*. Higher surface binding antibodies were protective and correlated to reduced active TB disease [176, 177]. These studies suggest that antibody responses against purified intracellular antigens are different than whole TB bacterium.

Despite being an intracellular pathogen, *Mtb* is potentially susceptible to various antibody-mediated immune mechanisms. IgAs, in addition to neutralization of the pathogen, also bind with the mycobacterial cell wall component Gal-3, and interfere with the interaction of mycobacteria with the phagosomal membrane, resulting in decreased replication of mycobacteria in the phagosomes [179]. Opsonization with Fcγ receptor was shown to promote phagolysosomal fusion and intracellular killing, and enhanced cellular responses through uptake and processing of mycobacterial antigens. Antibodies also promote phagocytosis and killing by complement receptor activation [180]. Several studies have shown a strong antibody response against a wide range of *Mtb* antigens, with an IgG2a ~ IgG2b > IgG1 > IgG3 predominance [181].

In addition to direct antibody-mediated mechanism, B cells also play a crucial role in modulating the host immune responses in TB infections by antigen presentation, co-stimulation of T cells, cytokine production, enhanced antibody-mediated cellular cytotoxicity (ADCC) and recruitment of immune cells in lungs [182, 183].

1.9.2.2 Cellular Immune Responses

Components of cellular immune responses include $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T cells (CTLs), which recognize peptides bound to MHC class II and MHC class I molecules on antigen presenting cells (APCs), respectively.

$CD4^+$ T Cells

$CD4^+$ T cells develop in the thymus as either natural T regulatory cells or naïve $CD4^+$ T cells. These T cells are termed as helper T cells because of their role in direct activation of macrophages, providing co-stimulatory signals to B cells to mature them into effector plasma cells and activation of $CD8^+$ T cells. Upon mycobacterial infection, APCs engulf the *Mtb*-infected, apoptotic or necrotic macrophages, or mycobacterial antigens, process and present them to T cells expressing TCR and $CD4^+$ molecules. Thereafter, activated $CD4^+$ T cells can polarize into different subsets including Th1, Th2 and/or Th17 cells, which secrete distinct pattern of cytokine, and control the fate of $CD4^+$ T cells mediated immune responses in TB [184].

$CD4^+$ T cells have been suggested to play a central role in protection against *Mtb*. HIV-infected people are highly susceptible to TB due to a decrease in $CD4^+$ T cell counts [185]. In addition, it has been demonstrated that mice that are $CD4^+$ T cell depleted and MHC-II deficient poorly control mycobacterial infection [186]. $CD4^+$ T cells influence the immune responses mainly by the induction of cytokines. Under the influence of IL-12 from DCs, naïve $CD4^+$ T cells

get polarized to Th1 type cells that are the primary source of IFN-γ during the acute phase of infection [187, 188].

IFN–γ is a critical cytokine required to control mycobacterial infection. Mice lacking IFN–γ as well humans with a genetic mutation in IFN–γ signaling is most susceptible to mycobacterial infections [189]. In addition to IFN–γ, Th1 cells also secrete TNF–α and IL-2. IFN–γ synergizes with TNF–α to recruit and activate macrophages, and promotes their anti-microbial effector functions [190]. IL-2 directly activates the T cells, increases their proliferation and survival. Recent studies have suggested that the polyfunctional *Mtb*-specific $CD4^+$ T cells (IFN–γ, TNF–α, IL-2) correlate with immune protection against *Mtb* [191, 192].

In contrast, Th2 responses regulate the differentiation of plasma cells by the production of IL-4, IL-5 and IL-13, which are associated with intracellular persistence of mycobacteria. Thus, dominant Th2 responses can undermine Th1 mediated protection. The Th2 response also promotes the polarization of alternatively activated M2 macrophages, which abrogate effective immune responses required for protection against *Mtb* [193, 194].

Th17 cells produce IL-17, IL-22 and IL-23 and lead to the activation and recruitment of neutrophils into the lung parenchyma [195, 196]. In several studies, a reduced number of Th17 cells were found in active TB patients. Th17 response has been shown to induce memory responses in mice [197]. However, the role of Th17 responses in *Mtb* protection is controversial.

$CD4^+$ T Regulatory Cells (T_{regs})

$CD4^+$ T cells can also function as regulatory T cells, and help in the maintenance of immune homeostasis. There are mainly two types of Tregs: natural (nTregs) and induced Tregs (iTregs). Natural Tregs originate from the thymus with high avidity for self-antigens and express the IL-2α chain (CD25) receptor together with the transcription factor FOXP3 and are not

influenced by cytokines. In contrast, iTregs originate from naïve $CD4^+$ $CD25^-$ T cells in the presence of cognate antigen and immunoregulatory cytokines such as TGF-β, IL-4 and IL-10 and differentiate into $CD25^+$ and $FOXP3^+$ expressing cells. iTregs are further categorized into Tr1 or Tr3, based on IL-10 and TGF-β secretion, respectively [198, 199].

Tregs have generally been shown to play a significant role in the establishment of persistent infection, and in suppressing the induction and proliferation of effector T cell responses. However, the exact role of Tregs in *Mtb* infections is not clear and controversial reports have been published. Studies in humans and mice suggest that Tregs are increased in the lungs during TB infection and they inhibit antigen-specific T cell responses [200, 201]. Tregs also influence the development of active and latent TB in humans. It has been shown that Tregs are high in peripheral blood in active TB while during latent TB infection they return to a normal level. In contrast, one recent study demonstrated that Tregs could down-regulate the inflammatory responses induced during active TB [202, 203]. Interestingly, another study has shown that Tregs ($CD4^+FOXP3^+$) induces IFN-γ production during pulmonary infection and are associated with reduced bacterial load in mice [204]. However, it is not clear whether they should be classified as Tregs or effector T cells, since FoxP3 have also been shown to be transiently induced in effector T cells. Whether Tregs are a causative factor for active TB or a response to inflammation remains to be elucidated.

$CD8^+$ T cells

$CD8^+$ T cells are referred to as cytotoxic T cells (CTLs). They recognize 8-9 amino acid long peptide antigens bound to the MHC class I molecule. $CD4^+$ T cell mediated activation is required to trigger the differentiation and maturation of $CD8^+$ T cells. CTLs induce destruction of infected cells through cytotoxic granules (granzyme or perforin), Fas-FasL interaction and

secretion of effector cytokines (IFN–γ and TNF–α). In the case of mycobacterial infections, CTLs play an important role in the clearance of intracellular bacteria [205]. CTLs mediate lysis of *Mtb*-infected macrophages through perforin or Fas-FasL mediated mechanisms [206]. In non-human primates, depletion of $CD8^+$ T cells led to reduced protection against *Mtb*. A number of studies have also shown that depletion of perforin and granulysin expressing $CD8^+$ T cells were associated with an increased mycobacterial burden. Further, individuals with active TB were found to have impaired expression of perforin and granulysin on $CD8^+$ T cells in the lungs, thus dysfunctional CTL responses are associated with disease progression [207]. Besides direct killing of the infected cells, $CD8^+$ T cells also produce IFN–γ and TNF–α that activate macrophages to inhibit mycobacterial replication [208]. Whether poly-functional *Mtb*-specific $CD8^+$ T cells are needed to provide protection from *Mtb* is not clear.

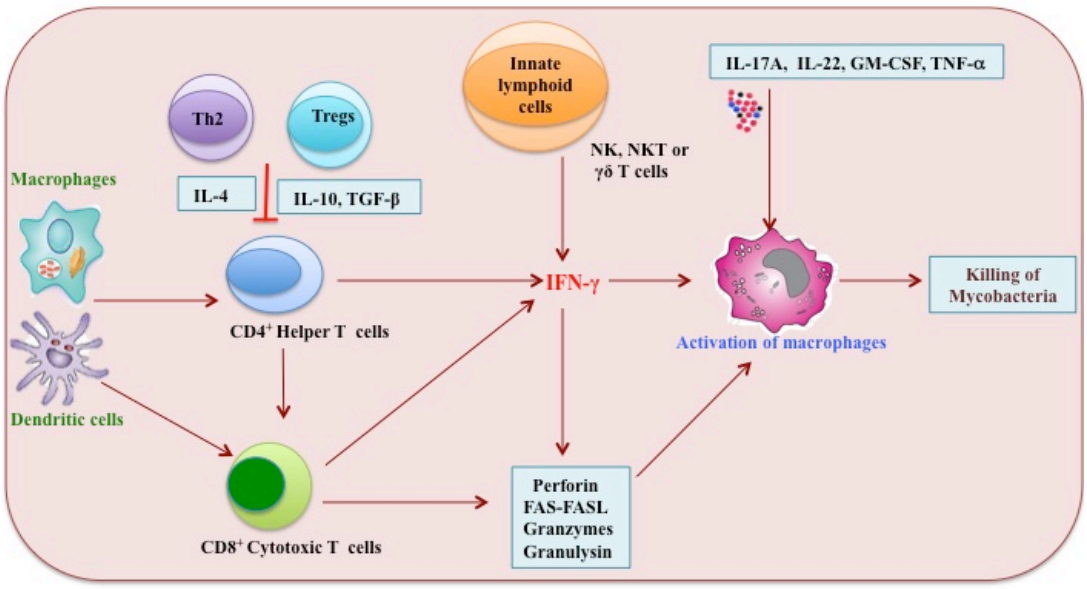

Fig 1.4: Immune responses to *Mycobacterium tuberculosis.*

1.10 Treatment of TB Disease

Various chemotherapeutic drugs have been used not only to cure the disease but also to interrupt the transmission and to prevent relapse. There are currently almost 20 drugs approved by the U.S. Food and Drug Administration (FDA) for the treatment of TB. Of the approved drugs, the first-line anti-TB agents that form the core of treatment regimens include isoniazid, rifampicin, pyrazinamide and ethambutol [209-211]. *Mtb* is a slow growing bacterium and difficult to kill, and therefore, TB treatment requires a minimum of six months therapy in two phases: two months of four drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) in the intensive phase followed by four months of isoniazid plus rifampicin in the continuation stage (the so-called short-course chemotherapy). The current recommended six months treatment for drug-susceptible TB can provide cure rates of > 95% when administered under directly observe therapy (DOT). To treat drug-resistant TB, second-line anti-TB agents are used. The second-line anti-TB drugs include aminoglycosides (amikacin, kanamycin and gentamicin), polypeptides (capreomycin), thioamides (ethionamide and protionamide), oxazolidinone (linezolid), para-aminosalicylic acid, fluoroquinolones (oflaxacin, levofloxacin, gatifloxacin and moxifloxacin), nitromidazoles (delamanid) and cycloserine. However, the second-line drugs possess lower efficacy, demonstrated unfavorable pharmacokinetic profile with more serious side effects, and have restricted use. Anti-tuberculosis drugs that are currently used to treat TB are summarized in **Table 1.1** [212-215].

Table 1.1: First- and second line anti-TB drugs

Drug Name	Route	Daily Doses (range) mg/kg	Mechanism of Action
First-line TB drugs			
Isoniazid	Oral	5 (4-6)	Inhibit synthesis of cell wall component mycolic acid
Rifampicin	Oral	10 (8-12)	Inhibits DNA-dependent -RNA polymerase required for transcription
Pyrazinamide	Oral	25 (2-30)	Inhibits mycobacterial enzyme fatty acid synthase required for fatty acid synthesis
Ethambutol	Oral	15 (15-20)	Inhibit arabinosyl transferase enzyme vital for cell wall component arabinogalactan synthesis
Second-line TB drugs			
Capreomycin	IM or IV	15-20	Inhibits protein synthesis (binds to ribosomal subunit 16S and 23S rRNA
Amikacin	IV	15	Inhibits protein synthesis (binds to the bacterial 30S ribosome)
Gentamicin	IV	7	Inhibits protein synthesis via 30S ribosomal protein and 16 S RNA
Streptomycin	Oral	15	Inhibits protein synthesis
Cycloserine	Oral	10	Inhibits peptidoglycan synthesis (D-alanine racemase)
Ethionamide	Oral	10-15	Inhibits mycolic acid synthesis
Ofloxacin	Oral	15-20	Inhibits DNA replication and transcription by inhibiting DNA gyrase
Levofloxacin	Oral	7.5-10	Inhibits DNA replication and transcription
Para-amino salicylic acid	Oral	250	Inhibits folic acid and iron metabolism
Bedaquiline	Oral	200-400	Inhibits ATP synthase required for energy production
Linezolid	IV	300	Inhibits protein synthesis
Delamanid	Oral	100-200	Inhibits biosynthesis of keto-mycolic and methoxy-mycolic acids

1.11 Vaccine Development

Successful cure of TB has been hampered due to long-term treatment with multiple drugs and their associated cost, side effects, non-compliance and increasing incidence of drug-resistant TB cases. Consequently, vaccination is the most effective approach for global control and elimination of TB. However, despite huge advancements in TB immunology, we are still awaiting an

effective vaccine against TB. The current vaccine BCG provides only partial and inconsistent protection against pulmonary TB. Thus, development of an effective and reliable vaccine for TB is a high global priority. Development of a new TB vaccine is challenging due to a lack of clear understanding of the immune-correlates of protection, disease pathogenesis and availability of relevant animal models [217]. Studies conducted so far suggest that broad and multi-specific $CD4^+$ T cell responses are required and have been correlated with protection against *Mtb* infection. In this regard, several vaccine strategies focused on generating strong multi-specific cellular immunity against immune-dominant antigens of *Mtb* have shown promise in preclinical models [218-220].

An ideal TB vaccine should provide protection against all stages of the mycobacterial life cycle. It should prevent new infections, latent infections and reactivation, and eliminate mycobacteria completely from the host. Multiple approaches are being used for the current TB vaccine development, including a live mycobacterial vaccine to replace BCG, subunit vaccine to boost BCG and therapeutic vaccine as an adjunct to chemotherapy [221, 222]. The most advanced therapeutic vaccine against TB is a whole inactivated environmental non-tuberculous *Mycobacterium vaccae* (MV). However, conflicting results have been observed in its clinical trials [223]. The proportion of patients with sputum smear conversion was 3-fold higher in vaccinated group compared to placebo (31.8 vs. 9.5%); however, the difference was not statistically significant ($p = 0.07$). Also, other secondary endpoints such as erythrocyte sedimentation rate, leukocyte counts and hemoglobin content were not affected in the vaccinated cohort compared to placebo [223]. A few studies have shown the effectiveness of MV as a preventive vaccine in small groups; however, large clinical trials including people of different age groups, regions and populations are required to confirm whether MV is efficacious as a prophylactic vaccine [224].

Different prophylactic and therapeutic vaccines currently being tested in clinical trials are summarized in the following table [225]:

Table 1.2: Prophylactic and therapeutic vaccines in clinical trials

Vaccine Name	Composition	Clinical Status	Sponsors
Preventive Vaccines			
Live vaccines			
VPM 1002	rBCG expressing listeriolysine and lacking urease gene	Phase IIa	MPIIB, VPM, TBVI, SII
MTBVAC	*Mtb* MT 103 strain with deleted of phoP and fadD26 gene	Phase I	U of Zaragoza, Biofabri, TBVI
Subunit recombinant fusion protein based vaccines			
H1:IC31	Fusion protein Ag85B-ESAT-6 in IC31 adjuvant	Phase IIa	SSI, TBVI, Intercell
H4:IC31	Fusion protein Ag85B-TB10.4 in IC31 adjuvant	Phase IIa	SSI, SP, Aeras
H56:IC31	Fusion protein Ag85B-ESAT-6-Rv2660c in IC31adjuvant	Phase IIa	SSI, Intercell, Aeras
M72F:ASO1E	Fusion protein *Mtb*32a-*Mtb*39a in ASO1 adjuvant	Phase IIb	GSK, Aeras
Subunit modified viral vectors based vaccines			
MVA85A	Modified vaccinia ankara virus expressing Ag85A	Phase I	University of Oxford, TBVI
AdHu5Ag85A	Recombinant human adenovirus type 5 expressing Ag85A	Phase I	McMaster university
Ad35/AERAS-402	Recombinant human adenovirus type 35 expressing Ag85A, Ag85B and TB10.4	Phase II	Crucell, Aeras
ChAdOx-1.85A	Recombinant chimp adenovirus type 68 expressing Ag85A	Phase II	University of Oxford
Therapeutic /Immunotherapeutic vaccines			
RUTI	Detoxified fragment of *Mtb* in liposome	Phase IIa	Archivel pharma
M.indicus pranii	Whole cell *M. indicus pranii*	Phase III	Cadila pharmaceuticals
M. vaccae	Whole cell *M. vaccae*	Phase III	AnHui Longcom

1.12 Animal Models of Mycobacterial Infection

Several models have been developed to study TB disease pathology, immune responses and drug testing. However, they vary in aspects of human TB pathology, disease progression and immune responses. Major research progress in the field of TB vaccine and drug development has been hampered by a lack of animal models that closely mimic the human conditions of mycobacterial infection [226]. Some of the most commonly used animal models in TB research, drug and vaccine development are summarized in **Table 1.3** with their advantages and limitations:

Table 1.3: Different models used in TB research [227-230]

Model	Route of infection	Stage of infection	Advantages	Disadvantages
Non-mammalian models				
Amoeba (*Dictyostelium discoideum*)	Phagocytosis of *M. tb* or *M.marinum*	• Intracellular mycobacterial growth in vacuoles	• Both species replicate similarly in mammalian macrophages. • Suitable for macrophage-pathogen interactions. • Identifying gene involved in phagocytosis.	• Single-cell model with limited applications
Fruit fly (*Drosophila melanogaster*)	Injection of *M. marinum*	• Progressive disease wasting phenotype	• Innate immunity well conserved. • Physiological aspects of disease similar to human TB. • Genetic tool.	• Mycobacteria are not natural pathogen.
Zebra fish larvae and adult Zebra fish	Injection of *M. marinum*	• Early granulomas in larvae. • Active disease (necrotic granuloma) • Latent infection (fibrotic granuloma)	• Disease progression and pathology similar to humans. • Suitable for studying mechanism of latency, dormancy and reactivation. • Good for innate (larvae) and adaptive immunity (adult). • Good for early stage drug and vaccine studies. • Small, cost-effective, suitable for large-scale screening. •	• Physiological and anatomical differences between human and zebra fish. • Lack of cell lines.

Model	Route of infection	Stage of infection	Advantages	Disadvantages
Mammalian models				
Mouse	Aerosol, intratraceal, intravenous, intranasal infection with *Mtb*	• Chronic disease with non-hypoxic granulomas. • Latency (antibiotic required)	• Immune system similar to humans. • Small, cost-effective. • Availability of extensive range of immunological reagents. • Transgenic, knockout and diverse range of genetic backgrounds mice are available.	• Mice are not natural host of *Mtb.* • Granulomas are loosely organized. • TB pathology is different from humans.
Guinea pig	Aerosol infection with *Mtb*	• Progressive disease with caseous granulomas	• Highly susceptible to *Mtb.* • Closely resemble to the human disease. • Suitable for drug and vaccine studies.	• Expensive. • Lack of reagents.
Model	**Route of infection**	**Stage of infection**	**Advantages**	**Disadvantages**
Rabbit	Aerosol infection with *Mtb* or *M. bovis*	• Latent infection with caseous granulomas	• TB pathology and disease progression are similar to humans. • Excellent model for studying pathology and cell-mediated immune responses.	• High cost. • Large animals. • Genetic changes are difficult. • Lack of reagents.
Cattle	Aerosol infection with *M. bovis*	• Latent infection with hypoxic granulomas	• Natural host of *M. bovis*. • Disease pathology similar to human TB. • Suitable for BCG vaccine studies.	• High cost. • Large animals. • Genetic changes are difficult. • Moderate availability of reagents.
Non-human primates	Aerosol infection with *Mtb*	• Active disease with caseous granulomas • Latent infection with hypoxic granulomas	• Naturally susceptible to *Mtb.* • Mimics disease progression, pathology and human immune responses. • Suitable for studying clinical aspects of disease including therapeutics and diagnostics.	• High cost. • Large animals. • Space requirements. • Ethical concerns. • Not suitable for large-scale experiments.

1.13 Rationale and Hypothesis

Despite the availability of a preventive vaccine and anti-TB drugs, TB still remains a global health threat and an economic burden. The current multifaceted TB epidemic continues to grow at an alarming rate. The control of TB has become challenging because of the limited efficacy of the current vaccine, the lengthy treatment regimens with multiple drugs that possess serious side effects, the prevalence of HIV and TB co-infection and the emergence of multi- extensively- and totally-drug resistant strains of mycobacteria. Therefore, investigation of novel vaccine and therapeutic approaches to address this deadly disease is a global priority.

Significant efforts are being focused on the development of preventive and/or therapeutic vaccines. Several vaccines targeting either early stage antigens or late stage mycobacterial antigens formulated in adjuvants are in development. Early secreted antigenic target 6kDa protein (ESAT-6) is one such antigen, which has been shown to be an important target for protective T cell immunity against TB. ESAT-6 is expressed in pathogenic *Mycobacterium* species but absent in BCG vaccine and environmental mycobacteria. Recombinant ESAT-6 protein and synthetic overlapping peptides have been reported to provide strong Th1 responses in TB patients and also in patients recovered from TB after antimycobacterial drug treatment, suggesting that ESAT-6 is a key antigen for immune protection. Synthetic lipopeptide based cancer vaccines have demonstrated improved immunogenicity in clinical trials with very good safety and tolerance profiles. Therefore, T cell epitopes of ESAT-6 antigen identified from the literature that correspond to dominant human T cell epitopes associated with mycobacterial clearance, conjugated to palmitoyl-lysine chain, may have a strong potential as an effective TB vaccine candidate. Further, simultaneous stimulation of toll like receptors (TLRs) through agonists acting as adjuvants, such as polyI:C (TLR-3), monophosphoryl lipid A (MPL) (TLR-4) and

gardiquimod (or resiquimod) (TLR-7/8), may provide enhanced antigen-specific responses against the lipopeptides of ESAT-6.

In addition, investigation of novel host-directed immunotherapeutic approaches could provide one of the potential strategies to combat the emerging TB pandemic and improve treatment outcomes. It is very clear that besides adaptive immunity, host innate immune responses play a definitive role in protection and/or defense against TB infections. Multiple strategies are being used to boost the host immune system to fight TB disease. One such approach is the use of live and heat-inactivated mycobacterial species such as *Mycobacterium indicus pranii* and *Mycobacterium vaccae.* However, these bacteria may cause infections as genomic analyses have revealed their potential to become pathogenic in a host. Further, they may interfere with antigenic responses induced by TB vaccine. Therefore, immunotherapy with a non-pathogenic bacterium unrelated to mycobacteria genus could provide a unique approach to stimulate innate immune responses to treat and/or eliminate mycobacterial infections from a host. Heat-killed forms of a non-infectious, non-pathogenic fresh water bacterium *Caulobacter crescentus* HKCC, are being studied in my laboratory as a novel immunotherapeutic agent, and may have such potential.

Based on the above rationales, my hypotheses are as follows:

I. Palmitoylated-lipopeptides of ESAT-6 antigen can induce strong protective immune responses against *Mtb* infection, which can be further enhanced by selection of an appropriate adjuvant and route of immunization.

II. Heat-killed *Caulobacter crescentus* (HKCC) can stimulate and/or modulate various human innate and adaptive immune cells including DCs, NK, NKT and B cells.

III. HKCC alone or as adjunct to isoniazid can control TB infection through host-mediated mechanisms, and provide a novel immunotherapeutic approach.

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Chapter 2

Novel lipopeptides of ESAT-6 induce strong protective immunity against *Mycobacterium tuberculosis*: Routes of immunization and TLR agonists critically impact vaccine's efficacy[*]

[*]A version of this chapter has been published: ***Gupta N et al. 2016. Vaccine 34(46):5677-88.*** I designed and performed all of the experiments and wrote the manuscript arising from this chapter. Dr. R. Kumar (supervisor) and Dr. D.Y. Kunimoto (co-supervisor) contributed to the concept for these studies, the data analysis and manuscript composition. Dr. B. Agrawal also contributed to concept formation and manuscript composition.

2.1 Introduction

Tuberculosis (TB) is a re-emerging disease and vaccination is the most effective approach for global control of TB [1-4]. Unfortunately, the currently licensed BCG vaccine provides only partial and inconsistent protection against pulmonary tuberculosis [5-7]. To restraint the current and future outbreaks, and eradicate TB disease, a comprehensive vaccine approach is needed. Therefore, investigation of an effective and reliable new and/or improved vaccine against tuberculosis is a global priority.

Early secreted antigenic target 6kDa protein (ESAT-6) is a potent T-cell antigen expressed in pathogenic *Mycobacterium tuberculosis* (*Mtb*) but absent in BCG and environmental mycobacteria [8]. Recombinant ESAT-6 protein and synthetic overlapping peptides have been reported as strong Th1 inducing antigens in TB patients and also in patients recovered from TB after antibiotic treatment, providing a clue to a potential protective immune correlate. Even with its small size of 95 amino acids, ESAT-6 comprises an unusually high number of T cell epitopes spanning the entire sequence, supporting the potential of ESAT-6 as a putative vaccine candidate [9,10]. Moreover, peptides derived from ESAT-6 can permissively bind to multiple MHC molecules to activate T cell responses across HLA-types, supporting their role as vaccine candidates [11-13]. Consequently, ESAT-6 based subunit vaccine was found to provide significant protection against *Mtb* in mice and non-human primates [14-16].

Ligands/agonists for toll like receptors (TLRs) efficiently induce innate and adaptive immunity [17]. Stimulation of TLRs is essential to induce adaptive immunity. Vaccines that co-target TLRs can induce adaptive immune responses against pathogen-derived antigens, eliminating the need for complex toxic adjuvants and producing more specific and regulated immune responses. There are 11 known human TLRs, however, their expression and selectivity to

ligands dictate the final outcome regarding beneficial immune responses [18-20]. TLRs-3, -7, and -8 are all intracellular TLRs associated with recognizing nucleic acid products such as double stranded RNA (TLR-3) and single stranded RNA products (TLR-7 & -8) in late endosome lysosomes. TLR-4 is expressed on the cell surface and recognizes the active lipid A component of lipopolysaccharide. Patterns of TLR expression differ among subsets of dendritic cells (DCs) and other antigen presenting cells (APCs), and specific APCs can produce quite different responses to stimulation through a single TLR; accounting for diversity in TLR based regulation of innate and adaptive immunity [21]. Agonists of TLR-3 (polyI:C), TLR-4 (monophosphoryl lipid A, MPL) and TLR-7/8, (gardiquimod and resiquimod) are among the most widely used adjuvants that provide excellent antigen-specific responses and promote Th1 type immune responses [22].

The route of immunization and selection of adjuvant play critical roles in the induction of strong cellular responses. The subcutaneous route (s.c) of immunization is the most commonly used in mouse studies with peptide-based vaccines [23]. Immunization via the natural entry route (mucosal) would be preferred to induce protective local and systemic immune responses [24-27].

In this study, promiscuous immunodominant epitopes of ESAT- 6 antigen were identified that are recognized by both helper and cytotoxic T cells, and were modified by covalently linking them with a palmitate chain. The rationale for this work was based on the concept that attaching a natural fatty acid to peptides will enhance their immunogenicity due to increased stability, micelle forming properties, depot effect, efficient presentation by both MHC class I and class II and ability to activate PAMP receptors [28]. Peptides of varying lengths (15-25 aa) were chosen to allow natural processing and presentation of epitopes by antigen presenting cells. To the best of our knowledge, palmitoytated peptides of ESAT-6 antigen have not yet been explored as vaccine candidates for *Mtb*.

The immunogenicity of individual and combined ESAT-6-derived lipopeptides in mice immunized subcutaneously (s.c.) and/or intranasally (i.n.) was examined. The effect of TLR-3, TLR-4 or TLR-7/8 agonists admixed with ESAT-6 lipopeptides delivered intranasally or subcutaneously, on the induction of cellular immune responses, and on their role in reducing mycobacterial loads after intravenous *Mtb* (H37Ra) challenge was also determined.

2.2 Materials and Methods

2.2.1. Synthetic Peptides and Adjuvants

Synthetic lipopeptides derived from ESAT-6, were custom synthesized by Genscript Inc. (NJ, USA) (Table 1). All lipopeptides were prepared at 10 mg/ml in DMSO, stored at −20°C, and diluted with PBS prior to use. Toll-like receptor agonists PolyI:C (TLR-3), MPL (TLR-4) and gardiquimod (TLR-7/8) (Sigma Aldrich) were used as adjuvants.

Caulobacter crescentus (Cc) was kindly provided by Dr. B. Agrawal and was grown on solid PYE agar media (ATCC 43427) plates containing chloramphenicol (2 µg/ml, Sigma Aldrich) as a selection antibiotic. A single colony was transferred to liquid PYE medium supplemented with 2 µg/ml chloramphenicol and bacteria were grown at 25^{o}C. Logarithmically growing culture was centrifuged at 6000 rpm for 15 min, then concentration of bacteria was determined by measuring optical density at A_{600} nm and confirmed by plating serially diluted bacterial suspension on PYE agar. The following formula was used to determine bacterial colony forming units (CFU) per mL (optical density at A_{600} of 1.000 = 3 x 10^{9} CFU/mL). The pellet, or whole cells, of Cc was suspended in PBS and treated at 80^{o}C for 60 min to prepare heat-killed *Caulobacter crescentus* (HKCC). HKCC was stored at 4^{o}C until use and diluted in saline as required.

2.2.2 Mice Immunizations

All animal experiments used in this study were approved by the University Animal Care and Use Committee (ACUC) for Health Sciences, and conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). Five six-week old female BALB/c mice (purchased from Charles River Laboratories) were housed in a specific pathogen free animal facility. Mice were immunized twice, 14 days apart with individual lipopeptide (25 μg/mouse) or a pool of lipopeptides (lipopeptide mix: P1, P4, P5, P6 and P6, each at 12 μg/mouse, total 60 μg/mouse) in the absence or presence of toll-like receptor agonist MPL, PolyI:C, gardiquimod (GDQ) or HKCC. For s.c. immunization, each mouse received 100 μl of lipopeptide(s) and 25 μg of MPL, 20 μg of GDQ, 20 μg of PolyI:C, 50 x 10^6 CFU of HKCC or PBS. For i.n. immunization, 30 μl (15 μl in each nostril) of lipopeptide(s) and 10 μg of PolyI:C or MPL or GDQ, 50 x 10^6 CFU of HKCC or PBS were administered to each mouse. Control mice were immunized with an equal quantity of PolyI:C, MPL, GDQ, HKCC and PBS. Mice were euthanized using a CO_2 chamber, and various tissues (lungs, liver, spleen and lymph nodes) and samples (BAL, lung washes) were collected aseptically.

2.2.3 T Cell Proliferation Assay

Antigen-specific T cell proliferation assays were performed using nylon wool purified splenocytes and local draining lymph node cells as reported previously [29]. Respective lipopeptides were used as recall antigen at concentrations described in each figure. Plates were incubated for 4 days, culture supernatants were collected and cells were pulsed with 0.5 μCi/well [^{3}H]-thymidine (Amersham) for 12-18 h and harvested on filter papers. The levels of [^{3}H]-thymidine incorporated into the DNA of proliferating cells were counted in a Microbeta Trilux liquid scintillation counter (Perkin Elmer). Data are represented as the mean ± SD (standard

deviation) of counts per minute (cpm) for proliferation assays, or as the mean ± SEM (standard error of the mean) for stimulation indices (SI) of triplicate cultures. SD was used for cpm as it demonstrates the variations among replicate values. In the proliferation assay results where I presented data after calculating SI, SEM was used since the comparison in the means of SI is presented. Stimulation indices were calculated by dividing the mean of cpm of peptide-stimulated wells by the mean cpm of non-stimulated wells.

2.2.4 Cytokine ELISA

Cytokines secreted in culture supernatant collected from T-cell proliferation assay (described in section 2.2.3) and BAL were measured using sandwich ELISA kits (IFN-γ, TNF-α and/or IL-10) following the manufacturer's protocol (eBioscience, CA, USA). Briefly, 96-well ELISA plates (Corning Costar 9018, eBioscience) were coated with capture antibodies (1-4 μg/ml, 100 μl/well) diluted in coating buffer. Plates were sealed and incubated overnight at 4°C. The next day, plates were blocked with 1 x diluent buffer (eBioscience) at room temperature (RT) for 1 h, and 2-fold serially diluted recombinant standard or 100 μl/well of test samples were added in triplicates. The plates were incubated again at RT for 2 h. Biotinylated detection antibody (100 μl/well) in 1 x diluent buffer was added and plates were incubated for 1 h at RT. After incubation, 100 μl/well of avidin-horseradish peroxidase (Avidin-HRP) was added and plates were incubated at RT for 30 min. 1 x TMB solution (100 μl/well) was added and plates were incubated at RT for 15 min. The enzyme reaction was stopped by adding 50 μl/well of stop solution (2N H_2SO_4) to each well. Plates were washed three times with wash buffer (1 x PBS with 0.05% Tween-20) after each incubation step. The ELISA plates were read with an automated ELISA plate reader (Fluostar Optima, BMG Labtech GmbH, Ortenberg, Germany).

Data are represented as the mean ± SD of concentrations for each cytokine perfomed in triplicate wells.

2.2.5 Bronchoalveolar Lavage (BAL)

To harvest BAL fluid and cells, lungs were lavaged with 500 µl of ice-cold sterile PBS [with 0.3% wt/vol bovine serum albumin (BSA)] and two 500 µl PBS washes. Fluids were centrifuged at 1,500 rpm for 10 min, and RBC lysis was performed on cell pellets. For RBC lysis, the cell pellet was resuspended in 500 µl of sterile distilled water and vortexed briefly. Immediately after, 500 µl of 2 × PBS was added, the tube was vortexed briefly and the volume was made to 2 ml with 1 × PBS. The obtained lymphocytes were used for staining. The supernatants of the initial 500 µl BAL fluid were used for cytokine analyses. Dr. Satish Vedi provided expertise by collecting and processing BAL from mice.

2.2.6 Flow Cytometry Analysis of Immune Cells

A total of 1×10^6 cells from spleen and BAL from immunized mice were stained with extracellular (anti-mouse CD3e-FITC, CD4-PECy-5, CD8-APC-Cy-7, CD11b-Alexafluor-700, Gr-1-Percp-e fluor 710) (eBioscience, CA, USA) and intracellular (anti-mouse Granzyme B-Alexafluor-647, FOXP3-PE) (Biolegend, USA) markers using established procedures [30]. For intracellular cytokine staining, splenocytes cultured for 5 days with peptide antigens were treated with ionomycin (1 µg/ml), PMA (50 ng/ml) and brefeldin A (1.5 µg/ml) 1 X; eBioscience) for 5 h at 37°C and subsequently stained for extracellular: CD3-PE Cy7, CD4-APC or CD8-APC-Cy 7 and intracellular cytokines IFN-γ-PE and IL-10-FITC. Samples were run on LSR Fortessa SORP flow cytometer and (analyzed using FACS-DIVA software Becton Dickinson, Mountain View, CA). Respective isotype-matched control antibodies were used to gate non-specific staining.

Ms. Dorothy Kratochwii-Otto provided assistance in managing and troubleshooting flow cytometry instruments.

2.2.7 Mycobacterial Challenge of Mice

M. tuberculosis (H37Ra) was obtained from ATCC (Rockville, MD). Briefly, 14 days after the second immunization, mice were injected with 5×10^5 CFU/mouse of H37Ra intravenously. Five weeks after H37Ra infection, mice were euthanized using a CO_2 chamber, and lungs, liver and spleen were removed aseptically and individually homogenized in 5 ml of saline. A 100 µl aliquot was taken from each organ homogenate of individual mice and was plated on 7H11 Middlebrook agar plates (BD Biosciences) in serial dilution. The plates were incubated at 37°C for 3-4 weeks prior to counting the colonies. The number of bacterial colonies was counted manually using a magnifying glass apparatus. The bacterial counts obtained were multiplied by the dilution factor to represent the total CFUs from the whole organ.

2.2.8 Statistical Analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software Inc., CA, USA). Data were presented as mean ± SD or SEM. Significant differences between two groups were determined using the Student's t-test. The difference among the means of multiple immunization groups on immune reponses or bacterial loads were compared by two-way ANOVA followed by Tukey's multiple comparison test. A p-value less than 0.05 ($P < 0.05$) was considered to be statistically significant.

2.3 Results

2.3.1 Individual modified lip peptides derived from ESAT-6 induce cellular immune responses upon subcutaneous immunizations

The immunogenicity of modified lipopeptides derived from ESAT-6 were examined and depicted in **Table 2.1**. Mice were immunized s.c. with individual lipopeptides (P1-P7) or PBS twice, 14 days apart and examined for antigen-specific T cell responses in spleen and inguinal lymph nodes one week after the last immunization. There was dose-dependent T cell proliferation in mice immunized with lipopeptides P1, P4, P5, P6 and P7, but not with P2 and P3 (**Fig. 2.1 A** and **2.1 B**). Splenocytes from all mice immunized with P1-P7 provided similar responses against ConA (a T cell mitogen), used as a positive control (**Fig. 2.1 A** and **2.1 B**). Induction of antigen-specific $CD4^+$ and $CD8^+$ T cells responses was determined by IFN-γ production in *ex vivo* antigen-stimulated splenocytes. Concordant with the proliferation assay, both $CD4^+$ and $CD8^+$ T lymphocytes from P1 and P4-7 immunized mice demonstrated significant production of IFN-γ, while P2 and P3 did not induce an IFN-γ response compared to PBS controls (**Fig. 2.1 C**).

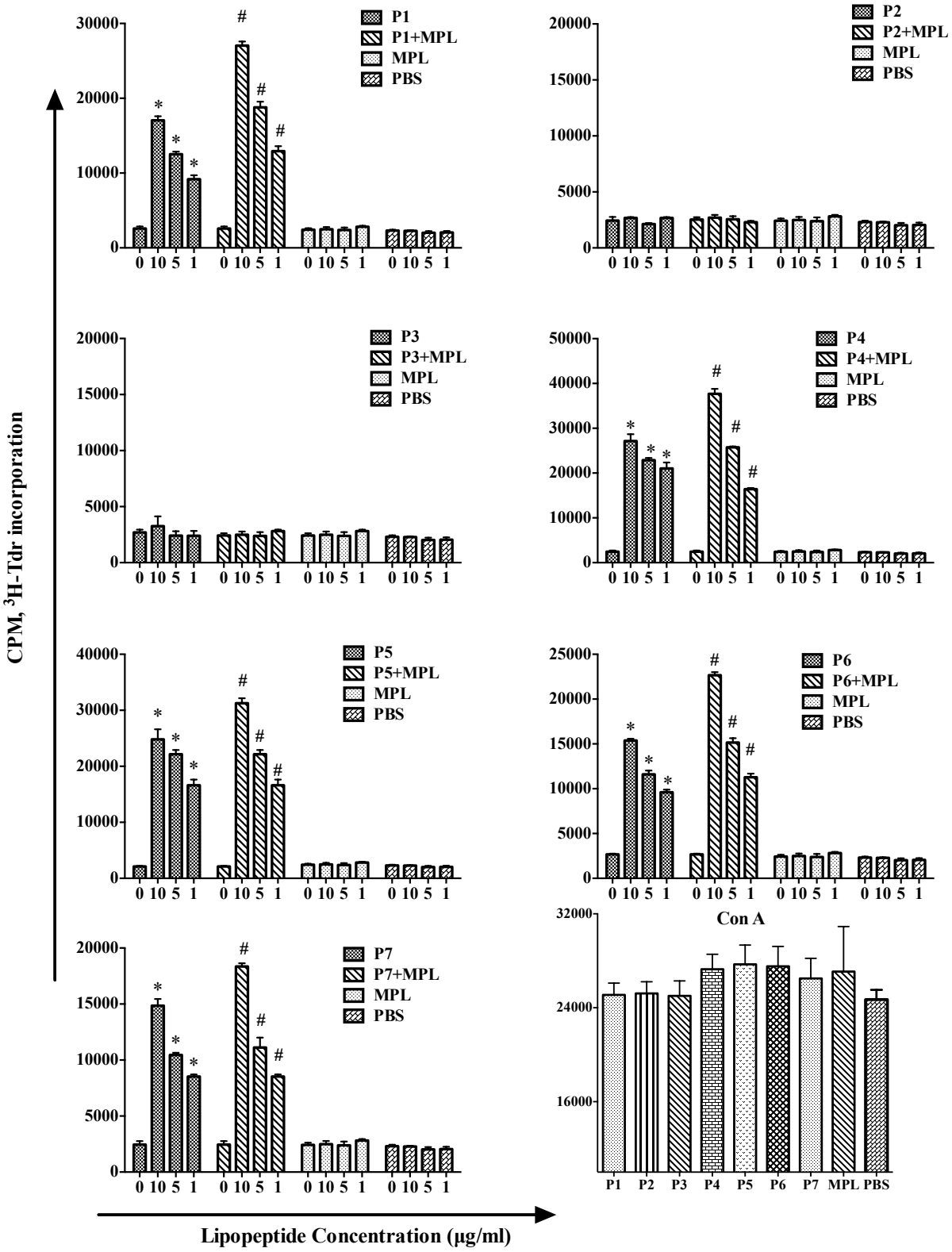
Table 2.1: ESAT-6 protein EsxA [*Mycobacterium tuberculosis* H37Rv], NCBI Reference Sequence: YP_178023.1

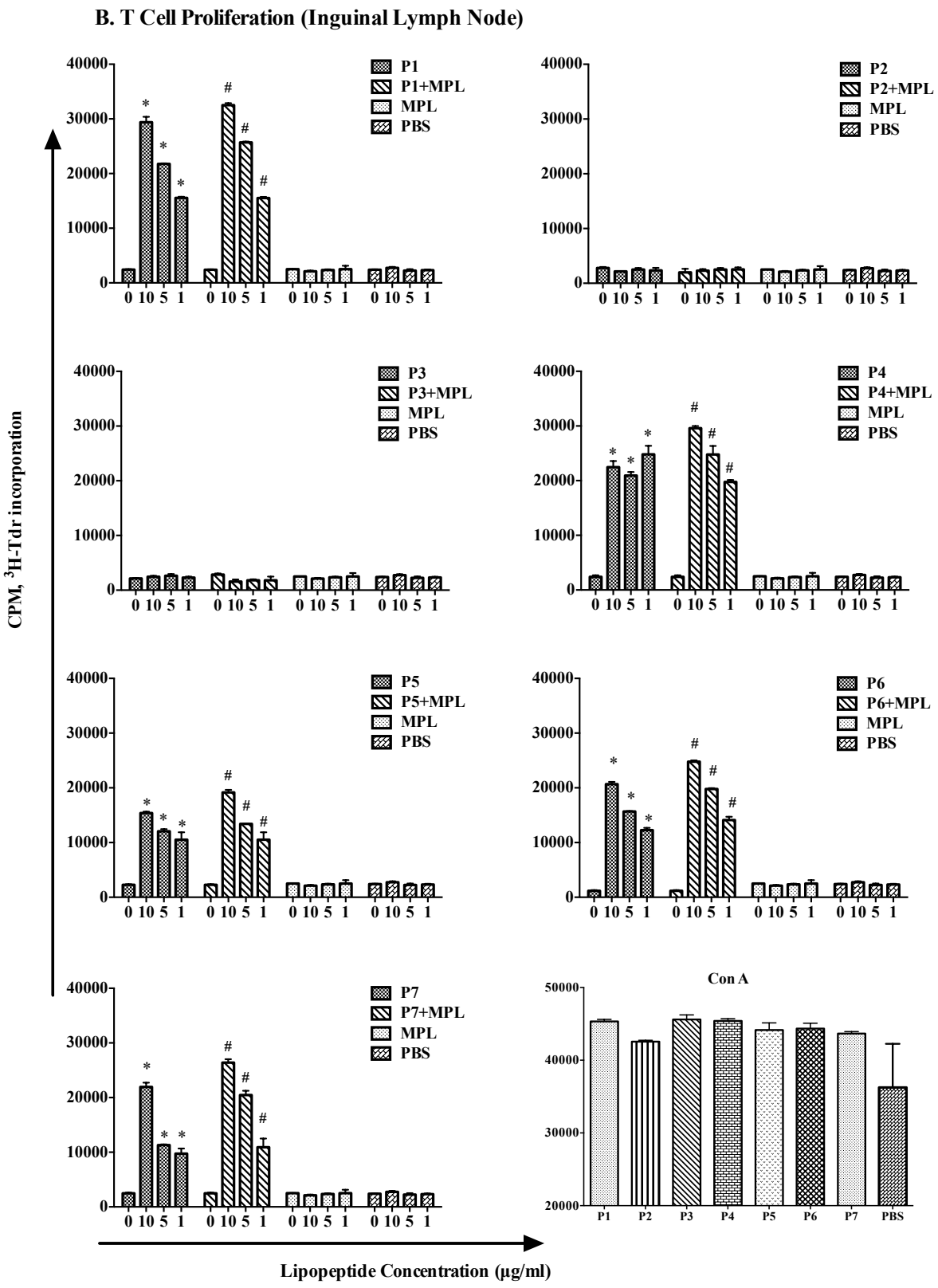
95 Amino acids from the N terminus to C terminus: MTEQQWNFAGIEAAASAIQG NVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWDATATELNNALQNLARTISE AGQAMASTEGNV TGMFA

Location of peptide in *Mtb* secretory protein	**Code**	**Amino Acid Sequence**
ESAT-6_{1-15}	P1	MTEQQWNFAGIEAAA **K(palmitate)G**
ESAT-6_{15-29}	P2	ASAIQGNVTSIHSLL **K(palmitate)G**
ESAT-6_{16-40}	P3	SAIQGNVTSIHSLLDEGKQSLTKLA **K(palmitate)G**
ESAT-6_{31-55}	P4	EGKQSLTKLAAAWGGSGSEAYQGVQ **K(palmitate)G**
ESAT-6_{46-70}	P5	SGSEAYQGVQQKWDATATELNNALQ **K(palmitate)G**
ESAT-6_{61-85}	P6	TATELNNALQNLARTISEAGQAMAS **K(palmitate)G**
ESAT-6_{76-95}	P7	ISEAGQAMASTEGNVTGMFA **K(palmitate)G**

Next, I determined whether the addition of the TLR-4 agonist MPL could increase the immunogenicity of lipopeptides P2 and P3. I found that inclusion of MPL with lipopeptides P2 and P3 did not affect their immunogenicity, whereas the T cell responses against lipopeptides P1 and P4-P7 were further increased with MPL (**Fig. 2.1 A** and **2.1 B**). The absence of a response in mice immunized with lipopeptides P2 or P3 proves that induced T cell responses are peptide-specific, not lipid-specific.

A. T Cell Proliferation (Splenocytes)




B. T Cell Proliferation (Inguinal Lymph Node)
CPM, 3H-Tdr incorporation
Lipopeptide Concentration (µg/ml)
P1
P1+MPL
MPL
PBS
P2
P2+MPL
P3
P3+MPL
P4
P4+MPL
P5
P5+MPL
P6
P6+MPL
P7
P7+MPL
Con A
0 10 5 1
P1 P2 P3 P4 P5 P6 P7 PBS

C. Antigen dependent IFN-γ production in spleen

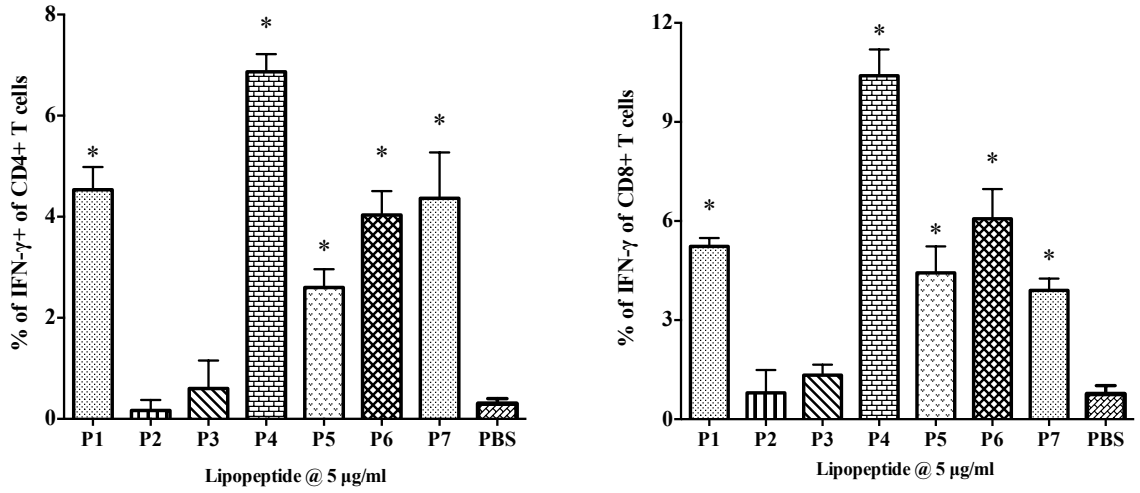
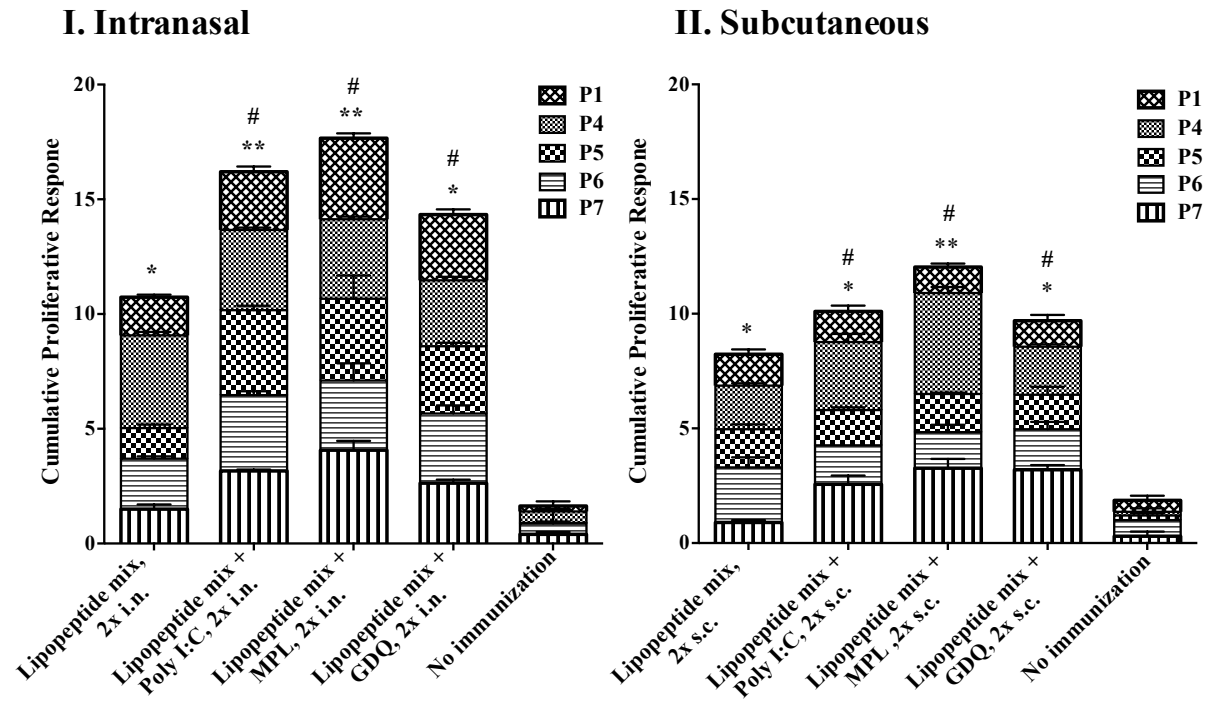


Fig 2.1: Cellular immune responses after immunizations with individual lipopeptides of ESAT-6. Female BALB/c mice (n = 5) were immunized subcutaneously twice, 14 days apart, with individual lipopeptides P1-P7 alone or with MPL as an adjuvant. MPL and PBS alone groups were used as controls. Eight days after the last immunization, T cells obtained from splenocytes and lymph nodes were cultured with irradiated APCs (splenocytes from unimmunized mice) and with respective lipopeptides P1-P7 at 10, 5 and 1 µg/ml concentrations for 4 days. T cell proliferation was measured by [^{3}H] thymidine incorporation in **(A)** spleen and **(B)** inguinal lymph nodes cells. Con A (1 µg/ml) was used as a positive control for all groups. Mean ± standard deviation of CPM (counts per minutes) from triplicate wells are shown. Spleen cells obtained from mice immunized with lipopeptide P1-P7 were cultured for 4 days with or without respective lipopeptides at 5 µg/ml concentration and labeled with antibodies against CD3, CD4 and CD8 for extracellular staining along with intracellular IFN-γ. The cells were gated for $CD3^+CD4^+$ and $CD3^+CD8^+$, which were subsequently analyzed for IFN-γ expression. Data are shown as the percentage of IFN-γ^+ of $CD4^+$ and $CD8^+$ T cells **(C)**. The peptide-specific response was calculated by subtracting the percentage of cells positive for IFN-γ expression in the absence of peptide (no peptide control). '*' and '#' indicates significant difference ($P < 0.001$) compared to the corresponding group in PBS and MPL immunized mice, respectively. Data are representative of three repeated experiments.

2.3.2 Immunization with a mix of ESAT-6 lipopeptides broadens the induced T cell responses in mice

To expand the induced T cell responses to multiple epitopes of ESAT-6 antigen, I determined the immunogenicity of the five immunogenic lipopeptides mixed together. I also investigated the impact of the route of immunization (s.c. and i.n.) and the addition of TLR agonists (MPL, polyI:C or gardiquimod) on induced T cell responses. Interestingly, mice immunized with pooled peptides by both intranasal and subcutaneous routes, elicited T cell proliferation (**Fig. 2.2 A**) as well as IFN-γ production (**Fig. 2.2 B**) against each of the lipopeptides present in the mix. Thus, the cumulative T cell response induced by the pool encompasses most of the T cell epitopes of ESAT-6. This experiment also conclusively demonstrated that lipopeptides induced significant systemic T cell immunity upon mucosal immunization (**Fig. 2.2 A, B**). Antigen-specific immune responses were also significantly increased with addition of an adjuvant to the lipopeptide mix (**Fig. 2.2**). Among the TLR-agonists used, MPL provided the highest T cell proliferation, whereas PolyI:C led to the maximum IFN-γ production by both routes of immunization. Interestingly, addition of GDQ led to T cell response higher than lipopeptide mix alone, but lower than the other two TLR agonists MPL and polyI:C. Overall, the magnitude of immune responses was higher with the intranasal route than the subcutaneous route, and addition of TLR agonists bolstered it.

A. T Cell Proliferation in Spleen



B. IFN-γ Production in Spleen

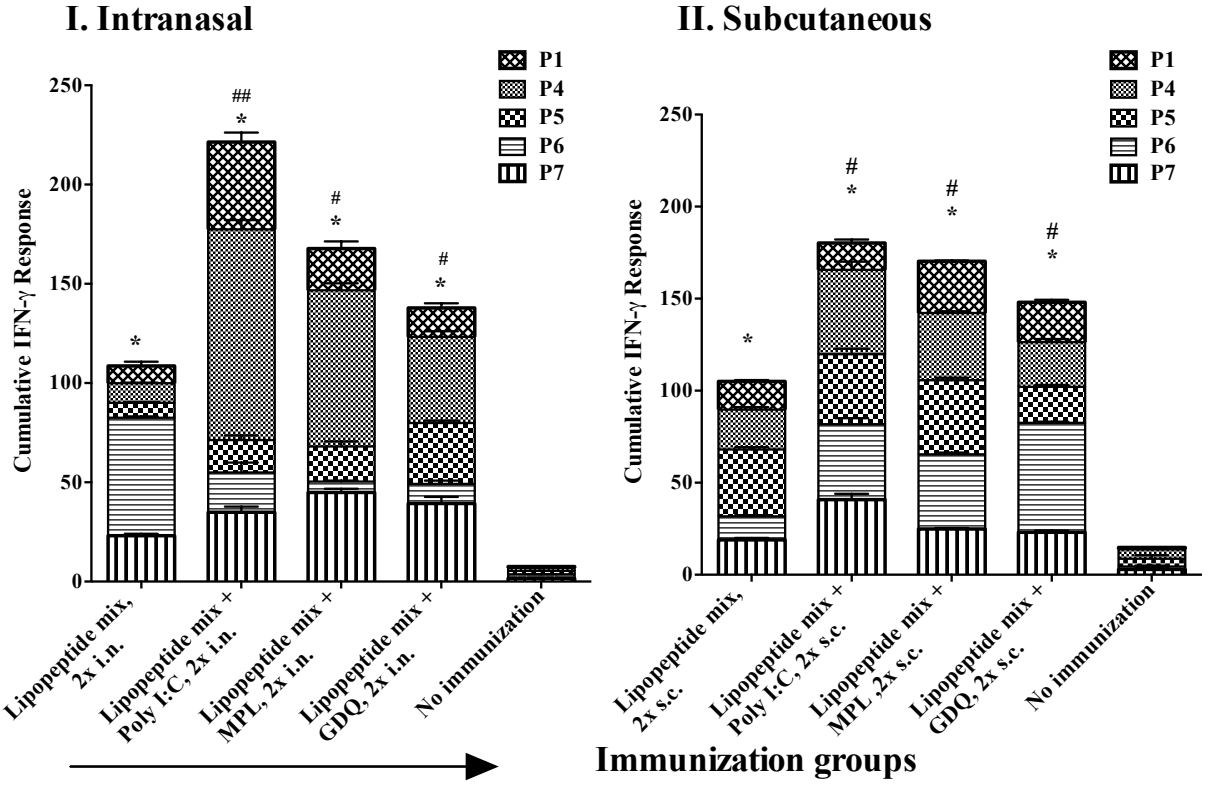


Fig 2.2: Cellular immune responses after immunizations with pool of immunogenic lipopeptides of ESAT-6 with or without an adjuvant. (A) Antigen specific T cell proliferation in spleen. Female BALB/c mice (n = 5) were immunized twice, 14 days apart with a mixture of P1 and P4-P7 alone or combined with Poly I:C, MPL or GDQ by intranasal (i.n.) and subcutaneous (s.c.) routes. PBS was used as a control. Eight days after the last immunization, T cells obtained from spleens were cultured with irradiated APCs and with the individual lipopeptide at 5 μg/ml concentration for 4 days**.** Proliferation was measured by [^{3} H] thymidine incorporation. Supernatants collected from antigen-stimulated T cell culture were used to determine **(B)** antigen specific IFN-γ by ELISA. Data with intranasal **(I)** and subcutaneous **(II)** routes are shown. Results are expressed as cumulative proliferative response to all immunizing lipopeptides. The stimulation index (SI) ± SEM from triplicate wells was calculated as follows: (CPM counts or Concentration (pg/ml) against respective peptide)/(CPM counts or Concentration (pg/ml) against no peptide control). Response against each peptide (P1, P4, P5, P6 & P7) was added (sum of SI for all lipopeptides) together to calculate the cumulative antigen-specific responses. '*', Indicates significant difference ('*', $P < 0.01$, '**', $P < 0.001$) compared to the corresponding group in PBS immunized mice and '#' compared to the group immunized with lipopeptide mix alone ('#' $P \leq 0.05$, '##' $P \leq 0.001$). Data are representative of three different repeated experiments.

2.3.3 Immunization with a mix of ESAT-6 lipopeptide along with Poly I:C, MPL or GDQ reduces growth of disseminated mycobacteria upon intravenous *Mtb* challenge

I then examined the protective efficacy of mucosal and parenteral immunization with the lipopeptide mix alone and in combination with a TLR-agonist. Immunization of mice with peptide mix alone by both subcutaneous and intranasal routes led to significant reduction in *Mtb* loads in all organs compared to PBS controls (**Fig. 2.3 A** and **B**).

Among the groups immunized with lipopeptide mix plus a TLR agonist, the maximum reduction in bacterial load was observed in the intranasal MPL-adjuvanted group, which was also significantly higher than lipopeptide mix alone group (**Fig. 2.3**). However, after subcutaneous immunization, bacterial load in the MPL-adjuvanted group was significantly reduced only in the lungs (~51%, **Fig. 2.3**). In contrast, Poly I:C- and GDQ-adjuvanted groups showed increases in bacterial burden in lungs and liver, and no difference in spleen, with both routes of immunization.

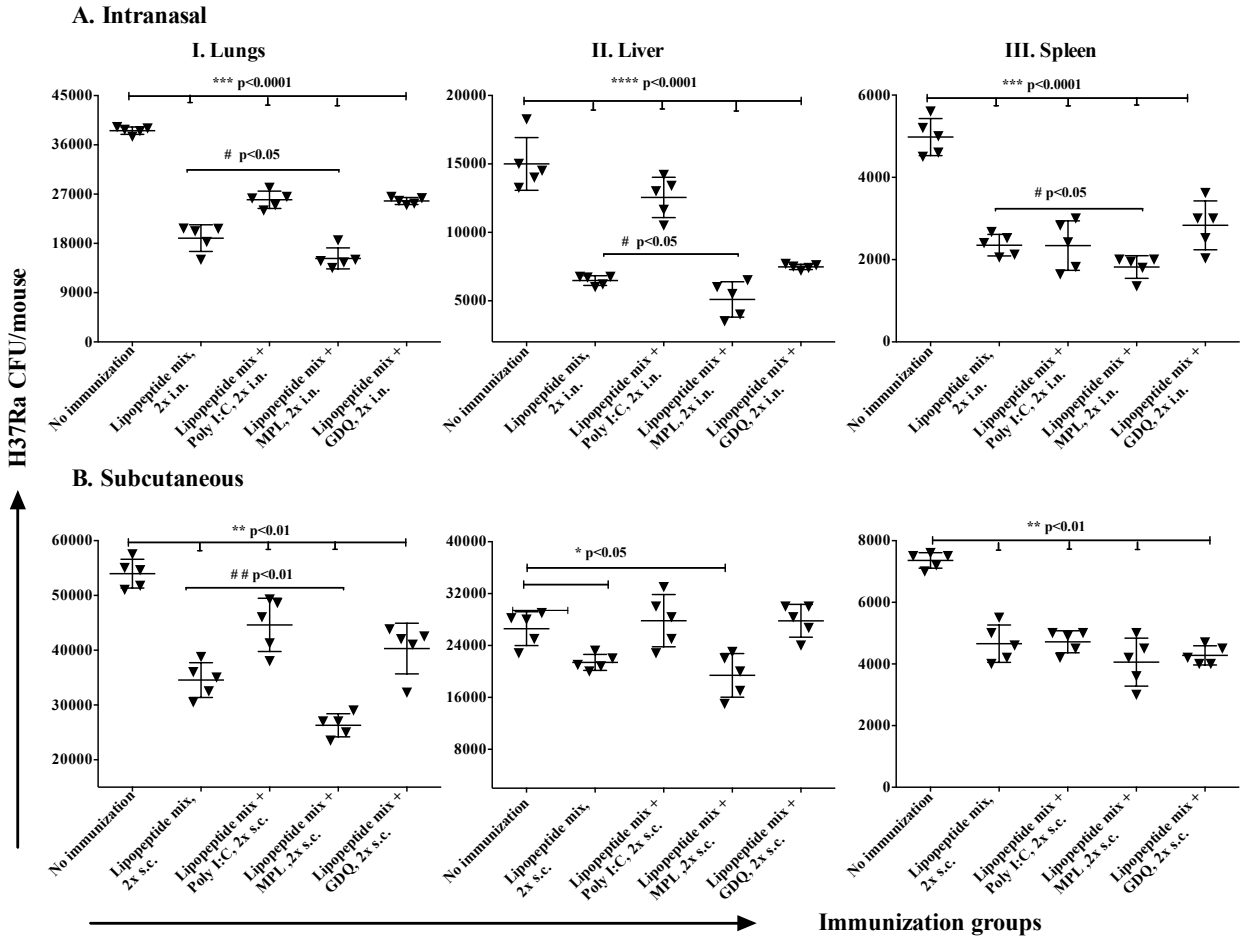


Fig 2.3: Immunizations with ESAT-6 lipopeptides reduce bacterial loads in mice after *Mtb* challenge. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice, 14 days apart, with a mixture of P1 and P4-P7 lipopeptides (12 μg each) alone and combined with Poly I: C, MPL or GDQ. Control mice were immunized with PBS. Eight days after the last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, *Mtb*-challenged mice were euthanized and lungs, liver and spleens were collected from **(A)** intranasally and **(B)** subcutaneously immunized mice. Bacterial loads were determined in **(I)** lungs, **(II)** liver and **(III)** spleen by CFU assay. All results are shown mean ± standard deviation of CFU (colony forming units) from individual mice. Data are representative of three different repeated experiments. '*', Indicates significant difference (*P < 0.05; **P < 0.01; ***P < 0.001) compared to the corresponding group in PBS immunized mice and '#' compared to the group immunized with lipopeptide mix alone ('#' P ≤ 0.05, '##' P ≤ 0.01).

2.3.4 Reduction in mycobacterial loads in mice immunized with lipopeptide mix and adjuvants is associated with local and systemic cytokine and/or systemic $CD4^+$ T cell responses

I measured the production of cytokines in lung washes and splenocytes, to assess if the protective effect of immunizing with ESAT-6 lipopeptide correlated with local and systemic immune responses. Interestingly, immunization with lipopeptide mix alone by both routes led to significantly (**P < 0.01) increased levels of effector cytokines IFN-γ and TNF-α and simultaneously reduced IL-10 levels compared to controls (**Fig. 2.4 A** and **B**). Co-immunization with MPL led to significantly increased IFN-γ by both routes, whereas the IFN-γ levels were reduced in polyI:C- and GDQ-adjuvanted groups, compared to lipopeptide mix alone and/or PBS controls (**Fig 2.4**). In comparison, TNF-α production was similar in groups adjuvanted with polyI:C (i.n. and s.c.), MPL (s.c) and GDQ (i.n.), while addition of MPL i.n. and GDQ s.c. led to reduced levels of TNF-α compared to the peptide mix group with no adjuvant. No significant reduction was observed in IL-10 levels among groups immunized with lipopeptide mix and PolyI:C, MPL or GDQ , except s.c. in the MPL-adjuvanted group.

In addition to soluble effectors (cytokines), I examined the intracellular expression of effector molecules in $CD4^+$ and $CD8^+$ T cells to determine their contribution in the observed reduction in mycobacterial loads. Concurring with cytokines present in BAL, the percentage of $CD4^+IFN\text{-}\gamma^+$ T cells was significantly higher and $CD4^+IL\text{-}10^+$ T cells was lower in the peptide mix alone and MPL-adjuvanted groups compared to all other groups (**Fig. 2.5 A** and **B**). Co-immunization of lipopeptide mix with PolyI:C and GDQ led to reduced IFN-γ production with both routes and increased IL-10 expression in $CD4^+$ T cells by s.c. immunization. There was no change in the percentage of $CD4^+IL\text{-}10^+$ cells upon immunization with PolyI:C and GDQ (i.n.) and MPL (s.c.) compared to lipopeptide mix alone. I also examined intracellular expression of

IFN-γ, Granzyme B and IL-10 in $CD8^+$T cells, but did not observe any significant difference between various experimental groups (**Appendix 1-3**).

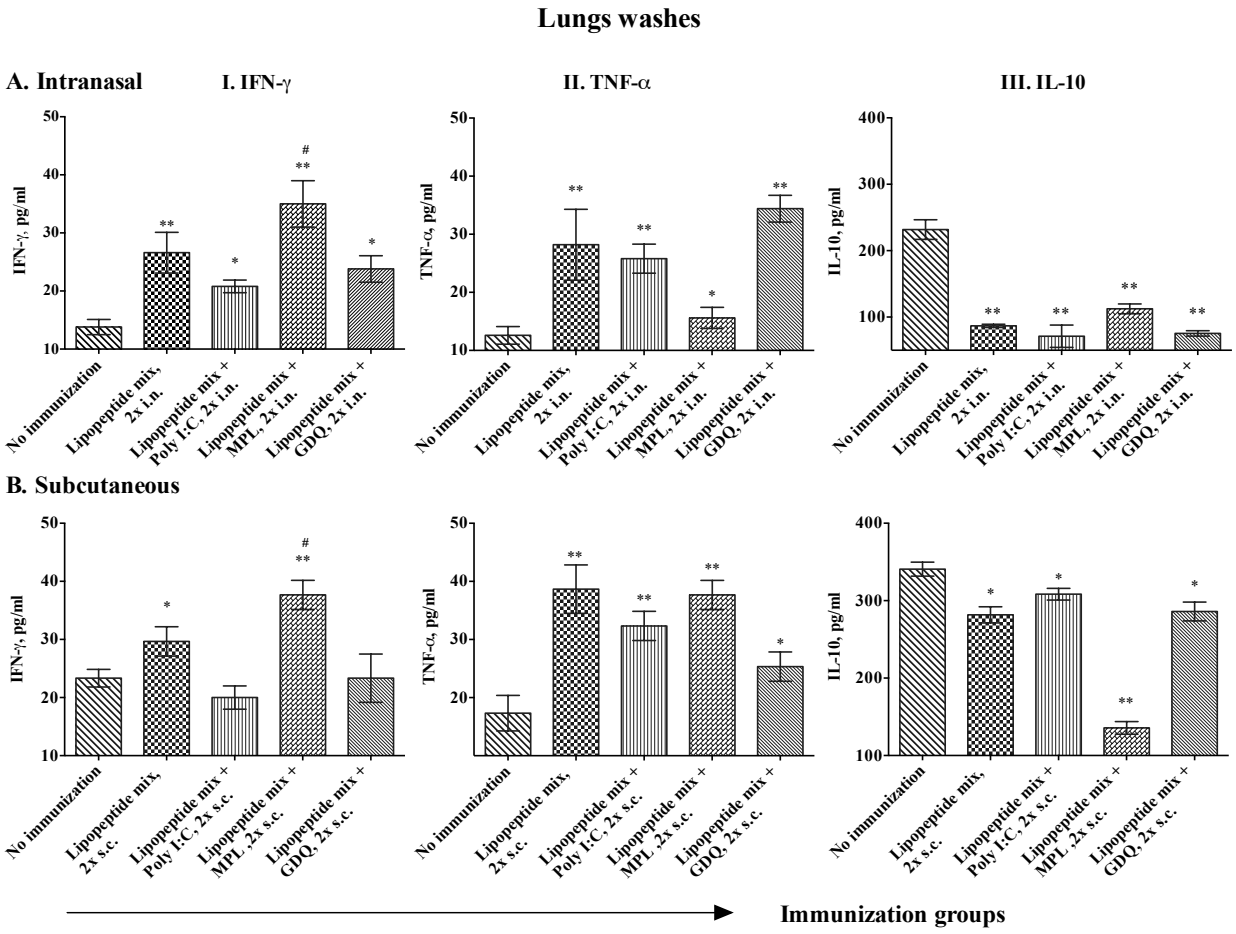


Fig 2.4: Immunizations with ESAT-6 lipopeptides with or without adjuvant lead to differential induction of cytokines in lung washes after *Mtb* challenge in mice. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice with a mixture of P1 and P4-P7 lipopeptides alone and combined with Poly I:C, MPL or GDQ. Control mice were immunized with PBS. One week after the last immunization, mice were challenged with H37Ra (0.5×10^6 CFU) intravenously. Five weeks later, *Mtb*-challenged mice were euthanized and lung washes were collected from **(A)** intranasally and **(B)** subcutaneously immunized mice to determine **(I)** IFN-γ, **(II)** TNF-α and **(III)** IL-10 by ELISA. Mean ± standard deviation of cytokine concentrations from individual mice are shown. '*', Indicates significant difference (*P < 0.05; **P < 0.01) compared to the corresponding group in PBS immunized mice and '#' compared to the group immunized with lipopeptide mix alone ('#' P ≤ 0.05). Data are representative of three different repeated experiments.

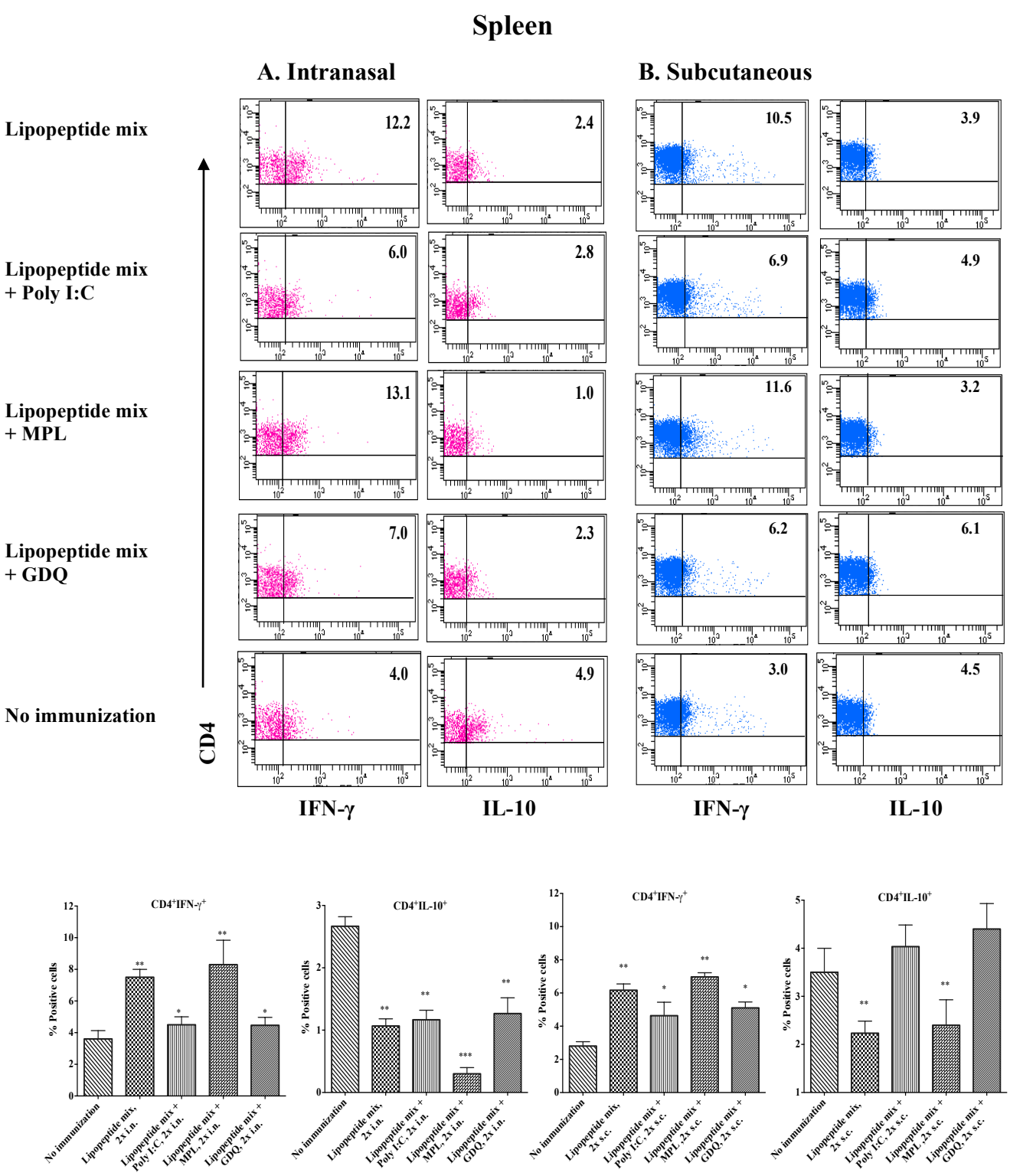


Fig 2.5: Intracellular IFN-γ and IL-10 are differentially expressed in antigen-specific CD4$^+$ T cells upon *Mtb* challenge in mice immunized with ESAT-6 lipopeptides with or without an adjuvant. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice

with a mixture of P1 and P4-P7 lipopeptides alone and combined with an adjuvant Poly I: C, MPL or GDQ. PBS immunized mice were used as a controls. One week after the last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, *Mtb*-challenged mice were euthanized and spleens were collected. Spleen cells obtained from immunized mice were cultured for 4 days with or without peptide pools and were labeled for surface expression of CD3 and CD4 and intracellularly for IFN-γ and IL-10. The cells were gated for $CD3^+CD4^+$ T cells that were subsequently analyzed for IFN-γ and IL-10 expression in **(A)** intranasally and **(B)** subcutaneously immunized mice. The percentage of IFN-γ^+ of $CD4^+$ T cells **(I)** and IL-10^+ of $CD4^+$ T cells **(II)** are shown. The peptide-specific response was calculated by subtracting the percentage of cells that were positive for IFN-γ and IL-10 production in the absence of peptide pool (no peptide control).'*', Indicates significant difference (*P < 0.05; **P < 0.01) compared to the corresponding group in PBS immunized mice. Data are representative of three different repeated experiments.

2.3.5 Increased myeloid-derived suppressor cells (MDSCs) in BAL and spleen is correlated with reduced protection in polyI:C or GDQ immunized mice

Next, I determined the basis of the observed reciprocal regulation of IFN-γ and IL-10 upon immunization with lipopeptide mix plus PolyI:C or GDQ following *Mtb* challenge despite high IFN-γ induction in immunized but unchallenged mice. There was no significant difference in percentages of $CD4^+FOXP3^+$ T_{regs} cells among various experimental groups. Intranasal administration of poly-L-lysine with carboxymethylcellulose alone has been shown to trigger the accumulation of MDSCs in lungs [31] so I quantitated $CD11b^+Gr\text{-}1^+$ myeloid cells in BAL and spleens. I found significantly increased infiltration and/or accumulation of MDSCs in BAL and spleen in PolyI:C- and GDQ-adjuvanted groups, upon i.n. and s.c. immunizations, respectively, compared to PBS, lipopeptide mix alone and MPL-adjuvanted groups (**Fig. 2.6**). Therefore, reduced protection after *Mtb* challenge in polyI:C- and GDQ-adjuvanted groups is associated with increased levels of MDSCs locally or systemically depending on the route of immunization, which may lead to enhanced IL-10 production and reduced IFN-γ production from $CD4^+$ T cells.

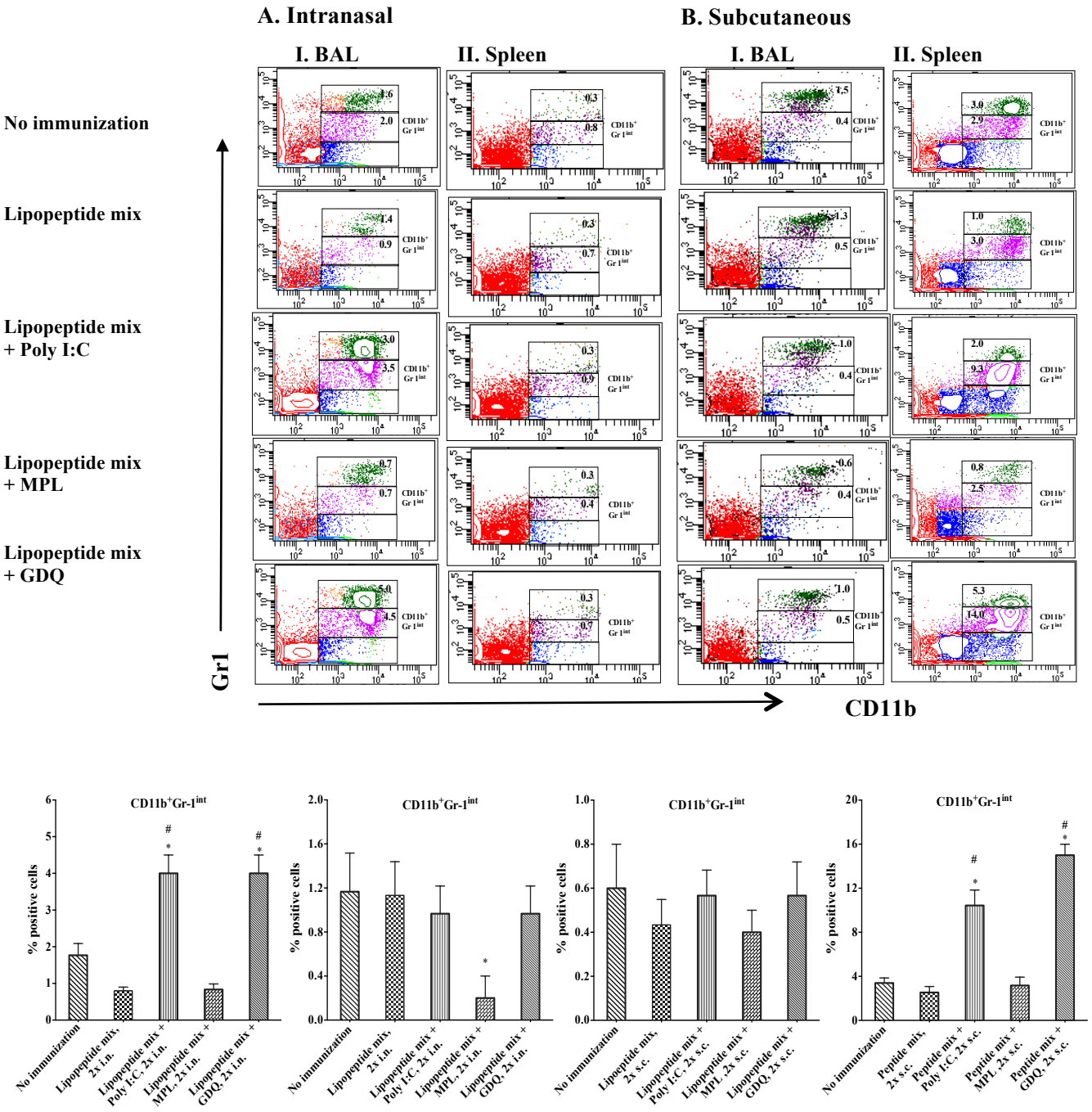


Fig 2.6: Increase in myeloid derived suppressor ($CD11b^{+}Gr\text{-}1^{+}$) cells in bronchoalveolar lavage (BAL) and spleen correlates with decreased reduction in *Mtb* loads in mice immunized with ESAT-6 lipopeptides with PolyI:C or GDQ. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice with a mixture of P1 and P4-P7 lipopeptides alone and combined with Poly I: C, MPL or GDQ. PBS immunized mice were used as controls. After 8 days of last immunization, mice were challenged with H37Ra (0.5 x 10^6

CFU) intravenously. Five weeks later, *Mtb* challenged mice were euthanized. Cells were obtained from BAL and spleen and labeled for surface markers CD11b and Gr-1. Percent positive $CD11b^{+}Gr\text{-}1^{int+}$ are shown from **(A)** intranasally and **(B)** subcutaneously immunized mice. Data are representative of three different repeated experiments.

2.3.6 Immunization with ESAT-6 lipopeptides mix along with a novel immunomodulator HKCC provides superior immune responses and reduction in *Mtb* loads

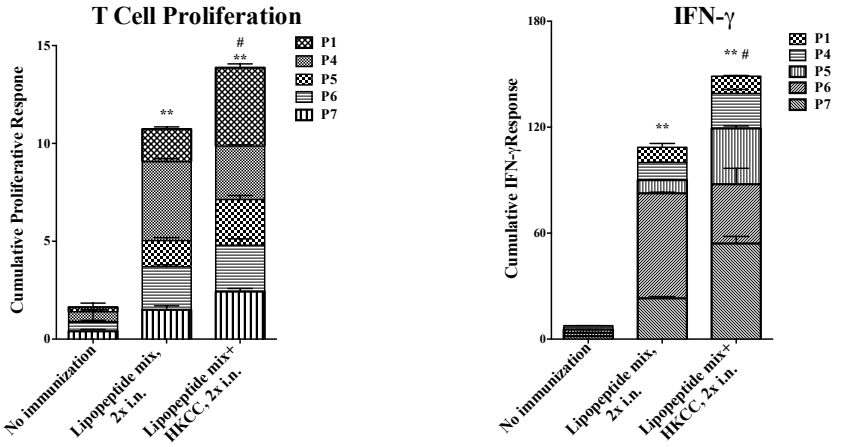
I also examined the effect of HKCC, (a novel immunomodulator being studied in Dr. Kumar's laboratory) on the immune responses induced by ESAT-6 lipopeptide mix, and their subsequent role in *Mtb* clearance by both intranasal and subcutaneous routes.

First I examined lipopeptide specific proliferation and IFN-γ production from splenocytes obtained from immunized mice. Interestingly, subcutaneous immunization of mice with ESAT-6 and HKCC did not lead to a significant increase in proliferation and IFN-γ production from splenocytes, whereas HKCC significantly enhanced T cell responses upon intranasal immunization (**Fig. 2.7**, **panel I** in **A** and **B**). Subsequently, I performed an *Mtb* challenge experiment in immunized mice. The addition of HKCC led to significantly ($*P < 0.05$) higher reduction in *Mtb* loads by both intranasal (lungs ~58%, liver ~50% and spleen ~60%) and subcutaneous (lungs 22%, liver 40% and spleen 43%) routes, compared to the lipopeptide mix alone (**Fig. 2.7**, **panel II** in **A** and **B**). In addition, HKCC also led to significantly increased IFN-γ production in lung washes by both routes, compared to the lipopeptide mix alone (**Fig. 2.7**, **panel III** in **A** and **B**). There was no statistically significant difference in TNF-α and IL-10 levels after immunization with HKCC by both routes. Interestingly, following *Mtb* challenge, the percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells in spleen increased despite no difference in T cell responses observed in subcutaneously immunized but unchallenged mice (**Fig. 2.7 B**, **panels I** and **IV**). In contrast, intranasal immunization with HKCC did not show change in the number of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells

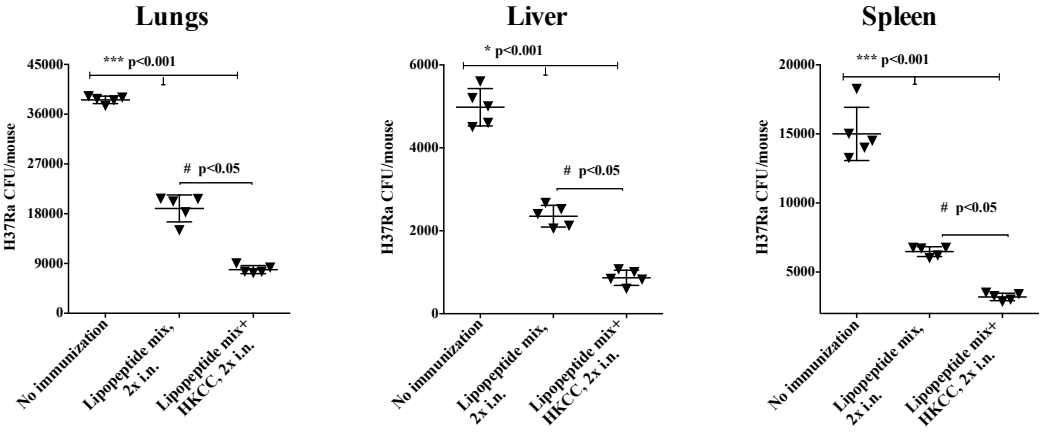
in challenged mice despite increased immune responses before challenge, compared to the lipopeptide mix alone (**Fig. 2.7 A**, **panels I** and **IV**). There was no significant difference in the number of $CD4^{+}IL\text{-}10^{+}$ T cells and MDSCs in the lipopeptide mix alone and HKCC-adjuvanted groups in mice immunized by both subcutaneous and intranasal routes. Therefore, protection after *Mtb* challenge in HKCC-adjuvanted groups is associated with increased levels of IFN-γ locally or systemically depending on the route of immunization, however it may be contributed from both $CD4^{+}$ T cells and possibly other cells such as NK and NKT.

A. Intranasal

I. Immune responses in spleen before *Mtb* challenge

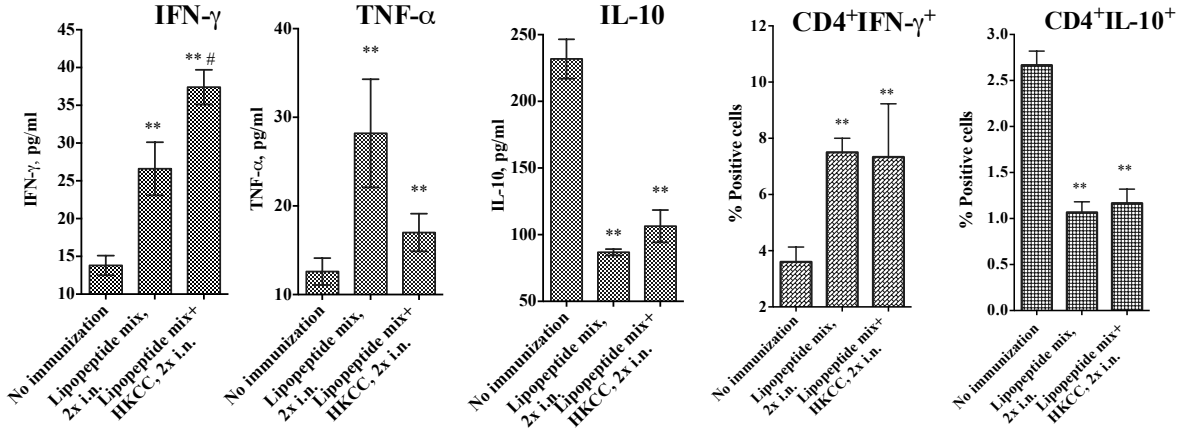


II. *Mtb* load



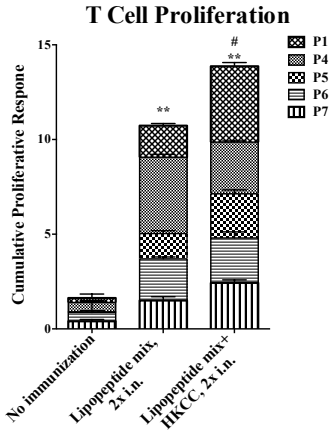
III. Cytokines in BAL after *Mtb* challenge

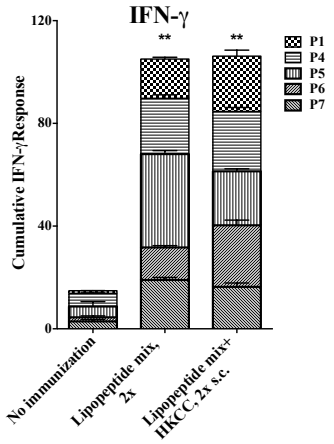
IV. Cytokines in spleen after *Mtb* challenge



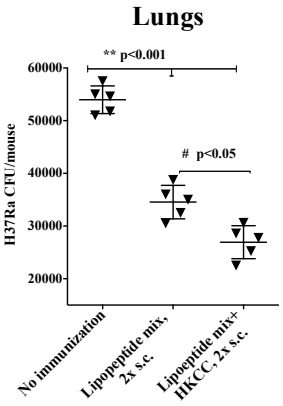
B. Subcutaneous

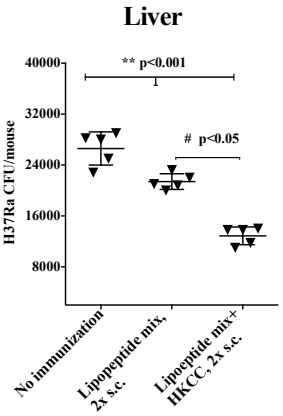
I. Immune responses in spleen before *Mtb* challenge

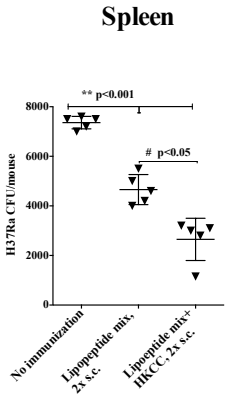




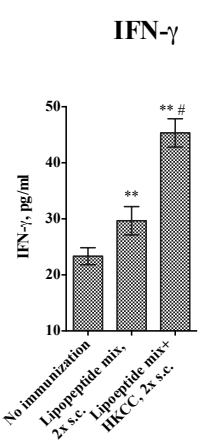
II. *Mtb* load

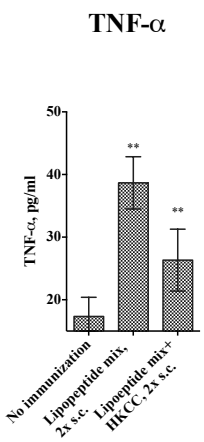


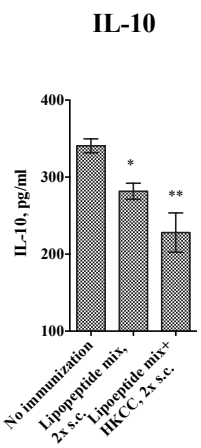




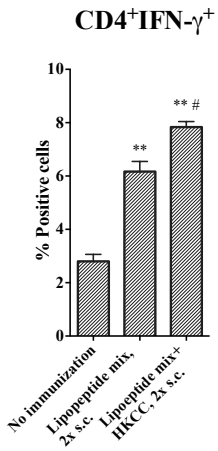
III. Cytokines in BAL after *Mtb* challenge







IV. Cytokines in spleen after *Mtb* challenge



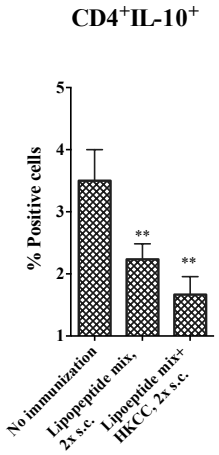


Fig 2.7: Immunization of mice with ESAT-6 derived lipopeptides along with HKCC leads to superior induction of immune responses and reduction in *Mtb* loads by [A] Intranasal and [B] Subcutaneous routes. (I). Antigen specific T cell proliferation and IFN-γ in spleen. Female BALB/c mice (n = 5) were immunized twice, 14 days apart with a mixture of P1 and P4-P7 alone or combined with HKCC (50 x 10^6/mouse) by intranasal (i.n.) and subcutaneous (s.c.) routes. Saline was used as a control. After 8 days of last immunization, T cells obtained from spleens were cultured with irradiated APCs and with the individual lipopeptide at 5 μg/ml concentration for 4 days**.** Proliferation was measured by [^{3}H] thymidine incorporation. Supernatants collected from antigen stimulated T cell culture were used to determine antigen specific IFN-γ by ELISA. Data with intranasal (A) and subcutaneous (B) routes are shown. Results are expressed as cumulative proliferative response to all immunized lipopeptides. For challenge study, after 8 days of last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, *Mtb* challenged mice were euthanized and lungs, liver and spleens were collected from intranasally and subcutaneously immunized mice. **(II).** *Mtb* loads in lungs, liver and spleen were determined by CFU assay. All results are shown as mean ± standard deviation of CFU (colony forming units) from five individual mice. Lung washes were collected from (A) intranasally and (B) subcutaneously immunized mice challenged with *Mtb* to determine cytokines IFN-γ, TNF-α and IL-10 in BAL by ELISA **(III)**. Mean ± standard deviation of cytokine concentrations from individual mice are shown. Spleen cells obtained from immunized mice after *Mtb* challenge were cultured for 4 days with or without peptide pools and were labeled for surface expression of CD3 and CD4 and intracellularly for IFN-γ and IL-10. The cells were gated for $CD3^+CD4^+$ T cells that were subsequently analyzed for intracellular IFN-γ and IL-10 expression in **(A)** intranasally and **(B)** subcutaneously immunized mice. **(IV).** The percentage of IFN-γ^+ and IL-10^+ of $CD4^+$ T cells are shown. The peptide specific response was calculated by subtracting the percentage of cells that were positive for IFN-γ and IL-10 production in the absence of peptide pool (no peptide control). '*', Indicates significant difference ('*', $P < 0.05$, '**', $P < 0.01$ '***', $P < 0.001$) compared to the corresponding group in PBS immunized mice and '#' compared to lipopeptide mix alone immunized group ('#' $P \leq 0.05$). Data are representative of three different repeated experiments.

2.4 Discussion

Development of a vaccine, which drives effective cellular immunity, is critical to combat deadly mycobacterial infections. Several vaccine strategies focused on generating strong multi-specific cellular immunity against immune-dominant antigens of *Mtb* have shown efficacy in preclinical models [32-36]. However, their efficacy in humans remains to be seen in clinical trials. Peptide-based subunit vaccine offers several advantages over whole antigen- or pathogen-

based vaccines, by strategically eliminating suppressive and/or non-immunogenic epitopes. However, weak immunogenicity and human genetic diversity have limited their use in vaccine development [37, 38].

Numerous studies have suggested that co-activating TLRs by either mixing or conjugating TLR(s)-agonists with peptides are effective in eliciting optimum immune responses [39-45]. A promiscuous $CD4^+$ T cell epitope of 16 KD antigen linked with the TLR-2 agonist Pam_2Cys was shown to protect mice from *Mtb* by inducing memory T cells. However, despite effectiveness, their poor solubility and stability are serious problems [46].

Synthetic peptides covalently attached with a simple palmitoylated lipid chain have demonstrated improved immunogenicity in clinical trials with very good safety and tolerance profiles [47-50]. Moreover, lipopeptides containing a single palimitic moiety are also known to activate TLR-2 and -4 providing moderate self-adjuvantation [51,52].

We therefore conjugated a palmitoyl-lysine residue to peptides of *Mtb* ESAT-6 antigen that correspond to dominant human T cell epitopes, as a potential TB vaccine. We also examined which combination of TLR-agonist and route of immunization could evoke optimum immune responses to protect from subsequent *Mtb* infection.

I first assessed the immunogenicity of seven individual lipopeptides covering the entire ESAT-6 antigen in mice after two s.c. immunizations. It was surprising to obtain strong cellular immune responses upon immunization with lipopeptides P1 and P4-7 without added adjuvants (**Fig 2.1**). On the other hand P2 and P3 were not immunogenic in BALB/c mice (**Fig 2.1**). Although we did not compare these results with those of free peptides, an earlier report showed free peptide P2 is immunogenic when given with CFA adjuvant in CB651 mice [53]. The reason for this discrepancy is not clear. Lipopeptides are internalized and cross-presented by DCs via an endocytosis-independent mechanism whereas non-lipidated peptides are internalized via an

endocytosis-dependent mechanism, which could lead to changes in immunogenicity [54]. Use of a strong adjuvant such as CFA, which is not approved for humans, may also break the barrier of immunogenicity or change a very weak, undetectable response to a detectable one. In our studies, addition of MPL as an adjuvant (a TLR4 agonist) bolstered the T cell responses generated against P1 and P4-P7, but did not affect the immunogenicity of P2 and P3 (**Fig. 2.1 A** and **B**). Lack of immunogenicity of P2 and P3 could be due to inefficient presentation by MHC of BALB/c mice (H-2^d) and/or inefficient recognition by the T cell repertoire of BALB/c mice. The binding of peptide epitopes to MHC and recognition of peptide-MHC complex by T cells are critically dependent on the amino acid sequences of the peptide epitopes. It is possible that peptide P2 and P3 do not contain anchor amino acid residues to bind at the antigen binding sites of MHC class I and class II molecules of H-2^d allele, or BALB/c mice lack T cells recognizing these peptide-MHC complexes.* To distinguish between these possibilities one could identify the binding of MHC class I and II molecules of H-2^d allele to these peptides, design the possible MHC-peptide tetramers and find their interactions with T cells isolated from BALB/c mice.

Interestingly, similar to $CD4^+$ T cell response, lipopeptides P1 and P4-P7 also provided a peptide-specific $CD8^+$ T cell response (**Fig. 2.1 C**). Although it has been widely accepted that $CD4^+$ T cells play a critical role against *Mtb* protection, the contribution of $CD8^+$ T cells also appears to be equally important in the containment and clearance of *Mtb*. It has been shown that *Mtb*-specific $CD8^+$ T cells possess effector functions, including direct killing of infected macrophages and production of cytokines (IFN-γ and TNF-α) to activate macrophages to inhibit replication of mycobacteria [55].

* In the peptide design, lysine residue was only used as a linker for palmitoylation and is not critical for ESAT-6 interaction with MHC.

A protective and successful immune response against *Mtb* infection requires consideration of quantitative and qualitative immune parameters against multi-epitopes, as well as site of action. Interestingly, we found that intranasal and subcutaneous immunizations with a mix of immunogenic lipopeptides alone induced systemic T cell responses against all epitopes of the mix, with an additive cumulative response (**Fig. 2.2**). In contrast, several studies have reported that after immunization with ESAT-6 protein or multiple immunodominant epitopes, immune responses are mainly directed towards dominant epitopes [56]. It is possible that the use of lipopeptides allows more epitope spreading due to efficient cross-presentation and T cell lipopeptide mix with TLR agonists PolyI:C, MPL or GDQ quantitatively enhanced the antigen-stimulation, compared to unconjugated peptides. Additionally, combining the immunogenic specific response by both subcutaneous and intranasal routes, as expected (**Fig. 2.2 A** and **B**).

Immunization with the lipopeptide mix along with different TLR agonists provided protection from *Mtb* challenge (**Fig. 2.3 A** and **B**). Intriguingly, both intranasal and subcutaneous immunizations with the lipopeptide mix alone significantly reduced bacterial burden in lungs, liver and spleen. Surprisingly, however, only the MPL-adjuvanted lipopeptide mix enhanced the efficacy of immunization in terms of reduction in bacterial burden (**Fig. 2.3**). In contrast, addition of PolyI:C or GDQ to the lipopeptide mix led to a reduction in effectiveness of vaccination (**Fig. 2.3**) including a reciprocal modulation of IFN-γ and IL-10 (**Fig. 2.4**), and higher IL-10 production correlated with increased bacterial load. Besides IFN-γ and IL-10 other cytokines/chemokines and/or unconventional T cells (NK, NKT and γδ -T cells) although not examined here, may have contributed to reduction in *Mtb* loads upon immunization with lipopeptide mix and various adjuvants, particularly because correlates of protection against *Mtb* are not clear. The mechanism underlying the decrease in protection could be attributed to the role of stimulation of different TLRs. TLR-4 agonists enhance antigen presentation by increasing

antigen internalization and delivery to the cytosol, while TLR-3 and TLR-7 enhance TAP-dependent antigen presentation [57]. Moreover, TLR-2 co-stimulation with TLR-4 or TLR-7/8 shifts the T cell responses to Th2 and Th17, respectively [58]. Further, suboptimal antigen presentation can also induce antigen-specific tolerance through the induction of Tregs instead of protection [59]. However, even though we observed enhanced IL-10 production in $CD4^+$ T cells (**Fig. 2.5**), we did not find evidence of increased percentage of $FOXP3^+$ Tregs (**Appendix 4**). Both PolyI:C and GDQ have been known to stimulate type 1 interferons. Several studies have reported the deleterious effect of Type I interferon on the outcome of *Mtb* infection *in vivo*, despite positive effects *in vitro* in *Mtb* infected cells [60]. In a mouse model of pulmonary bacterial pneumonia, both polyI:C and GDQ given intranasally, were shown to impair bacterial clearance [61]. Interestingly, we observed decreased populations of myeloid-derived suppressor cells ($CD11b^+Gr\text{-}1^{int}$) in BAL and spleen after intranasal and subcutaneous immunization, respectively, in *Mtb*-challenged mice immunized with lipopeptide mix only and lipopeptide mix plus MPL, compared to no immunization groups. However, upon combining polyI:C or GDQ with lipopeptide mix, populations of MDSCs were mostly increased, compared to lipopeptide only groups. This observation reveals a new pathway of inducing/recruiting MDSCs. Lung-residing MDSCs have been shown to provide a niche for *Mtb* survival and are associated with TB lethality [62, 63]. The mechanism of how these MDSCs are generated/recruited and lead to enhanced *Mtb* loads, will be investigated in future studies.

Several heat-killed pathogenic and non-pathogenic bacteria have been used as a delivery vehicle or vaccine adjuvant to induce antigen specific immunity in conjunction with their ability to stimulate innate immunity [64-66]. Therefore, I investigated the effect of heat-killed *Caulobacter crescentus* as a vaccine adjuvant to further bolster the protective immunity induced by lipopeptide mix in the mouse model of *Mtb* infection. Intriguingly, both intranasal and

subcutaneous immunizations with lipopeptide mix plus HKCC significantly enhanced the efficacy of lipopeptides with substantial reduction in bacterial burden and increased IFN-γ in BAL (**Fig. 2.7 A** and **B**). The mechanisms underlying superior protection by HKCC plus lipopeptide mix and the modulation of immune responses before or after challenge are not yet clear. I speculate that the enhanced effect with HKCC was due to induction/activation of multiple innate cells that regulate adaptive immunity resulting in protective immunity.

In conclusion, a mixture of simple lipid-modified peptides derived from ESAT-6 with or without MPL as an adjuvant, induces strong $CD4^+$ T cell immunity against multiple epitopes and provides very promising protection against *Mtb* infection; immunization by the intranasal route confers better protection than by the subcutaneous route. On the other hand, use of PolyI:C and GDQ was detrimental to protection induced by lipopeptides of ESAT-6 by both routes. In addition, HKCC as a novel adjuvant is also able to stimulate appropriate immune responses to provide protective immunity against *Mtb* infections. Further studies are required to determine the role of HKCC in reducing *Mtb* burden in mice. These results demonstrate the potential of a lipopeptide-based multiepitope TB vaccine and suggest a cautious approach in selecting routes of immunization and adjuvants.

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Chapter 3

Harnessing innate immunity to treat mycobacterial infections: Heat-killed *Caulobacter crescentus* as a novel immunomodulator[*]

[*] In this chapter I designed and performed all of the experiments. Dr. R. Kumar (supervisor), Dr. D.Y. Kunimoto (co-supervisor) and Dr. B. Agrawal (collaborator) contributed to the concept for these studies and the data analysis.

3.1 Introduction

The current TB epidemic continues to grow at an alarming rate due to four compounding factors: 1) the ineffectiveness of the current vaccine, BCG; 2) poor compliance associated with lengthy treatments with multiple chemotherapeutic drugs that have serious side effects; 3) the prevalence of TB-HIV co-infections and 4) the rapid emergence of multi/extensively/totally drug resistant strains of TB bacilli. These four factors have made the control of tuberculosis disease highly challenging. Extensive efforts to discover a new and/or improved TB vaccine have not succeeded yet [1-7]. Consequently, there is a need to investigate novel immunotherapeutic approaches to combat emerging TB pandemic and improve treatment outcomes.

Antigen-independent immunotherapy that boosts the host innate immune system to fight disease could be one of the potential approaches for the treatment and/or cure of chronic mycobacterial infections [8]. Although the precise contributions and fine-regulation of different innate immune mechanisms needed to fight against TB remain undefined, animal and human studies suggest that regulated activation of immune cells promotes mycobacterial containment. In contrast, uncontrolled activation/inflammation causes active disease with severe organ damage and immune deficiency as observed in reactivation of latent TB disease [9-12]. Several host-directed therapies that target different immune mechanisms including cytokines, corticosteroids, vitamin D3 and thalidomide, have been explored as potential immunotherapies with limited efficacy [13-16].

Along these lines, several live or inactivated microbes have had a beneficial effect in preventing and treating infectious diseases [17-22]. *Lactobacillus* species in live and heat-inactivated forms have been evaluated as an immunotherapeutic and vaccine adjuvant. However, poor induction of antibody and T-cell response has limited their use [23-30]. Environmental non-

tuberculous mycobacterial species have also been investigated alone and in combination with chemotherapeutics for treatment of cancers and *Mtb* infections [31- 33]. Live *Mycobacterium marinum* and heat-inactivated *Mycobacterium manresensis* were found to be effective immunotherapeutics in *in vitro* and *in vivo* models, respectively. Although environmental mycobacterial species do not lead to serious infections and disease, they are known to cause skin lesions, induce severe systemic immune responses and/or compromise the host's ability to respond effectively to other pathogens [34, 35]. Therefore, careful study of novel host-directed interventions is needed to offer safer and effective immunotherapeutic strategies to induce clinically relevant immune response against *Mtb*.

In recent years, research on non-pathogenic bacteria-based stimulation and regulation of innate immune mechanisms has opened new approaches for novel immunotherapeutic interventions. Many of the microbes studied activate innate immunological sensors, which result in aberrant production of inflammatory or anti-inflammatory cytokines that lead to either auto-inflammation or recurrent infections [36, 37]. As these results suggest, careful selection and identification of such agents are essential.

Caulobacter crescentus (*Cc*) is a non-pathogenic fresh water aquatic bacterium that is not known to cause any infection or disease in mammals. *Cc* has been comprehensively studied due to its dimorphic life cycle and high expression of the S-layer subunit protein RsaA [38, 39]. Recombinant *Cc* has been used as an expression vehicle because the abundant S-layer can be genetically modified to produce target molecules, antigens or antibodies [40-44-]. Live *Cc* has been shown to provide antitumor responses in mouse models, although the basis of this effect is not known [45]. But the heat-killed *Caulobacter crescentus* (HKCC) have not been investigated for immunotherapeutic intervention. Intriguingly, HKCC significantly enhanced protection from *Mtb* when combined with ESAT-6 antigen of *Mtb* (Chapter 2). While executing vaccine

experiments, I noted that administering mice with HKCC alone, in the absence of ESAT-6 lipopeptides, also reduced *Mtb* loads in mice.

In the present study, I have explored the role of HKCC in stimulating and modulating human innate immunity and ensuing adaptive immunity *in vitro*. Further, I have examined the effect of HKCC in controlling mycobacterial growth using intracellular macrophage and mouse models of mycobacterial infection.

3.2 Materials and Methods

3.2.1 Animals

All animal experimental protocols used in this study were approved by the University Animal Care and Use Committee (ACUC) for Health Sciences, and conducted in accordance with the guidelines of the Canadian Council of Animal Care (CCAC). Five- to six-week-old female BALB/c mice were purchased from Charles River Laboratories and housed in BSL 2/3 animal facility (HSLAS) at the University of Alberta.

3.2.2 Heat-killed *Caulobacter crescentus* (HKCC)

Caulobacter crescentus (*Cc*), containing plasmid vector p4A723/cmyc possessing a chloramphenicol resistance marker, was grown in PYE medium supplemented with chloramphenicol (2 μg/ml) at 25^{o}C. Logarithmically growing cultures were centrifuged at 6000 rpm for 15 min and the bacterial CFU was determined by measuring OD and confirmed by plating serially diluted bacterial suspensions on PYE agar. *Cc* was treated at 80^{o}C for 60 min to prepare heat-killed *Caulobacter crescentus* (HKCC), resuspended in saline and stored at 4^{o}C, prior its use.

3.2.3 *In vitro* PBMCs Stimulation

Peripheral blood samples were obtained from normal healthy donors 30–60 years old of both sexes after informed consent. Use of human blood samples was approved by the institutional Health Research Ethics Board at the University of Alberta. Human peripheral blood nuclear cells (PBMCs) were isolated from healthy donors blood by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation. Heparin-treated blood (20 ml) was layered carefully over Ficoll-Paque density gradient media (20 ml) without intermixing and was centrifuged at 2000 rpm for 30 min. After centrifugation, the intermediate buffy layer containing PBMCs was collected. For lymphocyte activation and cytokine production, 4×10^6 cells/ml were incubated in AIM-V medium in 24-well plates for 24 h and 96 h in triplicate, with HKCC (1E7) or other immunostimulators: LPS (a TLR-4 agonist, 1 μg/ml), Poly:IC (a TLR-3 agonist, 1 μg/ml), resiquimod* (a TLR-7/8 agonist, 1 μg/ml), or CpG (a TLR-9 agonist, 3 μg/ml). In all experiments saline and PHA (1 μg/ml) were used as negative and positive controls, respectively. At day 1 day and day 5, cells were stained for flow cytometry and supernatants were collected for cytokine analysis.

3.2.4 *In vitro* Differentiation of Monocyte Derived DCs from PBMCs

Briefly, PBMCs were isolated from peripheral blood by Ficoll-Paque and re-suspended at 5 x 10^6 cells/ml in RPMI 1640 (GIBCO), supplemented with L-glutamine, 1% human AB serum (Sigma), 1% sodium pyruvate (GIBCO) and 500 U/ml penicillin-streptomycin (GIBCO). The PBMCs were plated in 6-well plates (5 ml/well) and incubated at 37ºC (5% CO_2) for 2 h for adherence. After 2 h incubation, the non-adherent cells were removed and fresh RPMI media containing recombinant human GM-CSF (50 ng/ml) and recombinant human IL-4 (10 ng/ml)

* Resiquimod was used due to the unavailability of gardiquimod.

(Peprotech Canada Inc., Ottawa, ON, Canada), was added to the adherent cells and incubated for 5-6 days. On day 6, ~95% of the obtained cells were CD11c positive, suggesting the differentiation of myeloid DCs. DCs (2 x 10^5 cells/ml) were cultured with HKCC (1E6, 1E7 and 5E7), poly I:C (1 μg/ml), or medium for 24 h. On day 7, supernatant was collected from each well for cytokine analysis and DCs were harvested for staining for flow cytometry.

3.2.5 Allogeneic T cell Proliferation

DCs were prepared from human blood monocytes as described in section 3.2.4. Human T cells were purified using nylon wool columns from another donor (different HLA type, allogeneic). For allogeneic T cell proliferation, DCs pre-incubated with HKCC or LPS for 24 h were re-suspended in AIM-V medium followed by the addition of 10,000 and 20,000 DCs to 2 × 10^5 allogeneic T cells in triplicate in 96-well flat bottom plates. Control wells contained only media, only DCs, only allogeneic T cells, DCs plus allogeneic T cells or allogeneic T cells plus PHA (1 μg/ml). After 4 days of incubation at 37^{o}C, the wells were pulsed with 0.5 μCi/well [^{3}H]-thymidine (Amersham) for 18 h and harvested on filter papers (Perkin Elmer). The levels of [^{3}H]-thymidine incorporated into the DNA of proliferating cells were counted in a Microbeta Trilux liquid scintillation counter (Perkin Elmer). Proliferation is represented as the mean cpm ± SD (standard deviation) of triplicate cultures.

3.2.6 T cell and B cell Proliferation

Non-adherent T cells were purified using nylon wool columns according to previously reported procedures [46]. 2 × 10^5 T cells were cultured in triplicates in 96-well plates with HKCC (1E6, 1E7 and 5E7) in AIM-V media. Controls included T cells alone or T cells incubated with 1 μg/ml PHA. After 4 days of incubation at 37^{o}C, the wells were pulsed with 0.5 μCi per well [^{3}H] thymidine for 18 h, harvested and analyzed for ^{3}H-thymidine uptake. For B cell proliferation

assays, PBMCs were labeled with CFSE dye and cultured with HKCC (1E6, 1E7 and 5E7) or CpG (3 μg/ml) for 4 days and then stained for extracellular markers (CD3, CD4, CD8 and CD19), followed by examining dilution of CFSE in $CD19^+$ B cells.

3.2.7 Cytokine ELISA

The culture supernatants were tested for cytokines IL-2, IL-10, IL-17A, IL-22, IL-12, IL-6, IFN-γ, TNF-α, and granulocyte-macrophage colony stimulating factor (GM-CSF) using R & D Systems (Minneapolis, Minnesota) or eBioscience ELISA kits as per manufacturer's protocol. Breifly, for the detection and quantification of cytokines secreted from cells, 96-well ELISA plates (Corning Costar 9018, eBioscience) were coated with 100 μl/well of capture antibodies for the cytokine of interest diluted in coating buffer at a concentration of 1-4 μg/ml. Plates were sealed and incubated overnight at 4°C. The next day, after blocking with 1x diluent buffer (eBioscience) at room temperature for 1 h, a 2-fold serial dilution of recombinant standard and 100 μl/well of samples were added to the 96-well plate in 3 replicates and incubated again at room temperature for 2 h. A dilution of 1:2 to 1:50 was used for samples, with standards ranging from 5 to 2000 pg/ml. After application of samples, 100 μl/well of biotinylated detection antibody in 1 x diluent buffer was added and plates were incubated for 1 h. After incubation, 100 μl/well of avidin-horseradish peroxidase (Avidin-HRP) was added and plates were incubated at room temperature for 30 min. After washing, 100 μl/well of 1 x TMB solution was added and plates were incubated at room temperature for 15 min. Finally, the enzyme reaction was stopped after adding 50 μl/well of stop solution (2 N H_2SO_4) to each well. Plates were washed with wash buffer (1 x PBS with 0.05% Tween-20) after each incubation step. The ELISA plates were read with an automated ELISA plate reader (Fluostar Optima, BMG Labtech GmbH, Ortenberg,

Germany). Data are represented as the mean ± SD of concentrations for each cytokine perfomed in triplicate wells.

3.2.8 Flow Cytometry Analysis of Surface and Intracellular Markers

A total of 1×10^6 cells from spleen and bronchoalveolar lavage (BAL) from treated mice (see below) was taken for extracellular staining with multicolor fluorescently labeled mAbs (concentrations according to manufacturer's instructions). The cells were incubated with Fc mouse-serum (Sigma) to prevent non-specific binding and washed with fluorescence-activated cell sorter (FACS)-buffer [2% fetal bovine serum in 1 × phosphate-buffered saline (PBS)]. The cells were then incubated for 30 minutes at 4°C with anti-mouse CD3e-FITC, CD4-PECy-5, CD4-APC, CD25-PE-Cy7, CD8a-APC-Cy7, CD69-PECy-5, anti-CD49b-Alexafluor-700, anti-CD11c-FITC, anti-F4/80-efluor 450, anti-CD11b-Alexafluor-700, anti-CD40 PE, anti-CD86-APC, anti-MHC-II-PECy-5 (eBioscience) to detect extracellular markers. The cells were then washed twice with FACS buffer and analyzed using a LSR Fortessa SORP flow cytometer. Data analysis was conducted using FACS-DIVA software (Becton Dickinson, Mountain View, CA). Each marker was gated based on its respective isotype-matched control monoclonal antibody. Similar staining methodology was used with human PBMCs to determine various cells and activation markers anti-human CD11c-efluor 450, CD123-FITC, CD19-APC, CD40-Percp-efluor 710, CD80-PE, CD86-PEcy7, DEC-205-FITC, CD3-e-fluor 450, CD4-APC, CD8-PE, CD56-PE Texas Red, CD25-PECy-7, CD69-PerCP-efluor 710, CD11b-PECy-7, IL-17-PE, TNF-α-FITC, IL-10-PECy-7 and IL-22-APC (eBioscience) in flow cytometry experiments. Ms. Dorothy Kratochwii-Otto provided assistance in managing and troubleshooting flow cytometry instruments.

3.2.9 Mycobacterial infection of THP-1 cells and Treatment

THP-1 cells (human monocyte cell line) were grown in complete DMEM medium. Cells (1×10^6 cells/ml) were cultured in medium containing 50 ng/ml of PMA in 24-well plates for 24 h to allow differentiation into macrophages. Next day, cells were infected with *M. tuberculosis* (*Mtb*) H37Ra or *M. avium* (5×10^7) CFU/well for 4 h at 37°C and then washed with medium to remove extracellular bacilli. Cells were treated twice at 4 days intervals with supernatants collected from human PBMCs upon 24 h stimulation with HKCC, Poly I:C, LPS, resiquimod CpG, or with control drugs (rifampicin and clarithromycin). Five days after the second treatment, cells were lysed and plated on 7H11 agar plates for CFU determination.

3.2.10 *In vivo* Cytokine Induction

Groups of five C57BL/6 male mice were administered with saline or HKCC (50×10^6/mouse) orally (200 µl/mouse), or intranasally (30 µl/mouse). Five hours later, mice were euthanized using a CO_2 chamber and lung washes were collected.

3.2.11 Mycobacterial Challenge and Treatment in Mice

Female BALB/c mice were infected with *Mtb* H37Ra (0.5×10^6 CFU/mouse) intravenously. After 5 days, mice were treated intranasally or orally once weekly for four weeks with HKCC. Five days after the last treatment, mice were euthanized using a CO_2 chamber. Lungs, liver and spleen were collected from each individual mouse and were homogenized in 5 ml saline. A 100 µl aliquot was taken from each organ homogenate of each individual mouse and was plated on 7H11 Middlebrook agar plates (BD Biosciences). The plates were incubated at 37°C for 3-4 weeks, then the number of colonies were counted. The bacterial counts obtained were multiplied by the dilution factor to represent the total CFUs from the whole organ. Lungs washes, bronchoalveolar lavage (BAL) and spleens were also collected to examine cytokine

stimulation and activation of immune cells. Dr. Satish Vedi provided expertise by collecting and processing BAL from mice. Mr. Saurabh Garg assisted in setting up the CFU assays.

For combination studies, mice were treated subcutaneously with HKCC and sequentially with oral INH according to the schedule (**Fig. 14**). Three days after the last treatment, mice were euthanized using a CO_2 chamber and bacterial loads were determined in lungs, liver and spleen as described above.

3.2.12 Statistical Analysis

Data were analyzed by GraphPad Prism 6 software (GraphPad Software Inc., CA, USA). Data were presented as mean ± SD. Significant differences between two groups were determined using the Student's t-test. The difference among the means of multiple treatment groups on immune reponses or bacterial loads were compared by two-way ANOVA followed by Tukey's multiple comparison test. A p-value less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3.3 Results

3.3.1 HKCC induces a distinct pattern of cytokine production from human PBMCs

First, I examined the effect of HKCC on cytokine production from human PBMCs *in vitro*. Freshly isolated PBMCs from normal healthy human donors were incubated with HKCC at 1×10^7 and 5×10^7 CFU/ml for 24 and 96 h, and the amount of IFN-γ, TNF-α, IL-1β, IL-6, IL-12, IL-10, IL-17, IL-22, GM-CSF and TGF-β were measured in culture supernatants. Interestingly, HKCC induced rapid and sustained production of cytokines IFN-γ, TNF-α, IL-6, IL-12, IL-1β, IL-10, IL-17 and IL-22 in a dose-dependent manner with markedly different kinetics upon 24 and 96 h stimulation (**Fig. 3.1** and **3.2**). The production of IFN-γ, TNF-α, IL-6, IL-12, IL-1β, IL-10, IL-17A and IL-22 was significantly increased after 24 h stimulation (**Fig. 3.1**). After 4 days of

culture, the levels of TNF-α, IL-1β and IL-10 significantly declined whereas IFN-γ, IL-6, IL-12, IL-17A and IL-22 increased compared to the levels obtained upon 24 h culture (**Fig. 3.2**). GM-CSF was detected on day 4 only and not on day 1 (**Fig. 3.2**) and also with higher concentration of HKCC. TGF-β levels were only slightly increased over saline control upon stimulation of PBMCs with HKCC for 4 days (**Fig. 3.2**). These results indicate that HKCC can stimulate various innate and/or adaptive immune cells present in PBMCs to produce a diverse range of multifunctional cytokines in a dose-dependent manner.

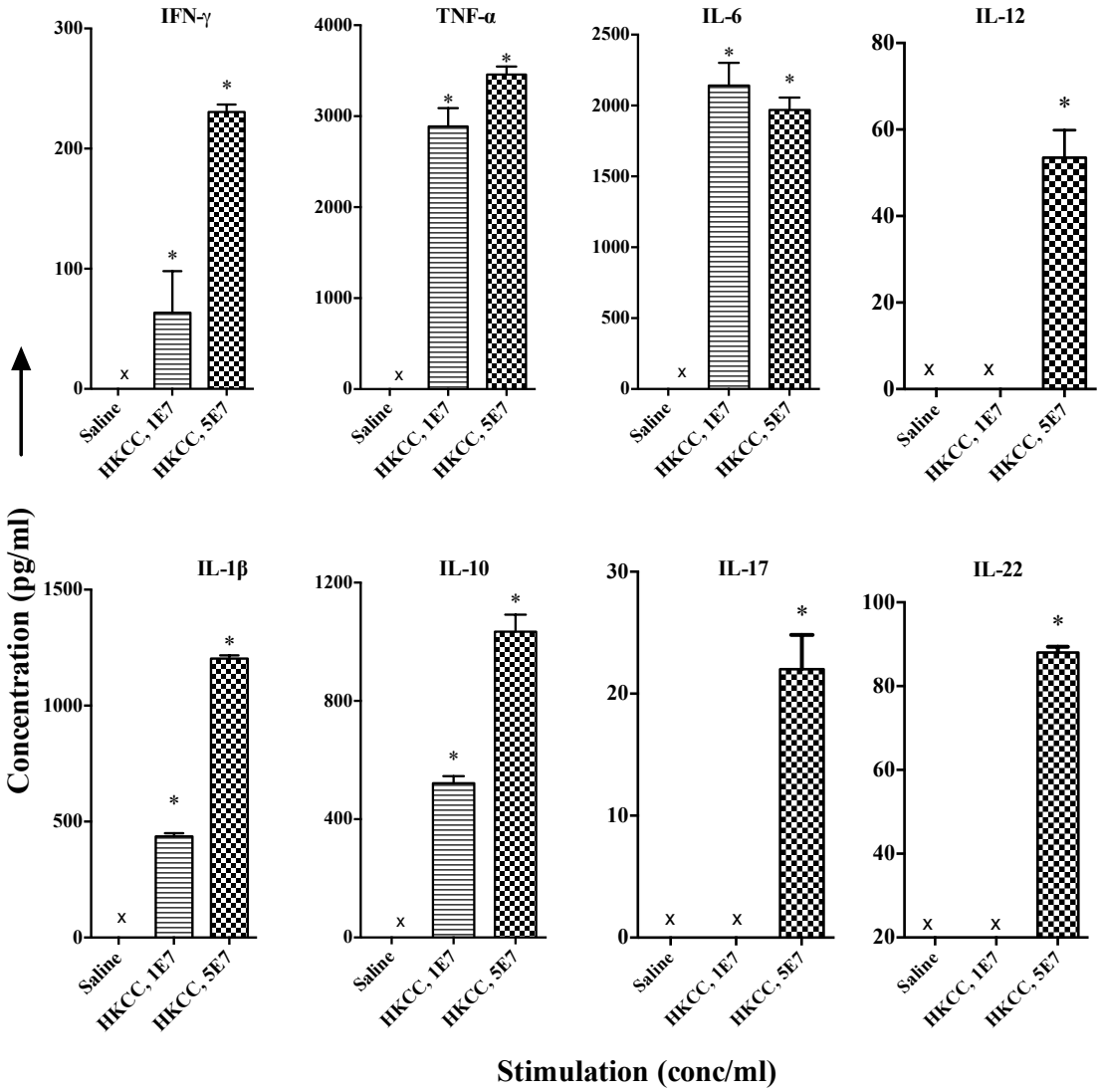


Fig 3.1. HKCC induces cytokine production from human PBMCs. PBMCs (4×10^6/ml/well) were cultured in 24 well plates with AIM V medium in the presence or absence of HKCC at two different concentrations (10×10^6 CFU/ml or 50×10^6 CFU/ml) for 24 h. Supernatants were collected, and analyzed for the presence of IFN-γ, TNF-α, IL-6, IL-10, IL-17A and IL-22 using sandwich ELISA kits. Results are shown as mean ± standard deviations of triplicate values. The experiment was repeated with four individual donors and data from a representative donor are shown.

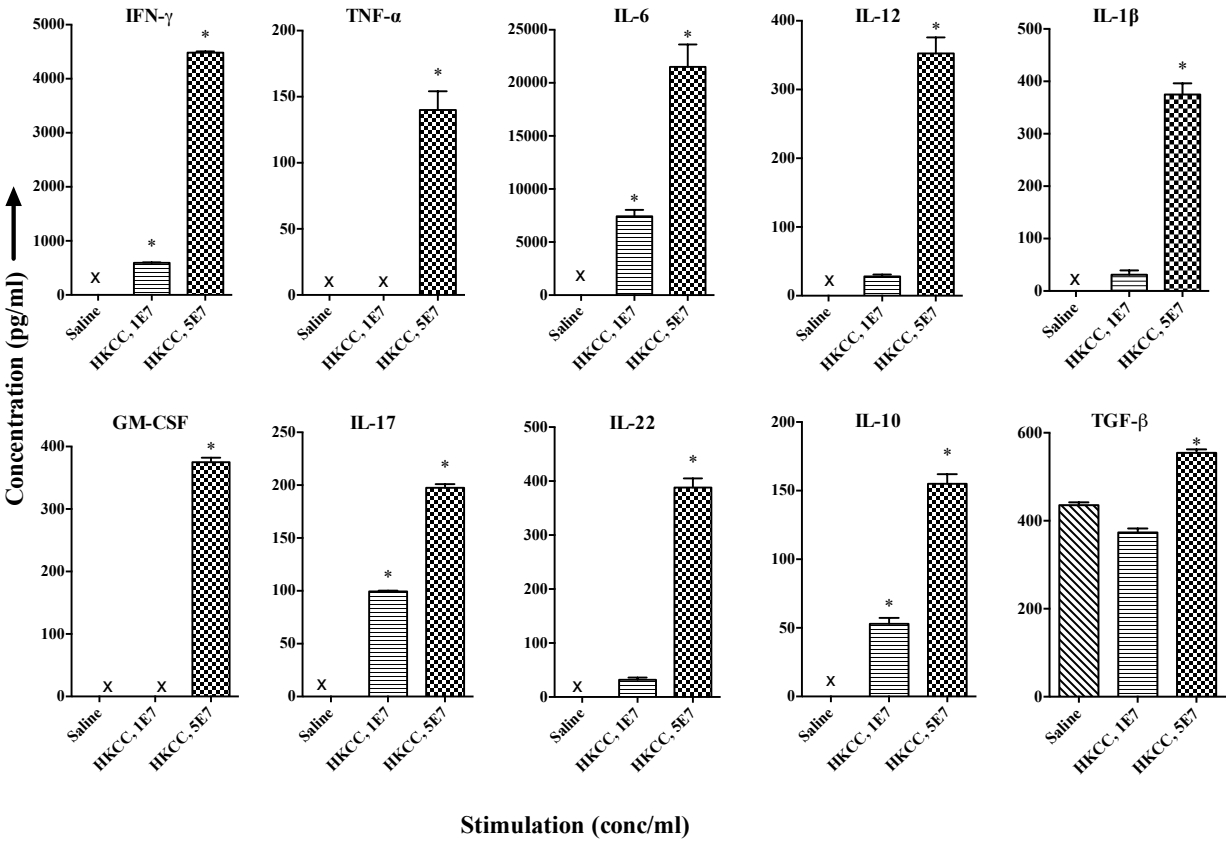


Fig 3.2. HKCC induces sustained cytokine production from human PBMCs. PBMCs (4×10^6/ml/well) were cultured in 24-well plates with AIM V medium with or without HKCC at two different concentrations (10×10^6 CFU/ml or 50×10^6 CFU/ml) for 4 days. Supernatants were collected, and analyzed for the presence of human IFN-γ, TNF-α, IL-6, IL-12, IL-1β, GM-CSF, IL-10, IL-17A, IL-22 and TGF-β, using sandwich ELISA kits. Results are shown as mean ± standard deviations of triplicate values. The experiment was repeated with four individual donors and data from a representative donor are shown. '*' $P < 0.05$, indicate significant differences compared to saline.

3.3.2 HKCC leads to the activation of both myeloid and plasmacytoid dendritic cells in human PBMCs

Next, I studied the effect of HKCC on the maturation of two major subsets of human DCs: myeloid (mDCs) and plasmacytoid (pDCs). Monocyte-derived mDCs were prepared from PBMCs using GM-CSF and IL-4 as described in Material and Methods and cultured with HKCC or Poly I:C for 24 h. DCs maturation was examined by surface markers and/or cytokine production. Stimulation with HKCC up-regulated the expression of co-stimulatory molecules CD40 and CD80 on mDCs. Interestingly, maturation marker DEC-205 was also upregulated on HKCC-exposed mDCs (**Figure 3.3 A**), whereas CD86 was only marginally (not significant) increased (data not shown). Further, a strong dose-dependent increase in the production of cytokines TNF-α, IL-12 and IL-6 and, to a lesser extent, regulatory cytokine IL-10, was induced by HKCC from mDCs (**Fig. 3.3 B**). Stimulation of PBMCs with HKCC also led to increases in the percentage and activation (CD40 and CD86) of pDCs (**Fig. 3.4 A** and **3.4 B**).

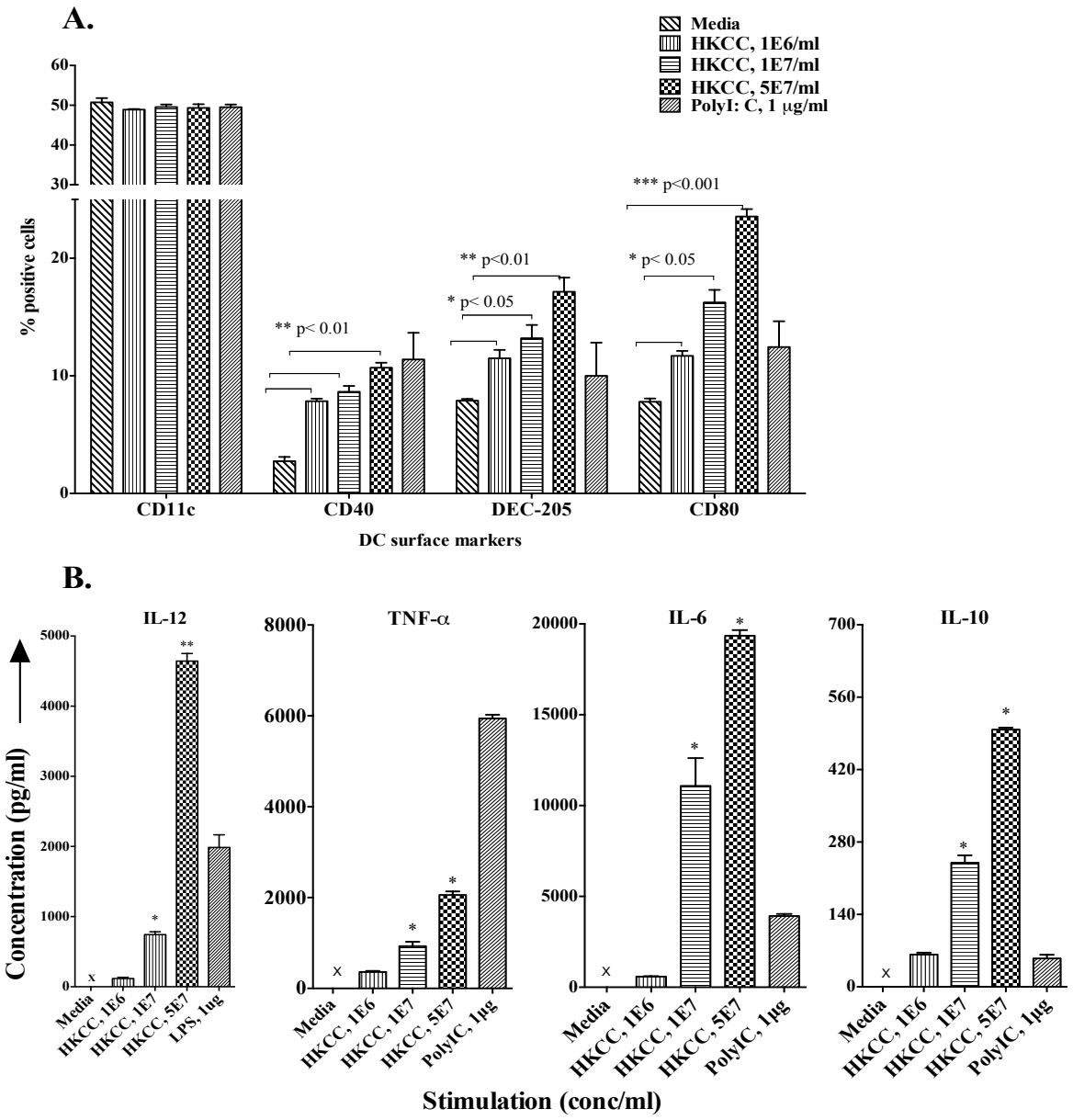


Fig 3.3. Treatment of monocyte-derived DC (mDCs) with HKCC leads to their maturation and cytokine induction. Immature monocyte derived DCs were cultured with HKCC or Poly I:C for 24 h. **(A)** Expression of CD11c, CD40, DEC-205 and CD80 was analyzed by flow cytometry. The cells were gated on the basis of side and forward scatter and then selected for CD11c-positive cells, which were analyzed for the expression of CD40, DEC-205 and CD80. **(B)** Cytokine profile induced upon incubation of mDCs with HKCC, LPS or poly I:C for 24 h. The concentration of IL-12, TNF-α, IL-6 and IL-10 was determined using sandwich ELISA kits. Results are shown as mean $\pm$ standard deviation of triplicate values. All experiments were performed with four different donors and representative data from a representative donor are shown. '*' $P < 0.05$, '**' $P < 0.01$ and '***' $P < 0.001$ indicate significant differences compared to controls of medium alone.

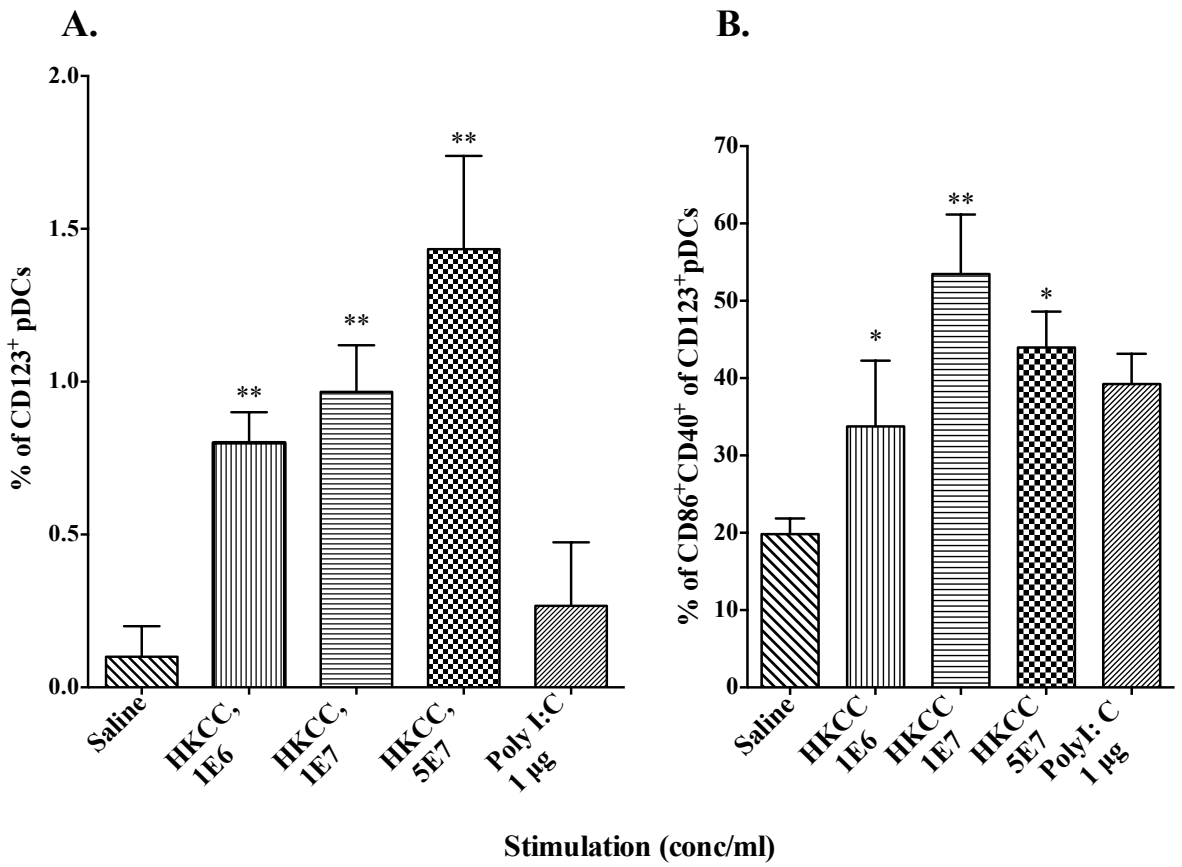


Fig 3.4. HKCC leads to activation of plasmacytoid DCs (pDCs). PBMCs (4×10^6/ml/well) were cultured with HKCC or Poly I:C for 24 h. **(A)** The percentage of $CD123^+$ pDCs was determined by gating for $CD11c^-$, $CD11b^-$, $CD19^-$ and $CD123^+$ cells. **(B)** Up-regulation of CD86 and CD40 are shown on $CD123^+$ pDCs. Results represent mean $\pm$ standard deviation of triplicate values. The experiment was performed with four different donors and a representative is shown. '*' $P < 0.05$ and '**' $P < 0.01$ indicate significant differences compared to saline controls.

3.3.3 HKCC augments allogeneic T cell proliferation through activation of mDCs but does not lead to direct proliferation and activation of human $CD4^+$ and $CD8^+$ T cells

T cell stimulation is a hallmark function of mDCs. T cells from MHC-unmatched donors recognize DCs from another donor as an allogeneic response. Allogeneic T cell response is strong and is influenced by stimulation provided by DCs. To examine the ability of HKCC to stimulate DCs and induce an adaptive immune response, I determined whether HKCC-treatment of mDCs

led to enhanced allogeneic T cell proliferation. I co-cultured mDCs pre-stimulated with different concentrations of HKCC with allogeneic T cells and measured their proliferation. Notably, allo responses were significantly enhanced by HKCC (**Fig. 3.5**).

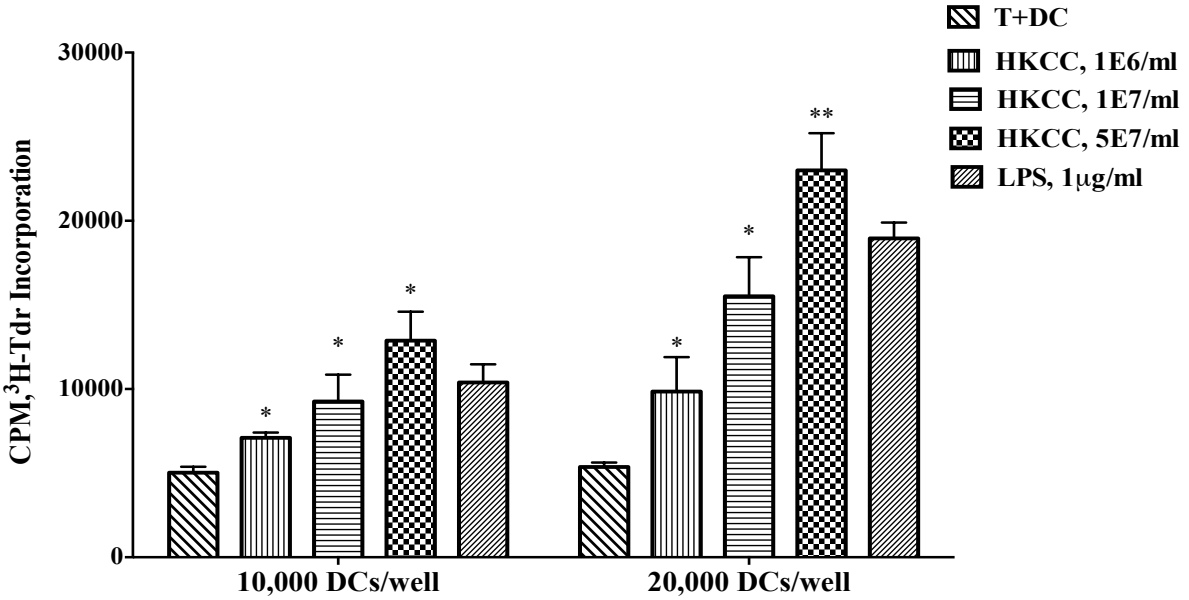
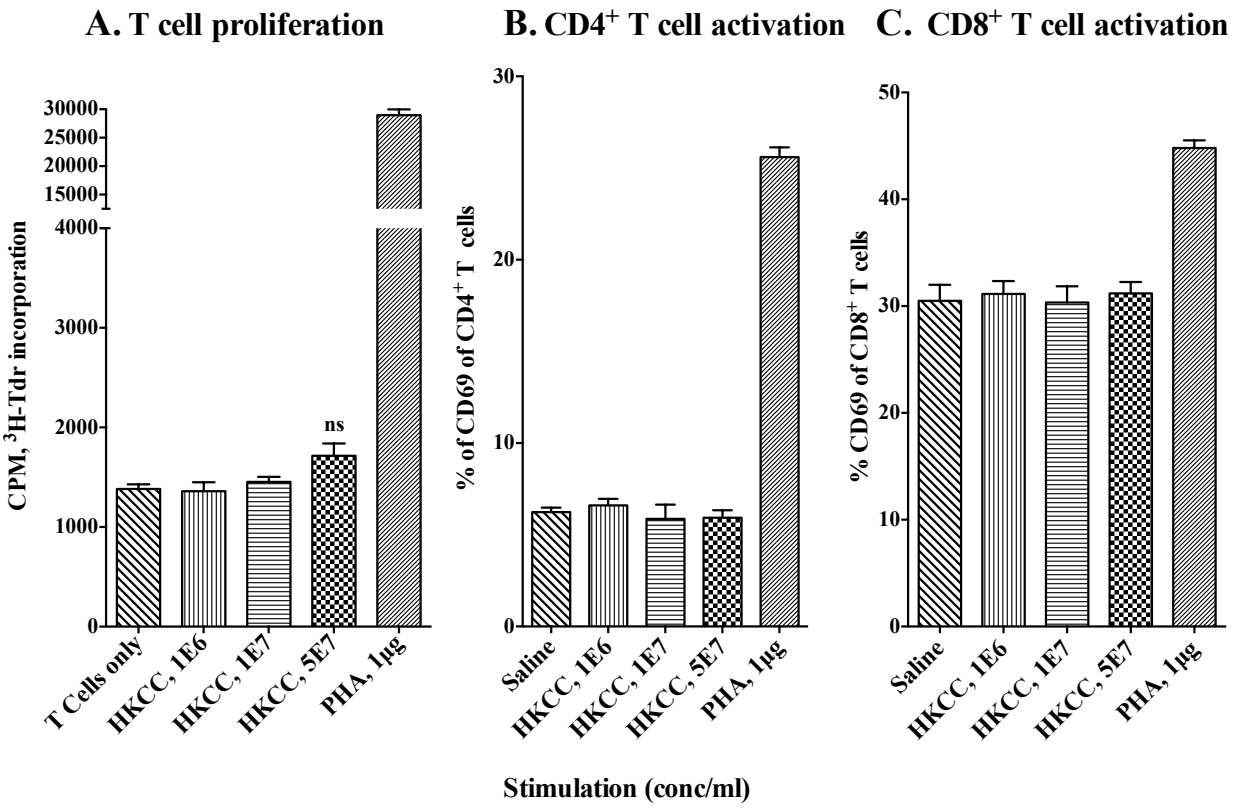


Fig 3.5. mDCs stimulated with HKCC augment allogeneic T cell proliferation. mDCs were plated in 96 well plates at 10,000 cells/well or 20,000 cells/well, and stimulated with HKCC, medium or LPS, followed by addition of 2×10^5/well allogeneic T cells. Plates were incubated for 4 days and proliferation was examined by ^{3}H-thymidine incorporation assay. Mean ± standard deviation of CPM from triplicate wells are shown. The data represent three repeated experiments with three different donors. '*' $P < 0.05$ and '**' $P < 0.01$ indicate significant differences compared to saline control.

To determine whether HKCC directly leads to proliferation and activation of T cells, non-adherent cells isolated from PBMCs were cultured with HKCC at different concentrations for four days, followed by examining proliferation and expression of early activation marker CD69. Interestingly, HKCC did not directly induce proliferation and activation of $CD4^+$ and $CD8^+$ T cells, unlike mitogen PHA (**Fig. 3.6**).



3.6. HKCC does not directly induce proliferation and activation of T cells. (A) Proliferation of T cells. Non-adherent T cells isolated from human PBMCs were plated (2×10^5/well) with or without HKCC or PHA at different concentrations in 96-flat bottom plates. Cells were incubated for 4 days and proliferation was examined by ^{3}H-thymidine incorporation assay. Mean ± standard deviation of CPM from triplicate wells are shown. Parallel cultures were set up for flow cytometry and cells obtained after 4 day culture were stained with CD3, CD4, CD8, and CD69 surface markers. Data are shown as the percentage of CD69$^+$ of CD4$^+$ **(B)** and CD8$^+$**(C)** T cells. The data represent four repeated experiments.

3.3.4 HKCC does not induce proliferation and activation of B cells but leads to enhanced expression of molecules associated with T cell co-stimulation and cognate T-B cell interaction

To determine the effect of HKCC on B cell activation, proliferation and expression of co-stimulatory molecules, CFSE-labeled PBMCs were cultured with or without HKCC for 4 days.

CpG was used as positive control. I found that HKCC did not induce proliferation of $CD19^+$ B cells from PBMCs and the activation molecule CD69 was not significantly upregulated whereas co-stimulatory molecules CD40 and CD86 were significantly upregulated on B cells (**Fig. 3.7**). In contrast, CpG led to increased proliferation and expression of all CD69, CD86 and CD40 molecules on B cells (**Fig. 3.7**). I also noted that HKCC did not affect the viability of B cells or induce apoptosis of B cells (**Appendix 5**).

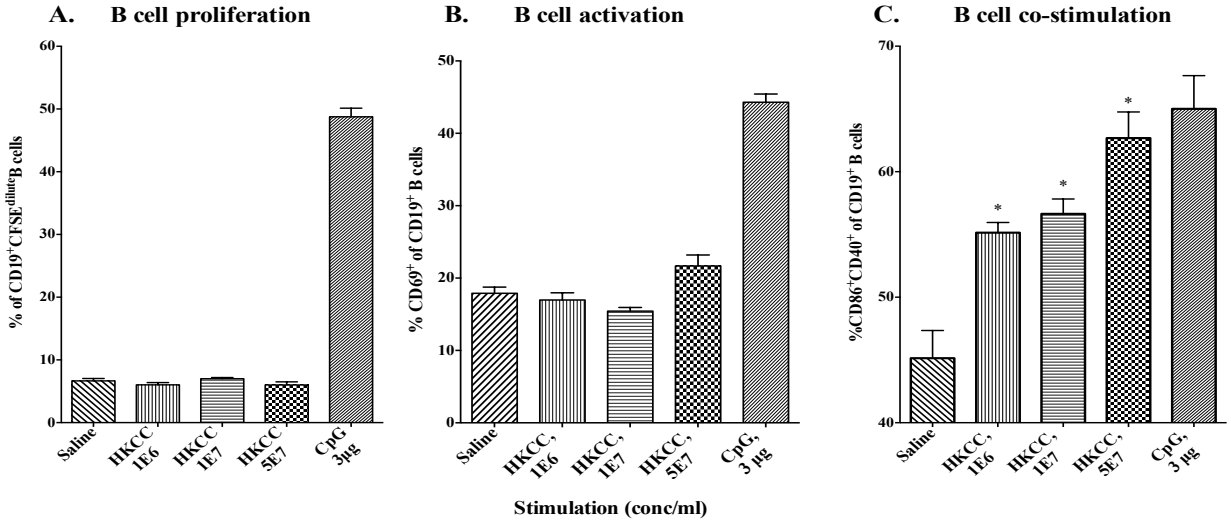


Fig 3.7. HKCC activates B cells but does not lead to their proliferation. PBMCs (2×10^6/well) were labeled with CFSE dye and treated with or without HKCC or CpG at different concentrations in 24-well plates. Cells were incubated for 4 days and labeled for CD3, CD11b and CD19 surface markers. The cells were gated for $CD3^-CD11b^-CD19^+$ cells and proliferation was examined by the dilution of CFSE dye. The percentage of CFSE diluted $CD19^+$ B cells is shown **(A)**. Unlabeled PBMCs were cultured with or without HKCC or CpG in 24-well plates and incubated for 4 days, and stained for CD3, CD11b, CD19, CD86, CD40 and CD69 markers. Data are shown as the percentage of $CD69^+$ **(B)** and $CD86^+CD40^+$ of $CD19^+$ B cells **(C).** Data represent mean $\pm$ SD of three replicates. '*' $P < 0.05$ indicates significant differences compared to saline control.

3.3.5 HKCC activates NK and NKT cells

The pattern of cytokines induced by HKCC upon co-culture with PBMCs (**Fig. 3.1 and 3.2**) pointed towards stimulation of innate lymphocytes. Therefore, I investigated whether HKCC activates NK and NKT cells. PBMCs were cultured with or without HKCC or PHA for 1 or 5 days. Activation was assessed by the expression of activation marker CD69 on $CD3^{-}CD56^{+}$ NK cells and $CD3^{+}CD56^{+}$ NKT cells after 24 h stimulation. I observed that HKCC significantly up-regulated the expression of CD69 on both NK and NKT cells after 24 h incubation (**Fig 3.8 A, B**). After five days of culture, HKCC substantially increased the percentage of NK and NKT cells in the culture **(Fig. 3.8 C, D**). This increase in percentage of NK/NKT cells does not appear to be due to increased proliferation, as there was only marginal (not significant) CFSE dilution in NK/NKT cell population after 5 days of stimulation with HKCC. It is possible that HKCC is directly or indirectly prolonging their survival. The survival of NK cells has been demonstrated to be prolonged by IL-15-IL-15R signaling [47].

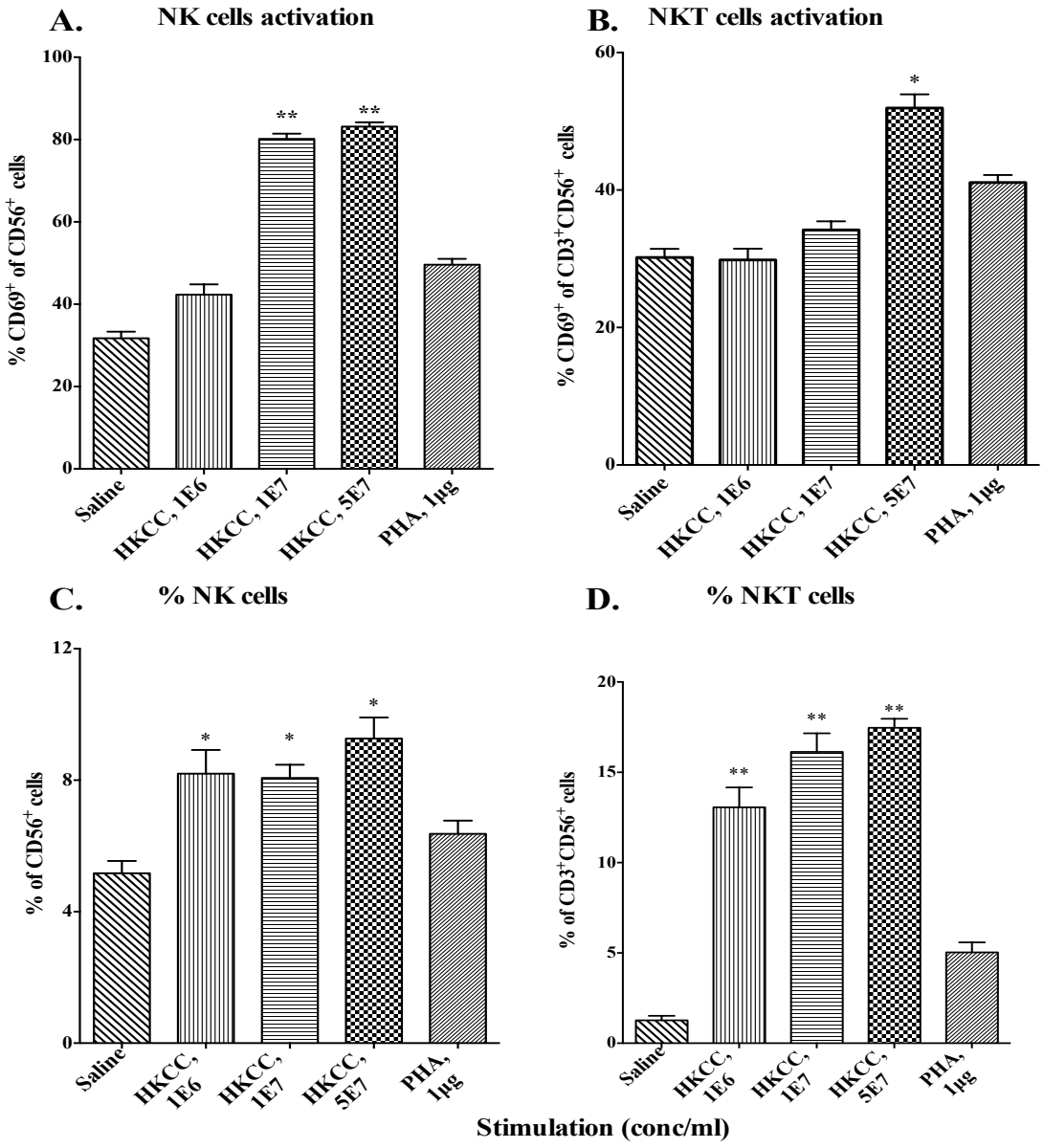


Fig 3.8. HKCC activates human NK and NKT cells and increases their percentage. PBMCs (2×10^6/well) were cultured with or without HKCC or PHA at different concentrations in 24-well plates. Cells were incubated for 1 or 5 days and stained for CD3, CD56 and CD69 surface markers. The cells were gated for $CD3^-CD56^+$ and $CD3^+CD56^+$, which were subsequently analyzed for CD69 expression. Data are shown as the percentage of $CD69^+CD3^-CD56^+$ NK cells (A), $CD69^+CD3^+CD56^+$ NKT cells (B) after 24 h of culture, $CD3^-CD56^+$ NK (C) and $CD3^+CD56^+$ NKT (D) cells after 5 days. Data represent mean $\pm$ SD of triplicates. '*' $P < 0.05$ and '**' $P < 0.01$ indicate significant differences compared to saline control.

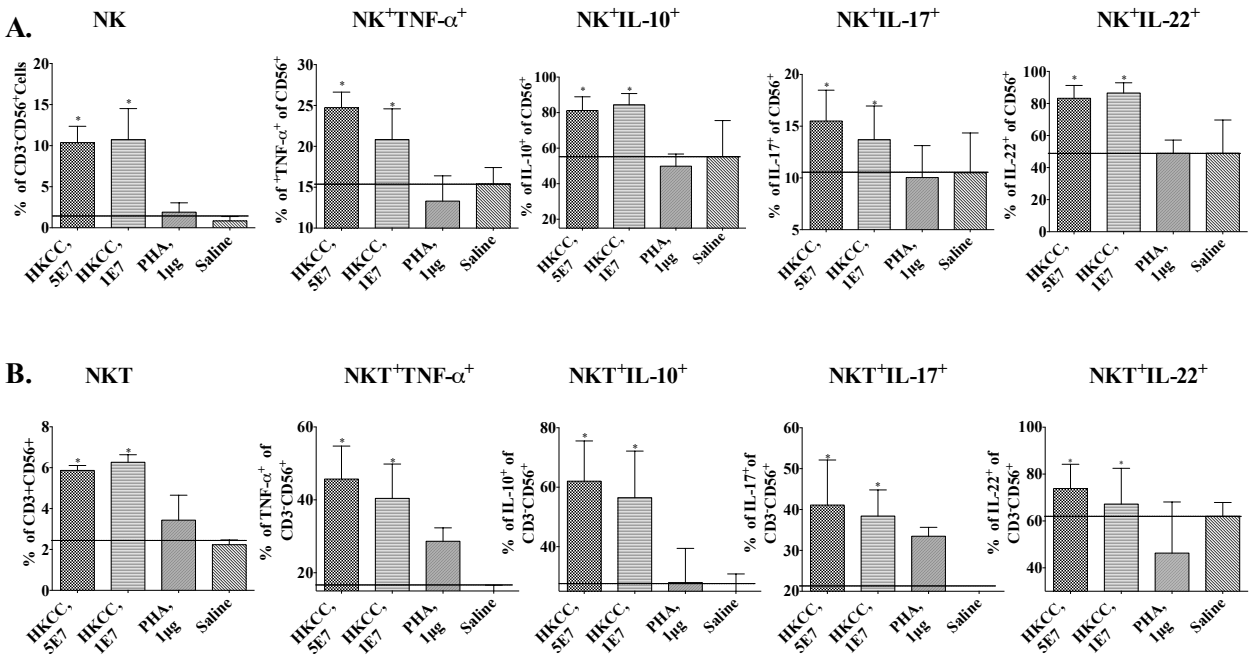


Fig 3.9. HKCC activates human NK and NKT cells to produce differential cytokines. PBMCs (4×10^6/well) were cultured with or without HKCC or PHA at different concentrations in 24-well plates. Cells were incubated for 4 days and stained for CD3 and CD56 surface markers along with intracellular cytokines. The cells were gated for $CD3^-CD56^+$ and $CD3^+CD56^+$, which were subsequently, analyzed for TNF-α, IL-10, IL-17 and IL-22 expression. Data are shown as the percentage of TNF-α^+, IL-10^+, IL-17^+ and IL-22^+ of CD3-$CD56^+$ NK cells and $CD3^+CD56^+$ NKT cells. **(A)** Activation of NK cells. **(B)** Activation of NKT cells. Data represent mean ± SD of three donors. '*' $P < 0.05$ indicate significant differences compared to saline control.

To determine the functional activity of HKCC-induced NK and NKT cells, PBMCs cultured with HKCC for 4 days were examined for intracellular TNF-α, IL-10, IL-17A and IL-22 production by flow cytometry. Intriguingly, HKCC-activated NK and NKT cells showed significantly increased expression of TNF-α, IL-10, IL-17A and IL-22 (**Fig. 3.9**). I also examined IFN-γ production. Interestingly, there was a significant increase in IFN-γ production from both NK and NKT cells after 24 h whereas at day 4 there was no significant difference compared to saline group.

3.3.6 Mucosal administration of HKCC induces cytokine production in mice

In order to investigate whether HKCC can induce cytokine production *in vivo*, C57BL/6 mice were administered with HKCC intranasally or orally. C57BL/6 mice were used in this experiment to examine the effect of HKCC in different haplotype ($H\text{-}2^b$) to determine its broad applicability across MHC-diverse populations. Mucosal routes were chosen instead of subcutaneous because DCs are more prominent at mucosal surfaces and more efficiently sense external stimuli or bacterial components. In addition, the efficient transcytosis of HKCC across the epithelium into the mucosal-associated lymphoid tissue may occur by microfold 'M' cells, a unique population of epithelial cells. Five hours after administration, cytokines were determined in lung washes. HKCC induced rapid production of TNF-α, IL-6, IL-12 and IL-1β in mice with both intranasal and oral routes (**Fig. 3.10**). Thus, HKCC has the ability to induce cytokine production *in vivo* after mucosal administration.

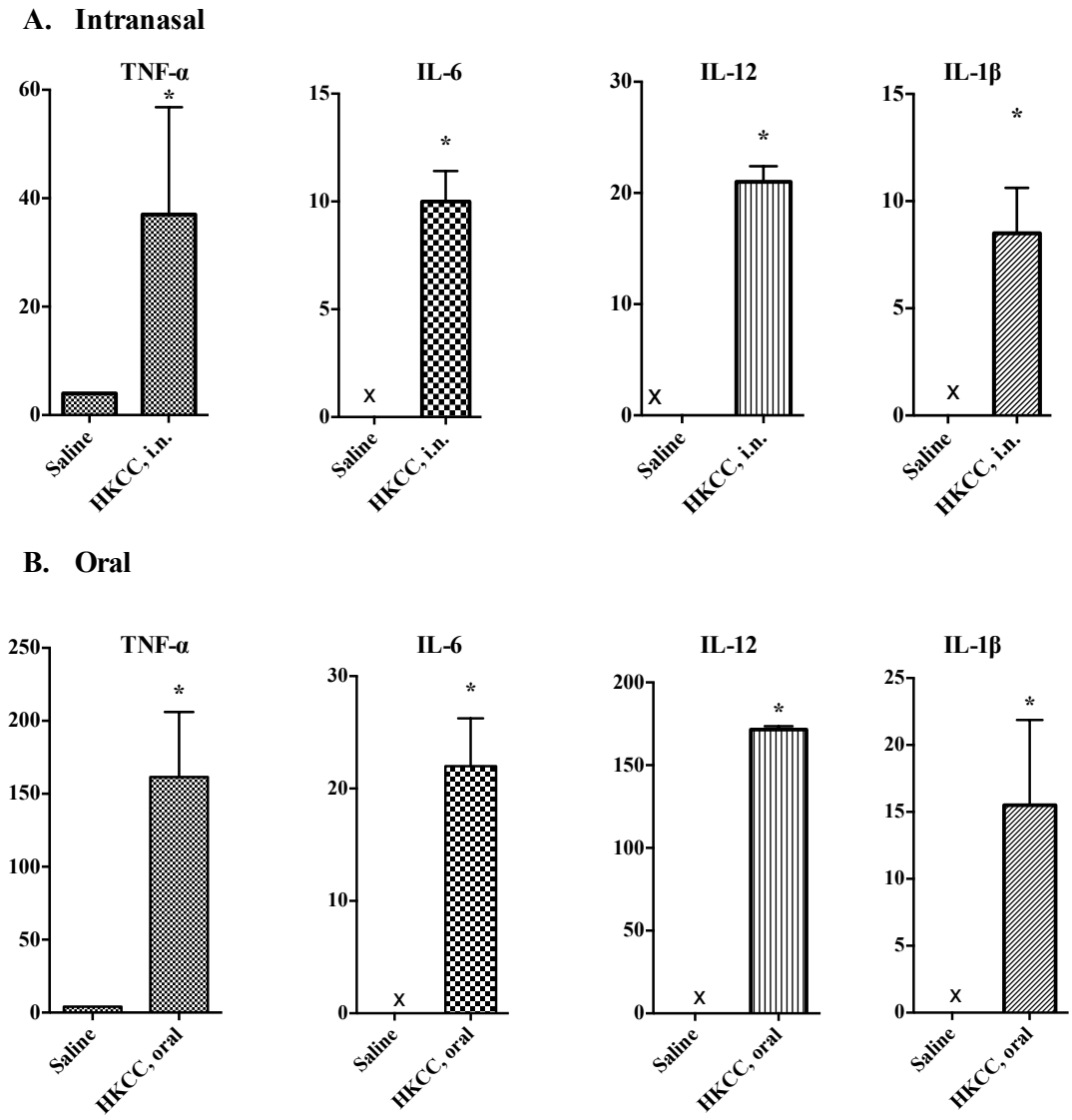


Fig 3.10. HKCC induces cytokines production after mucosal administration in mice. Groups of five C57BL/6 male mice were administered with saline or HKCC (50 x 10^6/mouse) orally (200 µl/mouse), or intranasally (30 µl/mouse). Five hours after administration, mice were euthanized and lung washes (1 ml/mouse) were collected (pooled for a group) and used to determine the presence of cytokines TNF-α, IL-6, IL-12 and IL-1β using a sandwich ELISA. Cytokines induced upon **(A)** Intranasal **(B)** Oral administration are shown. Results are shown as mean ± SD of triplicate values.

3.3.7 HKCC inhibits mycobacterial growth in human macrophages via host-mediated mechanisms

Mtb is an intracellular pathogen infecting macrophages, the phagocytic cells of the host that are the first defense against an invading pathogen. However, infection with *Mtb* incapacitates the ability of macrophages to destroy the bacteria they engulf [48]. There is ample scientific evidence to support immune-mediated clearance of *Mtb* infection [49, 50]. Therefore, I sought to determine whether supernatants obtained from PBMCs stimulated with HKCC would inhibit mycobacterial growth within macrophages. I infected THP-1 macrophages with *Mtb* (H37Ra) or *M. avium*, and treated then with supernatants from PBMC cultures stimulated with HKCC or other known immunostimulators (TLR agonists). Rifampicin and clarithromycin were used as positive controls for H37Ra and *M. avium*, respectively. Intriguingly, two treatments with supernatants from HKCC-stimulated PBMCs (data from three different donors are shown) led to significant host-mediated inhibition (~ 60-70%) of both *Mtb* and *M. avium* compared to saline treated supernatants (**Fig. 3.11**). In contrast, the addition of HKCC directly to THP-1 cells infected with *Mtb* or *M. avium* did not lead to the inhibition of mycobacterial growth**.** Supernatants from PBMCs stimulated with TLR agonists, PolyI:C, LPS, CpG and resiquimod did not provide significant inhibition of any of the mycobacteria within macrophages (**Fig. 3.11**).

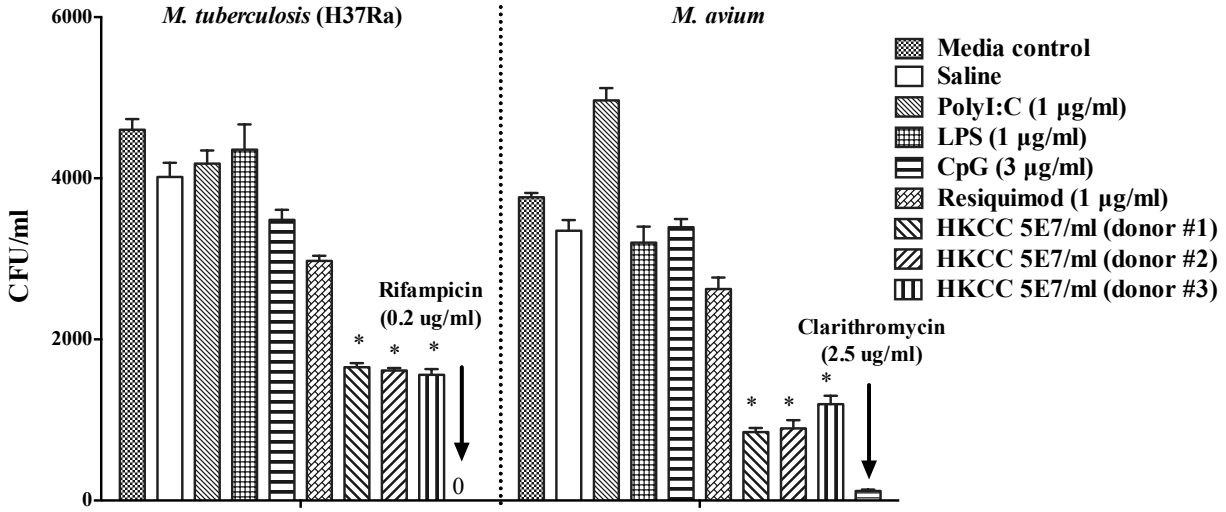


Fig 3.11. HKCC inhibits intracellular *Mtb* and *M. avium* growth via host-mediated mechanism in human THP-1 macrophage cells. Human monocytic cell line (THP-1) was infected with *M. avium* or *Mtb* H37Ra, followed by two treatments on days 0 and 4 with supernatants (50%) collected from human PBMCs treated for 24 h with HKCC, PolyI:C, LPS, CpG, resiquimod or saline in 24-well plates. As controls, clarithromycin and rifampicin were added directly to infected THP-1 cells. Five days after the second treatment, THP-1 cells were collected, lysed and plated on 7H11 agar plates to determine bacterial CFUs. Results are shown from three different donors and represent mean ± SD of triplicate wells.

3.3.8 Treatment with HKCC controls disseminated *Mtb* growth in a mouse model of *Mtb* infection and the protective effect is associated with both local and systemic innate immune stimulation

Next I examined the therapeutic potential of HKCC upon intranasal and oral administrations in *Mtb* infected BALB/c mice. BALB/c mice were used in these studies because earlier immune response and *Mtb* challenge experiments (Chapter 2) were conducted in BALB/c mice. Groups of 5 BALB/c mice challenged intravenously with *Mtb* (H37Ra) were treated once weekly intranasally or orally for a total of four weeks, starting from 3 days after infection. Five days after the last treatment, mice were euthanized and *Mtb* loads were determined in lungs, liver

and spleen of individual mouse by the CFU assay. Encouragingly, only four weekly treatments with HKCC substantially reduced *Mtb* loads in all of the organs compared to the saline-treated group (**Fig. 3.12**).

To investigate whether HKCC led to the modulation of local and systemic immune responses in *Mtb*-infected mice that correlated with its protective efficacy, I determined IFN-γ concentrations in lung washes. HKCC by both routes significantly (*$P < 0.05$) increased IFN-γ levels compared to the no treatment group (**Fig. 3.13 A**). IFN-γ is an effector molecule produced by both innate and adaptive lymphocytes. In my *in vitro* studies with human PBMCs I noted an effect of HKCC in activating innate immune cells, so I decided to also examine the activation of infiltrated NK and NKT cells in BAL. Interestingly, the percentage of activated NK and NKT cells, as determined by CD25 and CD69 expression, were found to be significantly higher in mice treated with HKCC by both routes compared to the saline treatment group (**Fig. 3.13 B**). These results coincide with the presence of IFN-γ in lung lavages.

Next, I determined the systemic innate immune stimulation upon HKCC treatment in *Mtb*-infected mice. Notably, HKCC treatment upregulated expression of CD86 and MHC-II molecules on $CD11b^{+}F4/80^{+}$ macrophages and $CD11b^{+}CD11c^{+}$ DCs, and CD40 expression on B cells (**Fig. 3.13 C**). In addition, treatment with HKCC was associated with increases in the percentage and activation (CD25 and CD69 expression) of NK and NKT cells in the spleen (**Fig. 3.13 D)**. Thus, therapy with HKCC in *Mtb*-infected mice provides significant local and systemic recruitment and activation of innate immune cells.

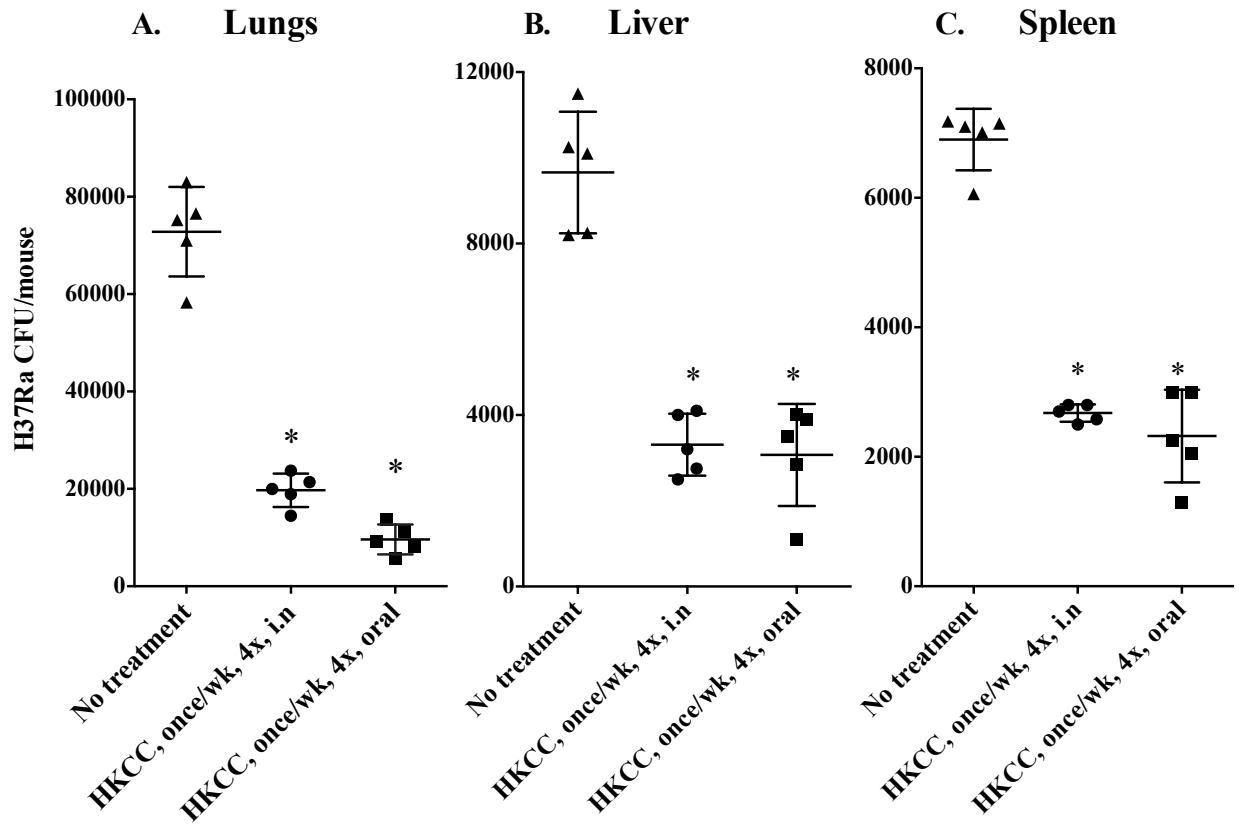


Fig 3.12. HKCC significantly reduces *Mtb* growth in lungs, liver and spleen. BALB/c female mice were challenged with H37Ra (0.5 x 10^6 CFU/mouse) intravenously. Starting three days after infection, mice were treated with HKCC intranasally or orally (50 × 10^6 CFU/mouse) once/week for 4 weeks. Control mice were treated with saline. Five days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU (colony forming units) from five individual mice. Data are representative of three different repeated experiments. '*' $P \leq 0.05$, indicates significant difference compared to the saline-treated mice.

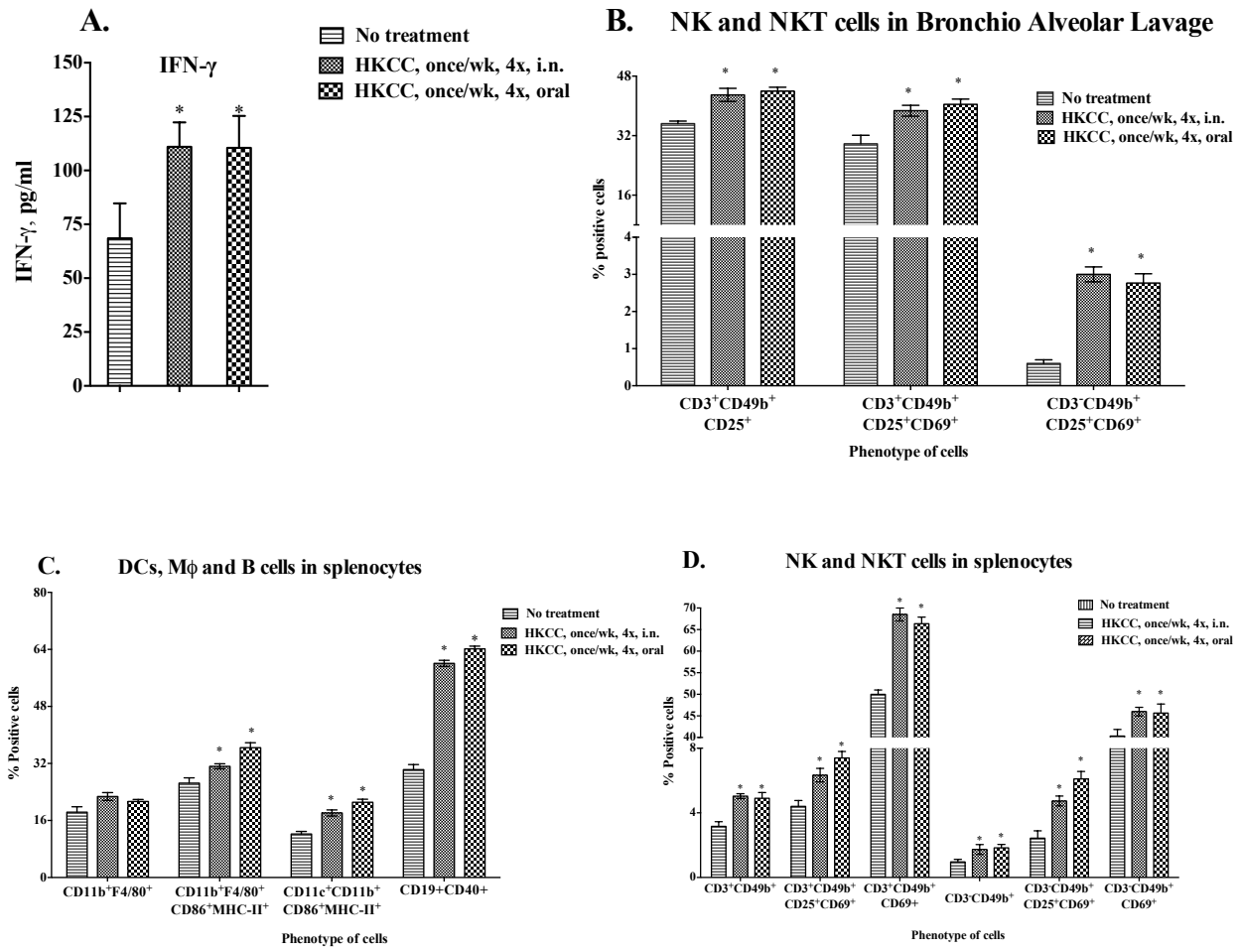


Fig 3.13. HKCC treatment boosts both local and systemic immune responses in *Mtb* challenged mice. BALB/c female mice were challenged intravenously with H37Ra (0.5×10^6 CFU/mouse). Starting three days after infection, mice were treated intranasally or orally with HKCC (50×10^6 CFU/mouse) once/week for 4 weeks. Five days after the last treatment, mice were euthanized and lung washes, BAL and spleens were collected. **(A)** IFN-γ in lung washes, by ELISA. Mean ± standard deviation of cytokine concentrations from individual mice are shown. Percent positive cells are shown: **(B)** NK and NKT in BAL, **(C)** DCs, macrophages and B cells in spleen, and **(D)** NK and NKT cells in spleen. '*' $P \leq 0.05$, indicates significant difference compared to the PBS-treated mice. Data are representative of three different repeated experiments.

3.3.9 Treatment with HKCC in conjunction with a low dose of first-line anti-TB drug isoniazid (INH) augments anti-mycobacterial effects in mice

The potential of immunotherapy for the mycobacterial infections could be enhanced if it could be used along with anti-TB chemotherapeutics. This could enhance the antimicrobials' therapeutic effects, reduce their doses and side effects, and prevent or delay drug-resistance problems. To determine the combinatorial effect of HKCC with available anti-TB drugs, *Mtb*-infected mice were sequentially treated with HKCC (subcutaneously) and a low oral dose of INH (1 mg/kg) using the schedule depicted in **Fig. 3.14**. It was interesting to note that only two intermittent subcutaneous treatments of HKCC with a low intermittent oral dose of INH led to very promising levels of inhibition of *Mtb* in lungs (90%), liver (94%) and spleen (98%). These effects were significantly superior than either agent used alone at the same dose and schedule (**Fig. 3.14**).

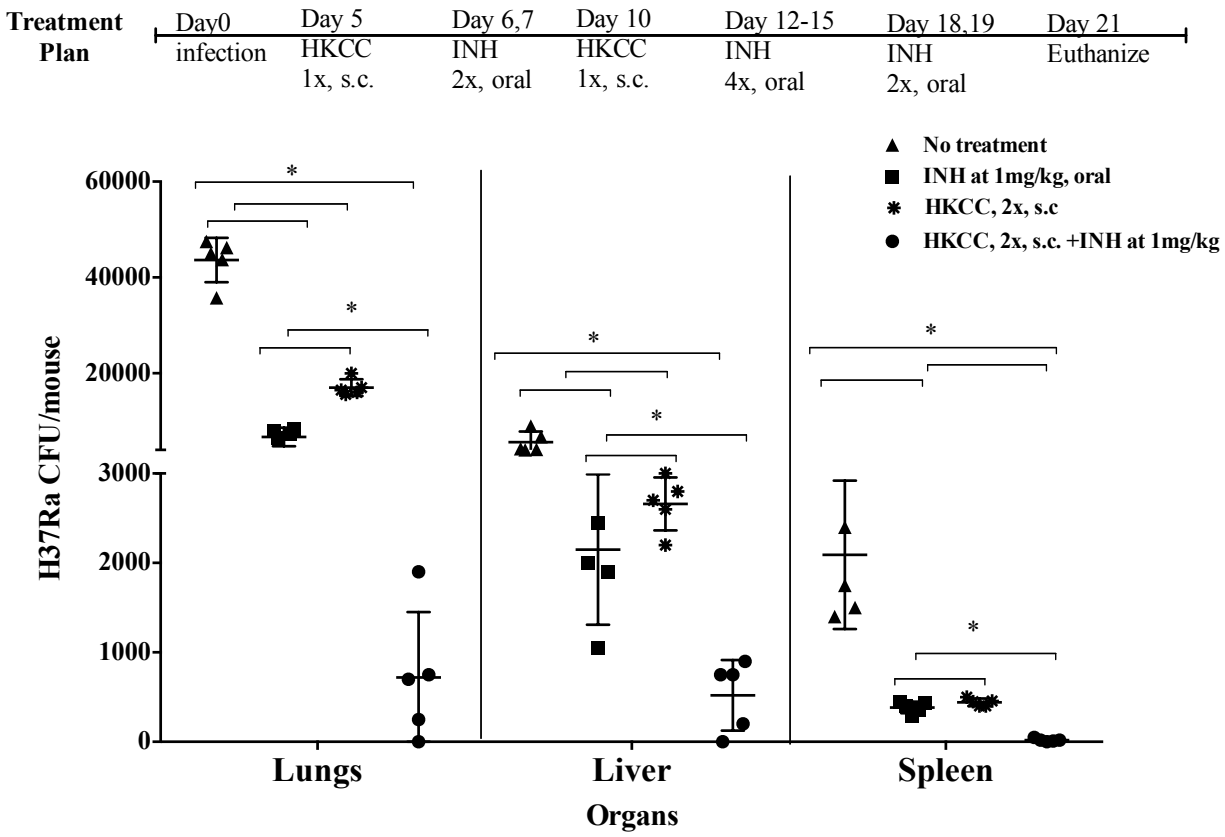


Fig 3.14. Treatment with HKCC in combination with isoniazid leads to higher reduction in bacterial burden than either agent alone. Groups of 5 BALB/c female mice were challenged with H37Ra (0.5 x 10^6 CFU/mouse) intravenously. From five days post infection, mice were treated with HKCC subcutaneously, and INH orally, or PBS (by the same route as a control) using a schedule shown in the figure. Mice were euthanized 2 days after the last treatment. Spleens, lungs and liver were collected to determine bacterial loads using the CFU assay. Results are shown mean ± standard deviation of CFUs from individual mice. Data are representative of three different repeated experiments. '*' $P \leq 0.05$, indicates significant difference compared to the PBS-treated mice.

3.4 Discussion

Tuberculosis is an ancient disease that is re-emerging worldwide in more dangerous drug-resistant forms. In the past two decades, significant efforts have been focused on developing an improved TB vaccine that targets the stimulation of antigen-specific adaptive immune responses.

To date, however, they have not led to measurable success clinically [51]. Our understanding of the role of innate immune cells is expanding. We now appreciate that they not only provide the immediate first line of defense but also extend well beyond that in their role in timelines, memory responses, immune regulation, control of inflammatory mechanisms and coordinating the downstream adaptive immune response [52-54]. This new knowledge has prompted many to investigate novel innate immunomodulatory agents that can be used to treat not only TB but also a number of other chronic infections. Our studies have revealed a heat-killed form of *Cc* (HKCC) to be a promising immunomodulatory/immunotherapeutic agent targeting such innate mechanisms.

I initially examined the induced secretion of various cytokines upon co-culture of HKCC with human PBMCs, a mixture of innate and adaptive immune cells, such as NK, NKT, monocytes, DCs, T cells, B cells etc. HKCC stimulated early production of IFN-γ, TNF-α, IL-1β, IL-6, IL-12, IL-10, IL-17A and IL-22 cytokines within 24 h, which was sustained for four days in culture, and delayed GM-CSF production (**Fig. 3.1** and **3.2**). These results were highly unexpected due to the non-pathogenic and non-infectious nature of *Cc* for humans. The obtained pattern of cytokine induction was indicative of direct and/or indirect activation of innate immune cells, followed by trans-activation of, and cytokine production by, a variety of innate and adaptive immune cells. Therefore, I analyzed the activation states of different cell types in PBMCs stimulated by HKCC. I first chose to use non-isolated populations of innate and adaptive cells present in PBMCs for the stimulation phase, followed later by examining individual cell types by flow cytometry. This way optimum interactions between different cells could occur in the PBMCs. Overall, my results reveal a previously unrecognized role of HKCC as a novel immunomodulatory agent stimulating mDCs, pDCs, NK and NKT cells, resulting in the

activation of $CD4^+$ and $CD8^+$ T cells but without direct T cell activation, and uniquely activating B cells (**Fig. 3.3-3.9**).

HKCC induces maturation of mDCs and leads to production of multiple cytokines, which are associated with the induction of an adaptive immune response. The co-production of IL-12, IL-6, TNF-α and IL-10 from mDCs indicated that HKCC induced a pro Th1 DC and DC-10 phenotype (**Fig. 3.3**). Interestingly, HKCC also increased the activation of pDCs, which have both stimulatory and regulatory effects on T cells (**Fig. 3.4**). pDCs have been shown to help balance T cell activation during autoimmunity and antiviral defense. Furthermore, enhancement of allogeneic T cell proliferation upon co-culture with HKCC-stimulated mDCs suggests the ability of HKCC to induce an adaptive immune response of an effector type, as required when encountering a pathogen. Different microbes as well as TLR agonists induce aberrant immune responses, including production of pro-inflammatory cytokines, and uncontrolled and abnormal polyclonal T and B cell proliferation and activation, which are linked to severe immunopathology and autoimmune inflammation [54-61]. In contrast, HKCC does not induce non-specific lymphoproliferation and activation of B and T cells (**Fig. 3.6, 3.7**), suggesting that HKCC would not lead to immune abnormalities associated with tissue damage or autoimmunity. Also specifically, HKCC did not upregulate CD69 expression on T and B cells; CD69 expression has been known to be associated with autoimmune and inflammatory responses [62, 63]. Interestingly, although HKCC did not cause B cell proliferation or upregulation in CD69 expression, it enhanced the expression of CD86 and CD40 (**Fig. 3.7**), which would result in the increased ability of B cells to interact with T cells or other immune cells to allow both higher antibody production and higher effector T cell responses [64, 65]. The reason and mechanism for this interesting property is not clear.

The early induction of multiple cytokines from PBMCs led us to examine NK and NKT cell activation by HKCC (**Fig. 3.8**). Accordingly, our results clearly demonstrated that HKCC activates innate lymphocytes NK and NKT cells (**Fig. 3.8**). In addition, intracellular cytokine staining identified NK and NKT cells as the source of TNF-α, IL-17A, IL-10 and IL-22 (**Fig. 3.9**). Interestingly, the induced cytokine signature suggests the activation of type II and/or heterogeneous NKT cells, implicating non-inflammatory characteristics of the induced innate immunity [66]. However, our experiments do not rule out the production of these cytokines from other cell types, which were not examined here. NK and NKT cells have recently emerged as playing important roles in immune regulation, immune homeostasis and memory responses. Besides classical mechanisms of activation, i.e., absence of MHC1 for NK cells and CD1d restricted activation of NKT cells, both NK and NKT cells have recently been shown to be activated by multiple non-classical mechanisms, which expands their role in overall immunity and immune regulation [67-69]. NK/NKT cells are able to induce maturation and functional activation of DCs, and NK/NKT-DC cross talk plays an important regulatory role both in innate and adaptive immunity [70]. NK and NKT cells are known to promote or suppress cell-mediated immunity in different conditions. In order to maintain homeostasis, NK and NKT cells can directly induce maturation of DCs or lysis of immature but not mature DCs [71]. Functionally distinct subsets of NK and NKT cells regulate these diverse biological functions. Both NK and NKT cells mediate potent immune regulatory functions in autoimmune diseases, cancer, infections and immune tolerance [72, 73]. The induction of TNF-α, IL-10, IL-17A and IL-22 by NK and NKT cells upon stimulation with HKCC provides an impetus to examine the potential role of HKCC in mediating immunoregulatory mechanisms. Consistent with our *in vitro* data with human PBMCs, I found that mucosal (intranasal and oral) administration of HKCC also

induces rapid production of cytokines in lung washes, suggesting the potential of HKCC in stimulating or modulating innate immunity (**Fig. 3.10**).

Recently, various innate cytokine pathways have been reported to influence the outcome of *Mtb* infection [74]. The cytokine signature induced by HKCC from human PBMCs was indicative of heterogeneous innate immune cells' stimulation (**Fig. 3.1** and **3.2**). Accordingly, supernatants collected from human PBMCs stimulated with HKCC for 24 hours showed very promising effects in reducing *Mtb* replication in a human macrophage cell line (THP-1) (**Fig. 3.11**). The exact role of individual or combinations of cytokines in producing this effect is not clear yet. Interestingly, supernatants collected from PBMCs stimulated with individual TLR agonists (CpG, polyI:C, resiquimod and LPS) did not have the same effect (**Fig. 3.11**). This observation could be attributed to a broad and distinct range of cytokines/innate cells induced upon stimulation with HKCC, compared to individual TLR agonists [75, 76].

Mucosal administration of HKCC in *Mtb*-infected mice led to a remarkable reduction in bacterial loads in lungs, liver and spleen (**Fig. 3.12**) and it was associated with activation of various innate and adaptive immune cells including NK and NKT cells (**Fig. 3.13**). Similarly, although I did not examine the role of the expanding universe of other types of innate lymphoid cells (ILCs) in our studies, they cannot be overlooked in providing protective immunity against *Mtb* upon treatment with HKCC. NK and NKT are prominent cells of innate immunity, but their contribution during mycobacterial infections has not been explored much and remains controversial. Depletion of NK cells in mice with antibodies against NK 1.1 and asialo-GM1 enhanced mycobacterial growth in mice [77]. Numbers and functional activity of NKT cells have been shown to be reduced in *Mtb*-infected patients, supporting their role in natural immunity [78]. In addition, Kulprannet *et al.* have shown a higher frequency of IL-4-producing NKT cells compared to IFN-γ-producing cells in TB patients [79]. Dhiman *et al*. demonstrated that IL-22

produced from NK cells but not by T cells inhibit intracellular growth of mycobacteria [80], and that NK cells have the ability to lyse the Tregs expanded during chronic infection, providing an important role of NK cells in immune defense against *Mtb* [81]. Our results demonstrate the *in vitro* and *in vivo* effect of HKCC correlates with the activation of NK/NKT cells and the cytokines they produce. Taken together, these findings suggest that the immunotherapeutic effects of HKCC in tuberculosis are at least partly dependent upon the functional activation of NK and NKT cells. Since, innate cells play a critical role in inducing, maintaining and regulating the downstream adaptive immune responses, the participation of adaptive immune responses in the observed anti-mycobacterial effect of HKCC, although not investigated in the current study, cannot be ruled out or ignored.

TB is a resilient chronic infection and extremely difficult to eradicate from a host . The best strategy for successful treatment and/or cure of TB could be envisioned that as *Mtb* replication is reduced by chemotherapy and concurrent immunotherapy, innate immune mechanisms will be harnessed to rid the body of *Mtb* completely. Our experiments using an HKCC-based immunotherapy, in conjunction with a low dose of first-line anti-TB drug isoniazid, (**Fig. 3.14**) were very promising and set the stage for such treatment regimens. Intriguingly, I observed ~98% reduction in viable *Mtb* in the lungs of mice treated concurrently with HKCC and INH (low dose), compared to 50-70% reduction in bacterial counts obtained with either agent alone. Similarly, in liver and spleen, a significantly higher reduction in *Mtb* counts was observed with concurrent HKCC and INH treatment, compared to either agent alone (**Fig 3.14**). Collectively, our data demonstrate tremendous promise of HKCC as a novel immunotherapeutic agent to treat a deadly chronic infectious disease TB. The immunotherapeutic potential of HKCC may also prove to be useful in stimulating host immunity to protect from a wide range of human pathogens. HKCC induces innate immunity to successfully eliminate a pathogen, however, its

role in altering the microbiome of a host must be determined. Since HKCC is a non-viable heat-killed form of the bacterium, it is unlikely to colonize the gut or compete with or displace host microbiota.

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Chapter 4

General Discussion

In this thesis, I have investigated novel palmitoyl-lysine conjugated T cell peptide epitopes of mycobacterial ESAT-6 antigen as potential vaccine candidate in the induction of antigen specific cellular immune responses and protective immunity against *Mtb*. I also examined the influence of various routes of immunization and adjuvants (Poly I:C, MPL and GDQ) in modulating the lipopeptides' induced immune responses and resulting protective immunity in mouse model of *Mtb* infection. In addition to known adjuvants, I have determined the effect of HKCC as a novel vaccine adjuvant on the protective immunity induced by ESAT-6 lipopeptides. While performing vaccine experiments, I discovered that administration of mice with HKCC alone in the absence of ESAT-6 lipopeptides also provided significant reduction in *Mtb* loads. I therefore, set out to study immunomodulation by HKCC and its subsequent role in reducing *Mtb* loads in mice. First, using human PBMCs in cell culture, I demonstrated unique innate immune stimulating properties of HKCC. Subsequently, I examined the ability of HKCC to control the growth of *Mtb* in *in vitro* and *in vivo* models through immune stimulation. In human macrophages infected with *Mtb*, I found that supernatants from human PBMCs stimulated with HKCC significantly inhibited mycobacterial replication. In mice, I demonstrated that HKCC alone could induce innate immunity that can significantly reduce mycobacterial load. Encouraged with these observations, I investigated the effect of HKCC in conjunction with the anti-tuberculosis drug isoniazid in a mouse model of *Mtb* infection which led to enhanced reduction in *Mtb* loads compared to individual agents alone. My studies have opened new avenues in vaccine and immunotherapy research for tuberculosis. My findings may have immense implications in preventing and treating serious tuberculosis infections, reducing the doses and duration of chemotherapeutic treatment and avoiding the emergence of drug-resistance.

4.1. Novel lipopeptides of ESAT-6 induce strong protective immunity against *Mtb* which critically depends upon route of immunization and adjuvant

A new generation of TB vaccine is urgently needed to control the global pandemic of TB, since the current BCG vaccine provides inconsistent and inadequate protection and is not very effective. Development of MDR, XDR and TDR strains of TB have further endorsed this need. Renewed efforts to develop new TB vaccines have led to investigation of various subunit, recombinant-vector based and modified BCG-based vaccines [1]. However, their clinical efficacy still remains to be seen. Subunit vaccine candidates that are in development can either boost BCG vaccine or can be used as a stand-alone prophylactic vaccine. An effective peptide-or protein based subunit vaccine requires an adjuvant that can increase the magnitude and durability of adaptive immunity. To date, several TLR agonists have been tested as effective adjuvants to induce cellular immune responses, however, none have been successful due to their potential toxicity and severe inflammatory side effects [2, 3]. Therefore, there remains a need for a safe and effective adjuvant capable of eliciting strong cellular and/or humoral immune responses against antigens, to be used in vaccine compositions.

Earlier studies have shown that strong multi-functional cellular immune responses against dominant antigens of *Mtb* play a critical role in the protection against *Mtb* infection [4]. In this study, I have designed a novel subunit vaccine by conjugating a palmitoyl-lysine chain to peptides derived from ESAT-6 protein corresponding to dominant human T cell epitopes. I examined their ability to stimulate antigen specific T cell responses and protective immunity against *Mtb* using intranasal and subcutaneous routes of immunizations in BALB/c mice. In addition, I determined the effect of known TLR agonists (PolyI:C, MPL and GDQ) and a novel immunomodulator HKCC for their ability to enhance the induced immune responses and resulting protective efficacy of our vaccine.

Upon subcutaneous immunization of mice with designed individual lipopeptides of ESAT-6, I observed that they induced significant cellular immune responses of their own. Interestingly, I found that intranasal and subcutaneous immunizations with a mixture of immunogenic lipopeptides alone induced systemic T cell responses against all epitopes of the mixture. Further, I noted that our newly designed subunit vaccine containing lipopeptides of ESAT-6 provided significant protection from *Mtb* infection when administered intranasally or subcutaneously.

Among the adjuvants investigated, I found that MPL (a TLR-4 agonist) and HKCC were particularly effective in further inducing antigen specific immune responses against lipopeptides of ESAT-6, and in providing enhanced protection from *Mtb* infection. In contrast, PolyI:C (a TLR-3 agonist) and GDQ (a TLR-7/8 agonist) led to reduced protection with increased number of myeloid derived suppressors cells and higher production of IL-10 from $CD4^+$ T cells.

PD-1 is a co-inhibitory molecule expressed on activated T cells and can dampen T cell responses upon engagement with its ligands PDL-1 and PDL-2. It has been shown that during *Mtb* infection, PD-1 expression is increased on T cells and its binding to PDL-1 expressed on APCs downregulates effector Th1 responses [5]. It is possible that differential induction of PD-1 on T cells and PDL-1 on infected macrophages also contributed in varying protection from *Mtb* upon immunization with different TLR agonists. Although in my project I did not examine the PD-1 and PDL-1 expression, their role could be determined by understanding their expression profiles and using anti-PD-1 or anti-PDL-1 antibodies along with or after immunization(s).

The discrepancy in protection by various adjuvants could be attributed to the role of stimulation of different TLRs. Although, members of the TLR family share certain structural and functional properties, the signal delivered by individual TLRs or their cross talk may elicit qualitatively and quantitatively different immune responses [6]. Further, how APCs and innate

immune cells are being activated during infection has an important impact on the quality and magnitude of adaptive immune responses. My findings suggest that signaling via TLR-3 (PolyI:C), TLR-4 (MPL), and TLR-7/8 (GDQ) agonists may have delivered qualitatively different signals to APCs, which would have affected the nature of the immune responses that were elicited as diagrammed in **Fig 4.1**.

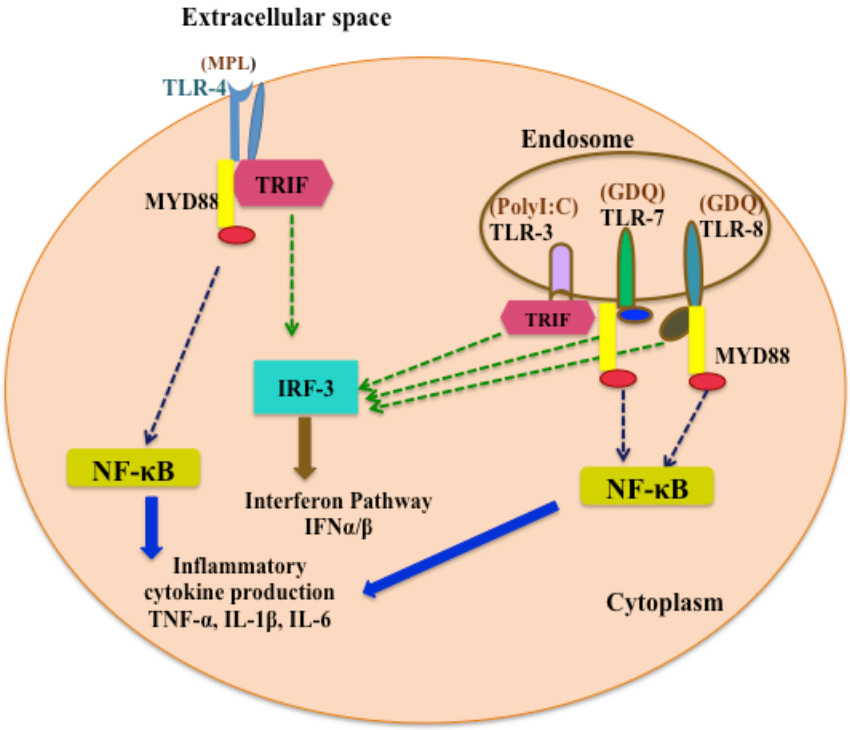

Fig 4.1: Location of TLRs and signalling pathways in antigen presenting cells

Additionally, the route of immunization also showed a significant influence on the induction of *Mtb* specific protective immunity. I found that intranasal immunization with lipopeptides of ESAT-6 by themselves and their co-immunization with adjuvants impart better protection in contrast to the subcutaneous route.

In conclusion, I demonstrated that lipopeptides of ESAT-6 could induce protective immune responses, which are critically affected by the adjuvant and route of immunization.

Inclusion of immunodominant human T cell epitopes of other *Mtb* antigens in the designed subunit vaccine can further broaden these protective immune responses. Overall, these studies suggest that the selection of an appropriate adjuvant and route of immunization is critical for a vaccine's success.

4.2. Heat-killed *Caulobacter crescentus* (HKCC) uniquely modulates host immune responses and provides effective protection against mycobacterial infections.

Host-directed therapy using immunomodulators may provide a promising strategy for treatment of chronic mycobacterial infections. Immunotherapeutic approaches can control mycobacterial growth, shorten the duration of drug treatments, reduce their doses and side effects, and may prevent the emergence of drug-resistant strains. Although precise contributions and fine-regulation of different immune mechanisms needed to fight TB remain undefined, animal and human studies suggest that regulated activation of innate immune cells promotes mycobacterial containment and stimulates adaptive immunity, while their uncontrolled activation and inflammation cause active disease with severe organ damage. Different strategies using live and inactivated mycobacteria or their components have been explored as potential immunotherapies, however limited success has been achieved [7-10]. In this regard, a whole inactivated *M. vaccae* (MV) has been studied as a therapeutic vaccine in clinical trials. A meta-analysis of clinical trial results showed that administration of MV with anti-TB drugs in never-treated patients improved the clinical symptoms of the disease [11].

In this study, I have investigated non-infectious gram-negative whole cell bacterium *Caulobacter cresentus* in heat-killed form (HKCC), as a novel immunomodulator, and I have examined its effect in activating human immune cells (APCs, NK, NKT cells, $CD4^+$ and $CD8^+$ T cells) and in the induction of cytokines. Interestingly, I found that HKCC stimulates mDCs,

pDCs, NK and NKT cells, the key players of innate immunity, and also induces cytokines' production. The cytokine signature induced by HKCC suggests the activation of type II and/or heterogeneous NKT cells. It has been shown that type I NKT cells are mainly associated with autoimmune and inflammatory diseases, while type II NKT cells cross-regulate the function of type I NKT cells and therefore have the unique ability to maintain homeostasis in infections or autoimmune conditions [12]. Further, my results demonstrated that HKCC does not directly lead to non-specific T cell activation whereas it uniquely activates B cells without leading to their proliferation and CD69 expression. These studies suggest that HKCC would not lead to immune abnormalities associated with tissue damage and autoimmunity.

Although I did not characterize the components of HKCC, it is plausible that various cell wall (such as S layer, LPS and peptidoglycans) and intracellular (such as bacterial DNA and cytoplasmic proteins) components of HKCC interact with innate patteren recognition receptors (PRRs) present on DCs, pDCS, NK and NKT cells to modulate their functions. Rapid production of cytokines after intranasal and oral administration of HKCC suggests that it can also interact with nasal and gut epithelium and/or mucosal immune cells through M cells and/or PRRs.

I also investigated host-mediated effects of HKCC in controlling the replication of intracellular *Mtb* (H37Ra) and *M. avium* in human macrophages. In a mouse model of *Mtb* infection, I found that mucosal administration (intranasal and oral) of HKCC alone was able to induce local (pulmonary) as well as systemic immune responses, and subsequent reduction in mycobacterial loads in lungs, liver and spleen. Treatment with HKCC in conjunction with a low dose of isoniazid provided a significantly enhanced effect in controlling disseminated growth of mycobacteria in mice.

In conclusion, my studies demonstrated that a non-pathogenic, non-viable bacterium unrelated to mycobacteria could be used as a novel immunomodulator to successful control mycobacterial infections.

4.3. Conclusions

Lipopeptide based subunit vaccines have many advantages due to their intrinsic adjuvanticity and efficient antigen delivery. My studies have focused on understanding the role of cellular immune responses stimulated by lipopeptides of *Mtb* ESAT-6 antigen in mycobacterial clearance. I have conclusively shown that lipopeptides derived from ESAT-6 antigen alone and with adjuvant MPL or HKCC can induce protective cellular immune responses locally and systemically, by both subcutaneous and intranasal routes, and could serve as a novel subunit vaccine for TB. Further, my studies revealed the immunological mechanisms induced by HKCC as a novel immune modulator. Intriguingly, immunotherapy with HKCC was able to induce innate immune responses in *Mtb* challenged mice upon mucosal administration and inhibit mycobacterial replication systemically. Additionally, HKCC along with low dose of isoniazid demonstrated enhanced reduction of mycobacterial load in mice.

Altogether, my studies have advanced our knowledge and provided important information in the field of vaccine and immunotherapy for TB.

4.4. Future directions

I have shown that palmitoyl-lysine chain conjugated peptides of *Mtb* ESAT-6 antigen that correspond to dominant human T cell epitopes can induce strong protective immunity in mice, and that the induced immunity can be plausibly optimized with the careful selection of route and adjuvant. I speculate that inclusion of similar lipopeptide constructs of other dominant antigens of *Mtb* would further broaden and enhance *Mtb*-specific protective immunity. Lipopeptide based

multi-antigen subunit vaccine need to be explored either as stand-alone prophylactic and/or therapeutic vaccine or as a booster to BCG vaccine. The protective potential of the immune responses induced by lipopeptides of ESAT-6 and/or multi-antigen peptides should be tested against an aerosol infection of virulent strain H37Rv in a mouse model or more rigorous animal models. The role of antibodies in protecting against *Mtb* is not clear; however, recent studies are emerging that describe the protective role of different isotypes of antibodies against *Mtb* surface antigens [13]. Therefore, the induction of humoral immune responses after immunization with lipopeptides of ESAT-6 and/or multi-antigen peptides should be determined to examine their role in clearing *Mtb* in future studies.

My studies demonstrated that HKCC as an adjuvant can induce antigen-specific immune responses against ESAT-6 lipopeptides and also protect the mice from subsequent challenge with *Mtb*. I further showed that HKCC has potential to stimulate and modulate the human innate and adaptive immune cells *in vitro* and control mycobacterial infection in *in vitro* and *in vivo* models. It will be interesting to examine whether immunotherapy with HKCC alone and/or in conjunction with anti-mycobacterial drugs is able to treat drug-resistant TB in animal models, whether combining HKCC as immunotherapy reduces the dose and duration of chemotherapy and whether addition of immunotherapy leads to reduction in emergence of drug-resistant *Mtb* strains.

I speculate that immune modulatory activity of HKCC is also due to activation of innate immunity, particularly innate lymphoid cells (ILCs), whose expanding universe is just beginning to be realized. The precise molecular nature of stimulatory/modulatory components of HKCC responsible for the interaction with immune cells should be further investigated.

Overall, my studies will have significant implications in the design of new subunit vaccine, development of new host directed therapies, and understanding the role of innate immunity in mycobacterial infections.

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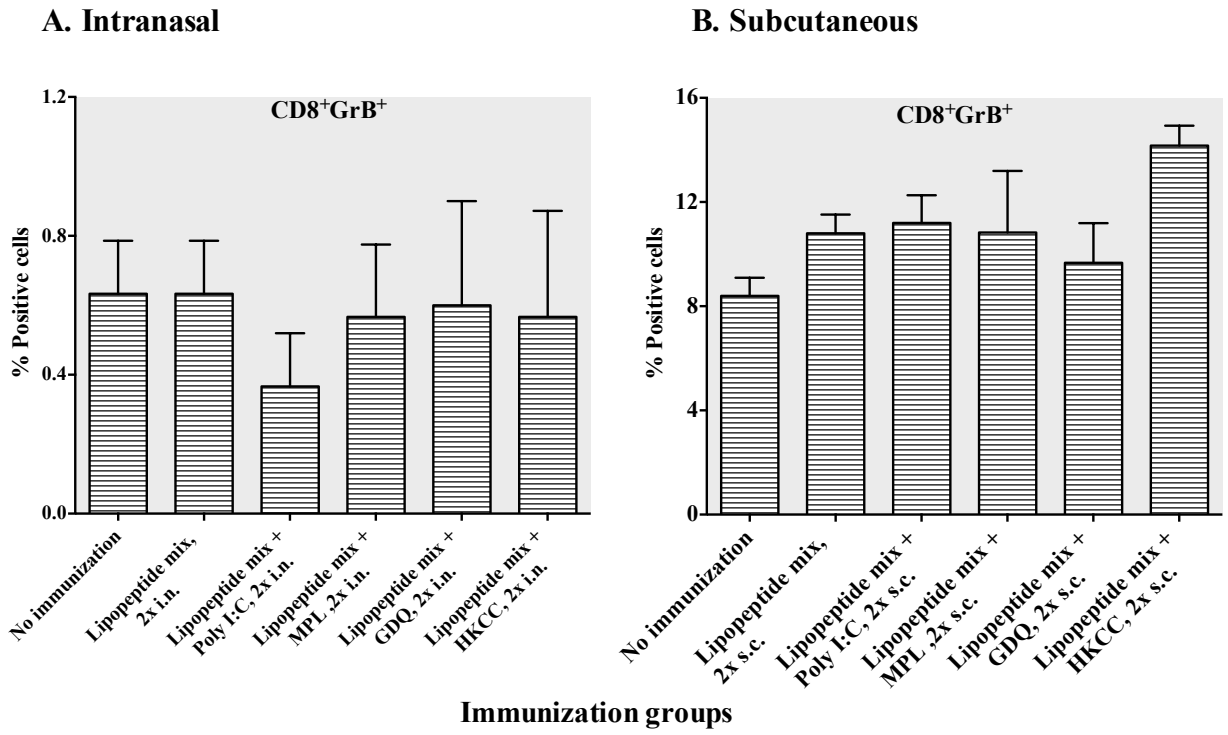
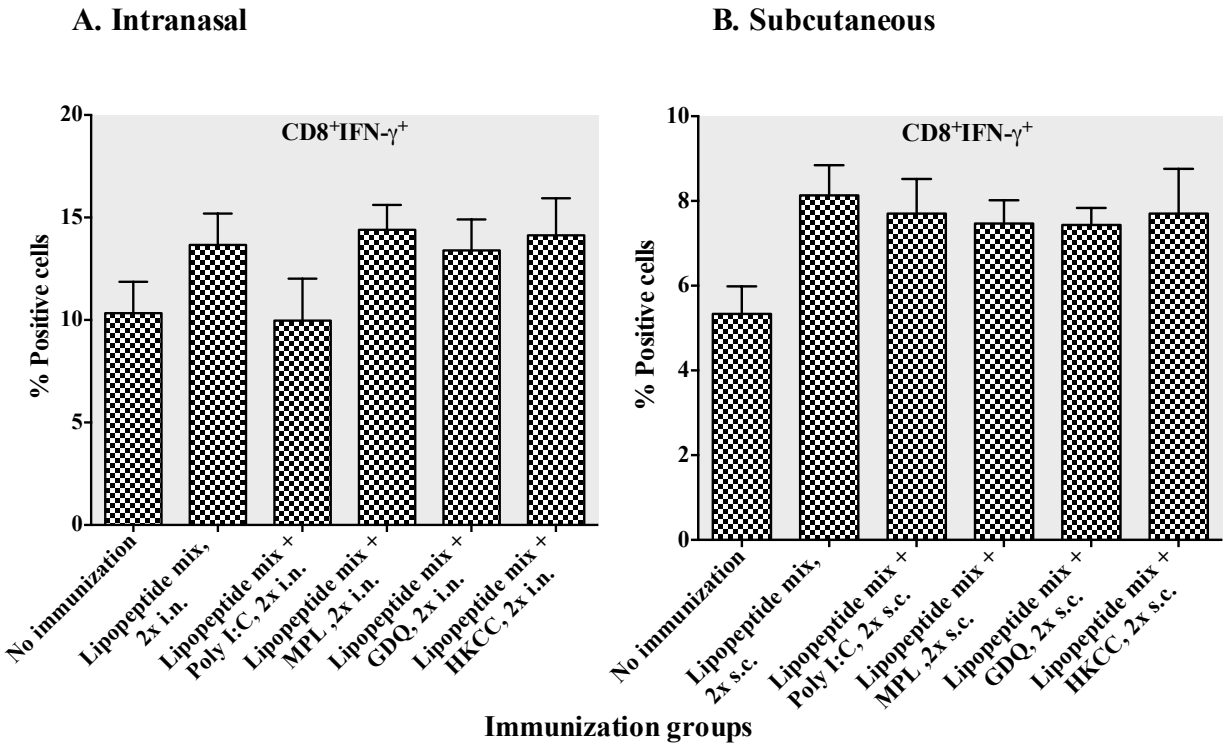
Appendices

Appendix 1



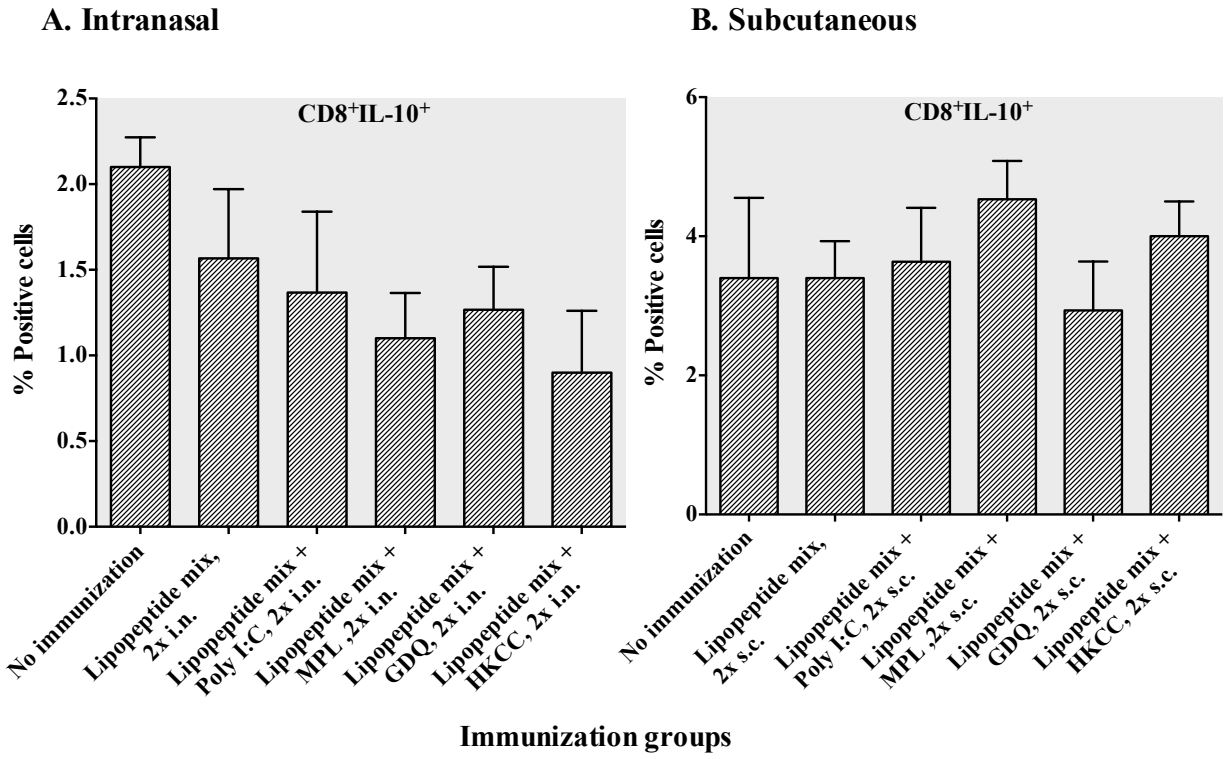
Granzyme B (GrB) expression on antigen-specific $CD8^+$ T cells upon *Mtb* challenge in mice immunized with ESAT-6 lipopeptides with or without adjuvants. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice with a mixture of P1 and P4-P7 lipopeptides alone and combined with an adjuvant Poly I: C, MPL, GDQ or HKCC. PBS immunized mice were used as a controls. One week after the last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, spleens were collected from *Mtb*-challenged mice. Spleen cells obtained from immunized mice were cultured for 4 days with or without peptide pools and were labeled for surface expression of CD3 and CD8 and intracellularly for GrB. The cells were gated for $CD3^+CD8^+$ T cells that were subsequently analyzed for GrB expression in **(A) intranasally** and **(B) subcutaneously** immunized mice. The percentage of GrB^+ of $CD8^+$ T cells is shown. The peptide-specific response was calculated by subtracting the percentage of cells that were positive for GrB expression in the absence of peptide pool (no peptide control). Data are representative of three different repeated experiments.

Appendix 2



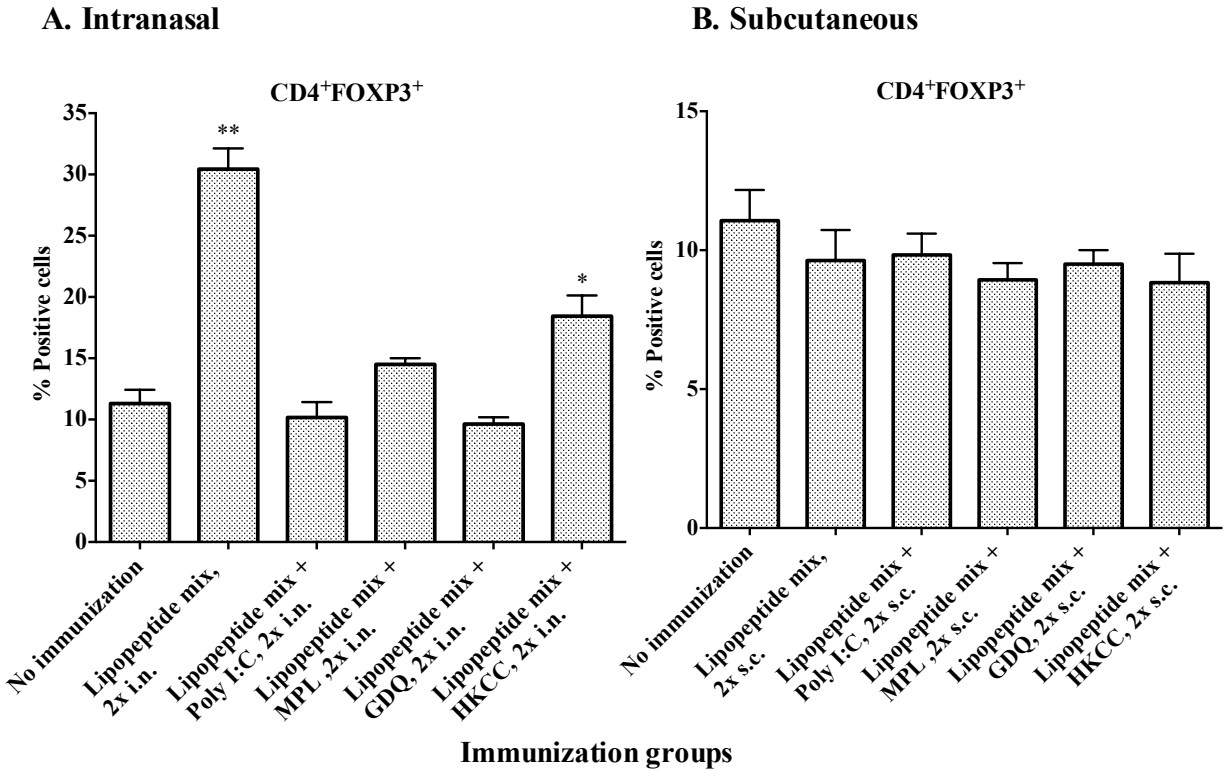
Intracellular IFN-γ expression on antigen-specific CD8$^+$ T cells upon *Mtb* challenge in mice immunized with ESAT-6 lipopeptides with or without adjuvants. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice with a mixture of P1 and P4-P7 lipopeptides alone and combined with an adjuvant Poly I: C, MPL, GDQ or HKCC. PBS immunized mice were used as a controls. One week after the last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, spleens were collected from *Mtb*-challenged mice. Spleen cells obtained from immunized mice were cultured for 4 days with or without peptide pools and were labeled for surface expression of CD3 and CD8 and intracellularly for IFN-γ. The cells were gated for CD3$^+$CD8$^+$ T cells that were subsequently analyzed for IFN-γ expression in **(A) intranasally** and **(B) subcutaneously** immunized mice. The percentage of IFN-γ$^+$ of CD8$^+$ T cells is shown. The peptide-specific response was calculated by subtracting the percentage of cells that were positive for IFN-γ production in the absence of peptide pool (no peptide control). Data are representative of three different repeated experiments.

Appendix 3



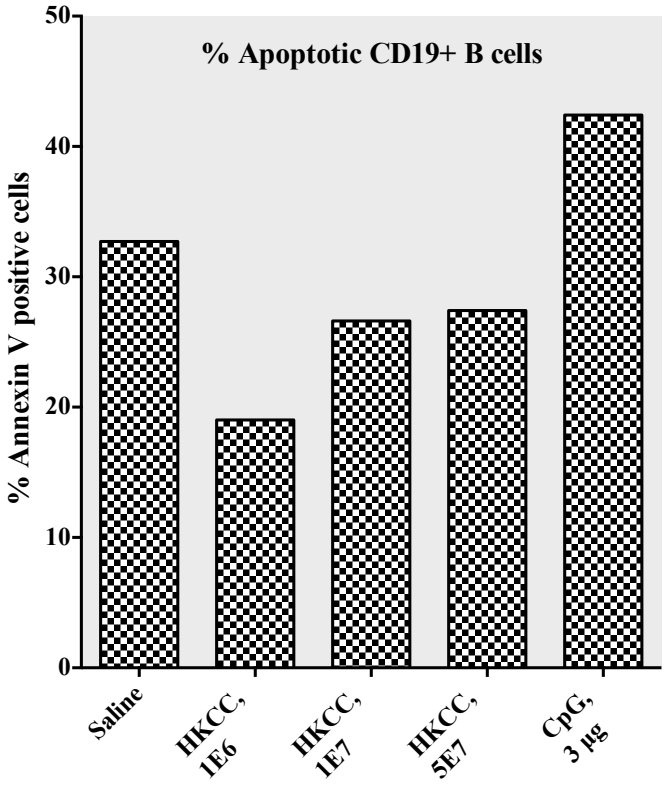
Intracellular IL-10 expression on antigen-specific CD8$^+$ T cells upon *Mtb* challenge in mice immunized with ESAT-6 lipopeptides with or without adjuvants. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice with a mixture of P1 and P4-P7 lipopeptides alone and combined with an adjuvant Poly I: C, MPL, GDQ or HKCC. PBS immunized mice were used as a controls. One week after the last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, spleens were collected from *Mtb*-challenged mice. Spleen cells obtained from immunized mice were cultured for 4 days with or without peptide pools and were labeled for surface expression of CD3 and CD8 and intracellularly for IL-10. The cells were gated for CD3$^+$CD8$^+$ T cells that were subsequently analyzed for IL-10 expression in **(A) intranasally** and **(B) subcutaneously** immunized mice. The percentage of IL-10$^+$ of CD8$^+$ T cells is shown. The peptide-specific response was calculated by subtracting the percentage of cells that were positive for IL-10 production in the absence of peptide pool (no peptide control). Data are representative of three different repeated experiments.

Appendix 4



FOXP3 expression on CD4$^+$ T cells upon *Mtb* challenge in mice immunized with ESAT-6 lipopeptides with or without adjuvants. Female BALB/c mice (n = 5) were immunized intranasally and subcutaneously twice with a mixture of P1 and P4-P7 lipopeptides alone and combined with an adjuvant Poly I: C, MPL or GDQ. PBS immunized mice were used as a controls. One week after the last immunization, mice were challenged with H37Ra (0.5 x 10^6 CFU) intravenously. Five weeks later, *Mtb*-challenged mice were euthanized and spleens were collected. Spleen cells obtained from immunized mice were labeled for surface expression of CD3 and CD4 and intracellularly for FOXP3. The cells were gated for CD3$^+$CD4$^+$ T cells that were subsequently analyzed for FOXP3 expression in **(A) intranasally** and **(B) subcutaneously** immunized mice. The percentage of FOXP3$^+$ of CD4$^+$ T cells is shown. '*', Indicates significant difference (*P < 0.05; **P < 0.01) compared to the corresponding group in PBS immunized mice. Data are representative of three different repeated experiments.

Appendix 5



HKCC does not lead to apoptosis of B cells. PBMCs (2 x 10^6/well) were labeled with CFSE dye and treated with or without HKCC or CpG at different concentrations in 24 well plates. Cells were incubated for 4 days and labeled for CD3, CD11b and CD19 surface markers. The cells were gated for $CD3^-CD11b^-CD19^+$ cells and apoptosis was examined by the expression of Annexin V dye. The percentage of Annexin V positive $CD19^+$ B cells is shown.