Modulations of immune responses induced by BCG vaccination

By

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I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree /diploma at this or any other Institution / University.

(**Sujay Gaikwad**)

Dedications

I am deeply indebted to my teachers, parents, and my wife who enlighten the way of my life with their efforts, care and love to shape and mould my scientific career. I wish to acknowledge my source of inspiration my brother Rahul, Uday, Vikrant, Rohit and Akshay, son Dhairyavardhan Gaikwad and friends Jatin Bhosle, Debasish Bhosle, Samarth Bhosle,Sureshchandra Pal, Chetan Patil, Abhishek Bansod and Maithili.

I dedicate this work to my loving parents ***Mrs Chhaya Gaikwad*** *and* ***Mr Sarjerao Gaikwad inlaws Mr Ajit Bhosle and Mrs Alka Bhosle.***

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Chapter 5. Conclusion

The attenuated strain of BCG has immense possibilities due to its potential favourable immune response generating ability. Despite its inability to protect adults of pulmonary TB, it is efficient enough to keep the disease away in children. The use of BCG is not just limited as a prophylactic agent against TB, but it is also used in cancer and other diseases for the immunological advantages it offers. Our work had a focus to diminish the drawbacks of the BCG vaccine to make it more effective in current usage settings. Our study focused both on immunomodulating the host by juglone or using antigenic-lipids from clinical isolates of *Mtb* as adjuvants to improve immune responses generated towards the BCG vaccine. The potential of juglone as an immunomodulator was demonstrated by various experimental studies in mice. Juglone immunomodulated BCG vaccine generated an immune response in mice by Th1 polarization. Th1 polarization was demonstrated by evaluating various Th cytokines like IFN-γ by *ex vivo* restimulation of mice splenic T cells. Juglone treatment led to a predominant increase in IgG2a isotype as compared to IgG1 isotype in mice serum which is an indirect measure of IFN-γ induced Th1 polarisation in juglone immunomodulated-BCG-vaccinated mice. Th1 polarization, along with the corresponding decrease in IL-10 derived from $CD4^+$ cells was due to reduced cell population of Treg cells. This reduction of Treg population along with Th1 polarisation due to juglone immunomodulation lead to enhanced NO, and IL-1β, enabling the development of M1 splenic macrophages. Enhanced T cell memory and $CD8^+$ cell population, suggested immunomodulated effector response induced by juglone in BCG vaccinated mice. Further, reduction in challenged bacterial CFU

counts as seen in BCG challenge test ultimately provided a proof of concept of juglone induced immunomodulation of BCG vaccine-induced immune response. The immunomodulatory dosage of juglone itself was not found to be toxic in mice.

Further, our other studies took into account bacterial non-peptide lipid antigens to induce enhanced immunomodulation of the host. These lipids were isolated from *Mtb* clinical isolates (Beijing, LAM-6, EAI-5 and control strain H37Rv and BCG) and were used to immunomodulate the mice. These studies were based upon our previous experience at our laboratory with *Mtb* clinical strains and their host responses, which lead to our hypothesis in which we propose, use of lineage-specific bacterial lipids antigens as an adjuvant to BCG vaccine. Diverse lipids from *Mtb* clinical isolates grown in liquid culture medium and in infected mice lungs were evaluated by LC-MS along with CD1d putative lipids as the mice have only CD1d presentation system. It was found that overall bacterial lipid expression varies when grown in culture medium and mice lungs. The expression of CD1d lipids also varied in expression across the clinical isolates in both growing conditions (culture medium and mice lungs). These CD1d restricted lipids like PI, PG and PIM_4 induced immune response through NKT cells which were studied by *ex vivo* co-culture studies. Our observation demonstrated differential expression of CD1d putative lipids in their known order of virulence in infected mice lung (unpublished work) or during their growth in the culture medium. This information provided insight for lineage-specific antigenicity of CD1d lipids in *Mtb* strains. Such comparative illustration shows that a simple study parameter like the estimation of Cd1-putative-*Mtb* lipid could help identify new vaccine adjuvant strategy and would not only help us to design lipid-based adjuvant to the vaccine but also help us in strengthening our understanding of the pathological evolution of *Mtb* strains.

Overall we conclude that vaccination under immunomodulation could be useful to harness better efficacy against tuberculosis or other diseases. This study is the first to testify use of juglone as an immunomodulator of BCG vaccine in C57BL/6 mice and the first one where CD1d putative lipids evaluation was used to prove lineage-specific antigenicity of the *Mtb* clinical isolates and their utility as an adjuvant to BCG vaccine thereby improving its efficacy.

Chapter 1. General Introduction

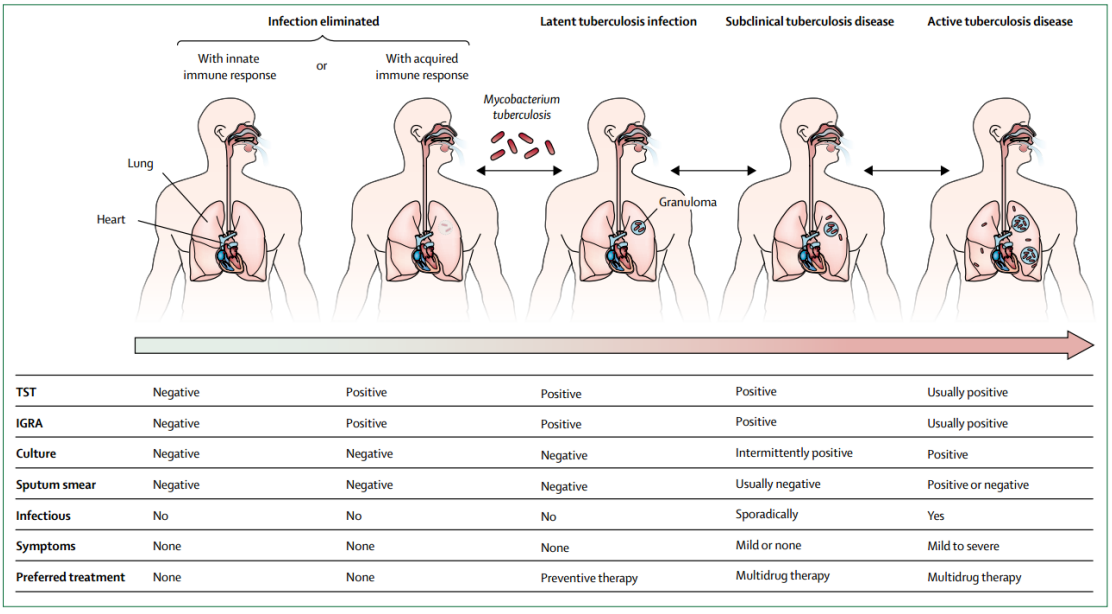
Tuberculosis (TB) was a known disease since prehistoric time, but the root cause of this disease was identified as a bacterium *Mycobacterium Tuberculosis (Mtb)* just a century ago, which was further isolated and characterized. Later, TB was recognized as a potentially controllable disease, against the previous consideration, that it was an ailment resulting from nature's fury and had no remedy; this realization that a solution is possible led to the development of a vaccine against this disease during the early 19th century. Despite these early interventions, TB still is the most significant cause of morbidity or mortality in human from a single infecting agent globally. Many epidemiological studies conducted by WHO determined that about 33% of the world's population was infected with *Mtb* annually (Kaufmann 2006b). In the year 2017, TB led to an estimated 1.3 million mortality (range, 1.2–1.4 million) in HIV-non infected individuals while around 300000 (range, 2,66,000–3,35,000) people died of HIV-TB co-infection. A new registry of TB cases accounted for 10.0 million (range, 9.0–11.1 million), i.e. 133 cases (range, 120–148) per 100000 population (WHO 2018). Apart from humans, TB in cattle is a major economic health issue which results in a reduction in productivity in chronically infected animals, restricted movement, culling of diseased animals, and trade limits. *M. bovis* is the causative agent of bovine tuberculosis which also infected humans before milk pasteurisation was not made compulsory in many countries. Almost 25% of cases of TB in children, were caused by *M. bovis* once upon a time (Roswurm and Ranney 1973). Report from 2012 described, Bovine TB as the major disease affecting livestock in Africa and South Asia, accounting to a loss of USD 300 million annually in these countries, while the global figure was USD 3 billion annually (Waters, Palmer et al. 2012). These facts

about TB warrant an insistent need to control this disease both in humans and animals. It also shows that the methods of control of TB like diagnosis or treatment from the end of 19th and the middle of the 20th century are not sufficient. For example, the methods developed by Robert Koch 125 years ago like sputum smear examination and PPD skin tests which are in use and are, however, inferior in sensitivity, detecting only half of the infected cases.

Further, these diagnostic tests do not differentiate environmental mycobacteria from pathogenic one. Prophylaxis of TB is dependent on a 98-year-old vaccine, the BCG, which was developed in the year 1921 by Albert Calmette and Camille Guérin at the Institut Pasteur, France. However, its efficacy remains controversial even today. Figure 1 shows the diagnostic and disease spectrum of TB. Thankfully *Mtb* is sensitive to antibiotics and the drugs discovered or synthesized during the 1940s, and 1950s can effectively heal the disease but with a cumbersome and the lengthy treatment procedure which usually discourages few patients from adhering to the prescription drugs continuously almost for a year. This poor compliance by defaulters develops drug-resistance in bacterial strains. Such multidrug resistance (MDR) in *Mtb* strains makes them immune to the first line of TB drugs (Isoniazid and Rifampin). The appearance of extensive drug-resistant (XDR) *Mtb* strains which develop resistance to both first-line and second-line anti-mycobacterial drugs makes the scenario scarier. Many countries have already reported the occurrence of these XDR *Mtb* strains in TB patients, with complicated picture arising with subjects with co-infection with HIV resulting into 100% fatality (Gandhi, Moll et al. 2006). Current bacterial drug resistance scenario brings us back to the same stand-point as Robert K and others were at more than a hundred years ago, with respect to control of TB is

considered. More efforts in terms of knowledge and funding are desirable for timely restricting the disease and the drug-resistance scare. Fast, specific, sensitive diagnostic tools and improvement of BCG vaccine efficacy or universal TB vaccine, more effective than BCG is urgently sought.

The incidence of TB in developed countries sharply reduced during the mid of the 20th century; this led to slow down of research on TB exclusively making it a disease of the poor. Due to this fact, both government funding and pharmaceutical companies' interest declined sharply towards TB research, as compared to research on other infectious diseases. Ex: The National Institute of Health (NIH) spends were 20-times lesser for TB research than for HIV research during the late 90s, even though both diseases claimed similar life toll of 2-3 million per annum (Check 2007).



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 1: Spectrum of tuberculosis infection and disease
Reproduced from Pai et al. (Furin, J., Cox, H., and Pai, M. (2019). Tuberculosis. The Lancet.doi:10.1016/s0140-6736(19)30308-3)

The annual survey by Treatment Action Group (TAG) said that the funding for research and development (R&D) for TB peaked in the year 2017 to $767.8 million

US dollars. These surveys were conducted by TAG since 2005. Major funding organizations included NIH, Bill and Melinda Gates Foundation and USAID, which gave impetus to TB research by presenting an opportunity to actualize existing know-how on basic immunology and biology of *Mtb*. Advanced R&D in TB may help to develop innovative and efficient diagnostic tools targeting the so-called "tricks" *Mtb* uses to evade an effective immune response in the host. It would also help in the proper knowledge of the different mechanisms, which help the bacteria in evading the host's immune response, further it would also assist in the development of new drugs and vaccines or may even help to improve the efficacy of the existing BCG as a vaccine. To improvise BCG vaccine efficacy or developing an altogether an alternate vaccine, it is essential to value the primary doctrine of immunization. An administration of vaccines stimulates the innate and adaptive immune system which is protective in nature to a consequent infecting pathogen. Commercially available vaccines mostly ensure the rapid production of high titer of antibodies upon the following infection by the pathogen in the vaccinated subject. These antibodies keep a check on the growth of extracellular bacteria and viruses and certain bacterial toxins by neutralizing them. The underlying immunological principle in successful vaccination is the capacity of the vaccine to boost the scale and pace of the protective antibody response during any natural infections. This increase in magnitude and speed of the developed immune response protects the vaccinated subject in the battle between the infectious agent developing into pathology or disease, and containment of the pathogen by host immunity (Gebreyohannes 2007). Unfortunately, intracellular pathogens causing tuberculosis, leprosy, leishmaniasis and others are not cleared by this strategy and induce chronic infections. This suggests that the high and rapid

immune response generated following infections with intracellular pathogens is not the only factor determining protection but, the quality of the immune response developed is more important in the clash between host and pathogen at the cellular level (Gebreyohannes 2007). Careful analysis of the reaction developed by healthy individuals, following natural exposure to virulent strains developing a progressive disease or those who survive or combat infection elucidates quality of the protective immune response against *Mtb*. Such recognition is crucial, which provides a cogent basis for vaccine design. It was reported that only 5-10 million people of the 100 million people infected every year, develop progressive disease. The remaining, 85-90%, individuals control infection, if not completely eradicate the infection, and remain infected by the bacterium in a latent state for throughout their life (Kaufmann 2006b). Such people with latent TB are typically known as healthy contacts and, which are known to develop and protective type of Th1 immune response. Therefore, the major challenge to develop a new vaccine or to improve the existing one lies in the determination of the immunological correlates of the defence system in the healthy contacts and developing such response through vaccination. Clinical studies have shown that exclusive or predominant cell-mediated Th1-response is vital to contain TB and other diseases where the infections are intracellular in nature. A proper understanding of what protective immune responses are made of and how they are generated, the further step will enquire for a way of vaccination that can assure the generation of a desirable response. Further, sections describe more about the principles of Th1 imprinting and the associated cellular mechanisms.

Chapter 2. Literature Review

This section includes all the explanation of the scientific observations and findings that progressed into the hypothesis were conceptualized in this thesis. The main objective of this research work was to investigate vaccination strategy design which could be efficient in a genetically diverse population against the causative agent of TB. My interest prevailed in various methods to prevent or cure TB, which is responsible for a large scale human morbidity and mortality from a sole infection-causing agent. I believe that the dimensions of this work could receive great appreciation if it is seen as a work with a potential implicate TB as a public health problem, so my attempts were to give a wide platform to understand TB as a public health hazard since the ancient times. I also assessed the current global TB burden with its economic and social imprint. In this upcoming section I have tried to provide a brief idea by presenting the facts arising from the existing academically published literature on *Mtb*, explaining the manner in which it develops TB (pathogenesis) and the multitude of responses generated by the host towards mycobacterium. The intrinsic worth and restrictions of the control measures from the past to combat the implications of TB would also be highlighted. In the purview of this, I would propose multiple intervention strategies, involving immunization or immunotherapy, which could assist in endeavouring ways, important in prevention or curing natural infection with the bacilli. The scientific basis for the proposed objectives of this study would be presented sequentially.

2.1 History of Tuberculosis

Many ancient civilizations worldwide have references to tuberculosis and its symptomatic suffering. The earliest of which could be traced back into the *Sanskrit* language. The ancient Indian scripture, *The Vedas*, intermittently refer TB as *Yakshma* (a disease which leads to wasting). A description of a disease similar to tuberculosis can be seen in the ancient Chinese, Arabic literature and even Bible (Sharma and Mohan 2013). These descriptions could be indistinguishable to the cardinal symptoms of tuberculosis as we describe TB today. Greek descriptions used by many philosophers right since the era of Hippocrates mentions using the words like Phthisis or consumption, which describe nothing but the wasting nature of TB (Daniel 1997). However, written historical documents are sometimes confusing as many symptoms overlap with those of the other diseases, but archaeological findings do support various claims of the disease existence and acknowledgements made by the respective ancient cultures. The most convincing evidence has been witnessed from a 3000-year-old mummy of a girl from Egypt (Zimmerman 1979), similarly ten-year-old boy from Peru (Allison, Mendoza et al. 1973). In these findings, the archaeologists distinguished microscopic acid-fast bacilli in bone samples. There have been many other reports confirming Mtb complex infected animals as early as seventeen thousand years in history (Rothschild, Martin et al. 2001). Such findings in animals have led to the perception that TB first developed in animals as *M. bovis* before jumping species or infecting humans right since they settled in villages and domesticated of wild animals for farming purpose thousand years ago. However, currently, we know from the fine genetic analysis of the bacterium that the *Mtb* complexes have evolved from common ancestors prototuberculosis (Brosch, Gordon et al. 2002; Gebreyohannes

2007). Although, this human-bacterial co-evolution has led to a selective adaptation of the *Mtb* during the course of the evolution leading to the generation of diverse strains. In spite of all the evidence, it can be still assumed that this infectious disease, remained rare in humans during ancient times due to the scattered living conditions, unlike today. Many epidemiological studies say that that TB requires a social network of around 150 to 450 persons living in close vicinity to become endemic in a society (McGrath 1988). Therefore, this disease was not a major public health concern until the 1st TB epidemic in Europe, until the industrial revolution took off, which also witnessed a rise in the number of cities, creating ideal conditions for the human-to-human transmission of *Mtb*. The infamous, outbreak of TB - "the great white plague"- occurred in the big cities of Europe. During this era, every European individual was infected with the bacilli resulting in around 25% deaths due to *Mtb* infection (Bloom and Murray 1992; Gebreyohannes 2007).

2.2. Global Burden and Epidemiology of Tuberculosis

Any epidemic due to an infectious agent follows a predictable route in the society; where primary diseased subjects are the utmost susceptible individuals of the community, predominantly children. Infected subjects develop the disease while the susceptible subjects perish, while the most resistant individuals survive, who develop protective immunity and inducing this genetically predisposed trait into the next generation. This phenomenon controls the epidemic in the community, and it declines as the number of individuals who are susceptible to the disease gradually reduce creating herd immunity till a fresh pool of disease-prone people arise in the community and reinstating the TB back into the community. Tuberculosis may remain

as endemic, infecting a very few (susceptible due to age, immunosuppression, malnutrition or due to concurrent disease) in the society between two outbreaks. TB is known for its persistence and the infected individuals are known to survive for many decades as the infection progresses into the disease and subsequently to mortality or recovery. To understand the importance of epidemiological terms, defining them would help the reader identify the "terms" in their correct context and meaning as these terms are usually used throughout this section of the thesis. "*The mortality rate is the number of deaths due to a given disease in a population at risk during a given period of time, while case fatality rate refers to the ratio of the number of deaths caused by a given disease to the number of diagnosed cases of that disease. Incidence refers to defined as the number of new cases in a population over a given period of time" while the "Prevalence is defined as the total number of cases in a population at a given point in time"(Gebreyohannes 2007).* World Health Organization (WHO) recognizes these rates as expressed per 1 lakh people per annum for all kind of TB related statistics in monitoring and control guidelines made available by them in every report. WHO has a unique method to estimate every statistic arising from the data collected throughout the region centres depending upon the quality of existing data. TB epidemic in Europe peaked during the 17th century with an incidence rate of around 1000 cases per 100,000 persons annually. Approximately 20% of all deaths in London were due to TB, and therefore few writers of that time described it as "Captain of all these men of death" (Daniel 1997). The *Mtb* infected Europeans did spread the disease further to all corners of the world due to immigration. The epidemic was at its maximum in USA in the 19th century, causing death to approximately 1500 per 100,000 people annually (Dutt AK 1999).

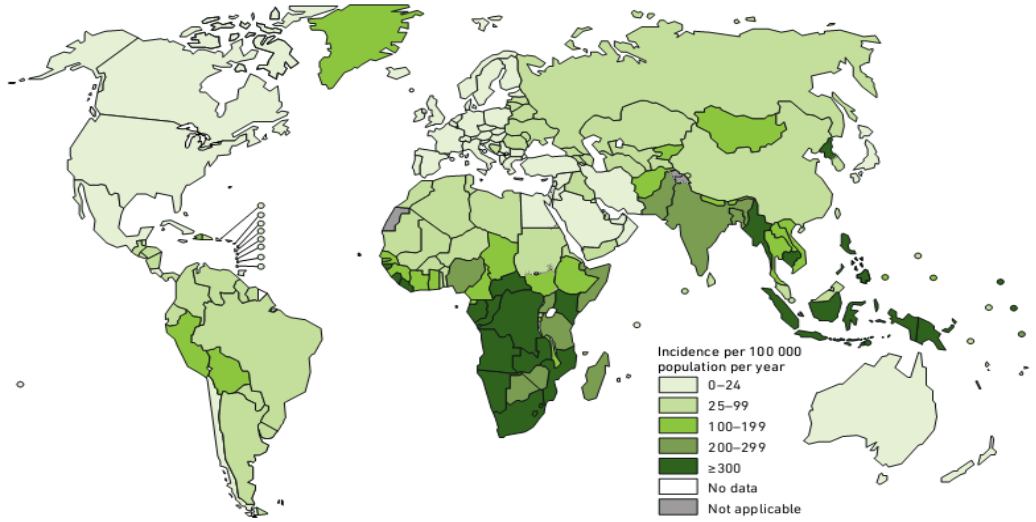


Figure 2. The global incidence rate of TB (2017). *Globally in 2017, there were an estimated 10.0 million incident cases of TB (range, 9.0–11.1 million), 2 equivalent to 133 cases (range, 120–148) per 100000 population. Estimates rates per capita are shown in Fig. Ref: Global TB report*

The outbreak is at its peak in various parts of Latin America, Africa and Asia today. The TB epidemic saw an enormous surge at an alarming pace globally, in the late 1980s, coaxing the World Health Organization to declare TB as a "global emergency" in 1993 before it was beyond control which fortunately leads to a focused political and public debate and created an awareness about the magnitude of the disease (Daley 2019). WHO annual report, in the year 2017, stated that 6.4 million cases of TB were registered globally, accounting for an estimated 13 lakhs fatalities. Figure 2 represents the global incidence rate of TB reported in the year 2017 by WHO. The incidence rate has been on a surge since the year 2013, following the year 2009–2012 in which 5.70–5.80 million new cases were registered per year, this increase was majorly due to improved reporting system in private sector which usually went unreported in countries like India and Indonesia. The TB mortality among non-

HIV infected patients has fallen by approximately 29% since the year 2000, from 1.8 million in 2000 to 1.3 million in 2017, and by 5% since 2015 which was also the baseline year of the End TB Strategy (WHO 2018). These statistics definitely show the public health tragedy that TB is likely to cause in the anticipated future. TB is not the only public health or socioeconomic burden, but also affects individual work productivity being a disease common in individual ageing between 15 to 45 years. (Dye, Scheele et al. 1999; Gebreyohannes 2007).

2.3 Tuberculosis Infection and the Associated Risk Factors

We have discussed previously that only 10% of those infected with *Mtb* develops the full-blown disease. The remaining ones develop an effective immune response limiting bacterial multiplication developing pathology. These observations depict the role of immunological factors and other conditions that can influence the result of infection and the same would be briefly discussed, which can distinguish factors and their importance in the susceptibility or resistant phenotype in the host (Gebreyohannes 2007).

2.3.1 Genetics of the Host

The mean life span threat of developing TB in the infected person is approximately 10% as described by few reports where an increased threat of disease development the TB is reported in few individuals of a population living in comparable conditions (Raviglione, Snider et al. 1995). High consistency of TB rate does exist among monozygotic versus dizygotic twins (Comstock 1978) while few studies acknowledge environmental factors more than the host genetics to conclude susceptibility (van der Eijk, van de Vosse et al. 2007). A set of studies have described

heritability of immune phenotypes concomitant to TB, ranging from approximately 30% to 68% (Stein, Guwatudde et al. 2003; Stein, Nshuti et al. 2005). A similar study from our laboratory also determined that host immunity is determined by the pathogen and not just the host (Chakraborty, Kulkarni et al. 2013). Sufficient laboratory proof from murine experiments has shown the importance of the genetic factors of the host in determining susceptibility or resistance in host to many intracellular pathogens, including *Mtb*. Such results and conclusions indicate the importance of host genes and their importance in the outcome of TB pathogenesis. NRAMP1 gene (Natural resistance-associated macrophage protein) encoded by the SLC11A1 gene, has been reported to have multiple effects on macrophage activation is often associated with TB infection (Li, Yang et al. 2011). Further, many research groups have shown that host susceptibility/resistance to TB is an outcome of multiple gene regulation and not a single gene event, these genes regulating the outcome could be present both within MHC or outside MHC region (Bellamy 2003).

An good substantiation from murine research that MHC genes influence the susceptibility or resistance phenotype to TB in spite of many variations between studies on the involvement of the exact alleles, depending on the dose or route of infection, bacterial virulence, and various criterions used to describe a disease in a particular study (Gebreyohannes 2007). Resistance to infection was linked with an increased level of *Mtb* antigen-specific IFN-γ generation, and better delayed T-type hypersensitivity (DTH), signifying the importance of a Th1-type response to resistance (MacMicking, Taylor et al. 2003). Although it is difficult to associate MHC genes to their role in determining the immune Th1/Th2 imprint against a particular antigen, speculations could be made on the mechanisms involved based on evidence

and studies performed earlier. Combination of antigen dose and the number of antigen-specific $CD4^+$ T-cells in an unprimed animal is believed by some to determine the Th1/Th2 nature of the immune response which would be generated (Wangoo, Sparer et al. 2001). If the role of MHC genes in determination of the Th1/Th2 nature of the immune response is to be considered, we must assume few hypotheses based on the resistance/susceptibility phenotype of the host. One such hypothesis advocates that the susceptibility to *Mtb* infection can be either due to feeble Th1 response or significant Th2 element. The weak Th1 response denotes susceptibility; then we can say that susceptibility is a recessive phenotype; due to a very fragile peptide-MHC interaction in all the alleles is an obligation for the susceptibility phenotype to be expressed. Likewise, susceptibility due significant Th2 response denotes the governing character of the phenotype, as sturdy relation of peptides is regulated with the allele presiding inside the MHC which determines susceptibility irrespective of the nature of association occurring with the leftover allele (Gebreyohannes 2007).

The function of MHC and xMHC (extended MHC) genes do definitely control the immune response owing to its participation in presentation of the antigen and activation of *Mtb* specific CD3 positive T-cells, but there other antigen-specific genes which are involved engaging immune response which includes genes encoding complement, cytokines and major heat shock proteins mapped within the MHC region (Consortium 1999; Horton, Wilming et al. 2004). Genes present outside the MHC region also perform a significant role in immunity against *Mtb* infection. Mice studies have shown numerous loci on chromosome one, three, and seven known as Trl-1, Trl-2 and Trl-3, respectively as TB resistance locus, are recognized to manipulate the pathology or bacterial replication rate in the lung (Kramnik, Dietrich et al. 2000;

Mitsos, Cardon et al. 2000; Mitsos, Cardon et al. 2003; Gebreyohannes 2007). In humans, however, case studies during a disease outbreak have shown that several genes, like Mannose-binding protein (MBP), The vitamin-D receptor, IFN-γ and IL-1 receptor genes add-up to the threat of developing TB subsequent to the *Mtb* exposure (Marquet and Schurr 2001; Gebreyohannes 2007).

2.3.2 Social and Economical Aspects

Social, as well as economic factors, contribute to the risk of developing TB. Urbanization is a major reason referred to be the determining factor for the development of this endemic to an epidemic disease. Overcrowding in the human settlement region was supposed to be the primary cause for the elevated incidence rate of TB in Aboriginal-Canadians at the end of the 18th century (Hoeppner and Marciniuk 2000). Deprived social and economic factors, lesser income, meagre hygiene and living circumstances in urban settlements and overcrowding, develop an ideal niche for the human-to-human spread of *Mtb*. The decline in medical facilities due to economic crunch in various regions of the former USSR were also found partly guilty for the revival of TB in the western world (Shilova 2001; Gebreyohannes 2007). Additionally, malnutrition, homelessness, alcoholism and drug addiction are the major factors facilitating the disease progression from *Mtb* infection (Brudney and Dobkin 1992; Gebreyohannes 2007). The inter-relation between malnutrition and TB infection is comprehensively discussed previously (Gebreyohannes 2007; Boloor, Iqbal et al. 2014). However, malnutrition as a consequence of TB or predisposition to the disease is not clearly understood. Also, sufficient clinical evidence is available to show macronutrients like dietary protein, lipids, nutrients like vitamins (A, C, D), minerals like iron and zinc could negatively influence cell-mediated immunity

(Chandra 2002). Hence, it could be claimed that malnutrition can predispose people to TB, and the disease is best controlled by cell-mediated immunity. Additionally, there is a high chance of developing TB among people with the poor social and economic situation as these group of people also show high noncompliance during treatment, which may increase the danger of emergence of the multidrug-resistant pathogen (Mishra, Hansen et al. 2005; Gebreyohannes 2007). Therefore it can be said that TB is the disease of economic well being and the condition of the country's healthcare system.

2.3.3. Concomitant Diseases

Tuberculosis risk is hiked by existing chronic debilitating diseases like cancer and diabetes. Apart from the diseases, any treatment involving immunosuppressive therapy might also increase the risk of developing active TB. Currently, HIV infection has been verified to be the clear risk factor for developing active TB (Sharma, Mohan et al. 2005). In the life span of any healthy individual, the risk of developing TB is just 0.1-0.2% annually considering an average life expectancy as fifty years (Bloom and Murray 1992; Raviglione, Snider et al. 1995; Gebreyohannes 2007), Overall, poor social, financial conditions existing in the third world nations along with the HIV pandemic does influence the TB related epidemiological outcome in Saharan Africa and South-East Asia complicating management measures employed in TB endemic countries (Gebreyohannes 2007).

2.4. Etiology of Tuberculosis

In various countries, TB was considered as a hereditary disease for many centuries. Many renowned medical practitioners declined the idea that TB could be an infectious disease until Robert Koch isolated the disease-causing agent before

identifying it as infecting bacilli. This inspiration came after the discovery by Jean-Antoine Villemin, the first demonstrator of the infection causing ability of TB by conducting multiple research experimentations with rabbits, Robert Koch himself followed the path to identify the aetiology of TB (Daniel 1997). He was also successful in isolating the organism in culture medium and was able to stain it and observe this particulate matter under the light microscope. He reported a rod-shaped bacterium in 1882 naming it as "Tubercle Bacillus" (Gebreyohannes 2007).

2.4.1 Classifying the Mycobacteria

In 1896, Lehman and Neuman introduced the group bacillus. Currently, the group bacillus has been put under genus mycobacterium. (Sneath 1986). This genus represents a little curved rod-shaped particle with dimensions measuring 0.9-10.5 μm in length and 0.25-0.65μm in width. This genus consists of several pathogenic, opportunistically pathogenic and saprophytic species including the typical species of mycobacteria that cause disease in man and animals like *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. paratuberculosis* and *M. avium* (Gebreyohannes 2007). Generally, Mycobacterium is categorised into 2 wide groups: i.e. fast growers forming visible colonies which takes seven days to grow, and slow growers which take more than seven days, to form visible colonies on culture medium. The one which infects human causing TB in human and animals are the slow-growing bacteria which are classified as *Mtb* complex (MTBC). This category consists of human pathogens *M. tuberculosis* and *M. africanum* (a native of Africa); the bovine mycobacterium *M. bovis*, which also includes BCG; and *M. microti* which causes disease in rodents. In spite of the fact that humans are naturally susceptible to TB of human infecting mycobacteria and

some extent by the cattle infecting mycobacterium and additional species of mycobacterium like *M. avium* complex (*M. avium*, *M. intracellulare*, *M. scrofulaceum*, and *M. asiaticum*) does develop TB in immuno-deficient people (Gebreyohannes 2007).

Mycobacterium has an enhanced presence of complex lipids in their cell wall, accounting to around 25-45% of the dry mass of the bacterium; generated by around 35% of the bacterial genome, which is dedicated to lipid production pathways and metabolism (Cole, Brosch et al. 1998; Gebreyohannes 2007). This high lipid content makes the cell waxy, and the major components involved in this includes, peptidoglycans, glycolipids, mycolic acids and various other proteins, lipids and carbohydrates. These waxy components are partly accountable for the vigour of the bacterium which opposes the host defences, harsh chemical and physiological environments, and conventional bacterial stains (Russell 2001). The mycobacterium stains violet in colour after gram staining and therefore it is classified under gram-positive. The mycobacterium, therefore, can be properly visualized after Ziehl-Neelsen carbol fuchsin (ZNCF) staining, where the basic fuchsin from the stain binds to the acidic groups of the unsaturated mycolic acid side chains in the mycobacterial cell wall. The steamed-hot carbol-fuchsin enhances dye penetration into the lipid-rich cell wall of the mycobacterium. The basic fuchsin cross-linked to the acidic side chains in the mycobacterial cell wall providing stability to the dye-complex and thereby increasing its resistance to de-colourisation agents like mineral acids (ethanolic HCl). Therefore the terminology 'acid-alcohol-fast' is usually preferred to depict the bacillus (Collins and Grange 1985; Gebreyohannes 2007) .

2.4.2 Mycobacterium Culture and Characteristics

In the whole mycobacteria genus, the MTBC, in particular, is complex to grow in any culture media. They have a special requirement which needs growth-medium high in serum albumin, glycerin and various other growth factors and micronutrient elements. The egg-based (Löwenstein-Jensen or Stone Brink's) medium or agar-based Middlebrook 7H9 medium are among the commonly used culture medium for culturing mycobacterium. Mycobacterium is known to optimally thrive in a liquid culture medium like Middlebrook 7H9 broth, which is free of egg and agar but rich in glycerol, albumin and micronutrient-elements. Glycerin probably suppresses the growth of *Mycobacterium bovis* and BCG, which thrive and flourish better in the presence of pyruvate based medium instead of glycerol (Reichman and Hershfield 2000; Gebreyohannes 2007). The mycobacteria, classified under MTBC group, have a very slow-growing rate with a generation time of around 18-24 hr. The single cells of the clinical specimen or culture after plating the on the culture agar plate takes several weeks of incubation time to form visible colonies. The slow growth of mycobacteria is generally due to the hydrophobic nature of the cell wall. This results in clumping of the bacteria together, thereby becoming lesser permeable to any nutritious material into the cell (Sneath 1986). Therefore, surfactant Tween 80 is added to cultures of mycobacteria which wet the surface of the cells and further leads to the dispersal of the clumps easily inducing a rapid growth of the bacilli. Tubercle causing bacillus is an obligate aerobe, but its growth is optimal in a humidified incubator in the presence of 5-10% CO_2. The optimal growth temperature of this bacteria is 37^{o}C except for the avian type, *M. avium*, which requires a temperature of 40-42^{o}C. This intracellular bacterium is facultative; while human leprosy causing bacteria, *M. leprea*, is also

intracellular but cannot survive in artificial media or culture. Many attempts were made to grow this bacterium in culture media once isolated from the mice or guinea pigs, but everyone was unsuccessful due to the unique temperature requirements and nutrition requirement for its optimal growth. The Armadillo (nine-band), having a lower body temperature similar to most of the mammals, were found to be the perfect host and animal model to study the course of the disease experimentally (Kirchheimer 1975).

2.4.3 Mycobacterial Biology

The mycobacterial chromosome is composed of 4,411,500 bp which harbouring around 4100 genes. Most of the genetic material is dedicated to the generation of enzymes and lipids implicated in the development of its complicated cell wall (Cole, Brosch et al. 1998). Almost 52% of the genes can be assigned to a function for the thousands of proteins encoded by those genes. Around 367 genes are unique to the virulent *Mtb* strain (Camus, Pryor et al. 2002; Gebreyohannes 2007). A number of these genes were supposed to play a vital task in the distinctive biology of the bacilli, which allows them to sustain one of the most hostile conditions of the host's body. In adverse conditions, due to lack of oxygen, nutrition or host's immune response, the bacillus can enter into dormancy by suppressing the transcription of all the genes involved in growth, while up-regulating various latency genes controlled by the dormancy regulon (DosR) (Boshoff and Barry 3rd 2005; Gebreyohannes 2007). Whenever the situation is appropriate for the replication of the bacteria, and other genes, encoding for revival-promoting elements, are usually up-regulated. Such re-activation of the dormancy in mycobacterium leads to replication and infection in nearby macrophages which is known as reactivation disease (Cohen-Gonsaud, Barthe

et al. 2005). Therefore it is important to understand the biology of mycobacteria during the dormant and the replicative stage, and it is important to identify genes expression and lipid composition which are prevailing in every stage, and this might consequently help in developing an effective vaccine against *Mtb* in every stage of the pathological progression.

2.4.4 Structure and functions of Mtb cell wall components and their role in immune regulation.

The structural definition of the cell wall of Mycobacterium species begun during the 60s and 70s followed by the recent development in NMR and Mass spectroscopy and exhaustive bioinformatic data leading to deeper insights unfolding the structure of the mycobacterial cell and the associated genetics and biosynthesis pathways (Brennan 2003). The expression of lipids in the cell wall of *Mtb* determines the course of infection. Figure 3 represents the cell wall structure of the mycobacterium. Multiple findings indicate that *Mtb* can re-mould the lipid composition of the cell wall as an adaptive mechanism against host-imposed stress. The lipid species (trehalose dimycolate, diacylated sulphoglycolipid, and mannan-based lipoglycans) have the ability to initiate an immunopathologic response, whereas others (phthiocerol dimycocerosate, mycolic acids, sulpholipid-1, and di-and polyacyltrehalose) have the ability to reduce the host immune responses. Therefore a detailed understanding of the important mycobacterial lipids and their role in immune regulation is essential, and hence it would be discussed ahead.

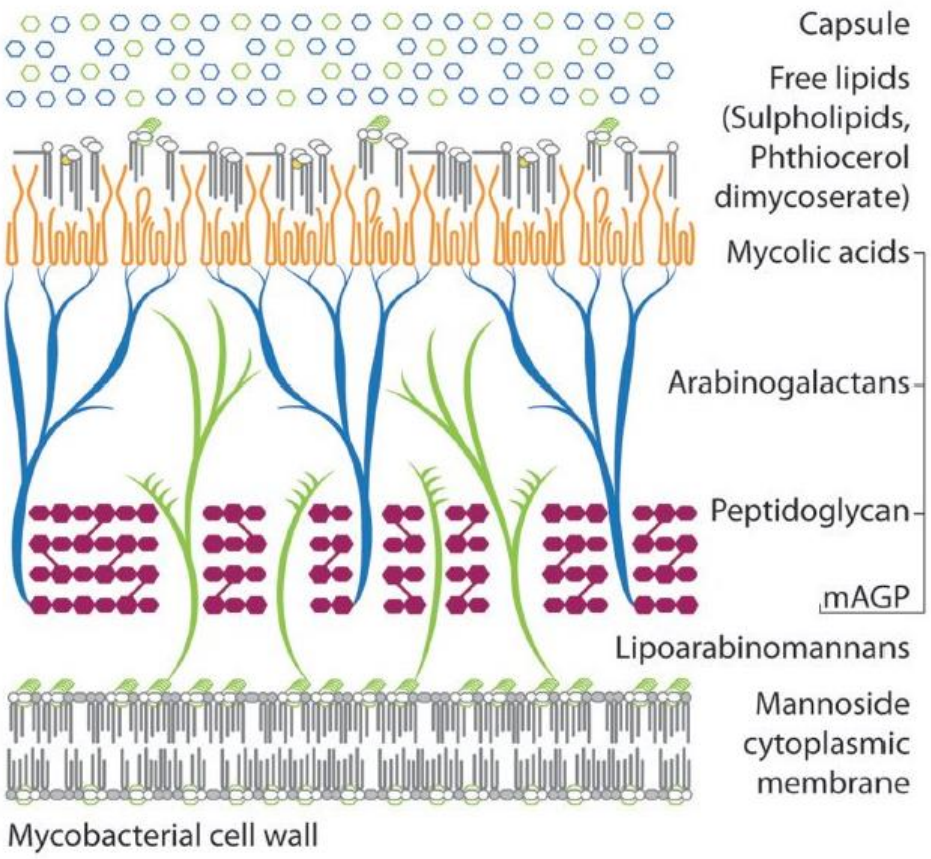


Figure 3: Schematic representation of the mycobacterial cell envelop*e. MPM, mannosyl-b-1-phosphomycoketides; PDIM, phthiocerol dimycocerosates; PIM, phosphatidylinositol mannosides; LM, lipomannan; LAM, lipoarabinomannans. Reference:* Gordon and Parish, Microbiology, 2018;164:437–439.

Table 1 represents *Mtb* lipid-induced immune response and associated metabolic pathways in lipid biosynthesis. The major cell wall lipids and their role in eliciting an immune response in the host are discussed ahead. The cell wall mycolic acids (MAs) are usually covalently linked to several molecules, like TDM and arabinogalactan in Figure 3 (Cantrell, Leavell et al. 2013). Mycobacterial TDM acts as a receptor for, macrophage inducible C-type lectin (Mincle) (Ishikawa, Ishikawa et al. 2009). Generation NO and subsequent production of TNF and MIP-2 (CXCL2) by macrophage occurs by activating mincle, leading to granuloma formation (Ishikawa, Ishikawa et al. 2009). Alternative macrophage activation occurs by a MyD88-

dependent response to TDM (Sakamoto, Kim et al. 2013). Macrophage receptor MARCO (class A scavenger receptor) along with TLR-2 and cluster CD14 recognises TDM; thereby results in an efficient inflammatory response to *Mtb* (Bowdish, Sakamoto et al. 2009). Figure 4 shows the effects of lipids on the immune responses of phagocytic cells.

2.4.4.1 Mycolic acids (MAs)

The MAs were not considered as lipids having any importance in the early immune response to the mycobacterial infection, as the fact that many free mycolic acids in the mycobacterial cell wall are generated as the hydrolysis product of TDM (Yang, Kulka et al. 2014), which might interact with host cells inducing innate immune response. Although the fact is, MAs are the most important structural lipids which makeup cell wall and envelope with a protective role. In *Mtb*, MAs are hydrophobic C54–C63 fatty acids with C22–C24 alpha side chains (Takayama, Wang et al. 2005). The 3 major class of *Mtb*-MAs prevalent are alpha, keto, and methoxy-MA (Barry 3rd, Lee et al. 1998; Takayama, Wang et al. 2005). These classes vary in the functional groups attached to the meromycolate region of the MA. Research has shown that free MAs could lead to inhibition of TLR-2-mediated pro-inflammatory response in RAW264.7 cells and in A549 human lung epithelial cells (Sequeira, Senaratne et al. 2014). MAs may comprise of a scaffold for the stimulating CD1-restricted T cells by mycobacterial lipid antigens (Layre, Collmann et al. 2009). The antigenic property of glycerol monomycolate (GroMM) depends upon, the hydroxyl -group of glycerin and mycolic acid carbon number, which was a vital trigger for T cell responses (Layre, Collmann et al. 2009). Many researchers have shown that free MAs stimulates CD1b-restricted T cells forming stimulatory complexes with CD1b

(Beckman, Porcelli et al. 1994; Zhao, Siddiqui et al. 2015; Van Rhijn, Iwany et al. 2017). Any small structural changes in trehalose-MA have been critical in the induction of pro-inflammatory response in mice while the MA-cyclopropane ring was shown to be long-term persistence determining factor for a pathogenic mycobacterial strain in rodents (Glickman, Cox et al. 2000). Furthermore, the variations in the quantity of keto- and methoxy-MAs also manipulate the growth rate of *Mtb* in the macrophage (Yuan, Zhu et al. 1998) while the loss of keto- and methoxy-MA has an association with the bacterial weakening in rodents (Dubnau, Chan et al. 2000). Further, the abundance of MA in the *Mtb* cell wall defines the progression and course of the disease in the infected individuals.

2.4.4.2 Mannan-based lipoglycans

The lipids which are involved in wall-regulation, such as phosphatidyl-myo-inositol mannosides (PIM), glycolipids lipomannan (LM) and lipoarabinomannan (LAM) modulate, the host reaction during any infection by facilitating the interaction with various receptors on macrophages and DCs (Schlesinger, Hull et al. 1994). Several reports have shown that these lipids molecules have an imperative role in mycobacterial cell wall integrity (Fukuda, Matsumura et al. 2013) and growth (Haites, Morita et al. 2005), while the lipid molecules like PIMs, LM, LAM, and mannose-capped lipoarabinomannan (Man-LAM) were shown to be significant in eliciting host defences response. Further, LM, LAM, Man-LAM and PIMs have been confirmed for their role in modulating the host immune responses. Phospho-myo-inositol-dimannoside (PIM2) and phospho-myo-inositol-hexamannoside (PIM6) were shown as TLR-2 agonists (Gilleron, Quesniaux et al. 2003) while LM was shown to stimulate IL-12 production and apoptosis unlike PIM2 (Dao, Kremer et al. 2004). Furthermore,

LAM was shown to inhibit IL-12 generation by DCs while it modulated *Mtb*-induced macrophage apoptosis in human (Nigou, Gilleron et al. 2002). Also, Man-LAM mimics MR, which enable *Mtb* to evade host defence mechanisms by inhibition of phagosome maturation and IL-12 and TNF and simultaneously increasing IL-10 cytokine by DCs or monocytic cells (Jozefowski, Sobota et al. 2008). In the *Mtb* cell wall, the LM: LAM ratio has the ability to affect the virulence of the bacterium (Dao, Kremer et al. 2004). Krishnan et al. analysed the reaction of BMMs, DCs, and rodents to whole lipid extract from the 6 main genetic lineages of *Mtb* and confirmed that the cell envelope lipids affect *Mtb* virulence and innate immunity parameters (Krishnan, Malaga et al. 2011). This is a clear indication of *Mtb* cell wall lipids determining the outcome of initial phase *Mtb* infection. If *Mtb* survives initial phase from antimicrobial host effector cells, the chronic infection phase begins. The other cell wall lipids well-known for eliciting or manipulating an immune response include PDIM, DAT, and polyacyltrehalose (PAT) (Karakousis, Bishai et al. 2004). Researchers have shown the beta-oxidation pathway to be the major source of *Mtb* lipids in response to host-induced stress during the chronic phase of infection (Mendum, Wu et al. 2015). The DAT, PAT, sulpholipid-1 (SL-1) and PDIM are the beta-oxidation products. These lipids are imperative in reducing the host immune response, thereby protecting the bacillus.

2.4.4.3 Di-and polyacyltrehalose

Mtb glycolipids (Diacyl-trehalose DAT, polyacyl-trehalose PAT and triacyltrehaloses), localized into in periphery of the cell wall are known to possess di- and tri-methyl side-chain of fatty acids. These glycolipids are known to be present in several pathogenic species of mycobacteria (Hatzios, Schelle et al. 2009). The

production of PAT from DAT is a distinct mechanism by which *Mtb* protects itself against the bactericidal host in an infected cell. The isolated, purified form of DAT from *Mtb* H37Rv and M.fortuitum ATCC 6841 inhibit the propagation of mouse T-cells in a concentration-dependent manner *in vitro*, suggesting its pragmatic role in T cell hypo-responsiveness and immunosuppression involving *Mtb* infections (Saavedra, Segura et al. 2001). Mutating *Mtb* msl3 gene defines the importance of polyacylated trehaloses in mycobacterial virulence. The msl3 gene mutant can infect both activated, and resting macrophages from BALB/c mice 2-4 fold more efficiently as compared to msl3 intact H37Rv (Rousseau, Neyrolles et al. 2003).

2.4.4.4 Sulpholipid-1 (SL-1)

Sulpholipids were discovered by Middlebrook et al. (Middlebrook, Coleman et al. 1959) during the half of 20th century. Multiple *in vivo* and *in vitro* experiments have shown that the complex lipids are instrumental in determining the virulence of the *Mtb* (Queiroz and Riley 2017). The role of diacylated sulfoglycolipids (Ac2SGL) and SL-1 in immunopathogenesis by *Mtb* could be found in the literature (Goren, Hart et al. 1976). Nonpathogenic mycobacterium species lack SL-1 while it is the major lipid on *Mtb* surface of the pathogenic mycobacterium. Therefore SL-1 and its precursor, Ac2SGL, have become the central target for the development of new biomarkers. Isolated and purified SL-1 from *Mtb,* H37Rv strain inhibits the formation of mature phagolysosome in murine peritoneal macrophages cultured *in vitro*. It was previously shown by research laboratories that the sulfatide leads to imbalance in the phagosomal or lysosomal membranes in the host cells (Queiroz and Riley 2017). SL-1 inhibits macrophage priming, leading to a decreased release of superoxide anion (O_2^-),

subdued phagocytosis and lesser release of IL-1 (Pabst, Gross et al. 1988), inferring the role of neutrophil signal transduction pathway (Zhang, English et al. 1991). Studies have shown that SL-1 causes mitochondrial surface damage in association with TDM, without inducing any toxic effect in mice if injected intraperitoneally with SL-1 (Kato and Goren 1974). Studies have shown that Ac2SGL has the ability to attach to the MHC-like receptor specific for lipids known as CD1b and thereby produce IFN-γ and IL-2 post-stimulation (Gilleron, Stenger et al. 2004; Queiroz and Riley 2017)

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2.4.4.5 Phthiocerol dimycocerosate (PDIM)

Cox et al. isolated *Mtb* transposon mutants which were unable to replicate within the mouse lung (Cox, Chen et al. 1999). These transposons induced mutation in the mas, fadD28 and mmpL7 genes which are known to code several enzymes and proteins essential for the synthesis and subcellular localization of PDIM (Cox, Chen et al. 1999; Queiroz and Riley 2017). Several enzymes were shown to be involved in PDIM biosynthesis (Trivedi, Arora et al. 2005). It was observed that a PDIM-deficient *Mtb* H37Rv mutant does not replicate properly and elicits lesser lung tubercles as compared to a PDIM-producing H37Rv strain in guinea pigs (Goren, Brokl et al. 1974). Earlier, a study has shown that *Mtb* mutant strains which were not able to synthesize PDIM were weakened in murine model (Cox, Chen et al. 1999). Such strains were not only defunct in cell wall synthesis but showed anomalous colony characteristics on a solid culture medium, as compared to the wild type, this also impaired its growth in mouse lungs (Cox, Chen et al. 1999; Queiroz and Riley 2017).

PDIM also has a role in cell wall permeability, and therefore sub-cellular translocation of PDIM led to enhanced cell wall permeability (Queiroz and Riley 2017).

The generation of phenolphthiocerol-based DIM variants (PGLs) has been linked to a hyperlethality phenotype in a mouse model of TB (Reed, Domenech et al. 2004). The DIM variants are produced by *M. bovis*, *M. kansasii*, *M. marinum*, *M. ulcerans*, *M. microti*, *M. africanum*, *M. haemophilum*, and *M. gastri* (Onwueme, Vos et al. 2005) which are pathogenic to humans (Katoch 2004; Velayati, Boloorsaze et al. 2005) and are associated with more severe clinical manifestations in a rabbit model of *Mtb* meningitis (Tsenova, Ellison et al. 2005). On the other hand, a PGL-deficient *M. bovis* mutant is attenuated in a guinea pig model of infection (Collins, Skou et al. 2005). The overexpression of PGL by *Mtb* or addition of purified *Mtb*-PGLs to macrophages decreases the release of pro-inflammatory cytokines, whereas loss of PGLs is correlated with enhanced release of cytokine by *Mtb*-infected macrophages (Reed, Domenech et al. 2004). The PGLs of *M.bovis* reduces pro-inflammatory cytokines while PGLs from *M. leprae* induces nerve demyelination, an important factor contributing to the nerve function impairment (Rambukkana, Zanazzi et al. 2002) and invasion of Schwann cells in leprosy patients (Ng, Zanazzi et al. 2000). *M. leprae.* PGLs also obstruct antigen-presentation and suppress the proliferative ability of T-cells to mitogens (Onwueme, Vos et al. 2005). The PGLs might also contribute to virulence in other mycobacteria, as seen in the recent observations in which DIM-deficient mutant of *M. marinum* is attenuated in a goldfish model of infection (Ruley, Ansede et al. 2004).

Lipids	Features of the biosynthetic pathway/Possible biosynthetic pathway	Related immune responses	Induced immune activity
Trehalose dimycolate	Not described	• Inhibits leukocyte migration • Elicits granulomatous response • Activates macrophage by C-type lectin and MyD88	Enhanced immunopathology
Mycolic acid	Utilizes acetyl-CoA and malonyl-CoA as substrates/Beta-oxidation (glycerol monomycolate)	• Induces macrophage cholesterol accumulation • Stimulates CD1-restricted T cell • Not recognized by TLR-2 and TLR-4	Mostly dampened immunopathology
Mannan-based lipids	Not described	• PIM is recognized by TLR-2 • LM induces IL-12 production and apoptosis • LAM inhibits IL-12 production and modulates macrophage apoptosis • Man-LAM is involved in host defense evasion; suppresses the production of IL-12 and TNF	PIM and LM enhance immunopathology while LAM and Man-LAM dampen immunopathology
Di-and polyacyltrehalose	Utilizes propionyl-CoA and methylmalonyl-CoA as substrates/Beta-oxidation	• Inhibit T cell proliferation • Inhibit macrophage uptake	Dampened immunopathology
Diacylated sulphoglycolipid	A sulpholipid-1 precursor. It uses propionyl-CoA and methylmalonyl-CoA as substrates. Beta-oxidation	• Recognized by CD1b receptor • Stimulates expression of IFN-γ and IL-2 in $CD8^+$ cells	Enhanced immunopathology
Sulpholipid-1	Utilizes propionyl-CoA and methylmalonyl-CoA as substrates. Beta-oxidation	• Inhibits phagolysosome formation • Inhibits macrophage priming, phagocytosis and IL-1 release.	Dampened immunopathology
Phthiocerol dimycocerosate	Utilizes both malonyl-CoA and methylmalonyl-CoA as substrates/Beta-oxidation	• Plays a role in cell wall permeability and in protection against bactericidal activity.	Dampened immunopathology

Table 1: Mycobacterium tuberculosis lipid-associated immune response and major metabolic pathways in lipid synthesis

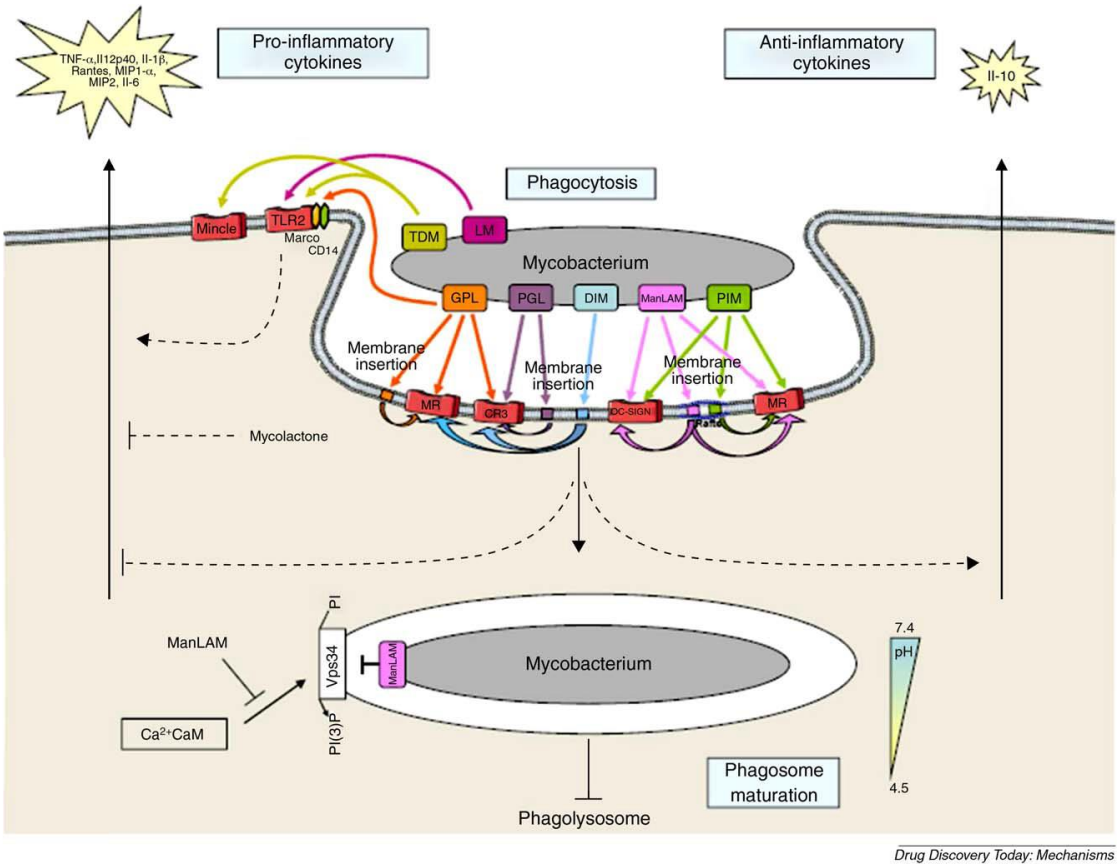


Figure 4: Effects of lipids on the immune responses of phagocytic cells. *This schema summarizes several data reported in the literature. Several lipids at the surface of mycobacteria are recognized by phagocytic receptors: complement receptor 3 (CR3), mannose receptor (MR) or DC-SIGN. They act either directly as ligands or indirectly by insertion into the host cell membrane, notably into raft domains (LAM or PIM), leading to a rise in the efficiency of receptor-mediated phagocytosis. Once internalized, pathogenic mycobacteria accumulate in pathogen-friendly phagosomes which do not mature toward fusion with lysosomes. These vacuoles are characterized by incomplete luminal acidification and poor bactericidal functions. Phagosome maturation arrest includes the action of ManLAM, TDM, DIM. ManLAM interferes with membrane trafficking initiated by $Ca2^{+}$ fluxes. The infection of host cells by mycobacteria also modulates their cytokine responses. Mycobacterial lipids participate actively in this process either through the engagement of phagocytic receptors or the recognition of activation receptors such as TLR2.*

2.4.5 Molecular Typing & Diagnosis of Mycobacteria

Identification of mycobacterium governs effective control and prevention measures to TB, which depends excessively on accurate diagnosis. There has been a lack of new dependable techniques which makes a compulsory dependence on the hundred-year-old methods like light-microscopy, mycobacterial culture and the Mantoux-tuberculin skin test. The easiest, economical and fast method of direct identification is the microscopic examination of clinical specimens; however, it differentiating between the pathogenic and the non-pathogenic species of mycobacteria is difficult by this method. This method also lacks the sensitivity of, as it requires around 9,000-60,000 per ml of mycobacteria in every sputum sample to generate a positive diagnosis (Collins and Grange 1985; Gebreyohannes 2007). The examination of culture developed from the clinical samples can take up to 4-8 weeks of time before it reaches the medical examiner, providing a definitive but a delayed diagnostic of TB infection which at times it too long and late for the treatment. The Mantoux-skin test is a routine test used for cattle, although the specificity of this test is very less. Further, this test cannot determine infection to the host from pathogenic or non-pathogenic species of mycobacterium, between new and chronic infections or recovered patients. This test also gives false-positive reactivity in BCG vaccinated population (Gebreyohannes 2007). The advances in research methodology have to lead to the development of techniques based on molecular biology, which have ascertained faster detection of the bacteria directly from clinical samples. Such identification not only ascertains mycobacterial species or strain but also provides information on transmission dynamics of TB, which could help discriminate between an external infection and latent reactivation of TB.

PCR or Polymerase-Chain-Reaction is based upon the amplification of mycobacterial DNA, and it has been used in swift detection of the bacteria directly in the clinical samples, be it man (Siyar, Uddin et al. 2018) or animal (Song, Tan et al. 2018). DNA Fingerprinting techniques were developed in the year 1993 for both detection and identification of species/strains of mycobacteria involved in TB (Van Embden, Cave et al. 1993). Restriction-fragment-length-polymorphism (RFLP) is based upon the DNA fragment length and repetitive DNA elements, i.e. Insertion sequences (IS) detected after digesting the genomic DNA with a particular restriction endonuclease (Gebreyohannes 2007). The common IS considered for studies is *IS6110* which can be found in almost every species of the MTBC group and, is absent in other organisms like non-pathogenic environmental mycobacteria (Van Soolingen, De Haas et al. 1994). The copy number of *IS6110* defers from 0 to 25 and the relative number and position of the IS differentiate between various strains/species of mycobacteria. However, molecular epidemiology study using *IS6110* for *M. bovis* in cattle is vulnerable since, most of the strains carry just a single copy of *IS6110*, which is mostly found at a unique single chromosomal locus (Collins, Erasmuson et al. 1993). DNA fingerprinting (*IS6110* or other short repetitive DNA elements as molecular-probes) have been used in the multiple studies involving global epidemiology or mycobacterial transmission (Gebreyohannes 2007; de Viedma, Mokrousov et al. 2011) but also distinguish exogenous infection and endogenous TB reactivation (Flynn and Chan 2001). This method, with alternate genetic molecular-markers, are widely used to study M.bovis and its global epidemiology (Wilkinson, Bishop et al. 2017) and in the identification of the origin of infection in cattle herds (Perumaalla, Adams et al. 1996). These markers are also important in the

epidemiology and zoonotic studies of *M. bovis* in man and animals (Jemal 2016). In spite of all the positives the major restriction of DNA fingerprinting is its requirement for having a culture-grown bacteria far enough so that the DNA is extracted. Such DNA extraction requires months to pass before the result is available with the medical practitioner before identification of the *Mtb* into species/strains or their drug sensitivity to available antibiotics. Recently, many improved molecular techniques, such as spoligotyping, Mycobacterial Interspersed Repetitive Units (MIRU), and DNA microarrays have been formulated which has the ability to detect simultaneously, the types of various members of the MTBC group. These methods could be employed to discriminate *M. bovis* from *Mtb* infections (Duarte, Domingos et al. 2010), and to identify multi-drug resistant strains of *Mtb* (Luo, Jiang et al. 2011). The cross-species dissemination of *M. bovis* in wild and domestic animals was demonstrated earlier by using spoligotyping in a few studies (Aranaz, Liebana et al. 1996; Serraino, Marchetti et al. 1999; Gebreyohannes 2007). Spoligotyping is a molecular technique famed for its rapidness, sensitivity and reliability. These genetic tools are also widely used to distinguish *M. bovis* and the BCG vaccine, which are genetically close, and also in the formulation of new anti-TB vaccines (Jiang, Liu et al. 2015).

MIRU-VNTR typing utilises differences in repetitive sequences which are not under selection pressure and relatively evolve at a fast pace, making them a suitable prospect for molecular epidemiological investigations and surveillance. MIRU-VNTR genotyping utilises a standard set of 24 loci which is considered as the international gold standard, and it is currently in use for routine *Mtb* genotyping globally (Supply, Allix et al. 2006). The discriminatory power of MIRU-VNTR typing usually depends

upon the number and set of loci used and therefore could be enhanced by the inclusion of hypervariable loci especially in the prevalence of highly homogenous genotypes (East Asian lineage, e.g. Beijing) (Allix-Beguec, Wahl et al. 2013).

2.5. Immunity and Immunopathology in Tuberculosis

Each year *Mtb* infects around 0.1 billion individuals, out of those only 10 million individuals develop the clinical disease within a few years post-infection. These data suggest most of the individuals produce an immune response which may restrict but might not completely alleviate infection (Flynn and Chan 2001; North and Jung 2004; Gebreyohannes 2007). The incomplete removal of mycobacterium in the host is sustained as latent infection. These individuals have a higher chance of developing reactivation and bloom into a full disease anytime in the life of the host (Boshoff and Barry 3rd 2005). Also, TB prone people are at also at the risk of developing chronic inflammation as the host directs its immune responses towards the mycobacterium but ends up inflaming the infection site if the bacterium is not cleared. Post-infection with *Mtb*, the even the susceptible host generates a protective type of response to control the replication of the bacterium. However, the generated Th1-type response is sometimes too small to completely arrest the mycobacterial multiplication (Gebreyohannes 2007); which leads to the progressive growth of the bacterial population in spite of the limited immune response. This further leads to the intensification of Th1-type response induced bilateral tissue damage. This implies that the immune-reaction which protects against TB is also accountable for the pathology associated with *Mtb* infections. Therefore scientists infer it through the use of the term "double-edged sword" to describe such immune response developed against TB.

Another active immune-cell population of T-cells, better known as the Th17 cells, is described to have a role in the immunopathology of *Mtb* infection (Gebreyohannes 2007). Pro-inflammatory cytokine, IL-17 is secreted by Th17 cells, is a potent cytokine and is responsible in the pathogenesis of many infectious, autoimmune and inflammatory diseases. Thus, considering the pathogenesis of TB and the nature of the immune responses will be crucial in the development of universal and effective immunization which could kindle the development of protective immunity without affecting pathology (Gebreyohannes 2007).

2.5.1. Pathology of Tuberculosis

Mycobacteria can get the entry to the host body through different routes mainly via lungs through breathing and also through the stomach by ingesting contaminated food and water. Primary infection is established by the bacterium at the site of entry after accessing the host. Many textbooks and review articles discuss in details pathogenesis and development of lesions post-infection with the virulent *Mtb* strains. Therefore, I have discussed the pathogenesis of TB concerning pulmonary TB following aerosol infection. Infection with *Mtb* can even occur after inhaling 1-5 bacilli in a droplet nucleus from air exhalation of a coughing infected individual. Alveolar macrophage engulfs these bacilli in the lung alveoli and would clear the bacteria before the infection is established. However, if the alveolar macrophages fail to kill the bacterium, blood monocytes would be recruited to perform the same function at the site infection where differentiation of these monocytes into macrophages takes places.

The main focus of infection is established in any region of the lung, but it affects the vulnerable part the most, which is the “apex” or upper part of the lung by exponentially replicating bacteria while monocytes and other inflammatory cells continue to be recruited at the site. The periphery of the granuloma consists of Langerhans cells (multinucleated giant cells), epithelioid cells, alveolar macrophages, while a centre consists of a solid necrotic region(Gebreyohannes 2007). The necrosis at the centre of the granuloma is unfit for the mycobacterial replication, which is full of dead or latent bacteria located both inside and outside of the macrophages. Infiltrating lymphocytes (T-cells and B-cells) and secreted cytokines make up the granuloma (Ulrichs and Kaufmann 2006). Fibroblasts also lead to the creation of the granuloma and then limit the dissemination of the bacterium into the nearby tissue. Encapsulation of the granuloma is the process which is under the influence of macrophage-derived cytokines, mainly TNF-α (Saunders, Tran et al. 2005; Gebreyohannes 2007).

In many animal studies, anti-TNF-α antibodies resulted in reactivation of TB in the latent disease setting. Further macroscopic nodules known as tubercles are formed by formation of fibrosis around the granuloma and surrounding tissue. These lung tubercles are diagnosed by X rays examination and the serve as the primary diagnostic markers for human pulmonary TB. The macrophages upon activation kill most of the mycobacterium present inside the granuloma of the infected individuals; the granuloma then gradually then shrink and progressively calcify. However, many mycobacteria enter dormancy and get activated at a later phage, when the host is immuno-compromised, resulting in reactivation TB. The mycobacterium also can enter cells with restricted antigen-presenting ability, like endothelial cells or

fibroblasts and enter dormancy. Such a mechanism is developed by the bacterium through evolution to avoid immune surveillance, creating a reservoir for the bacilli during unfavourable replication conditions (Cassidy 2006; Gebreyohannes 2007). Many supporting evidence in patients from TB endemic regions show the presence of mycobacterial DNA in the cells other than macrophages in lung tissues who died of other causes having no visible TB lesion verify that *Mtb* is not just macrophage-specific (Hernandez-Pando, Jeyanathan et al. 2000). The bacterium is not well contained inside the granuloma in the susceptible individuals, and therefore the granuloma swells and grows in shape as well as in cellularity, creating caseous lesions at the centre mainly because of cellular necrosis. Such granuloma might liquefy and rupture, leading to cavitations in the lung, which are highly infectious. A persistent cough might be caused due to this, which produces blood-laced sputum harbouring multiple bacilli. The ruptured granuloma leads to spread of the mycobacterium into the multiple organs via blood or lymph, creating a granular-seed like lesions throughout the body known as “miliary tuberculosis”. Miliary TB has the ability to threaten life if a large number of bacteria move through the blood and spread all over the body. Further, the course of the disease or the clinical symptoms is so diverse that the diagnosis of miliary TB before death is not easily achievable (Gebreyohannes 2007).

2.5.2. Protective Immunity against Tuberculosis

Mycobacteria possess several protein antigens which are highly immunogenic and can induce both humoral as well as cell-mediated immunity (Aguilar and Rodriguez 2007). The role of humoral immunity or antibodies have been

debated in control of TB and inferences have been drawn to show that antibodies play a lesser role in protective immunity against mycobacteria as the evidence are hard to come by. Cell-mediated immunity, on the other hand, is widely considered to be prominent against chronic intracellular pathogens (Achkar and Casadevall 2013). Many studies with mice (Salgame 2005; Redford, Boonstra et al. 2010) and comparison between TB patient and healthy control in humans (Jasenosky, Scriba et al. 2015; Amelio, Portevin et al. 2017) supports the observation that cell-mediated immunity is governed by the Th1 cytokines, predominantly IFN-γ, which is effective in controlling the disease exacerbation. TB patients are generally reported to have higher levels of both Th1 and Th2 cytokines in their blood; even though Th1 cytokines are necessary for protection, their mere presence may not indicate protection, suggesting that Th2 cytokine probably suppress the protective role of Th1 cytokines (Gebreyohannes 2007). Further, in spite of high levels of Th1 cytokines in TB patient's effective control of *Mtb* infection requires additional effector cells, apart from the Th1-cells. Many cells apart from Th2 cells like regulatory T-cells participate in the down regulation of Th1 cells in TB patients (Shen, Zhao et al. 2013). In the upcoming sub-sections, I have tried to review various evidences showing the role of different immune cells and their relative importance during mycobacterial infection.

2.5.2.1. $CD3^+$T-cells

The T-cell repertoire in any healthy individual is formed as a result of thymic selection (positive and negative), giving naive $CD4^+$ and $CD8^+$ T cells a milieu to interact, and be primed, with potential "foreign" antigen in the secondary lymphoid tissues. Antigen engagement via the T cell receptor (TCR) further generates the

repertoire of antigen-specific T cells and most likely directs the functional attributes of the T cell. Many phenotypes of both $CD4^+$ and $CD8^+$ T cells have been identified to have varying functions. The fundamental understanding of the T cell immunity is to understand the different types of T cells and their mode of expansion and regulation during their encounter with antigen. Various cells of the adaptive immune system, conflict those of the innate immune system, interacts with the external agents in a highly specific way, i.e., they bear specificity, heterogeneity, and memory. Such functions are primarily taken care by two types of cells that in virtue, recognise the antigen. i) The thymus-dependent or T lymphocytes, involved in the cellular responses against intracellular pathogens, organ transplants, and malignant cells. ii) The bone marrow or bursal-dependent B lymphocytes, providing humoral immunity, i.e., antibody-mediated immune responses against extracellular pathogens, their toxins, and other environmental substances. The antigen-presenting cells (APCs) include dendritic cells, macrophages, and B cells which are instrumental in the presentation of antigen to T cells. APCs take up major protein antigens, process them into peptides, and get the peptides to bind the major histocompatibility complex (MHC) molecules, and present these antigens on their cell surface, where they could be easily recognized and bind antigen receptors on T lymphocytes.

The T cells can be experimentally identified by a surface cluster of differentiation (CD) molecule named CD3 which is a multimeric protein complex composed of four distinct polypeptide chains; epsilon (ε), gamma (γ), delta (δ) and zeta (ζ). The polypeptide chains assemble and function as three pairs of dimers (εγ, εδ, ζζ). Therefore anti-CD3 antibody can be used against this efficient T cell marker (Chetty and Gatter 1994). The CD3 complex serves as a T cell co-receptor

which non-covalently engages with the T cell receptor (TCR) (Smith-Garvin, Koretzky et al. 2009). The $CD3^+$ cells are comprised of two major groups: the CD4 and CD8 populations. The CD4 cells show helping activity (T helper or Th activity) to other populations of cells, and therefore they could be subdivided into at least Th1, Th2, Th9, Th17 and T regulatory (Treg) cells, each of which has a characteristic profile of cytokines which they generate. Figure 5 represents the major T helper cell populations, their required stimulus, their mode of action and targeted response.

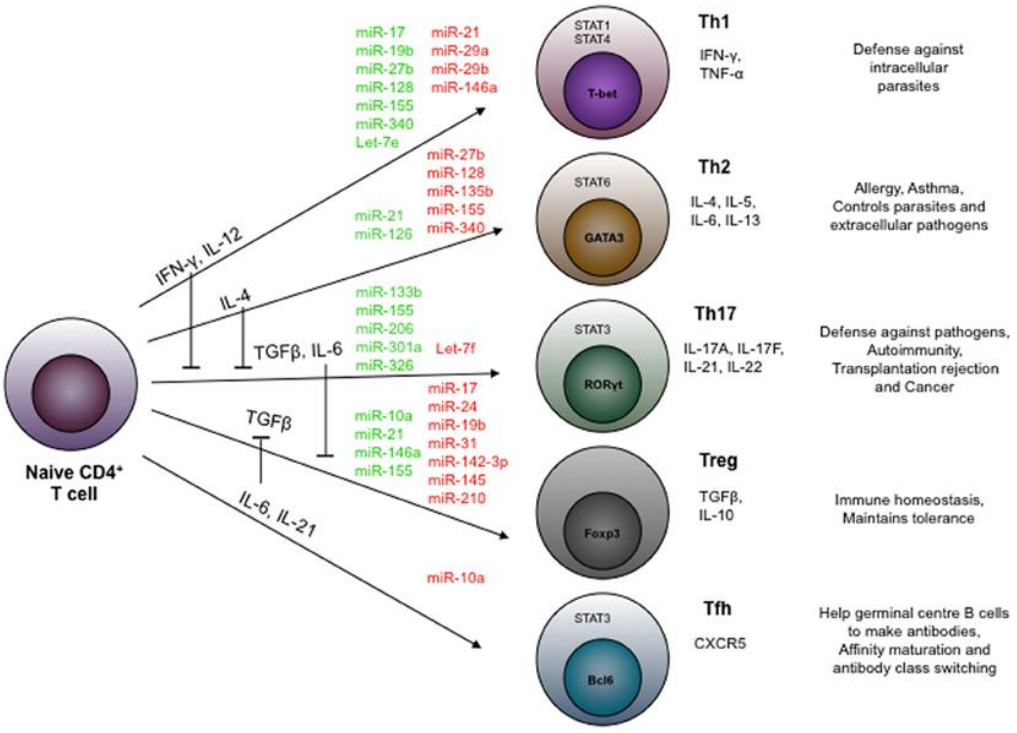


Figure 5: Brief information about the "Four" CD4 T helper cells and their fates, functions, unique products, characteristic transcription factors, and cytokines. *The different $CD4^+$ subsets are generated from the naive T cells by the different cytokines. Each $CD4^+$ subset produces a different type of interleukins. Zhu, Jinfang et. al. Blood 112.5 (2008): 1557-1569.*

The CD8 T cytotoxic population is the second prominent set of T lymphocytes that plays a role in killing target cells; they are sometimes categorized as Tc1 and Tc2 sub-populations similar to that of Th1 and Th2 cells. Together, the T lymphocytes

facilitate a central role in the orchestration of all major roles of the adaptive immune system and perform four central responsibilities. i) Promoting inflammation by cytokine production (Th1 and Th17 cells) ii) Helping the B lymphocytes (Th2 cells) iii) Regulating the cells by immunosuppressive responses (T regulatory cells) iv) Killing of unwanted cells (CTL). Many such observations support the concept that T-cells are crucial in protective immunity against mycobacterial infections. In humans, the enhanced vulnerability of HIV/AIDS infection ($CD4^+$ deficiency) to co-infection with TB demonstrates the role of $CD4^+$ T-cells in protective immunity against mycobacteria.

2.5.2.1.1 $CD4^+$ helper cells

$CD4^+$ T helper cells do have a central role in immune protection by helping B cells make antibody, inducing macrophage to increase microbicidal action, recruiting neutrophils, eosinophils, and basophils to the site of infection and inflammation, through cytokines and chemokine production. Distinct $CD4^+$ Th cells have diverse roles depending upon the cytokines secreted by them once being stimulated. $CD4^+$ Th cells are classified into Th1 and Th2 on the basis of the cytokines they produce or require. Th1 cells require IL-12 for their stimulation, and they produce type 1 cytokines, like IL-2 and IFN-γ. On the other hand, Th2 cells require IL-4 which leads to their stimulation and produce type 2 cytokine, such as Interleukin-4, -5,-10 and -13 (Mosmann 1991; Gebreyohannes 2007; Raphael, Nalawade et al. 2015). There is compelling evidence in the research literature that the central source of IL-10 is *Mtb* exposed macrophages and the Tregs. The Th1 and Th2 cells are known for their distinct functions. Th1 cells and the cytokines they produce helps in the activation of cells which effectively controls intracellular pathogens whereas the Th2 cells and their

respective cytokines are efficient against exogenous pathogens (Bretscher, Ismail et al. 2001; Gebreyohannes 2007). This, therefore, infers that the vaccination should guarantee the induction of Th1 cells while subduing the generation of Th2 cells upon a subsequent mycobacterial infection to be operative in controlling the development of TB. This is further confirmed by clinical findings in healthy individuals present in TB endemic areas who show elevated levels of mycobacterial antigen-specific Th1 cytokines and as compared to antagonist IL-4 producing Th2 cytokines (Demissie, Abebe et al. 2004). This does not mean that CD4 T cells are the single set of the cell, and rather they represent a chain of individual cell populations with different functions (Mosmann, Cherwinski et al. 1986). Few $CD4^+$ T-cell populations are distinguished from one another immediately after they emerge from the thymus, as "natural" regulatory T (nTreg) cells (Sakaguchi 2004; Shevach 2006) and natural killer T cells (NKT cells) (Bendelac, Savage et al. 2007). Naive conventional $CD4^+$ T cells have four or possibly more distinct fates which are determined by the signal they get during their early interaction with antigen. These four cell populations are Th1, Th2, Th17, and induced regulatory T (iTreg) cells. Mossman and Coffman recognised the Th1 and Th2 phenotypes, with Th1 cells being considered as critical for the immune response generated to intracellular microorganisms while Th2 cells for immunity to several extracellular pathogens, like helminths (Mosmann and Coffman 1989; Paul and Seder 1994). Abnormally activated Th1 cells generally lead to organ-specific autoimmune diseases, while Th2 cells were accountable for allergic inflammatory diseases.

The pro-inflammatory nature of IL-17 shows that it has an active role in pathogenesis and lesion development during *Mtb* infections. The available evidence,

from mouse experiments lacking IFN-γ, have shown increased IL-17 cytokine and corresponding Th17 cells causing tissue injury post-BCG infection while the immunopathology was not severe in normal mice infected with BCG (Cruz, Khader et al. 2006). Exogenous IFN-γ lead to a lesser frequency of BCG-specific IL-17-producing T-cells in vitro, this suggests that IFN-γ regulates the secretion of IL-17 to reduce any tissue damage during mycobacterial infections. A contrast to this finding seemed in a study in mice which deciphered that IL-17 to be important in the early immune response during *Mtb* infections by recruiting IFN-γ producing T-cells into the lung and thereby enhancing granuloma formation. An IL-17 deficient mouse was reported to produce lesser IFN-γ with impaired granuloma formation (Umemura, Yahagi et al. 2007).These data suggest that IL-17 has a protective role during the initial phase of infection, thereby contributing to the immunopathology during the chronic stage of the disease if not counter-regulated by Th1 cells.

Further, studies on the Th17 cells show their critical role in protection against microbial challenges, especially the extracellular bacteria and fungi. Few studies infer autoimmune responses by Th1 cells (Skapenko, Leipe et al. 2005), while studies with autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and few forms of inflammatory bowel diseases (IBD), attribute it to Th17 cells (Louten, Boniface et al. 2009). Also, the regulatory arm of $CD4^+$ cells, i.e. Treg cells are established as an inducible cell population which controls diverse CD4 population functions which are of prime importance in TB pathology and anti-TB vaccine; and therefore due to their contrasting functions, they are described separately.

2.5.2.1.2 Regulatory T cells (Tregs)

Another significant subset of T cells in TB pathogenesis and immunity is the Treg cell population, which limit the magnitude of antigen-specific immune responses Tregs, are important regulators of the immune system which maintains homeostasis between immune activation and immune suppression (Coffer and Burgering 2004). An overactive immune response against self and non-self antigens is physiologically harmful and may trigger the development of various chronic inflammatory and autoimmune diseases. Tregs leads to inhibition of activation and proliferation of self-reactive T cells and thereby inhibit the immune response of self-reactive T cells against self-antigens (Hori, Nomura et al. 2003; Coffer and Burgering 2004).Multiple Tregs, subsets like natural, inducible, and IL-10–producing Tregs, have been reported (Hori, Nomura et al. 2003). It is thought that Tregs saves the host from autoimmunity by elevating the Th2 phenotype while decreasing the autoreactive T cells (Paust and Cantor 2005). However, the presence of regulatory cells minimizes collateral damage to the host tissues, which in the absence of Tregs would have resulted in a strong immune response against the pathogen. Subsequently, the number of the T-regulatory-$CD4^{+}$CD25hi T-cells (Ribeiro-Rodrigues, Resende Co et al. 2006) cells increases during TB infection. This lead to an increased level of, IL-10 and TGF-β which is produced by regulatory T cells in the primary or secondary lymphoid organs (Olobo, Geletu et al. 2001) during active TB infection in human.

Any tissue damage caused by activated Th1 cells is thereby opposed by the regulatory T cells. This suppression of Th1 cells by T-reg cells results in a pathology allowing uninhibited multiplication of the pathogen, leading to a clinical illness of the host. Treg cells have been studied in a very comprehensive manner, and yet the

markers which define this subset of immune cells are ambiguous, and no agreement has been reached on this among the scientific fraternity. The Treg cell identification marker used by several studies is CD25, but it has also been found to be expressed by Teff cells and hence CD25 is not an optimal marker to study this subset of T cells (Baecher-Allan, Brown et al. 2001). The transcription factor FoxP3 is a guide control which determines the development of the functions of $CD4^+CD25^+$Treg cells (Fontenot, Gavin et al. 2003). This marker is considered along with the conventional markers $CD4^+CD25^+$ for more proper identification of this subset. The sub-categorization of Treg as natural (nTreg) or induced (iTreg) is decided on the development of nTreg ($FoxP3^+$) in the thymus, while the fate of iTreg is decided during the immune response to an infection in the peripheral lymphoid organs, i.e. gut-associated lymphoid tissue, spleen and lymph nodes. The classification of iTreg cells is described on the basis of TGF-β and IL-10 secretion.

Further, it was demonstrated that the IL-10 from $CD11b^+$ myeloid cells is essential for the maintenance of FoxP3 expression as well as Treg function, (Murai, Turovskaya et al. 2009) through direct interactions via the IL-10-receptor (IL-10R) expressed on the Treg. An additional level of immune regulation is provided by a positive feedback loop for the generation of IL-10 by Treg populations. Effect of IL-10 on T cells is not yet clear and more studies would shed some light on the significance of IL10. Further, the role of IL-10 from non-myeloid origin can be studied by T cell-specific *Il10ra* gene deletion (Pils, Pisano et al. 2010). Inactive pulmonary TB, increased levels of IL-10 was shown to be elevated in the human lungs (Almeida, Lago et al. 2009) and serum (Deventer, Deutekom et al. 1999).Various studies on PBMC obtained from pulmonary TB patients nullification of endogenous

IL-10 resulted in an increase in T-cell proliferation and IFN-γ production (Zhang, Gong et al. 1994). The cytokines IL-10 and TGF-β both were found to inhibit $CD4^+$ T-cell proliferation and IFN-γ production in PBMC of healthy PPD^+ patients (Rojas, Balaji et al. 1999) mostly by inhibiting antigen-presenting cell function.

Available literature illustrates that macrophage in mice infection precisely over-expressed IL-10, leading to a substantial reduction in RNI, increase in arginase-1, and overall reduced macrophage function. (Schreiber, Ehlers et al. 2009). The cytokine IL-10 was found in abundance during mycobacterial infection, collectively controlling arginase-1, and nitric oxide, which are important in mycobacterial cell killing. An IL-10 transgenic mice study shows a distinct inability of the mice to combat mycobacterial infection irrespective of IL-10 source, which kills the mice earlier than the control mice as the disease progresses. These observations were true in-spite of intact T-cell IFN-γ response. (Schreiber, Ehlers et al. 2009). This shows that cytokine IL-10 from both innate and adaptive sources is dynamic and regulates the responses against *Mtb* infection depending upon the stage and location of the infection. Thus, overall studies involving *Mtb* infection show that endogenous IL-10 from any cellular source may inhibit protective Th1 response through an indirect effect on macrophages or DCs and therefore lead to chronic infection although the main goal of it is to limit any immunopathology. However, these studies could not showcase the inter-relationship between IL-10 and its suppressive effect on pathogen obliteration

2.5.2.1.3 $CD4^+$/ $CD8^+$ memory T cells

Secondary effector cells for T-cell lineages, having classical memory and secondary in responses to Th1 and Th2 subsets are well characterized. The early

examination of memory in the Th17, T follicular helper (Tfh), and Treg lineages was challenging, owing to a lack of consistent fidelity in the lineage. Now, phenotypic plasticity in these primary and secondary effectors from these groups is recognized as factual understanding. The memory T cells are fundamental in coordinating antigen-specific recall responses in humans and animals. The memory T cells respond more rapidly to cognate peptide-MHC complex as compared to naïve T cells with a short lag time before it could enter the cell cycle for executing effector functions. However, superior "responsiveness" of memory cells as compared to naïve cells is not the only attribute of these cells, which results in rapid and robust induction of inflammation, but the memory cells are better armed as compared to naïve cells. Unlike naïve T cells, memory T cells are made of various subsets possessing trafficking patterns and localizations unique to them. For example, the tissue-resident memory T cells can be found in formerly inflamed tissue and function as primary responders to cognate antigen re-exposure.

Furthermore, heterogeneous circulating T memory cells add up to inflammation by either swift migration to the inflamed tissue or by responding to cognate antigen within secondary lymphoid organs and recruiting or creating more effector T cells. Improvement of vaccine design and treatment of immune-mediated diseases could be defined by studying the mechanisms regulating T cell homing and trafficking and its influence on the development, maintenance, and function of memory T cell subsets. The model of separating T cells into 'central memory' T cells (TCM cells) and 'effector memory' T cells (TEM cells), on the basis of their expression of CC-chemokine receptor 7 (CCR7) or CD62L, along with CD45RA56, is important in understanding T-cell dynamics and its importance in

compartmentalization of the immune response mediated protection in lymphoid or non-lymphoid organs. Such varied expression of CCR7 and CD62L provides critical information on the ability of T memory cells to enter into lymphoid and non-lymphoid organs. In addition, grouping $CD4^+$ and $CD8^+$ T cells as TCM or TEM cells have also been linked to disease protection due to their distinct cytokine production pattern (mainly, IFN-γ and IL-2). Naïve CD4 T cells are mature, quiescent, post-thymic T lymphocytes bearing TCRs with single-antigen specificity, and CD4 receptors that restrict the antigen recognition capacity of the TCR to only peptides presented on MHC class II molecules. Naïve cells are guided by a transcriptional program, which maintains their capacity for homeostatic trafficking and antigen surveillance but does not endow the naïve cells with significant effector capacity. Classically, these cells bear the characteristic surface receptors CD62L and CCR7. These receptors are generally believed to promote localization of naïve CD4 T cells to central and regional antigen surveillance via continual trafficking through the secondary lymphoid organs (SLOs) via the blood, though a study has questioned the essentiality for CCR7 (Forster, Schubel et al. 1999; Caucheteux, Torabi-Parizi et al. 2013).

T memory cells have been considered to differ from naive lymphocytes by altering its cell surface marker expression, by upregulating CD44 while downregulating CD45RB and L-selectin (Budd, Cerottini et al. 1987; Akbar, Terry et al. 1988). Naïve T cells could be identified on the basis of higher expression of CD62L and lesser expression of CD44, while T-memory cells on the basis of up-regulated CD44 and down-regulated CD62L expression. In our study, we included antibodies against CD3, CD4, CD62L and CD44, which were used in combination to identify naïve and memory T cell subsets in the mice study. Changes in these subsets

are evident in older mice after antigenic stimulation. Precursor cell frequencies or phenotypic deviations are indirect parameters for analyzing immunological memory to any pathogens. These cellular dynamics and the effector functions in the $CD8^+$ T-cell memory subset have been studied with many models of viral infections, including the infection with lymphocytic choriomeningitis virus (Beura, Anderson et al. 2015) and influenza virus (Pizzolla, Nguyen et al. 2017). However, immunological memory in the $CD8^+$ T-cell subset and their consequence in the vaccination response is lesser understood. *Mtb* being an intracellular pathogen, the $CD4^+$ T memory cells are widely researched as they are the main mediators of protective immunity by building immunological memory and therefore they are of prime importance to study them to develop a newer and more efficient vaccine against TB (Orme, Andersen et al. 1993; Kaufmann and Andersen 1998; Zhu, Dockrell et al. 2018).

In many classical studies with murine models, it was observed that the memory cells are developed in animals healed from a primary infection with *Mtb* (or BCG) by chemotherapy. Such memory cells could also be isolated from the thoracic duct (Lefford and McGregor 1974; Sheridan and Lefrancois 2011) and were found to be long-lived $CD4^+$ T cells (Orme 1988; Sheridan and Lefrancois 2011), which accumulated in the infected organs rapidly, thereby leading in higher number of $CD45RB^{low}$, $CD44^{high}$ cells (Griffin and Orme 1994; Andersen, Andersen et al. 1995; Bell and Westermann 2008).

2.5.2.1.4 $CD8^+$ T cells

Another important subset of T-cells which is active participant in immunity against TB infection is the $CD8^+$cell population (Gebreyohannes 2007). There have

been conflicting observations describing the role of $CD8^+$ T-cells in protecting the host against TB in mice, as the mycobacterial peptide antigens are first processed via the exogenous antigen-processing pathway and presented on MHC class II to $CD4^+$ T-cells (Gebreyohannes 2007). The high susceptibility of $\beta 2m^{-/-}$ mice lacked $CD8^+$ T-cells as well as CD1 restricted NKT cells (Bendelac, Killeen et al. 1994). Nevertheless, the route of infection and the corresponding dosage would not equal a natural infection. $CD8^+$ cell effector function is realized through 2 various mechanisms: (i) Receptor-mediated killing, by up-regulating FasL on $CD8^+$ cytotoxic T-cells (CTL) on antigen exposure, which binds to the Fas. This type of killing of the infected cells involves apoptosis, but such a mechanism is not enough to slay the intrinsic pathogen and thus may lead to the pathology if none of the elicited macrophages is available for killing the mycobacterium (Santucci, Amicosante et al. 2000). (ii) Secretion of cytotoxic granules, in which CTL cells exocytosise their granular contents targeting cells, thereby killing both the cell and the pathogen (Gebreyohannes 2007). Perforin is known to inject itself onto the periphery of the targeted cell leading to the pore formation on the periphery of the *Mtb* infected cell providing granzyme-B the right of entry to the infected cell, which induces the death of the cell by activating a cascade of caspases (Cooper, D'Souza et al. 1997; Gebreyohannes 2007; Lopez, Susanto et al. 2013). Even granzymes-B slaying the pathogen is considered to be of lesser importance in the efficient killing of *Mtb* due to the complex and high lipid composition of the *Mtb* cell wall. This phenomenon might lead to release living *Mtb* to the extracellular milieu, which would be beneficial only if the activated macrophage is available to grab and slay the released bacillus. Granulysin is one more molecule that is found in the granular composition of human

cytotoxic T cell lymphocytes, which cooperate generously with the lipid membranes, thus activating lipid-remodelling enzymes present on the mycobacterial cell wall. Therefore the direct killing of mycobacteria inside infected macrophage is caused by the release of Granulysin by CTL (Stenger 2001; Gebreyohannes 2007).

$CD8^+$ T-cells play a vital role if the bacterium is intracellular in the cell not expressing MHC class II-like epithelial cells, endothelial cells and fibroblasts. However, the mechanism of mycobacterial antigens processing and peptide loading onto the MHC class I molecule before being presented to CTL is yet not determined accurately (Grotzke and Lewinsohn 2005). Although studies have suggested various mechanisms, one of which is the "cytosolic model", which says that microbial subunits from live mycobacteria form pores on the surface of the phagosome through which protein and lipid antigens and sometimes even bacilli is leaked into the cytosol. These leaked products are processed through an intrinsic antigen-processing pathway before they are presented on the class 1 MHC complex. Although this was shown only in macrophages which were exposed to live and virulent mycobacterium, indicating that it might be due to the bacterial virulence factors (Mazzaccaro, Gedde et al. 1996). (Gebreyohannes 2007) Schaible et al. had suggested that virulent bacilli elicit apoptosis leading to the formation of apoptotic vesicles containing mycobacterial lipid and protein antigens against previous consideration that *Mtb* antigen penetrates the phagosomal membrane, accessing the cytosolic antigen processing pathway. Such apoptotic blebs are consumed by the DCs or bystander APCs, from the extracellular milieu and are loaded onto CD1 molecules and MHC class I and presented to CD1 and $CD8^+$restricted T cells, respectively (Schaible, Winau et al. 2003). These reports suggest their existence of multiple mechanisms by which *Mtb* antigens are

subsequently processed and presented on class 1 MHC leading to activation of cytotoxic T lymphocytes (Gebreyohannes 2007).

There exist a minor subset of T cells known as unconventional T-cells which are CD1 restricted. These CD1 restricted T-cells have the ability to distinguish glycolipids abundant on the bacterial cell wall, such as mycolic acid and lipoarabinomannans (LAM) and generate IFN-γ (Stenger 2001).

2.5.2.1.5 Natural Killer T cells (NKTs)

Natural killer T-cells belongs to the T lymphocyte family and are found in various tissues within the body (Bendelac, Savage et al. 2007; Bennstein 2018). Rearranged TCR of NKT cells recognises lipid antigens presented on CD1d, an MHC-like molecule, instead of MHC itself (Bendelac, Savage et al. 2007), unlike conventional T lymphocytes (Robertson, Berzofsky et al. 2014). The cellular homing pattern is governed by the expression of chemokine receptors expression, like CXCR3 (accumulation in inflamed tissues) and CXCR6 (liver homing). These NKT cells have an innate-like feature like the rapid generation of cytokines post-stimulation. Therefore, they are termed as "bridge between innate and adaptive immunity" (Robertson, Berzofsky et al. 2014). These cells also increase the recruitment of innate-like cells (Carnaud, Lee et al. 1999) and DC. They are also involved in B-cell maturation (Cerundolo, Silk et al. 2009). Natural killer T-cells are subdivided into two groups according to their TCR chains. Type I NKT cells, or invariant NKT (iNKT) cells, which have a distinct invariant TCR α-chain with limited TCR β-chain repertoires, while Type II NKT (NKTII) cells are known to express a wide range of diverse TCR chain permutations (Dasgupta and Kumar 2016). Different studies have

shown the existence of NKT-like cells, such as CD1-restricted T-cells and MR1-restricted mucosal-associated invariant T-cells cells (Godfrey, Stankovic et al. 2010).

Upon activation, Type I NKT cells produce a mixture of soluble and membrane-attached effector molecules. Since long, Type I NKT cells are known for their unique potential to generate both IFN-γ and IL-4 together influencing the generation of Th1 and Th2 immune response, respectively. In addition to IFN-γ and IL-4, Type I NKT cells are known to generate Th1 cytokines such as TNF-α and Th2 cytokines like IL-5 and IL-13. Type I NKT cells produce growth factors like IL-2 and GM-CSF, pro-inflammatory cytokines like IL-6 and anti-inflammatory cytokines like IL-10 and TGF-β. Apart from generating various cytokines, Type I NKT cells produce inflammatory chemokines like RANTES, MIP-1α, and MIP-1β (Matsuda, Mallevaey et al. 2008; Marrero, Ware et al. 2015). Ultimately, Type I NKT cells produce soluble cytolytic proteins like perforin, granzymes and in humans, granulysin and FasL (cell surface molecules involved in cytotoxicity) (Gansert, Kieβer et al. 2003; Matsuda, Mallevaey et al. 2008). Type I NKT cells can be further categorized into various sub-subsets based upon differences in the expression of CD4 and NK1.1. Table 2 shows diverse types of NKT cells and their distribution in human and mouse.

Type I NKT cells are known to possess the ability to regulate the function of essentially every type of hematopoietic cell. Type I NKT cells regulate the function of APCs such as DC, macrophages, B cells and other lymphocytes including like NK cells and many T cell subsets (Cerundolo, Silk et al. 2009; Doherty, Melo et al. 2018). Type II NKT cells stimulated with sulfatide interestingly can regulate the function of Type I NKT cells. This cross-regulation among NKT cell subsets was described to be as an evolving paradigm (Berzofsky and Terabe 2008; Dhodapkar and Kumar 2017).

Some mouse models of autoimmunity and antitumor and antimicrobial immunity supports the idea that an imbalance in NKT cell subset leads to the predisposition of the host to various diseases (Arrenberg, Halder et al. 2009).

	Type I NKT cells		Type II NKT cells
Feature	Mouse	Human	Mouse/Human
TCRα	Vα14–Jα18	Vα24-Jα18	Diverse, Vα3.2, Vα8, Vα1 bias
TCRβ	Vβ8.2, Vβ7, Vβ2	Vβ11	Diverse, Vβ8 and Vβ3 bias
α-GalCer reactivity	Yes	Yes	No
Antigens recognized	α-GalCer analogs, glycosphingolipids, diacylglycerols, phospholipids	α-GalCer analogs, glycosphingolipids, diacylglycerols, phospholipids	Sulfatides, lysosulfatide, lysophospholipids
Detection	α-GalCer-loaded CD1d tetramers	α-GalCer-loaded CD1d tetramers, anti- Vα24/anti-Vβ11 mAbs	Sulfatide-loaded CD1d tetramers, indirectly with a combination of CD1d-deficient and Jα18-deficient mice
Mature phenotype	NK1.1+	CD161+	NK1.1+/CD161+
Subsets	CD4+, DN, CD4−/NK1.1−	CD4+, DN, CD8+	CD4+, DN, CD8+
Frequency in thymus	0.2–0.5%	0.001–0.01%	ND
Frequency in blood	0.2–0.5%	0.001–0.5%	ND
Frequency in spleen	0.5–1.0%	0.01–0.5%	ND
Frequency in liver	20–40%	~1%	In humans, more abundant than Type I NKT cells
Frequency in intestine	0.05– 1.0%, enriched in lamina propria	<1.0%	ND
Cytokine production	Th1, Th2, Th17 (CD4−/NK1.1− subset)	Th1, Th2, Th17	Th1, Th2, Th17

*ND, not determined.

Table 2: Types of NKT cells and distribution

2.5.2.2. B cells

TB serology is a vast field, generating abundant data to show that, *Mtb* induces a humoral immune response to a wide variety of mycobacterial antigens in humans even though, it is an intracellular pathogen (Steingart, Dendukuri et al. 2009). Further, various studies show that intradermal-BCG vaccine induces IgG and IgM responses to many mycobacterial antigens, especially to the glycolipid lipoarabinomannan, a cell

wall antigen (De Valliere, Abate et al. 2005). Few studies had shown that the antibody responses could enhance both innate and cell-mediated immune responses against mycobacteria (De Valliere, Abate et al. 2005). The humoral immune response to mycobacterial antigens also varies depending on the state of infection (Kunnath-Velayudhan and Gennaro 2011). The LTBI individuals, who do not have active disease, possess a minor and partial range of *Mtb* antigens than those suffering from TB. Many reports in both human and animals show that the levels of antibody titers are a direct correlate of the mycobacterial burden (Kunnath-Velayudhan and Gennaro 2011; Achkar, Jenny-Avital et al. 2013). Anti-body titer against mycobacterial antigens in the serum of TB patients was a historic argument of the antibodies being non-protective.

Ironically, a similar argument is not used to challenge the current concept which says that immunity against TB is mostly cell and cytokine-mediated (i.e., IFN-γ), in spite of, most of the patients who develop TB have a normal T cell function and increased IFN-γ levels. Interestingly particular antibody titers do serve as markers for TB while the level of IFN-γ could show the progression of the disease from an infection into a disease (Diel, Loddenkemper et al. 2011). Although the fact is that not every antibody activated by *Mtb* is functional or even has a role in an inflammatory response. Many studies have shown or suggested that certain antibodies have a protective function against the disease. A false dichotomy which does not provide any role for humoral immunity but assigns all the credit to cell-mediated immunity may have been built up the due inability of the scientific community to come up with positive co-relates towards humoral immunity along with substantial evidence for a strong role for cell-mediated protection. However, such a simplistic scientific opinion

misses several mechanisms involving antibody-mediated immune response which could modify the verdict of intracellular bacterial pathogenesis with effects ranging from opsonisation to activation of FcR (Casadevall 2003).

2.5.2.3. Dendritic cells, Macrophages and NK cells

Dendritic cells (DCs) are the most effective professional antigen-presenting cells for priming naïve T cells, an important source of IL-12 and are very efficient cells in inducing antimicrobial and antitumor immune responses (e Sousa, Hieny et al. 1997). Various studies have shown that DCs fortify the cellular immune response against *Mtb* infection (Henderson, Watkins et al. 1997; Demangel and Britton 2000; Fortsch, Rollinghoff et al. 2000; Tascon, Soares et al. 2000). During the start of inflammatory response against *Mtb*, DCs are present in higher numbers at the sites of *Mtb* infection (Sertl, Takemura et al. 1986; Van Haarst, Hoogsteden et al. 1994). Immature DCs in the lung mucosa are specialized for antigen uptake and processing. As DCs interact with the pathogens, they mature and migrate in lymphoid organs where they prime T cells through cell surface expression of MHC, co-stimulatory molecules and the secretion of immune-regulatory cytokines like IL-12 (Banchereau and Steinman 1998). Mice depleted of DCs have impaired ability to develop effective $CD4^+$ T cell response which in turn results in a major loss of bacterial replication control, which results in a huge bacterial load in the lungs and spleen (Tian, Woodworth et al. 2005). Other studies with defunct DCs or deficiency have reported BCG-osis and spontaneous *Mycobacterium kansasii* infection (Hambleton, Salem et al. 2011). Research has shown that *Mtb* infected DCs produce a high level of chemokines such as CCL3, CCL4, CXCL8, CXCL9 and chemokine receptors such as CCR7, which is essential in the migration of NK and T cells

(Sasindran and Torrelles 2011). There have been studies which report a down regulation of surface molecules or generation of anti-inflammatory cytokines by *Mtb* infected DCs. *Mtb* also impairs DCs maturation and reduce the secretion of IL-12 by them, thereby inhibiting their ability to stimulate T cell proliferation (Hanekom, Mendillo et al. 2003). *Mtb* infects DCs with elevated frequency impairing their function *in vivo.*

Macrophages are a composite cell population with a high level of flexibility (Biswas, Chittezhath et al. 2012). Once activated, macrophages can show a variety of different, sometimes conflicting, functions like promoting inflammatory responses along with angiogenesis, tumour growth inhibition, tissue remodelling and cell destruction (Mantovani, Sica et al. 2004; Gordon and Martinez 2010). Earlier studies have shown that the function of macrophages is directly proportional to their micro-milieu, especially in terms of cytokine type and concentration. In line with the Th1/Th2 classification system, the terms M1 and M2 were common to understand macrophage functions and phenotype; these two categories are the polarised activation states of macrophages. In general, M1 polarization of macrophages is elicited by LPS or IFN-γ and is also known as classical activation, leading to the proinflammatory, increased microbicidal and tumoricidal response. In micro-milieu dominated by IL-4 or IL-13, macrophages undergo M2 polarisation which is also known as alternative activation, which is mediated by tissue repair, immune escape of pathogen and tumours, contributing to infection and tumour formation (Li, Katz et al. 2012). The humans, M1 macrophages are characterized by high levels of CXCL10, CXCL11, CCR7, TNF-α, IL-6 and IL-12, while M2 macrophages show elevated levels of CCL17, CCL18 and IL-10 (Mantovani, Sica et al. 2004). Many reports suggest *in*

vitro anti-mycobacterial activity in macrophages activated with IFN-γ and type 1 cytokine (Rook, Steele et al. 1986). This was shown in laboratory where it was demonstrated that a deletion mutation in gene NOS2 in the mice was lethal for it when infected with *Mtb* (MacMicking, North et al. 1997), while the mice with inability to generate reactive oxygen intermediates were affected to some extent in the capability to manage *Mtb* infection (Cooper, Pearl et al. 2000; Gebreyohannes 2007). *In vivo* studies shows that both mouse and human have reflected the vital task of IFN-γ which activates macrophages and thereby in the regulate the outcome of any infections with *Mtb*. Mice with mutated IFN-γ gene (Cooper, Dalton et al. 1993) and human individuals genetically mutant for IFN-γ receptor (Jouanguy, Altare et al. 1996; Gebreyohannes 2007) were observed to be ultra susceptible to mycobacterial infection and non-virulent BCG. Activated macrophages produce various cytokines like TNF-α and IL-1β. Mice depleted of TNF-α with a monoclonal antibody, or mice knocked out for TNF-α receptor has defects in the development of granuloma and are extremely susceptible to mycobacterial infections (Flynn, Goldstein et al. 1995).

Growth inhibition of mycobacteria is, in part, mediated by soluble factors, including IFN-γ and TNF-α produced by NK cells, and through monocyte apoptosis induced by NK cell contact (Yoneda and Ellner 1998; Brill, Li et al. 2001; Lu, Wu et al. 2014). NK cells are capable of destroying *Mtb* via the cytolytic proteins granulysin and perforin, in a contact-dependent manner without the help of accessory cells, such as monocytes (Lu, Wu et al. 2014). After *Mtb* infection activated NK cells secrete elevated levels of IFN-γ and perforin along with their increased number in the lung, suggesting their protective role in TB. Still, the depletion of NK cells, had a negligible effect on the pulmonary bacterial load, indicating that these cells might not have any

defining role which determines protective immunity (Junqueira-Kipnis, Kipnis et al. 2003). Apart from early innate immune functions, NK cells are predominant in granulomatous lesions of the lungs of *Mtb* infected patients (Portevin, Via et al. 2012). Direct contact between NK cells and *Mtb* that emerge from granulomas after cell lysis can start antimicrobial mechanisms, the release of granulysin, which has static or cidal effects on the *Mtb* growth (Portevin, Via et al. 2012; Lu, Wu et al. 2014). NK cells express NKp44 contributing to direct contact between the corresponding ligand on the cell-surface of *Mtb* infected macrophages, triggering IFN-γ production and increased macrophage activation (Portevin, Via et al. 2012; Esin, Counoupas et al. 2013). The depletion of NK cells in the mouse enhanced the growth of *M. avium* (Harshan and Gangadharam 1991). However, one such study in C57BL/6 mice has shown that NK cells proliferate in response to *Mtb* infection, while NK cells, if depleted, lead to no increase in bacterial load, suggesting the redundancy in the cellular response to mycobacterial infection (Junqueira-Kipnis, Kipnis et al. 2003). Interestingly, NK cells are required for mycobacterial resistance in mice infected with *Mtb* but deficient in T-cell, suggesting an imperative role of NK cells in fighting *Mtb* in immunocompromised patients (Feng, Kaviratne et al. 2006).

2.5.2.4 Presentation of Mtb lipids by CD1 complex

Ma+jor histocompatibility complex (MHC) class II presents mycobacterial peptides to $CD4^+$ T cells. Whereas; $CD8^+$ T-cell activation warrants, recruitment of MHC I complex by mycobacterial peptides in the cytoplasm (van der Wel, Hava et al. 2007). Similar to the MHC class I complex, CD1 molecules present several types of lipid antigens to be recognized by TCRs (Barral and Brenner 2007). Previously, lipids

were considered to elicit only innate receptors, but the studies involving TCRs showed that these complexes respond to lipids. Similarly, T cells were considered to recognize only peptide antigens through MHC complex bound to T cells, but studies with CD1 complexes expanded the range of natural T-cell antigens which also include lipids (Beckman, Porcelli et al. 1994), glycolipids (Moody, Reinhold et al. 1997), phospholipids (Sieling, Chatterjee et al. 1995), sulfolipids (Gilleron, Stenger et al. 2004), and lipopeptides (Moody, Young et al. 2004). Lastly, unlike the invariant, germline-encoded receptors of the innate system, TCRs formed by somatic rearrangements appear as crores-combination in any individual. This characteristic receptor diversity of T cells is a key requirement of acquired immunity. However, studies of T-cell response to CD1d and CD1b showed distinct conservation of TCRs which responds to CD1-lipid complexes (Fowlkes, Kruisbeek et al. 1987; Van Rhijn, Kasmar et al. 2013). These observations also raise several questions about TCRs asking whether, if they are always diverse, representing effectors of acquired immunity or they exist as 'innate T cells'. This increases the importance of human T-cell activation by mycobacterial lipids via the TCR as it contacts CD1-lipid complexes. New studies measuring the populations of human T cells in TB patients using CD1 tetramers becomes important as CD1 proteins do not vary in structure in individual to individual, increasing the projection for using lipid antigens as a new approach to immunomodulation, treatment and even immunodiagnosis.

Many lipid antigens of *Mtb* origin like MAs, lipoglycans (such as LAM), PIMs, lipopeptides, mannosyl-β-1-phosphomycoketides and sulpholipids can be potentially presented by CD1a, CD1b, CD1c and Cd1d in humans (Barral and Brenner 2007). *Mtb* is mainly a human pathogen. However, zebrafish, mice, guinea pigs,

rabbits, cynomolgus monkeys, rhesus macaques, common marmoset, and cattle have all been used as models and mimic certain aspects of human TB. Naturally occurring CD1 proteins in these models should be considered because the subset of CD1 varies from specie to specie. For example, all jawed fish, the zebrafish has MHC genes, but it has no CD1 genes (Wang, Perera et al. 2003; Miller, Wang et al. 2005). Rodents have CD1d-encoding genes, but lack CD1a, CD1b, CD1c, and CD1e-encoding genes (Dascher and Brenner 2003). The incomplete set of CD1 genes in these common animal models makes it significant to develop a new trans-species model. Mice that are transgenic for human CD1a, CD1b, CD1c, and CD1e have been created and used for experiments directed at immune recognition of mycobacterial lipids (Felio, Nguyen et al. 2009). Humanized-SCID mice also show certain CD1 expression patterns similar to humans. Therefore, spleen cells of these mice can present mycobacterial lipids to human T-cell lines in vitro (Lockridge, Chen et al. 2011). The CD1a, CD1b, or CD1c molecules evolved naturally in guinea pigs, and rabbits express and hence show at least 1 ortholog of each human CD1 isoform. These small animals are commercially available, and the experimental mycobacterial infection protocols in these animals have been well documented. Experimental mycobacterial lipid vaccination in guinea pigs have been performed, and some protection to TB was shown in them (Dascher, Hiromatsu et al. 2003).

The *Mtb* TLR2 agonist ligands like lipoproteins, lipoglycans, PIM (Banaiee, Kincaid et al. 2006), and LM (Nigou, Vasselon et al. 2008) are efficient enough to mount TLR based response. *Mtb* modulates lipids presentation to the T cells in various ways. T lymphocytes are modulated by *Mtb* through the presentation of lipid antigens by CD1 molecules. Till now, only a few mycobacterial CD1 ligands have been

identified. For example, glycolipids derived from mycobacteria such as mycolic acid (Beckman, Porcelli et al. 1994), glucose monomycolate (Sieling, Chatterjee et al. 1995), glycerolmonomycolate (Layre, Collmann et al. 2009) and phosphoglycolipids, such as LAM. However, these classes of lipids are present in both avirulent and in virulent mycobacteria. Gilleron et al. recognized *Mtb* specific sulfoglycolipid (Ac2SLG), which is presented by CD1b molecules (Gilleron, Stenger et al. 2004). Ac2SLG activated $CD8^+$-restricted T cells and showed strong immunoprotective functions in the form of release of pro-inflammatory cytokines and bactericidal activity in the mycobacteria-infected host cells (Gilleron, Stenger et al. 2004). They also showed that fatty acyl units of sulphoglycolipids administrate TCR recognition and lymphocyte activation (Guiard, Collmann et al. 2009).

Another antigenic mycobacterial lipid identified as Mannosyl-b-1-phosphoisoprenoid (MPI) (Moody, Guy et al. 2000) and Mannosyl-b-1-phosphomycoketide (MPM) (Matsunaga, Bhatt et al. 2004) is involved in the activation of CD1c-restricted cells (Moody, Guy et al. 2000). It is produced by a Pks12 dependent lipid biosynthesis pathway in mycobacteria such as *Mtb* and *M.bovis* BCG, but such a lipid biosynthesis pathway is lacking in mammalian species (Matsunaga, Bhatt et al. 2004). In a way, *Mtb* lipids contribute to host-resistance as it elicits pro-inflammatory molecules and antigens, while on the other hand, they assist immune evasion by suppressing the pro-inflammatory responses and down-regulating antigen presentation various antigen-presenting cells and T-effector cell responses. However, mice and other animal models only moderately define aspects of human infection like the bacterial ability to sustain in TB granuloma for a long time before reactivation. Therefore in vitro human model, for example, *in vitro* human granuloma

(Puissegur, Botanch et al. 2004) may help determine unknown functions of these lipids.

2.5.2.5 Immune Evasion Mechanisms by Mycobacteria

Enough evidence and work in the form of review are available, which discusses the detailed mechanistic approach used by *Mtb* to escape the host induced adversaries leading to the development of the disease. The prime goal explained in this paragraph is to help the reader understand the host-pathogen association, particularly in susceptible individuals. Mycobacterium residing inside macrophage can be killed if it is activated appropriately. Mycobacterium uses multiple receptors like the complement, mannose and lectin specific receptors, Fc receptors, scavenger receptors and TLRs on the macrophage cell membrane to access the host macrophages (Gebreyohannes 2007). However, some receptors are more frequently used by the mycobacterium, than others to avoid the activation of macrophages, ensuring their survival inside the macrophages. Mycobacterium tries for such "covert entry" to have access to the macrophages by avoiding activation of the oxidative burst in the host cell. To access entry via complement receptors and/or the mannose-binding receptors on the surface of macrophages, which are not linked to MyD88 provides an advantage to the bacterium. Reports suggest that MyD88-/- mice were unable to generate antigen-specific IgG2a, whereas generating an elevated level of IgG1 and IgE antibodies towards the similar antigen (Schnare, Barton et al. 2001). These findings strengthen the idea that the mycobacterial entry into macrophages mediated by receptors, which are usually not connected to the TLR signalling pathway, might elicit a Th2 type response, which is ineffective in regulating intracellular bacterial replication (Gebreyohannes 2007).

Mycobacterium is known to have developed multiple ways to bypass the host immune response to survive hostile intracellular milieu of the activated macrophages, by using proteins and glycolipids of the *Mtb* cell wall to its advantage. The protein from the host (tryptophan-aspartate-containing coat protein) is inducted by the virulent mycobacterium by retaining it inside the phagosome and consequently inhibiting phagolysosome formation leading to the killing of the bacteria. This observation was made in phagosomes having live bacilli but not with those containing heat-killed bacterium (Ferrari, Langen et al. 1999; Gebreyohannes 2007). *Mtb* employs numerous ways to defend against ROS and RNS, including direct scavenging of the reactive species and the repair and protection of proteins and DNA (Ehrt and Schnappinger 2009). Thick *Mtb* cell wall containing LAM, cyclopropanated mycolic acids and the phenolic glycolipid I (PGL-1), which act as potent scavengers of oxygen radicals provides the resistance to the generated ROS (Flynn and Chan 2001). In addition to these elements, *Mtb* produces the ROS scavenging enzymes like catalase (Ng, Cox et al. 2004), superoxide dismutase (Tullius, Harth et al. 2001). The structural configuration of LAM can induce or inhibit macrophage activation. Mannose-capped lipoglycans (ManLAM) are anti-inflammatory due to their ability to suppress the generation of IL-12 and TNF-α while inducing IL-10 which is independent of the TLR2 and MyD88 pathway (Nigou, Zelle-Rieser et al. 2001).

The lipoglycans, on the other hand, are capped with phosphoinositide residues (PILAM), and uncapped lipoglycans (LAM) are pro-inflammatory due to their stimulation of immune cells to secrete type 1 cytokines via a TLR2 dependent NF-κB pathway (Means, Jones et al. 2001; Gebreyohannes 2007). In spite of the facts stated about, how the bacteria evade the host through various mechanisms, the

epidemiological data suggests that only 10% of people exposed with *Mtb* progresses into disease thereby suggesting that the evasion mechanism is the phenomenon associated with a susceptible host (Gebreyohannes 2007).

Understanding the role of innate immunity in fighting TB in healthy individuals generally helps, identify the role of innate immune cells in the combat between host and the pathogen. Manipulating innate immune responses, therefore, contains or decreases the bacterial replication during the first month before the host generates an adaptive immunity against the infecting bacterium (Gebreyohannes 2007). A thorough comprehension on how mycobacterium dampens the development of effective immunity might help designing targets for the development of new pharmaceuticals, immunotherapy, and vaccines or by enhancing the efficacy of the existing one.

2.6. Diverse *Mtb* Strains and Differential Immune Response

Various strains and clades of *Mtb* differ genotypically and phenotypically and shows a diverse geographic distribution. These strains also show the difference in lipid profiles. *Mtb* lineage not only manipulates innate immune response but also virulence which is linked with unique cell envelope lipid profiles of *Mtb* strains (Krishnan, Malaga et al. 2011; Queiroz and Riley 2017). Apolar and polar lipids in *Mycobacterium canetti* were upregulated, while one in *Mtb* Beijing fractions down-regulated TLR2 and TLR4 expression on monocyte-derived macrophages. Even the expression of MHC class II is modulated by apolar and polar lipids from different strains of *Mtb.* Lipids from every mycobacterial strain-induced different cytokine pattern. Research showed that *Mtb* Beijing apolar lipid fraction induced the highest

levels of IL-10 (Rocha-Ramirez, Estrada-Garcia et al. 2008). Also, the TLRs recognize different *Mtb* strains variably impacting immune response differentially (Carmona, Cruz et al. 2013).

Mtb bacillus is classified under six major lineages, each strongly associated with strain predominance in different geographical locations, which are specific to the human population (Gagneux and Small 2007). On the basis of ancestry, lineage 1, 5 and 6 (Indo-Oceanic, West African 1, West African 2) are termed as ancient while lineage 2, 3 and 4 (Beijing, CAS/Delhi, Euro American) are termed as modern. Different lineages of *Mtb* show differential virulence pattern in an animal model and their behaviour in human disease (Bishai, Dannenberg et al. 1999; Tsenova, Ellison et al. 2005). We in our studies used representative clinical isolate strain East African-Indian (EAI-5), Beijing, Latin American-Mediterranean (LAM-6), which belongs to lineage 1, 2 and 4, respectively. H37Rv belongs to the lineage 4 which is a laboratory strain derived from a virulent clinical isolate from a patient while BCG used in our studies was a Moscow strain.

The modern strains are considered to be more virulent with more MDR stains inducing lesser pro-inflammatory and apoptotic responses compared to primitive strains (Romero, Balboa et al. 2012; Chakraborty, Kulkarni et al. 2013; Chen, Chang et al. 2014). Therefore strain derived lipids-antigenic presentation of such lipids is a dominant immune mechanism which determines host evasion or infection. Some lipids have been characteristically identified in W-Beijing family. Phenolic glycolipid and its association with the hypervirulent phenotype of the strains is also documented (Salgame 2005). PGL and its association with hypervirulent phenotype have been functionally identified in W-Beijing family (Salgame 2005). LAM from various *Mtb*

strains inhibits delivery of antigen presentation machinery (Lang and Glatman-Freedman 2006). The lipids from *M.bovis* BCG such as lipids (phosphatidylinositol di-mannosides (PIM2), phosphatidylglycerol, cardiolipin (CL), phosphatidylethanolamine, trehalose monomycolate (TMM), trehalose dimycolate (TDM), and mycoside B (MycB) can manipulate host macrophage (Rhoades, Hsu et al. 2003). These lipids show similarity in response in both *Mtb* and *M. bovis* eliciting the production of TNF-α, IL-1α, IL-1 β, IL-6, macrophage chemotactic protein-1, and IFN-γ-inducible protein-10. These cytokines are also involved in the recruitment of polymorphonuclear and mononuclear leukocytes and lymphocytes. Studies have shown that TDM caused the elevated levels of cytokines such as IL-1β, IL-6, and TNF-α and recruitment of neutrophils (Geisel, Sakamoto et al. 2005). Many *Mtb* specific lipids hinder in generating an adaptive response by developing inconsistent Th1-promoting cytokines (Salgame 2005).

2.7. Treatment and Control of Tuberculosis

Robert Koch first attempted a scientifically-based therapy at the end of the 19th century, where he tried to immunize patients with culture filtrates to treat TB (Kaufmann and Schaible 2005). In the course of time, Koch's lysate was later shown to have no beneficial quotient; it was discovered that it could be useful in diagnosis. Further, a high hope to end TB came up with the discovery of antibiotics during the late 1940s (Gebreyohannes 2007). Streptomycin, after its discovery by Selman Waksman in 1943 was successful in curing a 21yr old lady with complicated TB in the year 1945 (Pfuetze, Pyle et al. 1955). Likewise, the treatment with the likes of isoniazid was also exciting, which showed regression of clinical symptoms of TB in 45 patients treated with it (Selikoff, Robitzek et al. 1952). Since then, multiple

permutations from various antibiotics were tried on TB patients, expecting recovery of the *Mtb* infected patients. As the time passed with using these antibiotics, the confidence in them also enhanced and was appreciated to control or eradicate TB, on the global scale by prescribing regular treatment, yet the program was not successful in reducing the global burden of the TB. Therefore, WHO introduced DOTS mandate (Direct Observed Therapy, Short-course) in the year 1994. DOTS can be described as a focused short-term treatment of patients with multiple anti-TB drugs, administered through oral route under observation of the health care personnel to ensuring adherence to treatment course (Gebreyohannes 2007). In 1999 WHO and partner agencies launched DOTS-Plus as a comprehensive initiative to build upon the five elements of DOTS. Also, it would take into account specific issues such as the use of second-line anti-TB drugs.

The specific targets by WHO, set for the End TB Strategy include a 90% reduction in TB deaths and an 80% reduction in the TB incidence rate by 2030 compared with 2015; more immediate milestones for 2020 are reductions of 35% and 20%, respectively (WHO 2018). Another prime hurdle in the control of TB is the emergence of rifampicin-resistant TB (RR-TB) and (MDR-TB) multidrug-resistant TB (MDR-TB). Among RR-TB cases, an estimated 82% had multidrug-resistant TB. The MDR strains of *Mtb* do not respond to many first-lines antimycobacterial drugs. In 2017, 30% (2.0 million) of the 6.4 million new and previously treated TB cases notified globally were reported to have been tested for resistance to rifampicin. Globally, 1,60, 684 cases of multidrug-resistant TB and rifampicin-resistant TB (MDR/RR-TB) were notified in 2017 (up from 1,53,119 in 2016), and 1,39,114 cases were enrolled in treatment (up from 129 689 in 2016) (WHO 2018). Among

MDR/RR-TB patients notified in 2017, 50% were tested resistant, to both fluoroquinolones and second-line injectable agents, a considerable increase from the 39% tested in 2016.

Class	Mechanism of action	Drugs
Group A: fluoroquinolones		
Fluoroquinolones	Inhibition of DNA gyrase	Levofloxacin, Moxifloxacin, Gatifloxacin
Group B: second-line injectable anti-TB drugs		
Aminoglycosides	Inhibition of protein synthesis	Kanamycin, Amikacin, Capreomycin, (Streptomycin)
Group C: core second-line agents		
Thioamides	Inhibition of cell wall synthesis	Ethionamide, Prothionamide
Oxazolidinones	Inhibition of protein synthesis	Cycloserine, Terizidone, Linezolid, Clofazimine
Group D: add-on agents		
D1, various classes: isonicotinic acid hydrazide (high-dose isoniazid); nicotinamide analogue (pyrazinamide); amino alcohols (ethambutol)	Inhibition of mycolic acid synthesis	High-dose isoniazid
	Disruption of plasma membranes	Pyrazinamide
	Inhibition of cell wall synthesis	Ethambutol
D2, various classes: diarylquinoline (bedaquiline); nitro-dihydro-imidazooxazole (delamanid)	Inhibition of mitochondrial ATP synthase	Bedaquiline
	Inhibition of mycolic acid synthesis	Delamanid
D3, various classes: amino-phenol (para- aminosalicylic acid); carbapenems; thiosemicarbazone (thiocetazone)	Inhibition of DNA precursor synthesis	Para-aminosalicylic acid
	Inhibition of peptidoglycan synthesis	Imipenem plus cilastatin or meropenem plus clavulanate (available orally with amoxicillin)
	Inhibition of mycolic acid synthesis	Thiocetazone**

Table 3: First-line and second-line drugs used for the treatment of drug-resistant TB (WHO classification). *Streptomycin can be used when the isolate is susceptible, and none of the other injectable drugs is available. **Only use in HIV-negative individuals. Reference: Tuberculosis. Madhukar Pai et al. Nature Reviews Disease*

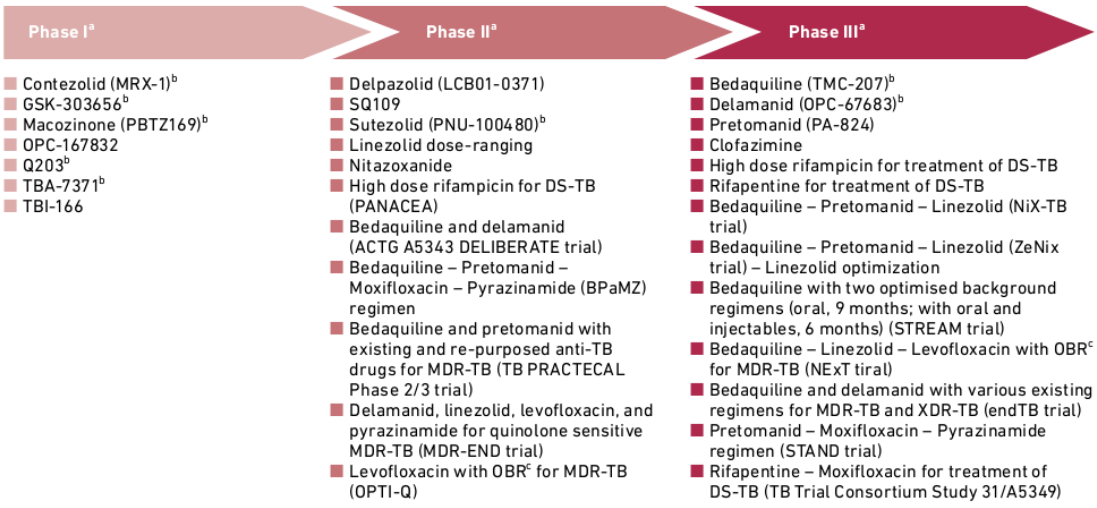
Primers volume 2, Article number: 16076 (2016) and WHO report 2018 (Updated data: August 2018)

Table 3 shows the list of anti-TB drugs and their mode of action. The *Mtb* strains are prone to the development of resistance to several antibiotics (Parwati, van Crevel et al. 2010). Further, reports from various countries have surfaced in which *Mtb* has been reported to show resistance to many first-line and second-line anti-TB drugs. These deviant mycobacterial strains are called as extensively drug-resistant (XDR), basically resistant to 2nd line of TB drugs making them highly lethal mainly in HIV positive TB (Centers for Disease and Prevention 2006) (Gebreyohannes 2007). Along with all these issues; several of which are not discussed here suggests that prevention would be the ideal means to control this disease.

Globally, the incidence rate is falling by about 2% per year, and mortality rate by 16%; by 2020, these figures need to be improved to 4–5% per year and 10%, respectively, to reach the first (2020) milestone of the End TB Strategy. Most deaths from TB could be prevented with early diagnosis and appropriate treatment. Millions of people are diagnosed and successfully treated for TB each year, averting millions of deaths (53 million 2000–2016), but there are still large gaps in detection and treatment. In 2016, 6.3 million new cases of TB were reported (up from 6.1 million in 2015), equivalent to 61% of the estimated incidence of 10.4 million; the latest treatment outcome data show a global treatment success rate of 83%, similar to recent years.

There were 4,76,774 reported HIV-positive-TB cases (46% of the total estimated incidence of TB), of whom 85% were on antiretroviral therapy (ART). A total of 1,29,689 people had started treatment for drug-resistant TB in 2017, with a small increase from 1,25,629 in 2015, which was just 22% of the total estimated

incidence of TB. This shows that treatment rate increased but the fact is that this rate needs to go further high to achieve the epidemiological milestones (WHO 2018). Recently the drug combination bedaquiline, pretomanid and linezolid (BPaL) was evaluated in Phase 3 clinical trial. The participants of this trial were XDR-TB and treatment intolerant or nonresponsive MDR-TB patients and were treated with BPaL regimen for 6-9 months. The results showed that of the first 75 participants' almost 89% of the trial participants had a positive outcome with their clinical infection cleared up with sputum negativity for TB after six months of treatment and post-treatment follow-up (Honeyborne, Lipman et al. 2019). FDA approved this drug regimen in August 2019, which presents a great promise. Since 1921, BCG has been employed for human use successfully and has lead to a reduction in cases of TB meningitis and miliary TB kids (Trunz, Fine et al. 2006).



[a]New drug compounds are listed first, followed by repurposed drugs and then by regimens. [b]New chemical class. [c]Optimized background regimen.

Figure 6: The global clinical development pipeline for new anti-TB drugs and regimens, August 2018. *Source: Adapted from the Working Group on New TB Drugs pipeline. http: //www.newtbdrugs.org/pipeline.php*

However, as discussed below, its efficacy against PTB remains controversial. There are various TB drugs and vaccines in clinical trials though no secure option has been the outcome. Figure 6 represents a clinical development pipeline for new anti-TB drugs and regimens.

2.8. TB prophylaxis: The Bacille-Calmette-Guérin (BCG) Vaccine

Globally, >90% of newborns are immunized with BCG annually, which is the only licensed vaccine to prevent the development of active TB disease. BCG policies and practices across the world are available at The BCG World Atlas (http://www.bcgatlas.org). The BCG vaccine was 1st used in humans in 1921 and has been evaluated in many trials and studies looking at manifestations of active TB disease (Zwerling, Behr et al. 2011). In clinical trials, the efficacy of the BCG vaccine against PTB in adults has been reported to be 0–80%. The reasons for this observed variability in BCG vaccine efficacy is not exactly understood.

2.8.1. History of BCG

Robert Koch was a pioneer to scientifically determine the immunity developed against TB after he discovered the causative agent of TB. He inoculated guinea pigs infected with TB, with live and heart killed mycobacterium or with a culture scum, he observed that the part of the host skin became black and slowly sloughed off, though later healed, curing the wound. This immune reaction was called as Koch's Phenomenon (Bothamley and Grange 1991; Gebreyohannes 2007). He had an intention to generate a therapeutic protocol based upon host immunity against TB by injecting the filtrates from *Mtb* culture, Koch was regarded as the first one to develop

a subunit vaccine if it is compared to modern era vaccines. However, this filtrate failed to treat TB in man (Kaufmann 2006; Gebreyohannes 2007). Koch's lysate, known as PPD or purified protein derivative, is still being in humans as a skin test to determine mycobacterial infections since for the last century until today to which help differentiate between that of the infected or exposed individual to *Mtb* from uninfected healthy person. The previous work of Jenner and Pasteur on immunization and the attempt made by Koch to heal TB, inspired Albert Calmette and Camille Guérin, the Frenchmen in 1900 to work with this disease (Gebreyohannes 2007). Figure 7 represents photograph of the major contributors to our understanding of vaccines namely Robert Koch, Albert Calmette and Camille Guerin. Their goal at the Pasteur Institute was to develop a vaccine against TB. They found the causative agent of bovine TB, *M. bovis*, as a suitable candidate for developing and potent vaccine. They allowed the mycobacterium to survive and proliferate on media containing ox bile and after ~214 passages, for over a decade it was found that the mycobacterium was no longer virulent in a variety of laboratory animals. The new vaccine was found to be safe to use but also it was found that this weakened strain was able to protect animals from challenge with virulent *Mtb* and *M.bovis* (Calmette and Plotz 1929; Gebreyohannes 2007).

Figure 7: L-R Robert Koch, Albert Calmette and Camille Guerin.

After confirming its safety in multiple laboratory and animal experimentation, it was finally used for humans. In 1921, a newborn baby whose mother died of TB with an assumption that the baby would develop TB and die was administered with BCG; the child grew up healthy with no TB (Daniel 1997). Later, millions of infants were vaccinated all across Europe and outside Europe with no reported side effects. The increased use encouraged the League of Nations to recognize, BCG as a safe vaccine for human use in 1929 (Calmette and Plotz 1929; Gebreyohannes 2007). The Lübeck catastrophe in Germany, which was caused by accidental injection to kids with a wrongly labelled *Mtb* instead of BCG, which bought a disgrace for using BCG worldwide (Kaufmann 2006b). Currently, BCG is in use in several endemic countries, and figure 8 represents BCG global coverage documented in 2017 by WHO.

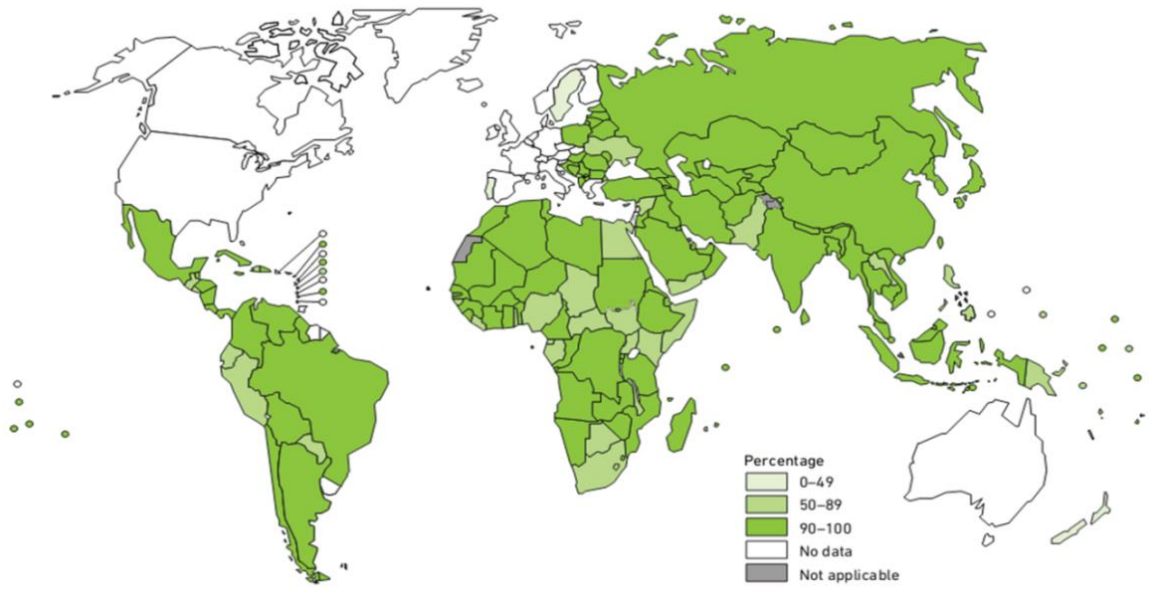


Figure 8: BCG global coverage (2017). *The target population of BCG coverage varies depending on national policy but is typically for the number of live births in the year of reporting.*

Source: http://apps.who.int/immunization_monitoring /globalsummary, 30 July 2018

2.8.2. Efficacy of BCG Vaccination

BCG has successfully immunized almost 3 billion people globally in spite of having considerable uncertainty in its efficacy to confer protection against TB (Kaufmann 2006). Many clinical trials were performed in many nations, which evaluated the protective effectiveness of BCG immunization. Though the effectiveness varies from person to person and many factors affecting the efficacy of BCG vaccination have been studied previously (Gebreyohannes 2007). There is extensive or systematic research to evaluate the efficacy of BCG immunization. The parameters vary from study to study, resulting in the disparity in the level of protection achieved in various trials. Efficacy of BCG vaccine depends upon the host factors like the host genetics is known to determine its susceptibility or resistance to TB infection. The efficacy is also dependent upon the environment of the host and the fact that the host is exposed to trans-reacting environmental mycobacterium, which is known to induce an immune response, which interferes with BCG induced immune imprint. Further, the efficacy of the vaccine also depends upon the BCG vaccine genotype. The genetic predisposes antigens which are present in BCG which are immunogenic. The genetic regions which are deleted in BCG, as a result of mutations occurring during the process of attenuation, varies strain to strain and such regions are designated as regions deleted (RD1, RD2 RD3 and so on) (Mahairas, Sabo et al. 1996; Gebreyohannes 2007). Exhaustive genetic studies, using many molecular PCR techniques which compared BCG with *Mtb*, have identified 16 deleted RD regions in BCG (Behr, Wilson et al. 1999). However, the RD1 region is the most important, as it is present in virulent strains of the MTBC but is absent in all BCG vaccine strains and other non-virulent species of environmental mycobacteria. Figure 9 shows the genetic

differences between the various BCG strains, which are in use currently. Proteins encoded by genes from RD1 regions, like ESAT-6 (6-kDa) and CFP-10 (10-kDa) are useful for improved development of vaccines which are effective against infections with the virulent strains of MTBC. The CFP-10 and ESAT-6 proteins are the most immunodominant antigens with the potential to elicit multiple cells of immune system to intervene protective immunity, and thus they play an imperial role in deciding mycobacterial virulence (Guinn, Hickey et al. 2004).

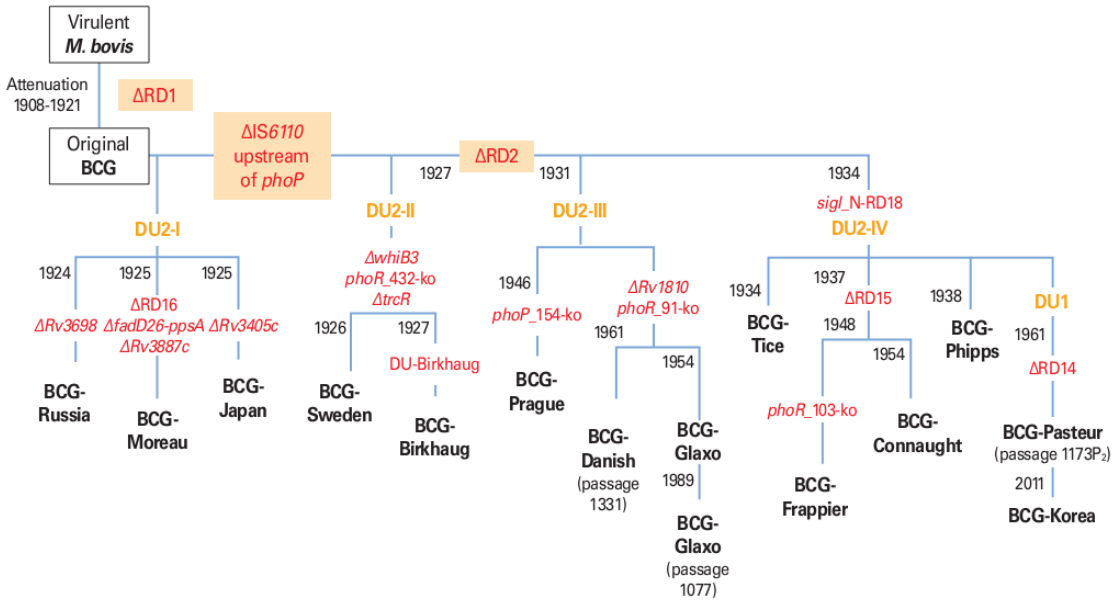


Figure 9. Refined genealogy of BCG vaccines. *Ref: Liu J, Tran V, Leung AS, Alexander DC, Zhu B. BCG vaccines: their mechanisms of attenuation and impact on safety and protective efficacy. Hum Vaccine 2009; 5:70-8. https://doi.org/10.7774/cevr.2013.2.2.83*

The RD1 gene deletion in the *Mtb* is known to reduce of mycobacterial virulence, similar to that of the non-virulent BCG strain in mice and cell lines (Lewis, Liao et al. 2003). ESAT-6 and CFP-10, down-regulate generation of Nitric oxide and inhibit the up-regulation of the B7 group of co-stimulatory markers in macrophages

infected with mycobacterium, thereby facilitating immune-evasion of mycobacteria (Miljkovic and Trajkovic 2004; Gebreyohannes 2007).

The original attenuated *Mycobacterium bovis* Bacille-Calmette-Guerin was continuously sub-cultured for many years until they were conserved and their stocks were preserved by lyophilisation. BCG was further distributed to various global locations where the culture resulted in what is now known as BCG Russia, Japan, Copenhagen, etc. Enhanced sequencing methods shows that additional genetic deletions occurred in these strains (Abdallah, Hill-Cawthorne et al. 2015). Many countries have only used one strain of BCG, while few have used two or more strains, thereby making strain implicated immune protection analysis in population difficult (Ritz and Curtis 2009). The different vaccine strains induce varying degree of T-cell response *in vitro* PBMC cultures from those immunised with different BCG vaccine strain (Andersen and Doherty 2005). Recently it was shown that the more virulent BCG strains (BCG-Phipps, BCG-Frappier, BCG-Pasteur, and BCG-Tice) exhibits highest levels of protection in severe combined immunodeficient mice (SCID) than the least virulent BCG strains (BCG-Sweden, BCG-Birkhaug) (Zhang, Ru et al. 2016). Finally the efficacy of BCG vaccine depends upon vaccination protocols**.** The efficacy of BCG vaccine varies depending upon other protocols related factors, involved in immunization. Many protocols vary due to the different route of vaccination and dose of vaccine bacteria administered (Gebreyohannes 2007).

2.8.3. Other qualities of BCG Vaccine

In spite of its lesser efficacy to resist PTB in adults, the BCG immunization programme is still the most outreached programme globally and is widely produced

and distributed with huge economics involved. The primal reason for its wide-scale use in vaccinating children is its higher efficacy against miliary TB and TB meningitis only in infants (Colditz, Berkey et al. 1995; Gebreyohannes 2007). There are few traits with BCG-vaccine, which other vaccines do not contain, which makes BCG a highly accepted choice. First of all, BCG is safe to human use, with nil adversaries', and therefore can be trusted for its use in neonates without any scare (Gebreyohannes 2007). Eventually, BCG is an economic choice and very easy to manufacture as an industrial pharmaceutical and therefore it is easily accessible and available to the people in remote regions of third world countries, which are actually the major contributors and benefactors of BCG vaccination and TB control.

2.8.4. Limitations of BCG as a vaccine

BCG vaccine efficacy differs from a country to country, race to race with its efficacy to be very little in adults as compared to the children (Colditz, Brewer et al. 1994; Mangtani, Abubakar et al. 2013). Even though the advantages of BCG vaccine outweigh its disadvantages, using BCG comes with some limits of using the BCG vaccine adding to its already limited efficacy against PTB. Its uses and cross-interference with the Mantoux skin test make it extremely hard to distinguish DTH to TB infection or BCG vaccination (Gebreyohannes 2007). This creates a problem in veterinary medicine in which the skin test is performed to detect bovine TB as a means of test and slaughter policy. This issue could be tackled by the genomic analysis of different mycobacteria, using diagnostic tests (Cockle, Gordon et al. 2002; Gebreyohannes 2007). Other restraint of BCG is the unfavourable response which comes from the observation of Lotte et al. which showed that BCG leads to life-threatening complications in about 1-3 individuals vaccinated per 10 lakh BCG

immunized subjects (Lotte, Wasz-Hockert et al. 1984). In a few studies, around 4-5% of immunized people had a complaint of adverse symptoms post BCG immunization (Turnbull, McIntyre et al. 2002). Unfavourable patch at the injection site, with or without the involving of nearest lymph node, were seen in almost half of the vaccinated cases (Gross, Kahan et al. 2004; Gebreyohannes 2007). The risk factors contributing to the tuberculosis are pre-existing metabolic illness, chronic smoking, and alcohol addiction, bad air quality, chronic steroid treatment, cancer, and malnutrition which adds up to further adds to the chance of BCG vaccine failure to protect against TB (Gupta, Shenoy et al. 2011).

2.8.5 Improving BCG Vaccine Efficacy

Newer approaches like genetic recombination, prime-boost vaccination, immunomodulation and use of organisms apart from *M.bovis* to prevent primary TB, reactivation of latent TB or better early clearance have been taken up for research. The advent of DNA recombination methods helped the scientific fraternity to integrate foreign DNA and its stable expression in mycobacteria (Burlein, Offutt et al. 1994), many attempts were directed towards using BCG as a recombinant vaccine delivery agent (Stover, De La Cruz et al. 1991; Gebreyohannes 2007; Stedman 2017). BCG was employed as delivery vehicle to deposit and initiate expression of the genes coding for immunodominant antigens from virulent *Mtb*, leading to the improvement in its efficacy (Nascimento, Rodriguez et al. 2017; Ahn, Tran et al. 2018). The virulent mycobacteria possess many virulent determinants which could be integrated with BCG (Forrellad, Klepp et al.).

A recombinant BCG::AB strain was developed which over-expresses immunodominant antigens Ag85A and Ag85B of *Mtb* have shown promising *results* protecting against TB as compared to parental BCG vaccine (Wang, Fu et al.). Another recombinant BCG::ESX-1^{Mtb} vaccine developed, was the one which expresses esx-1 locus of *Mtb.* The virulent *Mtb* having intact EXS-1 locus secretes ESAT-6 protein, which allows the bacteria, the entry to phagosome of the host cytosol. The *recombinant* BCG:: ESX-1^{Mtb} was more virulent and protective than BCG::ESX-1^{Mmar} (recombined from an esx-1 locus of *M. marinum*) against *Mtb*. The recombinant BCG::ESX-1 recombinant was able to induce phagosomal rupture, autophagy and lead to the elicitation of IFN-γ in THP-1 cells. BCG::ESX-1 induced higher protection in mice as compared to BCG by activating more $CD8^+$ T cells and $CD4^+$ Th1 cells specific to ESX-1 antigens (Groschel, Sayes et al. 2017). Recombinant BCG ΔureC::hly secreting listeriolysin toxin of L. monocytogenes with defunct urease-C (VPM1002) was able to rupture phagosome and induces apoptosis and autophagy by targeting inflammasome in macrophages. The deficiency of urease-C and concurrent acidification of phagosome caused by it allows listeriolysin to perforate membrane. The production rights of this vaccine is licensed to Serum Institute, India and is currently in Phase 3 trial conducted by **Indian Council of Medical Research (ICMR)**

Further, efferocytolytic processing of the recombinant BCG induced apoptotic vesicles carrying mycobacterial antigens which stimulate $CD4^+$ as well as $CD8^+$ T cells (Korns, Frasch et al.). Furthermore, the efficacy of BCG ΔureC::hly was improved by deleting anti-apoptotic virulence gene *nuoG*, encoding NADH dehydrogenase 1 subunit G resulting in reduced bacillary load in murine lungs,

ameliorating pulmonary pathology and thereby enhancing immune responses (Gengenbacher, Nieuwenhuizen et al.). Multiple immunizations by BCG ureC::hly did not improve the protection against TB (Gengenbacher, Kaiser et al.). Another important recombinant BCG (AEREAS-401, AREAS-422) is strain AFRO-1 expressing mutated perfringolysin-O. The mutated perfringolysin-O shows minimum cytotoxicity and can rupture phagosome in a wide pH range. AFRO-1 enhances immune responses and offers longer survival in mice against hypervirulent *Mtb* strain HN878 (Sun, Skeiky et al. 2009).. Clinical trials have earlier shown the role of NK and cytotoxic T-cell in inhibiting mycobacterial growth, and therefore they could be targeted for improved vaccines against TB. Simultaneously, prime-boost vaccination with BCG primed with alanine-proline-rich antigen (Apa) subunit, which itself is a cell-surface adhesion and secretory glycoprotein from mycobacteria improved protection against *Mtb* (Nandakumar, Kannanganat et al. 2016). It was shown that liposomized α-crystalline 1 a latency-associated antigen confers protection when used as BCG booster (Siddiqui, Amir et al. 2015). A replication-deficient recombinant vaccinia virus Ankara expressing antigen 85A (MVA85A) induced significantly higher levels of antigen 85A specific polyfunctional $CD4^+$ T cells (Beveridge, Price et al. 2007).

However, preclinical Phase 1/2a clinical studies shows that MVA85A did not enhance protection against TB disease better than by BCG, although it was a safe vaccine in infants (Tameris, Hatherill et al. 2013). Further, ChAdOx1.85A, a replication-deficient chimpanzee adenovirus expressing Ag85A was able to improve the protective efficacy of BCG vaccination (Stylianou, Griffiths et al. 2015). Similar virus-based booster VSV-846 led to enhanced cellular immune responses against

mycobacterium infection (Zhang, Dong et al. 2017). Other recombinants like SeV85AB (recombinant Sendai virus encodes Ag85A and Ag85B) vaccination-induced high-level protection exclusively through $CD8^+$ cells compared to BCG. It also induced high levels of lung-resident memory $CD8^+$ T (T_{RM}) cells and strong recall responses post-infection (Hu, Wong et al. 2017). Apart from recombined BCG, a heat-killed *Mycobacterium indicus pranii (*formerly known as *Mycobacterium w),* when used by the aerosol route, enhanced BCG induced Th1 and Th17 immune response in the lungs of infected guinea pigs along with polyfunctional T cells with effector function (Saqib, Khatri et al. 2016). This vaccine is named after Dr Pran Talwar the discoverer of this vaccine, it was extensively researched by National Institute of Immunology (NII), India and the production rights were licensed to Cadila Pharmaceuticals, Ahmedabad**,** India as "Immuvac" and is currently under phase 3 trial conducted by ICMR along with VPM1002.

Currently, BCG vaccine efficacy improvement did not convince or cleared any trial to be used extensively for human. Therefore, host immunomodulatory approaches which enhance the immune responses to conventional BCG could be considered.

2.9 Immunomodulator and host immunomodulation

An immunomodulator is a natural or synthetic substance that helps to regulate or normalise the immune system. Immunomodulator correct immune system which has lost their balance. Immunomodulation can correct weak immune systems and mend immune responses which are hyper or underactive, but they do not boost the immune system in the way immune stimulants do. An immunomodulator is recommended for someone with chronic autoimmune diseases, or they are used to restore the immune system in patients who have been on lengthy courses of antibiotics

or anti-viral therapies (Hardman G. Jeel 2001). There are varied potential targets when an immunomodulator may act involving different cells, target and mode of actions. Immunomodulator could be stimulators or suppressant, specific or nonspecific. These immunomodulators may interact with other medicines and affect their metabolism and action (Desai 2002; Pratibha M 2002).

Synthetic immunomodulator, such as azathioprine, 6-mercaptopurine, methotrexate, and mycophenolate mofetil, have been shown to suppress the immune system, thereby decreasing inflammation in the gut of the patients with inflammatory bowel disease, ulcerative colitis, and Crohn's disease. Tacrolimus was shown to be useful in Crohn's disease when corticosteroids are ineffective, providing any relief. In children, immunomodulator does not cause growth failure than corticosteroids (Jain 2001; Das 2004). Topical use of immunomodulators such as Tacrolimus and Pimecrolimus has been found to offer benefits in patients with eczema, vitiligo and nickel allergy. The herbal or natural molecule immunomodulators are widely used as immunosuppressive agents as they possess lesser side effects and have a lesser regulatory impact.

Bacterial extracts have been widely used as immunomodulators to prevent recurrent infections of the respiratory preparations containing bacterial lysates (e.g., Broncho-Munal ®, Luivac®) and are licensed for use in Europe (Arandjus, Black et al. 2006). In the case of TB, immunomodulation using pre-exposure subunit vaccines have been considered. It is thought that secretory proteins of living bacilli in the phagosome are the first antigens to be presented to the immune system during the early phase of infection resulting into an effective protective immune response (Andersen, Askgaard et al. 1991). Andersen et al. have described short term culture filtrate (STCF) rich in

secretory antigens from *Mtb*. Multiple research groups have reported the protective effect of immunization with culture filtrate proteins (CFP's) from log-phase *Mtb* cultures in mice as well as in guinea pigs (Pal and Horwitz 1992). They were also successful in showing that this protection was transferable by $CD4^+$ T cells. Extensive vaccine studies with ESAT- 6, have shown it to be an immunodominant target for IFN-γ producing T cells from infected mice (Pal and Horwitz 1992) and *Mtb*-infected patients (Ravn, Demissie et al. 1999).

Along with ESAT-6, another antigen, Ag85B, has also shown to induce partial protection in infected mice (Huygen, Content et al. 1996) and infected guinea pigs (Doherty, Olsen et al. 2004). In spite of these studies, vaccination with Ag85B-ESAT-6 in IC31® lead to a relatively weak ESAT-6 response (van Dissel, Arend et al.) generating a concern to reconsider ESAT-6 as a priority antigen. Many researchers focused on replacing ESAT-6 by TB10.4 (Rv0288/EsxH). TB10.4 was strongly recognised as ESAT-6 in TB patients, and BCG vaccinated donors as compared to ESAT-6) (Skjot, Oettinger et al. 2000). Vaccination with TB10.4 also induced significant protection in the mouse model (Dietrich, Aagaard et al. 2005) but none of these targets is yet employed, or FDA approved for human use. Adjuvants mentioned above may use oil-in-water emulsions. It has been shown that this can augment the efficiency of vaccination against *Mtb* (Lima, Bonato et al. 2001). In particular, cationic liposomes consisting immunostimulants such as monophosphoryl lipid A and trehalose dibehenate in cationic liposomes based on lipoid surfactants were shown to be useful in vaccination against *Mtb* (Holten-Andersen, Doherty et al. 2004). All of these adjuvants are strong inducers of Th1 immune responses. Many researchers have also focused on using *Mtb* lipids through various methods involving coating and

encapsulation for vaccination, and mixed efficacy was observed in the experimental animals (Owais, Masood et al. 2001).

Herbal formulations and compounds have been used as immunomodulators. Table 4 represents various natural product classes with established immunomodulatory potential. Substances derived from the plants' origin have a current commercial value for treating heart disease, high blood pressure, pain, asthma, and other problems. For example, ephedra and its active component Ephedrine is a herb used in traditional ancient Chinese medicine know to treat asthma and other respiratory problems (Nelson Jr and Ballow 2003; Spelman, Burns et al. 2006). Herbal anticancer, immunomodulatory properties of propyl gallate, a phytochemical polyphenolic compound, were highlighted by Yang et al. on how it affects migration of malignant glioma cells through inhibition of ROS and the NF-κB pathway.

S.No.	**Natural Compounds**	
	Low molecular weight compounds	
1	Alkaloids	(e.g., cocaine, vincristine)
2	Terpenoids	(e.g., sesquiterepnelactones, diterpenes, triterpens)
3	Phenolics	(e.g., flavonoids, coumarins, quinones, tannins)
	High molecular weight compounds	
1	Lectins	(e.g., Concanavalin A)
2	Polysaccharides	(e.g., lentinans, angelan, arabinogalactans, acemannans,)
3	Nucleotides	(e.g., poly I:C)

Table 4: Natural product class with proven immunomodulatory potential. *Ref: Kayser, et al. "Natural products and synthetic compounds as immunomodulators." Expert review of anti-infective therapy1.2 (2003): 319-335*

Many immunomodulatory plant-derived single chemical entities like curcumin, resveratrol epigallocatechin-3-gallate, quercetin, qolchicine, capsaicin, andrographolide, genistein have been extensively studied in clinical trials (Jantan, Ahmad et al. 2015). Cheng et al. had shown Th-1 selective immunomodulatory activity of crude leaf extracts from *Neolitsea* spp., Arruda et al. have reported the modulatory activity of *Mentha piperita* (peppermint) leaf hydroalcoholic extract on macrophages. Furthermore, S. Lewicki et al. explored the immunomodulatory properties of the *Rhodiola kirilowii* to stimulate innate immunity. Natural compounds from *Moringa oleifera* demonstrated protective effects against hepatitis B virus (HBV) infection, particularly for genotypes C and H. Another important group of natural compounds are naphthoquinones. The naphthoquinones such as phylloquinone, menadione and vicasol increased the immune response in rats after intensive physical activity. The most marked studied was phylloquinone for its immunomodulatory effects (Rybnikov, Laskova et al. 1997). Without any doubt, much work is yet to be done, in this respect as natural immunomodulators are highly promissory due even due to the cultural experience for thousands of years. As the research progresses, the more exciting role of the conventional chemicals is being unveiled, which can prove to be helpful in the prevention and treatment of inflammatory and immune-regulated diseases. Considering the multiple biological effects of natural compounds, it is lucrative to design future therapeutic strategies for inflammatory pathologies resulting out of *Mtb* infections with a synergistic combination of natural products, maybe along with conventional therapies.

2.10 Significant findings on the regulation of the immune response and our basis of working hypothesis.

Our work and the hypothesis was directed as per many theoretical generalisations from the literature, available in the public domain. I would discuss the evidence supporting these generalisations sequentially one by one. First of all, it is empirical to consider that any ratinonal approach to immunisation should find the ground in the information of the immune reaction in the people affected by the pathogen, stay normal as they develop an efficient immune response, especially in those who progress into the disease. There is enormous disagreement over the ideal immunological parameters used to identify and differentiate vaccine efficacy. Much evidence is available from other chronic diseases caused by intracellular pathogens showing majorly cell-mediated, Th1, responses. These observations give a strong correlate with protection against intracellular pathogens causing chronic diseases. In a mice model of study, resistant mouse strain like C57BL/6 generates a predominant cell-mediated, Th1, response with minor antibody fraction containing the infection, while the mouse prone to the strains like BALB/c suffer progressive disease, by generating Th2 or mixed Th1/Th2 response with substantial levels of serum antibodies during the initial phase of infection. Gradually both C57BL/6 and BALB/c mice succumb to the infection depending upon infecting dose of *Mtb*. A canonical cell-mediated, Th1 response is protective, but the disease would arise, either if the responses are not strong enough or are not of the right kind, i.e. having a noteworthy Th2 element. We can hypothesise that a strong and exclusive Th1 response is protective against TB, while a weak Th1 response or a response with a significant Th2 component is less protective or insufficient. This viewpoint could be justified by

taking the ratios of the T cell cytokines where IFN-γ:IL-4 ratio determines Th1:Th2 while IFN-γ:IL-10 determines Th1 and Treg balance.

The hypothesis was built up around on how to guarantee a strong Th1 response on natural infection using an immunomodulator; given the phenomenon of Th1 imprinting, it seems essential to successful vaccination. If an immunomodulator polarises BCG induced Th1 responses in mice, it would further lead to the development of effector functions which are indicative of Th1 responses. Study of macrophages, therefore, is a prime aspect of such an arms race between pathogens and the immune system. Macrophages differentiate into distinct effectors including classically (M1) and alternatively (M2) activated macrophages under the influence of $CD4^+$ helper cell cytokines, such macrophage cell type polarisation could be determined by measuring NO, TNF-α and IL-1β in vitro.

Complete $CD4^+$ T-cell priming needs cytokine signalling. During priming, the type and combination of the cytokines in the distant milieu does affect the proliferative and effector capabilities of the activating cell. The balance of cytokine signals helps drive the CD4 cell lineage depending upon the subset of cells, influencing their sustenance and develop antigenic memory (Pepper, Pagan et al. 2011; Oestreich and Weinmann 2012). A series of lineage-defining cytokines into memory $CD4^+$ cells by regulating their respective transcription factors have been described for Th1, Th2, Th17, Tfh, and Treg subsets, and are discussed previously in this thesis. Th1 cytokines IL-12, IFN-γ and Th2 cytokine IL-4 drive lineage commitment and their roles in the generation of CD4 T-cell memory is well documented. Therefore we hypothesise that a favourable Th1 and M1 response

induced by immunomodulator could, therefore, develop an antigen committed T cell memory.

An inverse relationship between cell-mediated and humoral immunity is pivoted by suppressor T-cells (T regulatory or Treg), which are induced under various stimuli. Tregs, which are developed due to natural infections, helps regulate the inflammatory response. Therefore they would be induced as an immune response to vaccination, particularly for live attenuated vaccines like BCG. The beneficial immunoregulatory role by which overwhelmed or exuberant immune response is checked by these cells. However, multiple studies indicate that Tregs possibly interfere with the generation of favourable vaccine-induced immunity. Some researchers had shown that antigen-specific Tregs induced by environmental mycobacteria suppress Th1 immune responses, thus compromising the response to BCG immunization in mice (Ho, Wei et al. 2010). Thus, depletion of Tregs before vaccination in the mouse model has been shown to enhance immune responses to some vaccines. Studies in a DEREG mouse, which can *in vivo* depleted of $Foxp3^+$ Treg cells by diphtheria toxin (Lahl and Sparwasser 2011), showed an enhanced anti-tumour response to immunization against existing melanoma (Klages, Mayer et al. 2010). In another study, transient depletion of Tregs in DEREG mice considerably improved vaccine-induced immunity against a solid tumour by recruiting and activating NK cells and CD8 T cell by IFN-γ (Mattarollo, Steegh et al. 2013). Depletion of $CD25^+$ Tregs by injecting anti-CD25 monoclonal antibody has been shown to induce more durable immunity in BCG immunized mice (Moore, Gallimore et al. 2005). Daclizumab has been used in many human breast cancer vaccine trials known to deplete Tregs and improve effector response. It is also known to reprogram

naïve Tregs to become IFN-γ generator (Rech, Mick et al. 2012). The human monoclonal antibody, Ipilimumab which inhibits Tregs by blocking CTLA-4, was approved by the FDA in 2011 for use in melanoma patients (Peggs, Quezada et al. 2009). Low dose, cyclophosphamide also have been described to transiently decreases Treg frequencies while preserving Teff functions, leading to improved responses to vaccine antigens in mouse and human cancer vaccine trials (Barbon, Yang et al. 2010).

The difficulty exists in translating murine experimental results to humans since mice Tregs are not phenotypically similar while depleting $FOXP3^+$ Tregs in healthy in humans becomes an ethical challenge. Still, only a few studies have investigated the role of Tregs in controlling vaccine immunogenicity in infants. Therefore the immunomodulator of choice which could improve BCG vaccine response should either improve antigen-specific Th1-M1-memory, T cell polarization or regulate or suppress the regulatory T cells without inducing any tissue-specific or general cytotoxicity *in vivo.* In the current pretext of immunomodulation, we selected a few naphthoquinones and studied their potential as an immunomodulator. We realised that naphthoquinone juglone held promissory potential with some preliminary experiments which are now out of the scope of this thesis. We performed several *in vitro*, *ex vivo* and *in vivo* experiments in the assumption that juglone would hold true to our hypothesis. Chapter 3 is dedicated to this work on the determination of juglone as an immunomodulator of BCG vaccine-induced responses.

Chapter 4, on the other hand, is dedicated to the understanding of *Mtb* lipids isolated from various clinical isolates and their utility as a vaccine adjuvant to improve BCG vaccine efficacy. The various experiments are reported in the literature,

which uses genetically distinct MTBC strains to infect human primary macrophages and mice. A clade-specific virulence pattern was reported in both of the models. Human-adapted *Mtb* lineages (clade I) comprising "modern" lineages, such as Beijing and Euro-American Haarlem strains, are known for their capability to grow faster as compared to that of clade II strains like "ancient" lineages, such as, e.g., East African Indian or *M. africanum* strains. (Reiling, Homolka et al. 2013). These clades and strains vary in virulence, growth rate and also determine disease pathology in the experimental animals.

A study from our laboratory showed that drug-resistant clinical isolates of *Mtb* having differences in their genotypes exhibited differential host responses in THP-1 cells for the studied parameters of intracellular growth, phagocytic index, induction of proinflammatory cytokines and apoptosis (Chakraborty, Kulkarni et al. 2013) although few studies suggested that the inflammatory profiles neither correlate with virulence in the experimental setting nor with epidemiological success (Reiling, Homolka et al. 2013) but these studies considered a single parameter unlike the study from our laboratory. Thus, more the number of parameters studied in both host and the bacterium enhance the range and prediction of virulence characteristics of clinical isolates. The mycobacterial virulence characteristics in macrophages and mice infected with the mycobacterium show that the MTBC genetic difference translates into pathogenic, differences in the host (Andrade, Amaral et al. 2012; Reiling, Homolka et al. 2013). Our hypothesis also revolves around the assumption that these genetic differences might also translate into their immunogenicity in an infecting host. Significant host-pathogen interaction and factors modulate the outcomes of infections, and therefore mycobacterial subunits might behave differently as compared to a live

bacterium in generating an immune response, keeping this in mind we also determined the variation in cytokine elicitation by the lipids from different clinical isolates and the live bacterium on the RAW267.4 cell line.

The importance of mycobacterial lipids in developing a disease in a host could be realised from a study which reported that the loss of lipid virulence factor was an instrumental factor in reduced the efficacy of the BCG vaccine (Tran, Ahn et al. 2016). Lipids and glycolipid molecules of *Mtb* origin are known to be presented to T-cells by CD1 cell-surface molecules in humans and other animals (Girardi and Zajonc 2012). These lipid-specific T cells have the cytolytic and bactericidal ability by secreting various pro-inflammatory cytokines (Dasgupta and Kumar 2016). There are studies described in the literature where lipids from *Mtb* were incorporated into liposomes with adjuvant and tested as vaccines in a guinea pig aerosol TB challenge model. The animals vaccinated with mycobacterial lipids showed reduced bacterial burdens in the lung and spleen at 1 month after infection with considerably lesser pathology and smaller granulomatous lesions as compared to control animals. The percentage of lung occupied by TB was notably smaller in lipid-vaccinated animals as compared to vehicle control guinea pigs (Dascher, Hiromatsu et al. 2003).

Further, in another rodent study in which a mice fed with lipid-encapsulated-BCG gave protection against aerosol challenge with *Mtb* (Aldwell, Brandt et al. 2005). Furthermore, the oral vaccination with lipid-formulated BCG induced a long-lived, multifunctional $CD4^+$ T cell memory immune response (Ancelet, Aldwell et al. 2012). Human studies also showed induction of *Mtb* lipid-specific T-cell responses by pulmonary delivery of mycolic acid-loaded polymeric micellar nanocarriers (Shang, Kats et al. 2018). These data support an imperative role for the lipid antigens in

developing the immune response to *Mtb* infection, probably through the generation of CD1-restricted T cells. Immunogenic lipids, therefore, represent a novel class of antigens that might enhance the protective effects of subunit-vaccine formulations. An effective subunit vaccine formulation should thus contain an array of protective antigenic elements to stimulate different T cell subsets possessing distinct but complementary effector-functions to control infection (Ellner 1997). Hence, studies are required to identify *Mtb* strains rich in the subsets of these immunogenic lipids in vitro (culture medium) and *in vivo* (mice infected diseased lung).

Therefore our studies were aimed at to make a comparison of CD1d (only class of lipids presented in mice) putative lipid status of the *Mtb* strains by comparing it to cytokine profile elicited by these respective *Mtb* strain lipids used to re-stimulate T cell co-culture from BCG vaccinated mice when used as a vaccine booster. Our assumption lies in the hypothesis that CD1d rich mycobacterial strain could be identified by such comprehensive lipids analysis conjoint with other vaccine efficacy parameters which could help bolster BCG vaccine development strategy. Our study refutes the assumption that there is a simple correlation between cytokine induction as a single functional parameter of host interaction and mycobacterial virulence while careful consideration of strain and lineage-specific characteristics could guide the attempts in deciphering immunological outcomes of infection with MTBC.

2.11 Hypothesis

"Naphthoquinone Juglone and Lipids from Mtb clinical isolates can improve BCG vaccine efficacy."

Chapter 3. Effect of Juglone immunomodulation on the BCG vaccine efficacy

3.1 Introduction

Juglone (5-hydroxy-1, 4-naphthalenedione) is a naturally occurring naphthoquinone, aromatic organic compound, found in the black walnut (*J. nigra)* and other plants of Juglandaceae family. The naphthoquinones known for their significant utility are juglone, lawsone, plumbagin, and lapachol (Babula, Adam et al. 2009). Juglone has a distinct phenyl group and is produced by produced by the walnut tree with other phenolic compounds like flavonoids, terpenoids, other naphthoquinones (gallic acid, caffeic acid, myricetin, and quercetin) (Nahrstedt, Vetter et al. 1981; Hirakawa, Ogiue et al. 1986; Sharma, Ghosh et al. 2009). The yellow pigmentation in the walnut tree is due to juglone (Inbaraj and Chignell 2004). Since ancient times juglone was known to humanity as a metabolite and allelochemical. Allelochemical is a compound not required for the growth, development, and reproduction of an organism, but is believed to have a biological effect on other organisms. Contrasting studies suggests that juglone may also play a role in plant development (Duroux, Delmotte et al. 1998).

The walnut tree had a widespread and geographically recognition, all around the world, mainly pertaining to the subjects of traditional medicine (Strugstad and Despotovski 2013). The hull of the walnut was being used as a remedy for parasites, ringworm, and other fungal infections, to heal ulcers and skin eruptions (Morton 1974). The American doctors during the early 20^{th} century prescribed juglone for the treatment of various skin diseases (Soderquist 1973), while southern Americans used

walnut husks to stun during fishing (Gries 1943). The Indian, Greek and Arab cultures, refers to the walnut tree for treatment of common illnesses and cancer (Sharma, Ghosh et al. 2009). In a few countries, walnut tree twigs have been used as a toothbrush or to make dye for colouring hair (Alkhawajah 1997). The concoctions from the walnut tree are known for keratolytic (Bezinger, Pinkas et al. 1990), antifungal (Nahrstedt, Vetter et al. 1981), antimicrobial (Sharma, Ghosh et al. 2009), antidiarrheal, anthelmintic, antihemorrhagic, anti-scrofulous, hypoglycemic (Strugstad and Despotovski 2013), diuretic, laxative, and with blood purifying, and detoxifying ability (Haque, Bin-Hafeez et al. 2003; Stampar, Solar et al. 2006). Furthermore, antiviral (Husson, Bilagines et al. 1986), and vascular protective (Perusquia, Mendoza et al. 1995) usefulness of walnut has also been discussed in literature before. Walnut has the highest significance in agroforestry system management due to its toxic effects on neighbouring plant growth (Von Kiparski, Lee et al. 2007). However, toxicity is not limited to the plant kingdom, but also in various protista, fungi, and animal groups, where they can either be controlled or killed by juglone or its extracts.

The mechanism for the toxic effects of juglone has not been clearly described. Many pathways have been suggested which include cell death, cell cycle disruption, DNA modifications (predominantly rapid dividing cells), inhibition of mRNA synthesis, alkylation of thiol or amine groups of essential proteins, and decreasing levels of p53 (tumour suppressor) (O'Brien 1991; Paulsen and Ljungman 2005). H^+-ATPase inhibition was also discussed as a possible mechanism of action (Hejl and Koster 2004), while some researchers discussed it as K^+ channel block (Varga, Bene et al. 1996). Juglone was shown to be reduced by mitochondrial and cytoplasmic enzymes to form a semiquinone radical (Inbaraj and Chignell 2004). Juglone was

cycled back by a process called redox cycling producing hydrogen peroxide, a strong oxidant which could cause oxidative damage to the cell (Inbaraj and Chignell 2004). Juglone had high toxicity as compared to other naphthoquinones due to its high redox potential of -93mV (O'Brien 1991). It has also been shown as highly electrophilic and thiol-reactive, causing irreversible protein complexation, especially the cysteine-rich proteins which are important in mitosis (Von Kiparski, Lee et al. 2007). Several naphthoquinones, along with juglone, have shown a broad range of toxic effects, associated with inhibition of growth, photosynthesis and respiration, reduced water transport in plants, larval development dysfunction, mitochondrial damage in insect muscles (Hejl and Koster 2004; Strugstad and Despotovski 2013). Further, the medical utility of juglone has also been studied by many research groups. Juglone has shown antifungal properties similar to some commercially available antifungal agents used to treat infections, such as athlete's foot and ringworm (Clark, Jurgens et al. 1990). One study showed that juglone inhibits three key enzymes from *Helicobacter pylori*, a causative agent of gastritis, peptic ulceration, and gastric cancer in humans, which could be used to treat infections from this bacterium (Kong, Zhang et al. 2008). Kiran et al. have shown 20 % mortality along with toxic symptoms in animals injected with 3 mg/kg BW juglone. The higher dose of 6 and 10 mg/kg BW juglone resulted in 60% and 100% mortality, respectively. They had established the LD_{50} dose for the juglone to be 4.2 mg/kg BW while a sub-lethal dose of 1 mg/kg BW juglone as an optimum dose for their anticancer studies (Aithal, Kumar et al. 2012).

Juglone is now, established as a cytotoxic agent, known as a natural inhibitor of Pin1 (peptidyl-prolyl cis/trans isomerase or PPIase) (Von Kiparski, Lee et al. 2007). Various other natural and synthetic inhibitors of Pin1 apart from juglone like

PiB, dipentamethylenethiuram monosulfide have also been identified (Moore and Potter). Since a long time, juglone was popular as an active compound from black walnut trees as the only allelopathic chemical, that inhibits germination or growth in other plants (Von Kiparski, Lee et al. 2007). In the later course it was realized to inhibit cancer cells showing anti-tumour effect in various pancreatic, ovarian, breast, cervical cancer and various other cancer cells (Fang, Qin et al.; Jin, Zhang et al.; Karki, Greenway et al.; Zhang, Liu et al.). Apart from its cytotoxic, genotoxic and anti-metastatic properties, ability of juglone to inhibit Pin1 determines functions of many phosphoproteins (Lin, Li et al.; Wulf, Liou et al. 2002; Lee, Tun-Kyi et al. 2009) having imperative role in the regulation of immunity (Esnault, Shen et al. 2008), cell cycle regulation (Wulf, Liou et al. 2002; You, Zheng et al. 2002; Yeh and Means 2007), ageing (Wulf, Liou et al. 2002) and cancer (Wulf, Liou et al. 2002; Yeh and Means 2007).

The bystander effects of conventional cytotoxic and antimetastatic drugs have shown immunomodulatory and immunostimulatory effects (Matar, Rozados et al. 2002). Nontoxic metronomic doses of paclitaxel (Taxol) (Machiels, Reilly et al. 2001; Zagozdzon and Golab 2001) and derivatives (Krawczyk, Å• uczak et al. 2005) , cisplatin (de Biasi, Villena-Vargas et al. 2014), cyclophosphamide (Brode and Cooke 2008; Sistigu, Viaud et al. 2011), cyclosporine (Choi, Shin et al. 2016), methotrexate (Li, Ling et al. 2015) , 5-fluorouracil (5-FU) (Cao, Zhang et al. 2014), doxorubicin (Inoue, Setoyama et al. 2014) have been repurposed as immunomodulators. These drugs either show increased dendritic cell activity or Treg suppression thereby up-regulating presentation of antigens to ag-specific T cells *in vitro* while drugs such as gencitabine selectively reduce myeloid-derived suppressor cell (MDSC) and enhance

the anti-tumour activity of $CD8^+$ T and NK cells (Nars and Kaneno 2013). Cyclophosphamide, an alkylating agent, at lower concentrations have shown to induce type I interferon to drive memory T lymphocytes phenotype, Th2/Th1 polarisation, cytokine shifts, and dendritic cell homeostasis along with differential suppression of Treg in mice. With the context that Pin1 modulates activity of TLR mediated signalling molecules (Kawai and Akira 2007) collectively in NF-κB and IRFs signalling pathways along with post-transcriptional regulation of Th1 cytokines by activated T cells (Esnault, Braun et al. 2007) it was speculated that natural and synthetic inhibitors of Pin1 like juglone, PiB, dipentamethylenethiuram monosulfide may act as an immunomodulator (Moore and Potter 2013). Immunomodulatory drugs act through a various stimulatory, suppressive or regulatory mechanism mediated by cells of innate or acquired immunity. These responses are generally propagated through various cytokines and chemokines assisted by $CD4^+$ T-helper cells. Though effector functions are carried out by other immune cells including $CD8^+$ T cells, B cells, macrophages, mast cells, neutrophils, eosinophils or basophils, their function depends upon the pattern of cytokine secretion and expression of specific transcription factors. Majorly recognized subsets such as Th1, Th2, Th17 and T regulatory (Treg) cells are the Th cell lineages those exist depending upon the cytokine they secrete or effector functions they undertake. These lineages could manifest switching from one subset to other by changing the course of immune response under the influence of immunomodulators. Such modifications of immune response with therapeutic intentions are not only desirable in the state of immunodeficiency but also sought to improvise or augment immune responses.

Various *in vivo* and *in vitro* experimental setups have been used to demonstrate compound induced immunomodulation (Sewell, Qing et al. 2003). To determine the immunomodulatory potential of juglone, we used ex-vivo BCG restimulation model. Modulation of BCG responses to determine the immunomodulatory activity of a pharmacological compound like lactoferrin has been previously demonstrated (Hwang, Arora et al. 2009; Sistigu, Viaud et al. 2011). Both Th1 and Th2 responses are generated by BCG immunization, with a dominant Th1 immune response leading to protective immunity against *Mtb*. Dendritic cell elicited vaccine response is based upon the balance of Th1 or Th2 along with Treg cell, which contributes to the vaccine imprint. The challenge is to sustain and modulate polarised Th1 induced responses which generally are rendered ineffective, in dominant or basal Th2 responses generated as a cross-reaction to exposure to helminths and non-tuberculous environmental mycobacteria which ultimately negatively impacts vaccine efficacy (Andersen and Doherty 2005; Poyntz, Stylianou et al. 2014). This is one of the major reason for ineffective vaccines against TB, leprosy and leishmaniasis where Th1 based cellular immunity is responsible for clearance of intracellular pathogens (Van Duin, Medzhitov et al. 2006). The variable protection of BCG arises due to generation of erratic immune responses in host followed by the waning of vaccine memory post-vaccination with time (Banatvala and Damme 2003; Heffernan and Keeling 2009). Protective cell mediated immunity is essential and more prominent than humoral immunity in controlling tuberculosis in human and mice. To overcome these adversities of BCG vaccine immunomodulators which may induce Th1 polarization and long term memory could be useful.

In this study, we evaluate the immunomodulatory activities of juglone. This was an attempt to demonstrate if juglone modulates Th1 responses by regulating Th1 cytokines in activated BCG specific T cells. Juglone had never been studied in the light of its ability to modulate vaccine-based immunity. We examined the role of juglone on RAW264.7 murine macrophage cell line, activated mice splenocytes and T cells. We also showed the effect of metronomic doses of juglone on BCG vaccine-induced immune response, its effect on antigen-specific cytokine shifts, IgG2a/IgG1 titre ratio, and splenocyte restimulation and tissue histopathology post-BCG vaccination

3.2 Study objectives

I. To determine if juglone generates a stable, predominantly cell-mediated/Th1 response against the mycobacteria in naïve adult and young mice immunised with BCG vaccine.

II. To determine whether the generation of cell-mediated responses following juglone treatment results in an M1 imprint in mice vaccinated with BCG.

III. To test the hypothesis that juglone could alter IgG2a/IgG1 switching, Treg, CD8, CD4 memory T cell population in BCG vaccinated mice.

IV. To determine whether juglone immunomodulation impacts an ongoing mixed Th1/Th2 response into a Th1 mode are effective in the mycobacterial system and to evaluate whether such modulation is associated with a reduction in bacterial burden.

3.3 Materials and Methods

3.3.1 Cell lines and reagents

Murine macrophage cell line RAW264.7 was procured from NCCS, Pune, India. Mice splenocytes, T cell co-cultures, splenic macrophages and RAW264.7 cell line was cultured and maintained in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, Sodium bicarbonate (2.0 g/L), antibiotic-antimycotic solution (HiMedia, Mumbai, India) and 10 % Fetal bovine serum (Gibco, Thermo Fisher Scientific, USA), at 37°C in 5% CO_2 incubator (Panasonic, Japan). All other chemicals were purchased from Sigma, Mumbai, India.

3.3.2 MTT assay for assessment of cytotoxicity

The cytotoxic potential of juglone was assessed using the MTT assay. Briefly, 1×10^4 cells per well were seeded in 96-well plates and incubated. Twenty-four hours later, cells were treated with juglone (0–12.5 μM) for 24 hr. Cells were washed with PBS and incubated further for 3 hrs with 100 μl of 0.5 mg/ml of MTT. The formazan crystals formed were dissolved in 100 μl of 10% SDS for 2 hrs, and absorbance was measured at 540 nm using a microplate spectrophotometer (Biotek Synergy HT Multimode Analyzer). Further, in another assay, RAW264.7 cells were pretreated with NAC (10 mM) and GSH (10 mM) and were consequently treated with juglone.

3.3.3 Measurement of intracellular ROS

The effect of juglone on the intracellular ROS levels was measured using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) fluorescence assay (Liu, Wang et al.

2012). Briefly, $5x10^4$ cells were pretreated with 0-20 μM DCFH-DA for 30 mins before treating them with different concentrations of juglone for 0-120 mins. Further, cells were washed, re-suspended in PBS, and the fluorescence intensity was measured using a microplate reader (Biotek) at excitation and emission wavelengths using 485/20 nm and 528/20 nm filter set, respectively. Results were expressed as arbitrary units of DCF fluorescence using KC4 software suite.

3.3.4 BCG Growth and Enumeration

BCG strain Moscow was purchased from Serum Institute, India. BCG stocks were propagated in Middlebrook 7H9 broth base (Hi-media, India) enriched with medium albumin supplement containing glycerol and ADC. The mycobacterial culture was further incubated at 37ºC in a humidified incubator environment with 5% CO_2 for 15-20 days. Mycobacterial cells were counted on the basis of their ability to form colonies by counting them while the multiply on the Middlebrook 7H10 agar media, as previously described (Gros et al., 1981). In short, the mycobacterial stock culture was subjected to shear in by using glass beads on vortexer in cold condition for 30 sec in a Branson Sonifier (Model 250) to dislodge the clumps of mycobacterium. Further the cells were passed through a 26 gauze needle and were diluted serially in 10 fold manner in PBS containing 0.05% Tween- 80. From each dilution, few drops of 10 μl each were seeded as a spot on plates containing Middlebrook 7H10 agar media base (Hi-media, India) supplemented with glycerin and OADC. The culture plates were incubated in a humidified, at 5% CO_2 incubator set at 37ºC. The mycobacterial colonies at the spot locus were enumerated 10-15 days after plating, and the average number of mycobacterial cells were calculated and expressed as cfu per ml. The

mycobacterial suspension was then stored in saline-tween 80 at -70^{o}C until used for injection (Gebreyohannes 2007)

3.3.5 Ethics statement and animal grouping

C57BL/6 mice were housed in a temperature and humidity-controlled polycarbonate cages and exposed to a 12h light-dark cycle. Each animal was randomly assigned to an experimental group. All procedures were reviewed and approved by the BARC Animal Ethics Committee, Mumbai, India (BAEC, 11/14). For the present study, animals were divided into three groups; Group A – Unvaccinated, group B – BCG-vaccinated and group C – Juglone treated BCG-vaccinated animals. Each group consisted of 4 animals each.

3.3.6 Immunization and juglone treatment

M. Bovis BCG, Strain Moscow (Serum Institute, India) was reconstituted in 0.9% physiological saline, and 0.1 ml ($1x10^6$ cells /ml) was administered to the experimental animals subcutaneously (SC) into the left inguinal region. The animals were rested for 9 or 65 weeks post-vaccination before being sacrificed for splenocyte isolation. In group C mice, 0.1 mg of juglone per kg body weight was injected intraperitoneally (IP) on two consecutive days before vaccination, while group A and B were injected with saline. Previously published work by (Aithal et al., 2012) have shown 20 % mortality along with toxic symptoms in animals injected with 3 mg/kg BW juglone while higher dosages of 6 and 10 mg/kg BW juglone resulting 60% and 100% mortality, respectively. They have established an LD50 dose as 4.2 mg/kg BW while a sub-lethal-optimal anticancer dose as 1 mg/kg BW for their anticancer studies.

We in our experiments have used a lower metronomic dose of 0.1 mg/kg BW of juglone in anticipation of a favourable immune response.

3.3.7 Preparation of splenocytes

Spleens of C57BL/6 mice were removed aseptically, and cell suspensions were made using sterile cell strainers (40 μm, BD Biosciences, USA) and RBCs in it were lysed subsequently. Non-adherent splenocytes passed through a nylon wool column (Polysciences, USA) were used as T cells and were adjusted at the concentration of 1×10^6 cell per well. Mitomycin C treated syngenic mice splenocytes were used as antigen-presenting cells (APCs) and were co-cultured with T cells at a concentration of 2.5×10^6 cells per well in 24-well plates (BD Biosciences, USA) (Hsieh et al., 2009). Splenocytes were also used at the concentration of 3×10^5 cells per well in 96 well plates for cell proliferation assay. Adherent splenocytes with enriched macrophage population were obtained after incubating splenocytes at a concentration of $2x10^6$ for 2h at 37°C to study macrophage response. Splenocytes at the concentration of $2x10^6$ were cultured with BCG-PPD (50 μg/ml) for 72 h at 37°C before staining with antibodies for flow cytometric analysis of T-helper memory and $CD8^+$ cells.

3.3.8 Splenocyte proliferation assay

Cell proliferation was studied by [^{3}H] thymidine uptake. Briefly splenocytes from control, juglone treated or untreated BCG-vaccinated mice were cultured at the concentration of $3x10^5$, re-stimulated with BCG-PPD (50 μg/ml) and cultured for 72 h

at 37°C. The activity was measured, and the results were expressed as the mean ratio ± S.D (N=3).

3.3.9 Cytokine and nitric oxide estimation

T cell co-cultures or splenic macrophages were incubated with culture medium with or without BCG-PPD (50 μg/ml) for a period of 72 h in 24 well plates. Supernatants were removed, pooled and analyzed for the presence of IFN-γ, IL-4, and IL-10 for co-cultured T-cells and IL-1β, TNF-α and IL-12 for splenic macrophages response, using sandwich ELISA sets (BD Biosciences, USA). The ELISA was performed according to the BD Biosciences manufacturer's instructions. The levels of NO were measured in cell-free supernatants by Griess reagent assay as described elsewhere (Dai et al., 2016)

3.3.10 BCG antigen-specific antibody assay

Mice were bled retro-orbitally; separated serum from the samples was pooled for every mouse within the group. ELISA plates (BD Biosciences, USA) were coated overnight with 10 μg/mL of BCG-PPD at 4°C, and blocking was done with PBS containing 2% bovine serum albumin. Sera were diluted (1:100) and added to the coated ELISA-plates before incubating it for 2h at RT. Rabbit anti-IgG1 and anti-IgG2a antibodies (Biolegend) were added, and the ELISA-plates were incubated for 1h at 37°C. Peroxidase-conjugated goat anti-rabbit antibody (Biolegend, USA) was added to each well, of the microplate and were subsequently incubated for 1h at 37°C,

followed by addition of TMB. The reactions were blocked by the addition of 1N H_2SO_4, and the ELISA-plates were read at 450nm.

3.3.11 Flow cytometry

Briefly, $1x10^6$ splenocytes or lymph node-derived cells were stained with mouse Treg flow kit (Biolegend) containing Foxp3-AlexaFluor®488/CD4-APC/CD25-PE. Cultured splenocytes ($1x10^6$) were treated with or without BCG-PPD and were stained with the mouse naïve/memory helper T cell panel containing CD3-APC/Cy7, CD4-PerCP/Cy5.5, CD62L-APC, and CD44-PE as per manufacturer's instruction. The cells were acquired on a flow analyzer (Sysmex Partec, Germany), and data were analyzed in FCS Express (De Novo Software, USA). The cells were gated on $CD3^+CD4^+$ population.

3.3.12 BCG challenge assay

Juglone treated or untreated C57BL/6 mice were vaccinated with $1x10^6$ cfu of BCG. These mice were BCG challenged after nine weeks by $1x10^5$ cfu into ear dorsum. mice were sacrificed by CO_2 overdose, and ear tissue was removed by dissection and homogenized. Tissues were bead-beaten in a mini-bead beater (BioSpec, USA) for 60 sec in 2 ml cryovial containing 1/3rd vol. zirconium beads and 1 ml of Dulbecco's phosphate-buffered saline (DPBS). Ten-fold serial dilutions of the tissue homogenates were made in PBS. The 100 µl of a neat sample and two dilutions

were plated onto 7H11 agar containing 10% OADC supplement (BD). Plates were incubated at 37°C for 3–4 week and BCG colonies (cfu) were enumerated.

3.3.13 Macroscopic pathological alteration, histopathological mice tissue analysis and biochemical analyses of the mice serum

Animals were sacrificed by an intraperitoneal overdose of thiopental sodium. Serum biochemistry parameters were analysed by Biochemical analyser (work outsourced). Serum bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total proteins, total albumin, blood urea nitrogen, creatinine, calcium, phosphorus, cholesterol, triglycerides, creatinine, Phosphokinase, lactate dehydrogenase were analysed in mice conducted in juglone treated and untreated mice. Lymph node, liver and lungs samples were collected and fixed in 10% neutral buffer formalin and processed by the routine paraffin-embedded block method. Tissue sections (3-5 µm) were stained with H and E stain and microscopic histopathological alterations evaluated by using a light microscope.

3.3.14 Statistical analysis

All experimental data were obtained from at least three independent experiments. Statistical analyses were performed using SigmaStat3.5 (SigmaStat, USA). Data for each experiment was expressed as a mean ±SD. Significant differences between the groups were determined by analysis of variance followed by

One Way ANOVA with Tukey's modification. Student T-test determined the difference between the two groups. A value of $p < 0.05$ was considered significant.

3.4 Results

3.4.1 Juglone induced cytotoxicity in the RAW264.7 cell line

Juglone was used in various concentrations on murine macrophage RAW264.7 cells, to determine the general effect of juglone on the cells. The cell survival was evaluated by MTT dye, and half-maximal inhibitory concentration (IC50) was measured. IC50 of juglone on RAW264.7 cells was estimated to be 6.52µM, as shown in figure 10A. This juglone concentration was further used along with antioxidants NAC and GSH. It was determined that NAC and GSH improved cell survival from 50% to 80% in RAW264.7 cells treated with juglone, as shown in figure 10B.

3.4.2 Juglone induced ROS in the RAW264.7 cell line

It the previous experiment it was observed that the cytotoxic effect of juglone was reduced in RAW264.7 due to presence antioxidants. Therefore, it was thought that juglone treatment might have induced reactive oxygen species (ROS). To determine the generation of the generated ROS, a time (0-120mins) and juglone concentration (5µm, 10µM, 20µM) dependent study was performed in RAW264.7 cells using cell-permeable fluorogenic probe 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) which is oxidized to highly fluorescent measurable 2', 7' -

Dichlorodihydrofluorescein (DCF). It was observed that juglone treatment leads to the generation of a considerable amount of ROS in RAW264.7 cells. This also suggests that cell cytotoxicity induced by juglone may be due to ROS generation. Figure 11 represents time and concentration-dependent ROS generation by juglone in RAW264.7 cells.

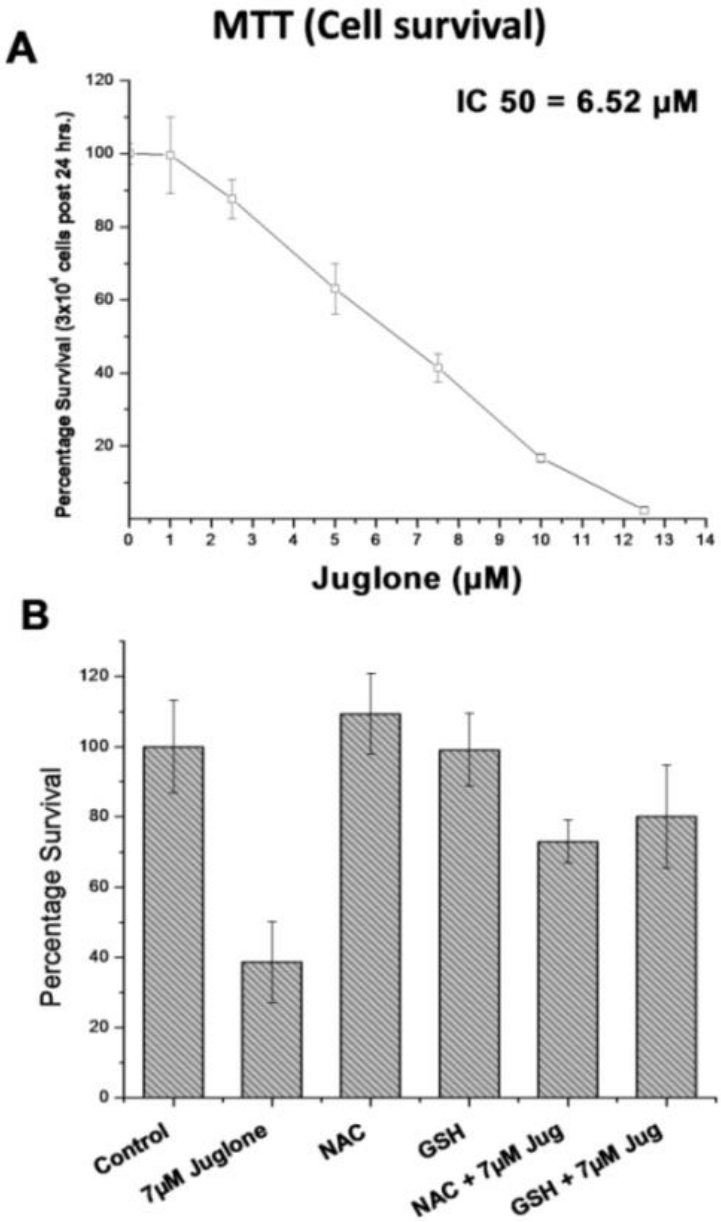


Figure 10. Juglone induced cell cytotoxicity measured by MTT assay. *Briefly, 1 × 10^4 cells per well were seeded in 96-well plates and incubated. Twenty-four hours*

later, cells were treated with juglone (0–12.5 µM) for 24 hr. Cells were washed with PBS and incubated further for 3 hrs with 100 µl of 0.5 mg/ml of MTT. The formazan crystals formed were dissolved in 100 µl of 10% SDS for 2 hrs, and absorbance was measured at 540 nm. IC_{50} values were determined from the concentration versus per cent viability curve (Fig. 10A). As shown in Fig. 10B, cells were pretreated with NAC (10 mM) and GSH (10 mM) and were consequently treated with juglone.

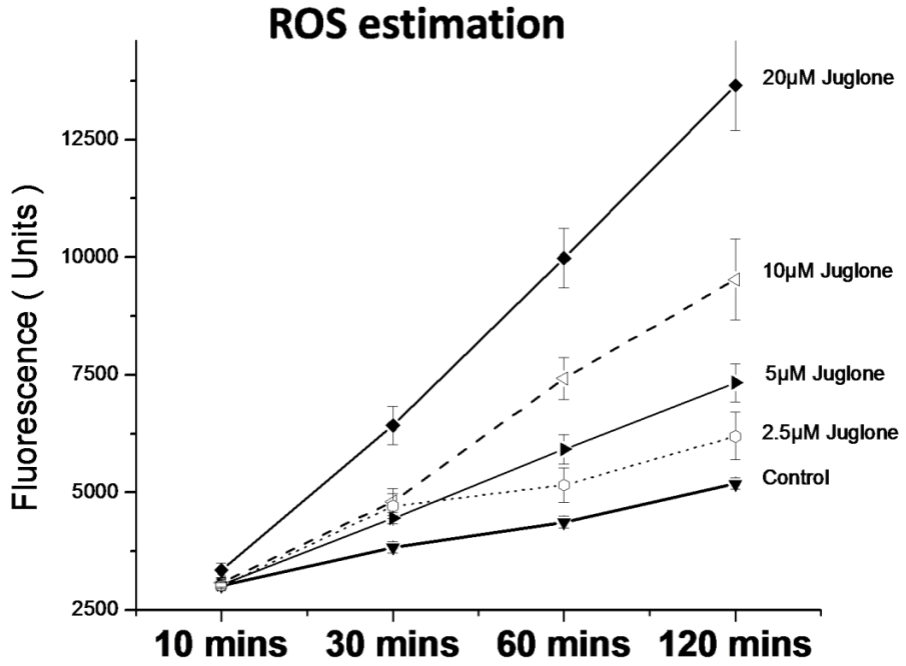


Figure 11. Time and concentration-dependent ROS generation by juglone in RAW264.7 cells. *The effect of juglone on the intracellular ROS levels was measured using the 2', 7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence assay. Results were expressed as arbitrary units of DCF fluorescence using KC4 software suite.*

3.4.3 Juglone induced Apoptosis in the RAW264.7 cell line

Apoptosis is characterized by membrane blebbing, condensation of cytoplasm and the activation of an endogenous endonuclease. This Ca^{2+} and Mg^{2+} dependant nuclease cleaves double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cell is due to DNA degradation and apoptotic cell death. ELISA was performed to detect the mono and oligonucleosomes, which is specific to the histones H1, H2A, H2B, H3 and H4.

Juglone increased enrichment ratio (cytoplasmic histone / nuclear histone) in cells with respect to controls. It was determined that juglone at IC_{50} concentration induces apoptosis in time progressive manner when incubated with RAW264.7 cells, as shown in figure 12.

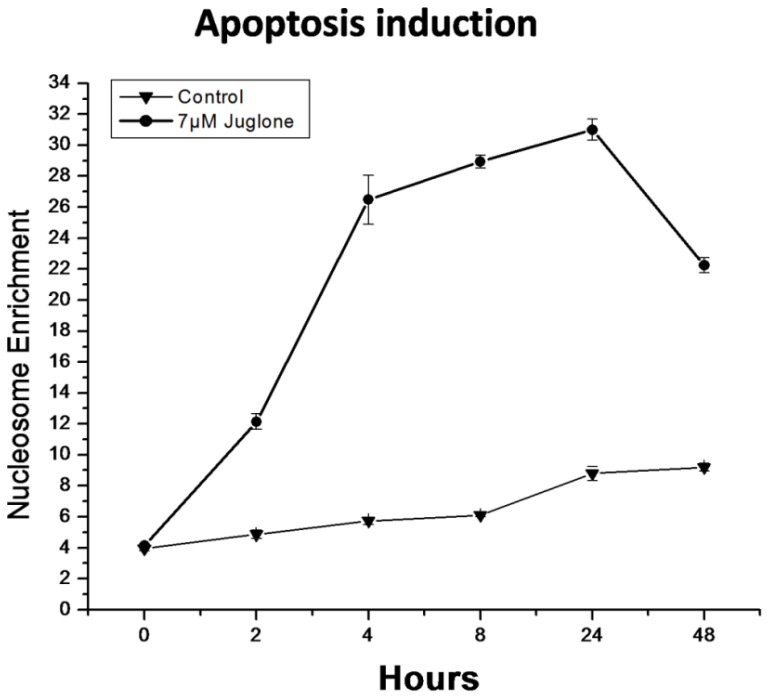


Figure 12: Apoptosis induction by juglone in RAW264.7 cells. *RAW264.7 cells ($3x10^4$ per well) were plated in 96-well plates and cultured either with or without juglone (7 µM). Enrichment of nucleosomes in juglone treated RAW264.7 cells at 24, 48 and 72 h incubation were evaluated. Results are expressed as the mean ratio ± S.E. of the absorbance from cells treated with juglone relative to the absorbance of untreated cells. Samples were run in triplicates.*

3.4.4 Juglone enhances Th-1 cytokines in BCG-PPD restimulated T cells co-cultured with APC

Recall immunity appears when primary vaccine response starts to wane off after 4-6 weeks post-vaccination (Nandakumar et al., 2016; Young et al., 2002). The recall immunity was evaluated by ex-vivo BCG- PPD restimulation of T cell co-

culture. Th1 cytokines were analyzed in T cell co-cultures from BCG-vaccinated mice treated with juglone and sacrificed nine weeks and 65 weeks. Fig.13 illustrates levels of Th1 (IFN-γ) and Fig.14 and 15 shows Th2 cytokines (IL4, IL-10) respectively in ex-vivo stimulated BCG-PPD T cells from various groups of mice which were treated or untreated with juglone and were BCG-vaccinated. Significantly higher levels of IFN-γ were seen in juglone treated BCG-vaccinated mice, stimulated with BCG-PPD compared to controls (unvaccinated and BCG-vaccinated untreated mice) sacrificed after nine weeks and sixty-five weeks post-BCG vaccination. Further, there was no significant difference in IL-4 levels between any of the mice groups.

Additionally, mice sacrificed after nine weeks, and sixty-five weeks post-vaccination showed higher and lower levels of IL-10 respectively in juglone treated BCG-vaccinated mice compared to controls. Immunomodulation was evaluated in terms of IFN-γ: IL-4 (Fig. 16) and IFN- γ: IL-10 (Fig. 17) ratio in co-cultured T cells restimulated with BCG-PPD. It was observed that juglone treatment leads to enhanced BCG vaccination efficacy which is reflected in higher IFN-γ: IL-4 ratio in mice sacrificed nine-week post-BCG vaccination. However, there was no difference observed between juglone treated and untreated, BCG immunized mice sacrificed sixty-five weeks post-immunization. Low levels of IL-10 and high levels of IFN-γ, both derived from T-cell, reflect better vaccine efficacy (Stober et al., 2005). Juglone treatment leads to high IFN-γ: IL-10 ratio in mice sacrificed nine and sixty-five weeks post BCG vaccination, thus suggesting beneficiary immunomodulation induced by juglone.

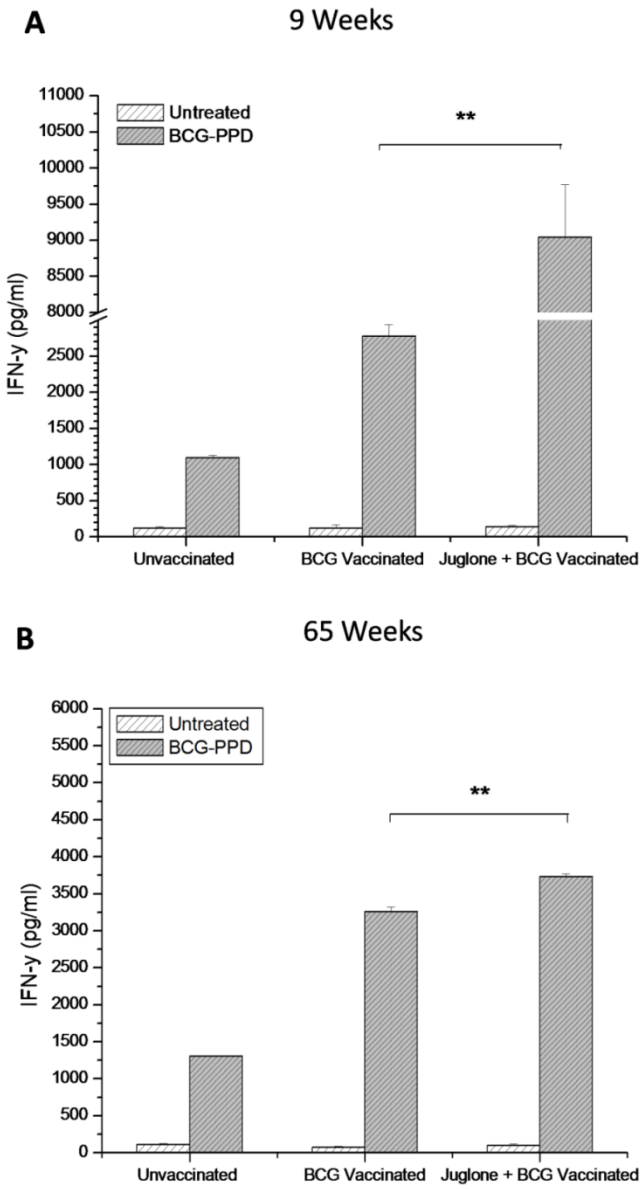


Figure 13: Levels of IFN-γ in T cell co-culture from, post 9 and 65 weeks BCG vaccinated mice treated with juglone. *Ex-vivo stimulated BCG-PPD T cells from various groups of mice which were treated or untreated with juglone and were BCG-vaccinated. Significantly higher levels of IFN-γ were seen in juglone treated BCG-vaccinated mice; stimulated with BCG-PPD compared to controls (unvaccinated and BCG-vaccinated untreated mice) sacrificed after 9 weeks (Fig. 13A) and 65 weeks (Fig. 13B) post-BCG vaccination. Results were expressed as mean ± S.D (N=3) **p < 0.001.*

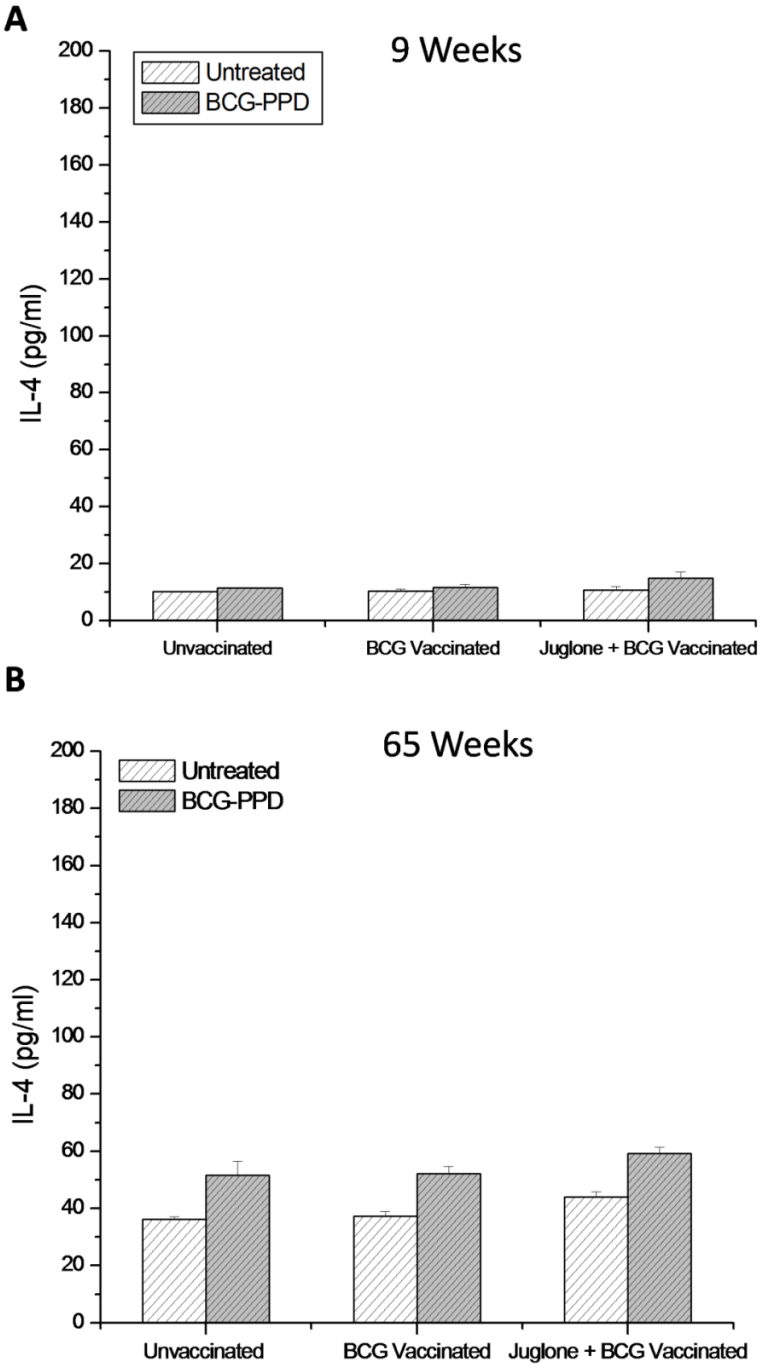


Figure 14: Levels of IL-4 in T cell co-culture from, post 9 and 65 weeks BCG vaccinated mice treated with juglone. *Ex-vivo stimulated BCG-PPD T cells from various groups of mice which were treated or untreated with juglone and were BCG-vaccinated. No significant difference in IL-4 levels was observed in juglone treated BCG-vaccinated mice, stimulated with BCG-PPD compared to controls (unvaccinated and BCG-vaccinated untreated mice) sacrificed after 9 weeks (Fig. 14A) and 65 weeks (Fig. 14B) post-BCG vaccination. Results were expressed as mean ± S.D (N=3)*

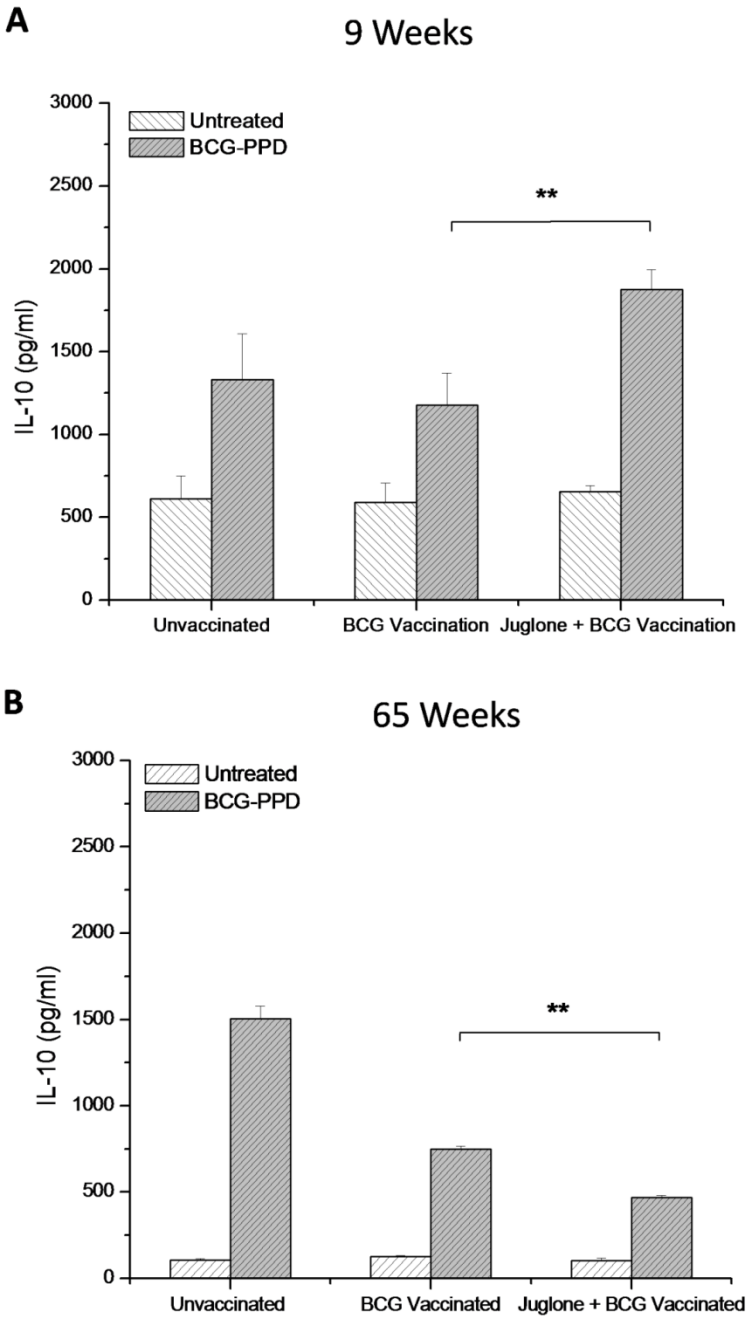


Figure 15: Levels of IL-10 in T cell co-culture from, post 9 and 65 weeks BCG vaccinated mice treated with juglone. *Ex-vivo stimulated BCG-PPD T cells from various groups of mice which were treated or untreated with juglone and were BCG-vaccinated. Significantly higher levels of IL-10 were seen in juglone treated BCG-vaccinated mice, stimulated with BCG-PPD compared to controls (unvaccinated and BCG-vaccinated untreated mice) sacrificed after 9 weeks (Fig. 15A) but not in 65 weeks (Fig. 15B) post-BCG vaccination. Results were expressed as mean ± S.D (N=3) **p < 0.001.*

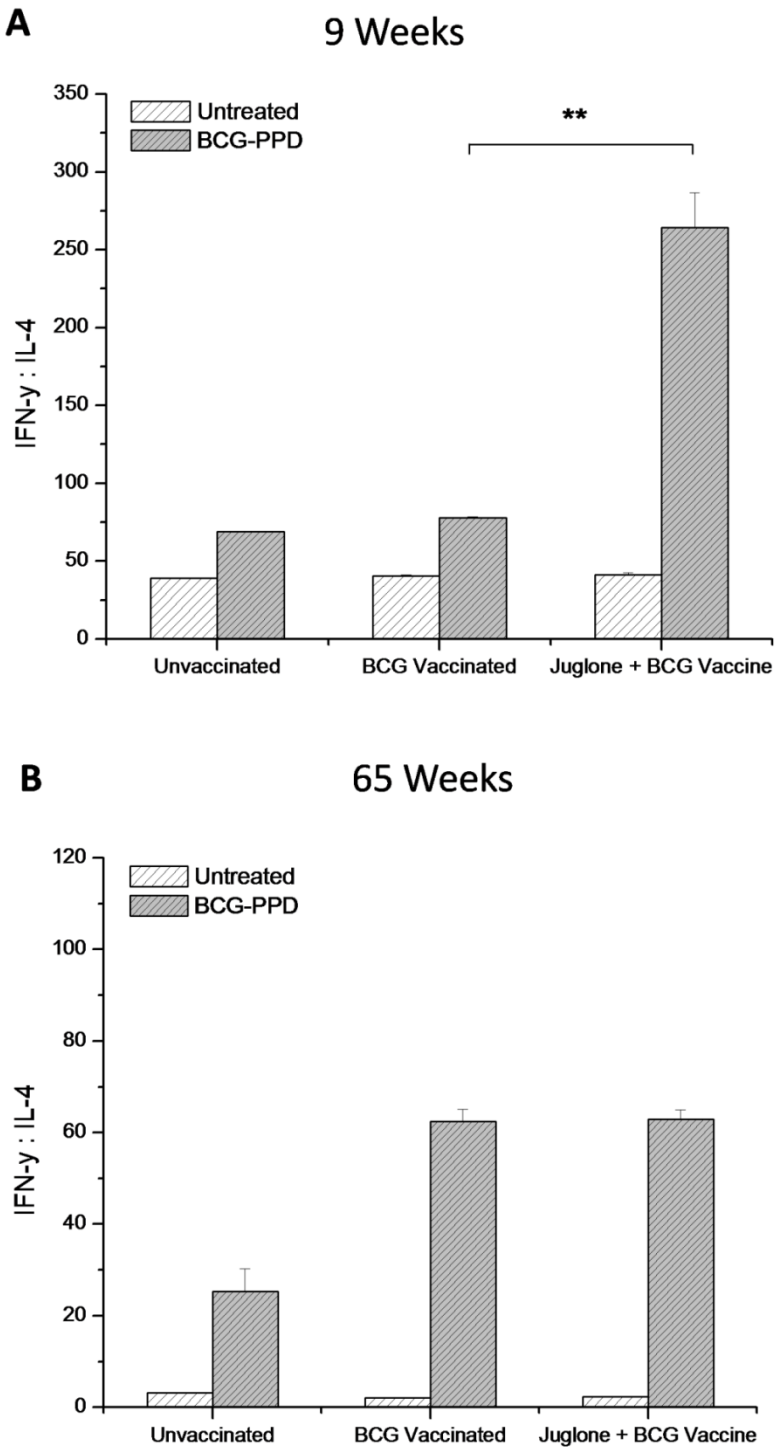


Figure 16: Levels of IFN-γ: IL-4 ratio in T cell co-culture from, post 9 (Fig. 16A) and 65 weeks (Fig. 16B) BCG vaccinated mice treated with juglone*. Results expressed as mean ± S.D (N=3) **p < 0.001.*

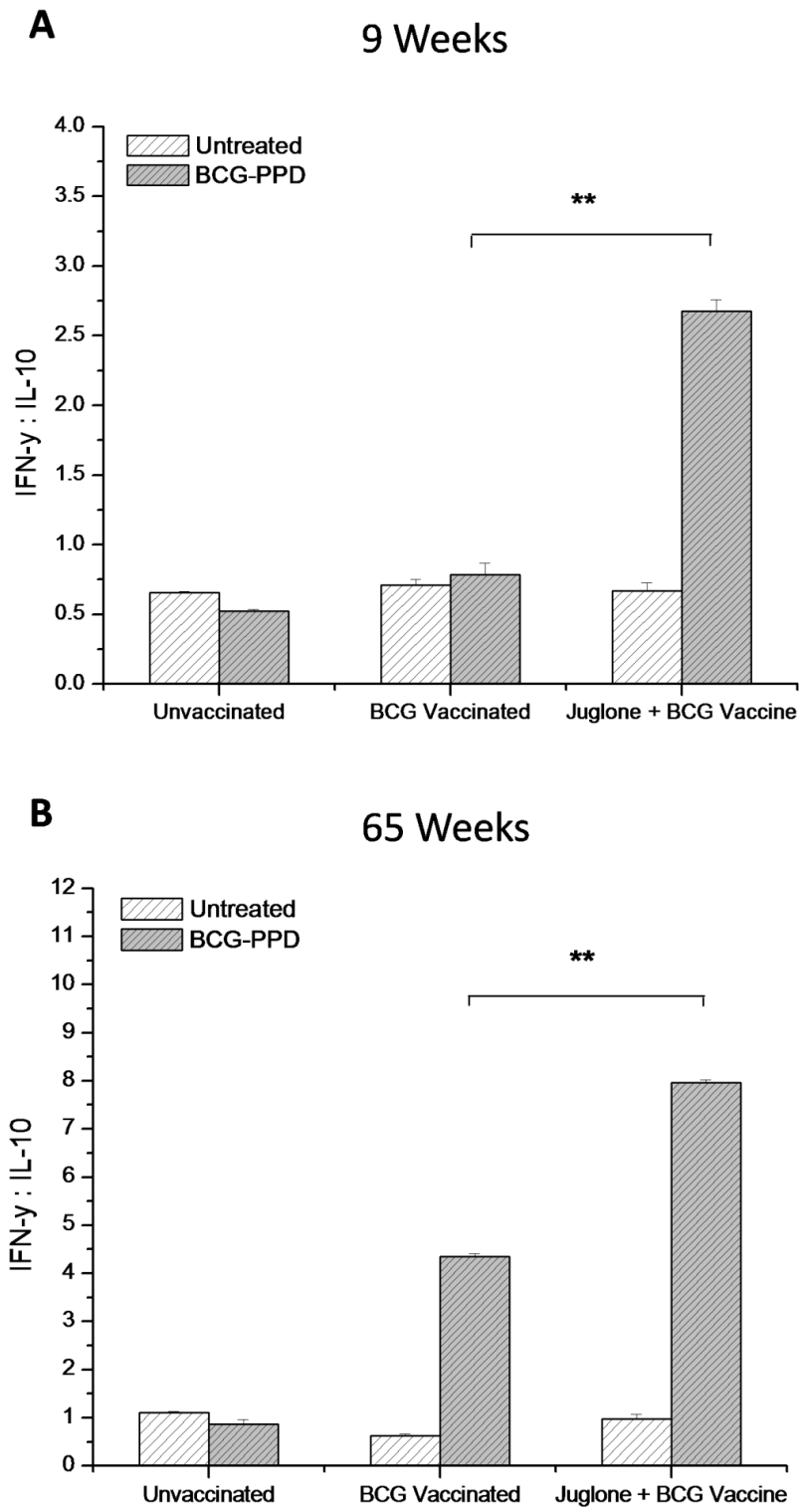


Figure 17: Levels of IFN-γ: IL-10 in T cell co-culture from, post 9 (Fig. 17A) and 65 weeks (Fig. 17B) BCG vaccinated mice treated with juglone*. Results expressed as mean ± S.D (N=3) **p < 0.001.*

3.4.5 Juglone treatment polarises splenic macrophage into M1 phenotype and also leads to antigen-specific IgG1 to IgG2a switch in BCG-vaccinated mice

The levels of NO, IL-1β, TNF-α and IL-12 were examined in culture supernatants of splenocytes stimulated with BCG-PPD to evaluate the development of M1 macrophages. A significantly higher level of NO and IL-1β was seen in the culture supernatant of splenocytes from juglone treated and BCG-vaccinated animals compared to that from control animals as illustrated in Fig. 18A and 18B, respectively. There was no significant difference in the levels of TNF-α amongst the different groups (Fig. 19A). Levels of IL-12 were also monitored in the culture supernatant of splenocytes (restimulated with BCG-PPD), and it was observed that IL-12 levels were higher in the culture supernatant of splenocytes from juglone treated and BCG-vaccinated mice compared to untreated BCG-vaccinated controls (Fig. 19B). Antigen-specific IgG2a isotype generation is under the regulation of IFN-γ, and thus it is definitive marker for Th1 cell activation *in vivo* (Else et al., 1993) while generation of IgG1 isotype antibodies requires IL-4 and T cell help; thus it is a measure of Th2 cell activation (Fischer et al., 2002).

To examine the effect of juglone on antigen-specific IgG isotypes, IgG1 and IgG2a were measured by ELISA in "two-fold" serially diluted serum nine weeks post BCG immunization. We observed significantly higher IgG2a levels in juglone treated BCG-vaccinated mice as compared to only BCG-vaccinated control (Fig.20A).

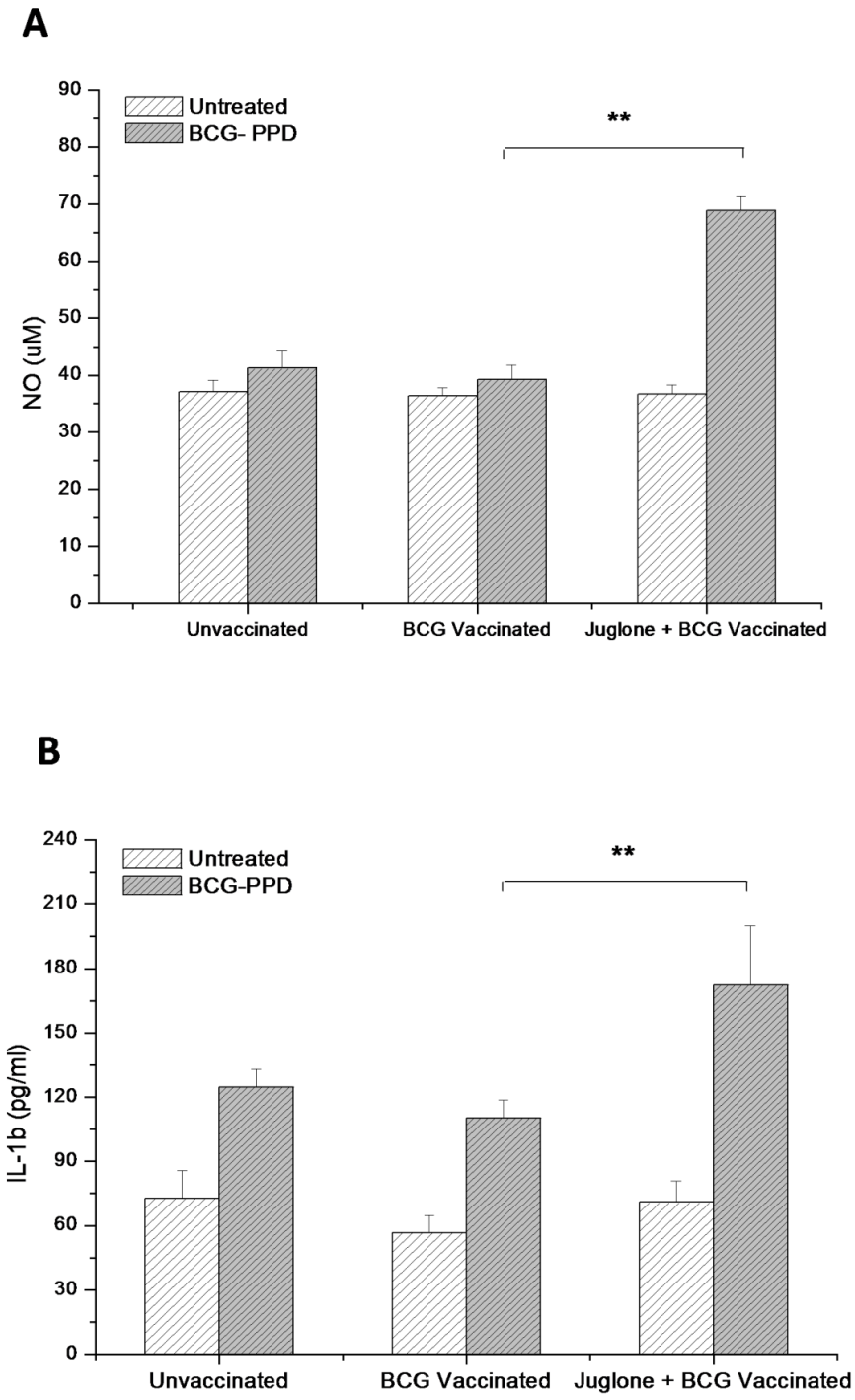


Figure 18: Levels of NO and IL-1β in splenic macrophage treated with BCG-PPD from, post 9 weeks BCG vaccinated mice treated with juglone. *C57BL/6 mice splenic macrophages were cultured with BCG-PPD (50 μg/ml) for 72 h at 37°C. Nitric oxide and IL-1β levels were estimated in culture supernatants from BCG-vaccinated mice treated with or without juglone and compared with unvaccinated control. Results were expressed as mean ± S.D (N=3) **p < 0.001.*

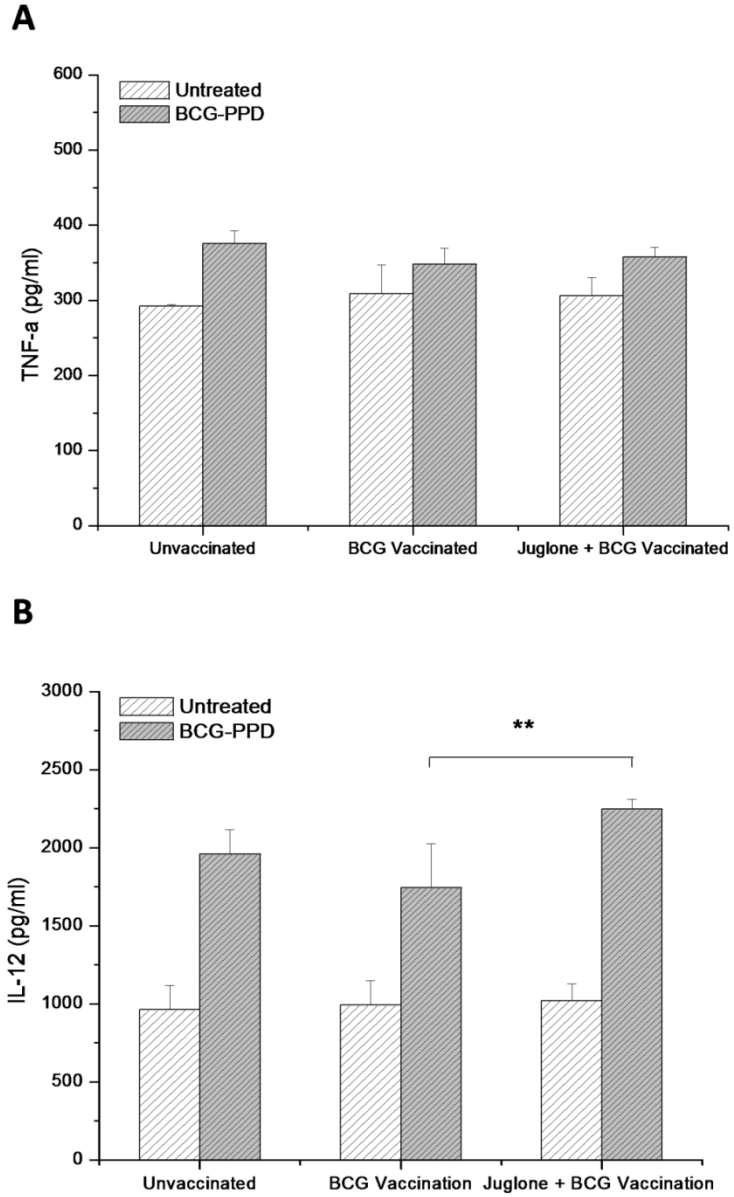


Figure 19: Levels of TNF-α and IL-12 in splenic macrophage treated with BCG-PPD from, post 9 weeks BCG vaccinated mice treated with juglone*. C57BL/6 mice splenic macrophages were cultured with BCG-PPD (50 μg/ml) for 72 h at 37°C. Level of TNF-α (Fig. 19A) and IL-12 (Fig. 19B) were estimated in culture supernatants from BCG-vaccinated mice treated with or without juglone and compared with unvaccinated control. Results were expressed as mean ± S.D (N=3) *p < 0.05.*

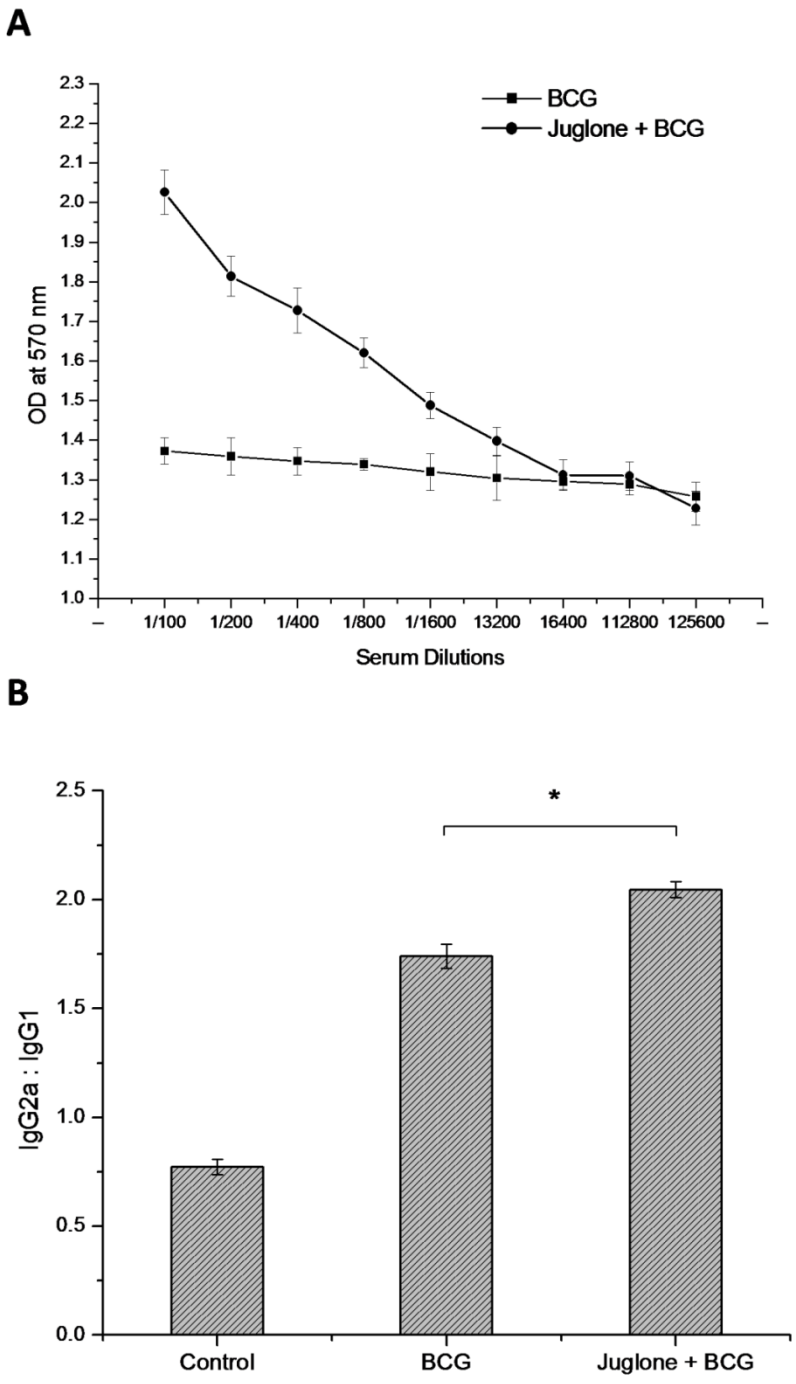


Figure 20: Levels of antigen-specific IgG2a and IgG1 isotypes in mice serum. *The serum was collected from juglone treated and untreated mice immunized with BCG and assayed by ELISA. Fig. 20A shows tenfold serial diluted IgG2a antibody ELISA (N=3) and Fig. 20B show levels of IgG1 and IgG2a antibody in the mice serum. Comparison of serum antibodies shows that mice vaccinated along with juglone had significantly higher levels of IgG2a and significantly lower levels of IgG1 than mice vaccinated with BCG alone No detectable levels of PPD-specific antibody were found in unvaccinated mice control. Results were expressed as mean ± S.D (N=3) *p < 0.05.*

However, there was no significant difference in IgG1 levels between juglone treated and untreated BCG-vaccinated mice (Data not showed). Higher IgG2a/IgG1 ratio is an indicator of Th1 dominance. It is clear from Fig. 20B that both juglone treated and untreated BCG-vaccinated mice groups show higher IgG2a/IgG1 ratio; however, juglone treated group illustrated significantly higher ratio as compared to the untreated unvaccinated group.

3.4.6 Juglone modulates Treg and T-memory cell population in BCG-immunized mice

Treg cells identified by the expression of CD4, CD25, and FOXP3 have an immunoregulatory function which maintains or modulate the immune system. To determine the immunomodulatory role of juglone treatment on Treg cell population, splenic and lymph node-derived $CD4^{+}CD25^{+}FOXP3^{+}$ cells were analyzed by flow cytometry. We observed a decline in Treg cell population in lymph nodes of BCG vaccinated mice treated with juglone treated as compared to control mice (Fig. 21). No significant difference was observed in splenic Tregs (Data not shown).

We also examined the effect of juglone treatment on T-memory phenotype in BCG-vaccinated mice. T-memory cells are identified by the expression of CD3, CD4, CD44, and CD62. Hence flow cytometric analysis of T-memory cells was performed by flow cytometry. Generally, $CD44^{lo}CD62^{hi}$ are considered as naïve cells, while $CD44^{hi}CD62L^{hi}$ and $CD44^{hi}CD62L^{lo}$ cells are considered as T central memory (Tcm) and T effector cells (Teff), respectively. The decline in CD62L expression in PPD restimulated cells gated on $CD3^{+}CD4^{+}CD44^{+}$ Tcm cell population in juglone treated

group may be due to the generation of Teff cells (Fig. 22). Juglone induces higher CD44 expression on cells gated on $CD3^{+}CD4^{+}CD62^{+}$ cells suggests the generation of higher Tcm population as compared to controls (Fig. 23).

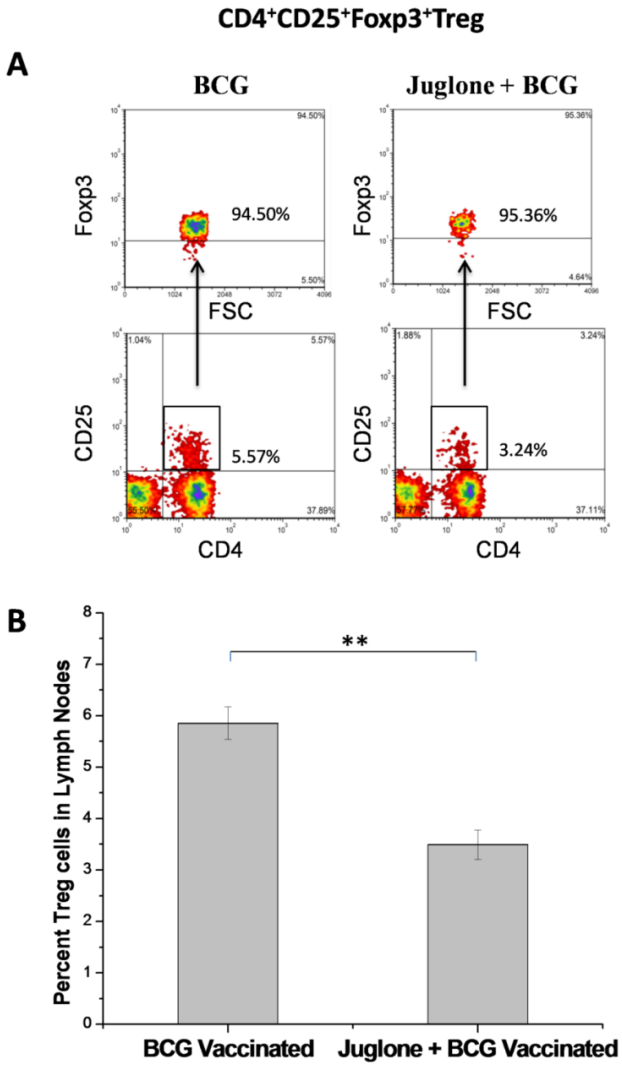


Figure 21: Flow cytometric evaluation of Treg cells. *The mice were sacrificed 3 weeks after juglone treatment, and BCG vaccination and lymph node were separated, and single cells were isolated. These cells were stained with fluorochrome labelled CD4, CD25 and Foxp3 antibodies and triple-positive cells were analysed using flow cytometer as shown in Fig. 21A. Fig. 21B represents bar graph plotted from the values obtained from the flow cytometric evaluation. Results were expressed as mean ± S.D (N=3), **p < 0.001.*

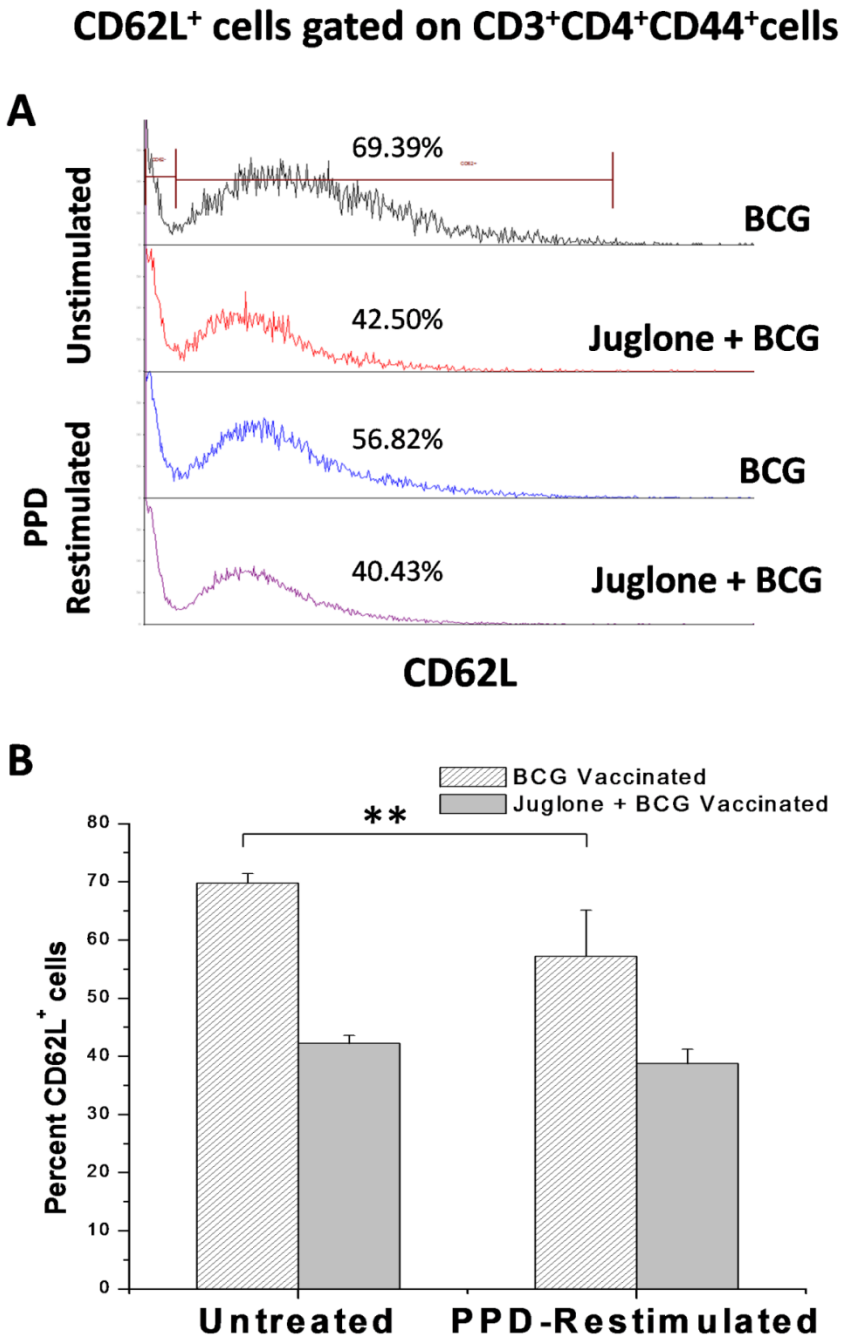


Figure 22: Flow cytometric evaluation of CD62L$^+$ T memory cells*. The mice were sacrificed three weeks post-BCG vaccination, and juglone treatment and cells were isolated from the lymph node. The cells were restimulated with BCG-PPD. Fig. 22A represents the CD62L$^+$ cells which were gated onto CD3$^+$CD4$^+$CD44$^+$ cells and Fig. 22B represents bar graph was plotted from the values obtained from the flow cytometric evaluation. Results were expressed as mean ± S.D (N=3), **p < 0.001.*

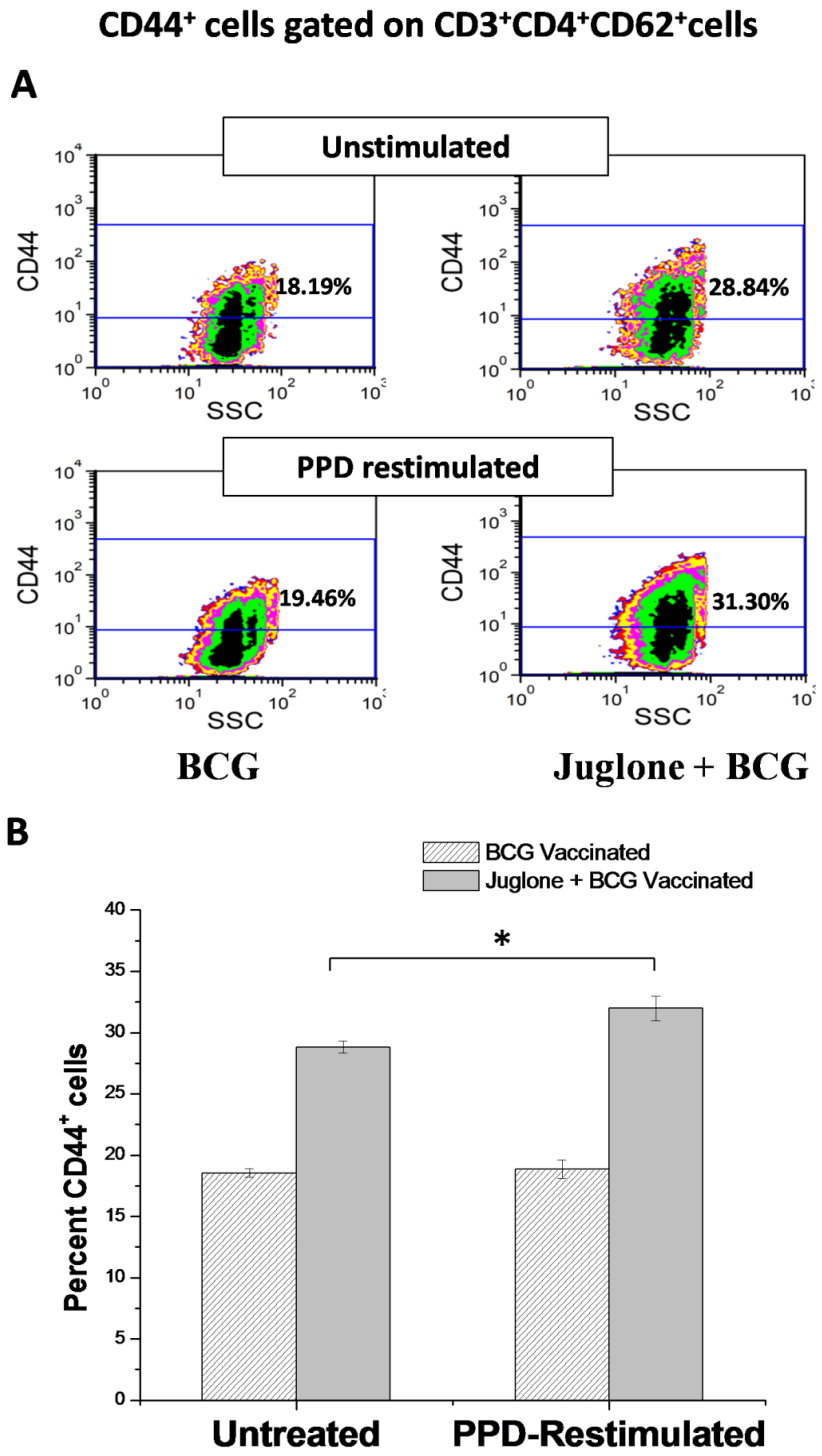


Figure 23: Flow cytometric evaluation of CD44⁺ T memory cells. *The mice were sacrificed three weeks post-BCG vaccination, and juglone treatment and cells were isolated from the lymph node. The cells were restimulated with BCG-PPD. Fig 23A represents the $CD44^+$ cells which were gated onto $CD3^+CD4^+CD62^+$ cells, and Fig.23B represents a bar graph which was plotted from the values obtained from flow cytometric evaluation. Results were expressed as mean ± S.D (N=3), *p < 0.05*

3.4.7 Juglone immunomodulates BCG vaccine efficacy in C57BL/6 mice

Splenocyte restimulation and proliferation show successful BCG immunization in mice as juglone treated or untreated BCG immunized mice showed a statistically significant difference with respect to control unvaccinated mice (Fig. 24, $p< 0.001$). The T-helper cell cytokines are not a direct co-relate of vaccine efficacy as they are only the mediators leading to ultimate effector cell functions. Effector cells like $CD8^+$ determine effector functions. Splenocytes were restimulated with BCG-PPD to determine $CD8^+$ population, from BCG vaccinated, juglone treated and untreated mice, and analyzed by flow cytometry. Our results demonstrated that $CD8^+$ T cells increased in the BCG-vaccinated animals treated with juglone (Fig.25A and 25B).

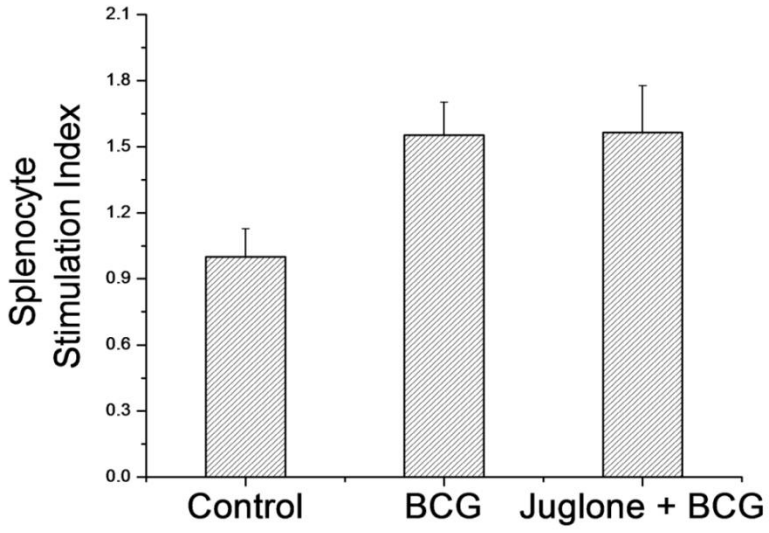


Figure 24: Splenocyte restimulation index. *Cell proliferation was studied by [^{3}H] thymidine uptake. Briefly, splenocytes from juglone treated or untreated mice vaccinated with BCG were restimulated with BCG-PPD (50 µg/ml) and cultured for 72 h at 37°C and the activity was measured. Results were expressed as mean ratio ± S.D (N=3) **p < 0.001 between BCG and Juglone + BCG as compared with control unvaccinated mice*

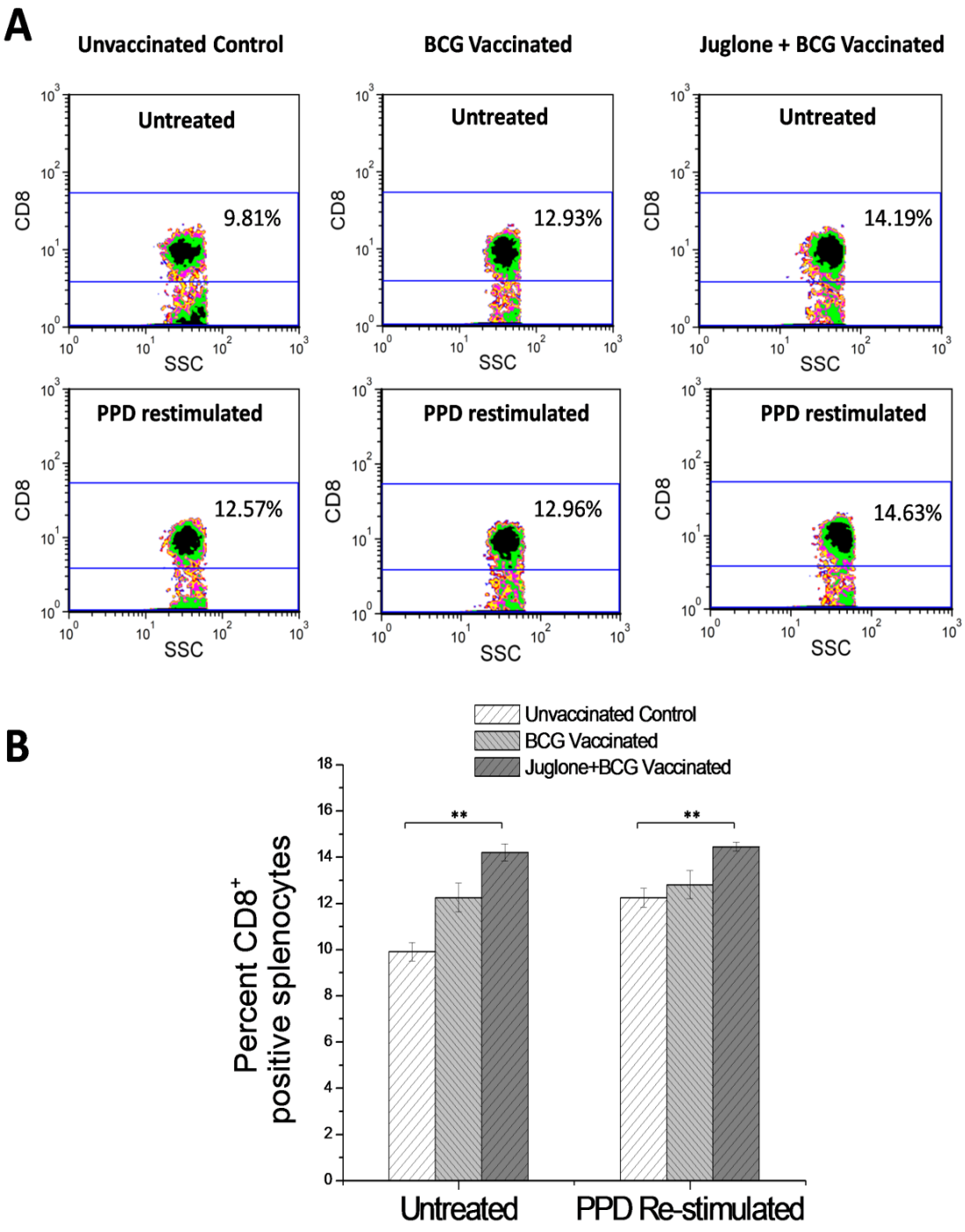


Figure 25: Percent of CD8⁺ cells in mice splenocytes. *Fig 25A represents a flow cytometric evaluation of CD8⁺cells obtained from the spleen of BCG vaccinated mice, treated with juglone following treatment with BCG-PPD. Fig. 25B represents the bar graph of the values obtained from flow cytometric evaluation. Results were expressed as mean ± S.D (N=3), **p < 0.001.*

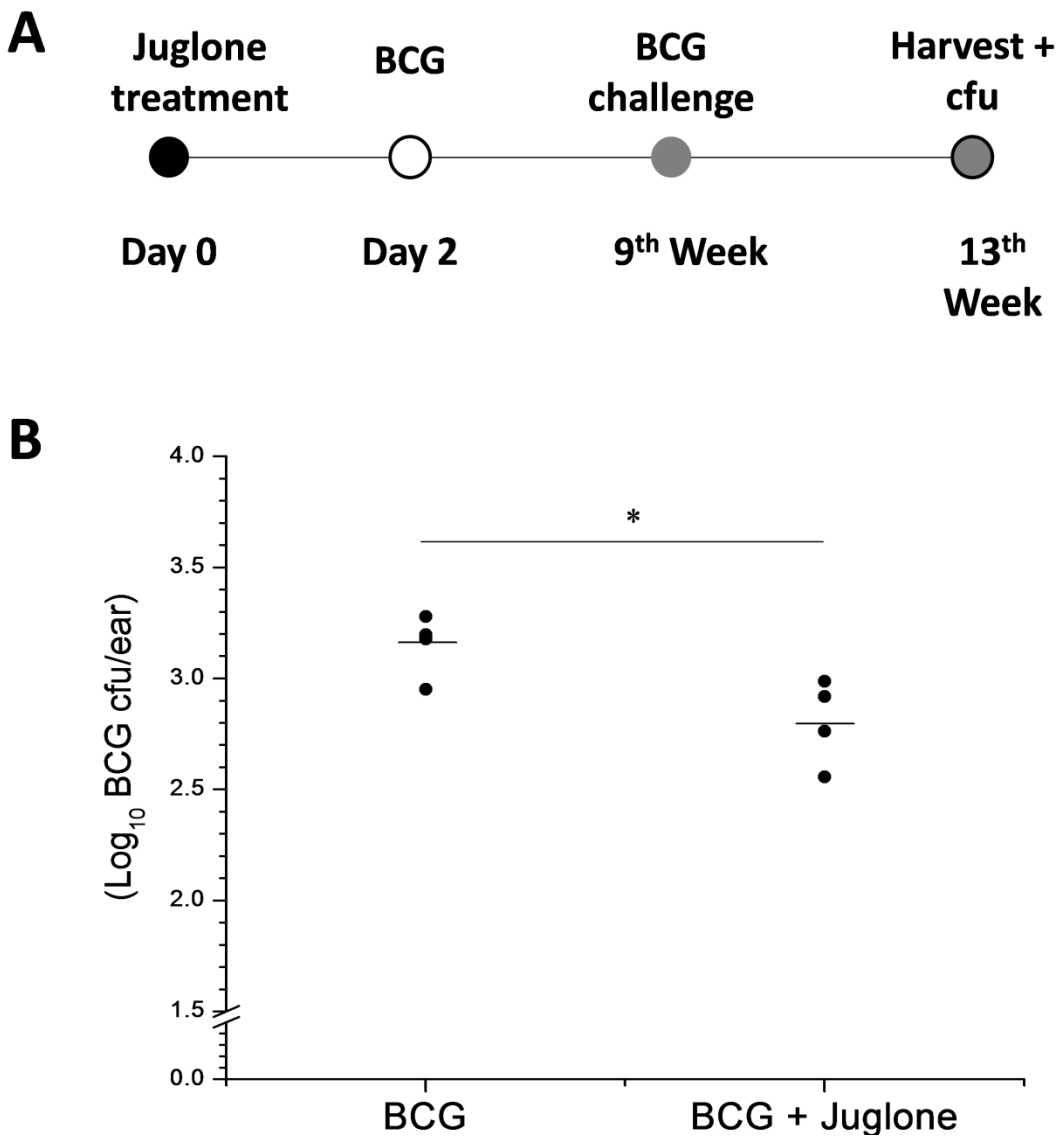


Figure 26: BCG challenge assay in mice. *Figure 26A is a schematic representation of the BCG ear challenge assay experiment. Briefly, juglone treated or untreated BCG-vaccinated mice were challenged with BCG. Ear dorsum was dissected and sheared before plating it onto Middlebrook 7H10 agar plates. Colonies or cfu's were counted after 3 weeks and results were plotted (Fig. 26B).*

A bacterial challenge to vaccinated animals determines the efficacy of vaccine response. Bacterial clearance was demonstrated by "BCG challenge test" as shown in Fig. 26A. Juglone treated and untreated BCG-vaccinated mice were challenged with

challenge-dose of BCG in-ear dorsum after nine weeks of vaccination. Four weeks later, BCG-CFU counts were quantified from sacrificed animals. Reduction in CFU count was observed between juglone treated and untreated mice vaccinated with BCG (Fig. 26B)

3.4.8 Histopathological evaluation of various organs from mice treated with juglone and vaccinated with BCG

Mice were treated with juglone dose of 0.5 mg per Kg body weight, (KW) to observe if juglone induces any structural changes at an immunomodulatory dose, which was used in this study. The BCG vaccinated mice treated with juglone were sacrificed, and various organs were isolated. Histopathological evaluation was performed on the mice organs. No evidence of structural changes was observed in the histopathological architecture of the liver and lung of BCG immunised animals treated with juglone as compared with unimmunised animals. The histological sections shown in figure 27, represents untreated normal mice (SS), BCG immunised mice (SB), juglone treated unimmunised mice (JS), and juglone treated BCG immunised mice (JB). Normal lymphoid follicles with normal cellularity were observed in the cortex and paracortical area in untreated, unimmunised mice, whereas a mild increase in cell density was observed in the cortical and paracortical area in JS and SB group of animals. Further, a moderate increase in cell density in the cortical and paracortical area was observed in juglone treated BCG immunised. The outcome of microscopic pathological evaluation of lung and liver tissues of different mice groups is shown in Fig. 27. Overall juglone did not induce toxicity in mice at immunomodulatory dose.

3.4.9 Serum biochemical analysis of mice treated with juglone

Serum biochemical analysis was performed in C57BL/6 mice injected with 0.5 mg juglone per kg BW of the mice and was compared with saline-treated control mice. Various parameters like serum bilirubin, aminotransferases, phosphatases, total proteins, creatinine, phosphorus, calcium and cholesterol, which are indicators of mice health, were evaluated. The serum from three animals of both the group was pooled for the study. This study was conducted to determine the toxicity of juglone in mice at a working concentration of juglone used for immunomodulation of BCG vaccine. No significant difference in any parameter was observed between juglone treated group as compared to control. This indicates that juglone did not alter mice serum parameters at working immunomodulatory concentrations used for this study. Table 5 shows serum biochemical analysis for various parameters of C57BL/6 mice treated with juglone.

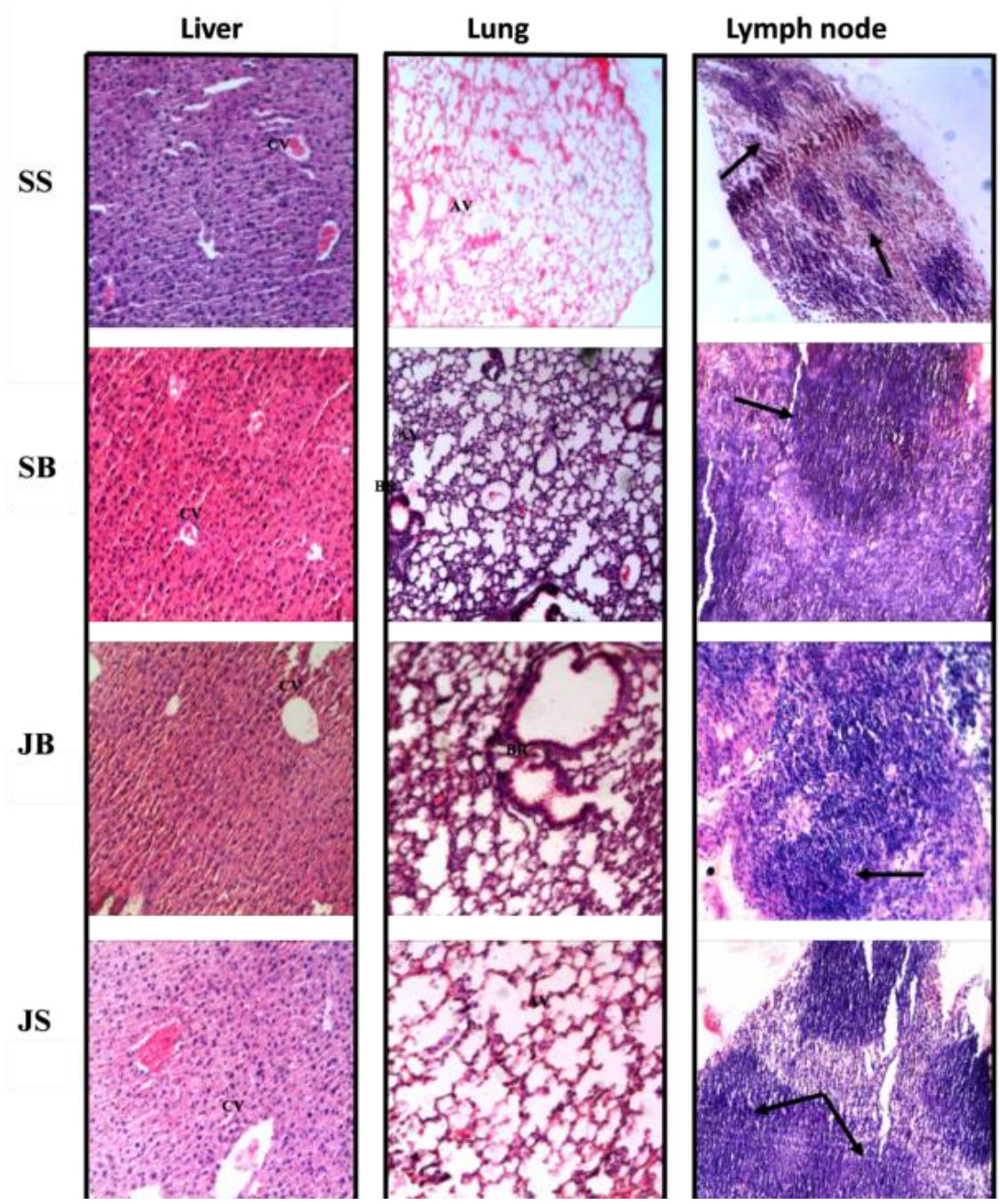


Figure 27: H and E staining of histological sections of liver, lung and lymphoid tissues were studied by simple microscope. *Central vein (CV) from liver, bronchioles (BR) and alveoli (AV) from lung micrographs respectively are marked (Fig. 27). Lymphoid changes are indicated by arrows**.** The sections are represented from untreated normal mice (SS), BCG immunised mice (SB), juglone treated unimmunised (JS), and juglone treated BCG immunised (JB).*

	C57BL/6 mice	Saline treated control mice	Juglone treated mice
1	Serum Bilirubin mg / dl	0.3	0.5
2	Aspartate Aminotransferase IU / L	121	166
3	Alanine Aminotransferase IU / L	61	51
4	Serum Alkaline Phosphatase IU/L	355	336
5	Serum Total Proteins g/dl	4.5	5
6	Serum Total Albumin g / dl	3.2	3.6
7	Blood Urea Nitrogen mg/dl	20.3	19.4
8	Serum Creatinine mg/dl	0.8	0.7
9	Serum Calcium mg/dl	8.2	8.4
10	Phosphorus mg/dl	5.2	5.7
11	Serum Cholesterol mg/dl	83	97
12	Serum Triglycerides mg/dl	86	83
13	Creatinine Phosphokinase (U/L)	259.9	176
14	Lactate dehydrogenase (U/L)	351	366

Table 5: Serum biochemical analysis of C57BL/6 mice treated with juglone.

3.5 Discussions

Immunotherapy involves healing infection, cancer or other illness by eliciting, improving or distorting an existing immune response (Sabado and Bhardwaj 2010; Hotchkiss, Monneret et al. 2013; Cobos, Figueroa et al. 2014). Immunomodulatory discourse generally involves minor adverse effects as compared to pharmaceutical drugs and reduces possibilities of drug resistance in microbes developed during treatment (Masihi 2001). Immunomodulation was shown to be therapeutic in chemo-resistant cancer (Shankaranarayanan, Kanwar et al. 2016). Cell-based immunotherapies, repurposed drugs, cytokine immunotherapy, are among many novel approaches which ratify immunomodulators as therapeutic agents.

We explored the potential of juglone as an immunomodulator. We found that juglone treatment in mice accounted for higher IFN-γ (Th1 cytokine) production in PPD restimulated T cells from mice sacrificed 9 and 65 weeks post-BCG vaccination, compared to Th2 cytokines like IL-4 and IL-10. The level of IFN-γ produced by the T cells has been the most used measure to verify vaccine-induced responses specific to infections requiring cellular immunity for protecting the host. IFN-γ has also been shown to have a role in effector function, i.e. clearance of different bacterial, parasitic, viral or fungal infections. However, there are many pieces of evidence which show that levels of IFN-γ producing T cells alone is not enough as an immune correlate of protection (Seder, Darrah et al. 2008). Coherent with this, still protective immunity against mycobacteria is directed by Th1 as opposed to Th2 cells. Further, the responses observed post 65 weeks confirmed that these responses might be long-lived. IFN-γ being a major Th1 cytokine is essential for host survival and enhances $CD8^+$ T cell function during *Mtb* infection (Orr, Beebe et al. 2015). We have observed that

juglone enhances $CD8^+$ cells in mice vaccinated with BCG. The *Mtb* directed effector function of the activated $CD8^+$ T cells may be dependent on the ability of these cells to secrete type-1 cytokines, mainly IFN-γ, which is known to activate macrophages, which end up killing the bacteria. Mostly, the $CD8^+$ T-cells tackles *Mtb* infection by directly killing infected macrophages. Further, IgG2a isotype generated in response to IFN-γ is a hallmark for macrophage-associated cell-mediated cytotoxicity while IgG1 is essentially IL-4 and T cell-dependent cytokine which has a role in humoral immunity (Fischer, Collins et al. 2002; Nguyen, Mouly et al. 2012). A predominance of IgG2a compared to IgG1 in BCG-vaccinated groups indicated higher IgG2a: IgG1 ratio. Similarly, it has been shown in leishmaniasis that the relative ratio of the amount of *L. major*-specific IgG1/IgG2a antibodies serves as an indication of the relative sizes of the Th2 and Th1 components of the immune response, as reflected in the number of parasite-specific IL-4 and IFN-γ-producing $CD4^+$ T cells in the spleen (Bretscher 1992; Rostamian, Sohrabi et al. 2017). Also, it has been known that in the resistant mice, a major Th1 response with IFN-γ generation was associated with high serum levels of antigen-specific IgG2a antibody whereas a Th2 response with IL-4 generation was associated with predominant IgG1 antibody (Pond, Wassom et al. 1989). These findings led us to explore the use of the IgG1/IgG2 ratio in indirectly assessing the Th1/Th2 phenotype of the response along with T-cell cytokines like IFN-γ (Th1), IL-4 (Th2) and IL-10 (Treg) generated following BCG vaccination in mice and post-restimulation of the splenocytes by PPD in vitro. Furthermore, IL-10 derived from $CD4^+$ cells is considered as a pre-challenge indicator of vaccine efficacy (Stober, Lange et al. 2005) with higher levels of IL-10 as marker of vaccine failure, as it is known to down-regulate Th1 responses induced by *Mtb* antigens (Couper, Blount

et al. 2008; Pitt, Stavropoulos et al. 2012). The cytokine IL-10 is responsible in the blocking of pro-inflammatory cytokines, such as TNF-α and the Th1-polarizing cytokine IL-12 thereby acting directly on APCs such as macrophages and DCs, ultimately affecting the immune response to pathogens (Fiorentino, Zlotnik et al. 1991; Mannino, Zhu et al. 2015). Further IL-10 is also responsible in inhibition of phagocytosis and microbial killing by limited production of ROS and RNI (pivotal for mediating immunity to intracellular pathogens) generated in response to IFN-γ (Gazzinelli, Oswald et al. 1992). It has been suggested that the effects of IL-10 are not only limited to direct effects on the APC's as IL-10 could also engage the differentiation of IL-10-producing inducible Tregs as shown in experimental autoimmune encephalomyelitis (Barrat, Cua et al. 2002).

Enhanced IFN-γ: IL-4, IFN-γ: IL-10 and IgG2a: IgG1 ratios in our study suggested immunomodulation by juglone. This Th1 polarization may indicate effective protection against *Mtb* infection. $CD4^+CD25^+Foxp3^+$ regulatory T cell is a dominant player in regulating peripheral self-tolerance and homeostasis of the immune system (Bettini and Vignali 2010). Treg cells suppress the differentiation and function of Th1 cells *in vivo* and in vitro (Shen, Zhao et al. 2013). BCG-directed generation of IFN-γ by $CD4^+$ T cells is inhibited by a high number of $CD4^+CD25^{hi}$ Treg (Li, Lao et al. 2007). Depletion or inhibition of Treg has shown to improve vaccine efficacy in mice (Casares, Rudilla et al. 2010) and enhancement of vaccine-mediated antitumor immunity in cancer patients (Dannull, Su et al. 2005). Similarly, we also observed a decrease in Treg population in BCG-vaccinated mice treated with juglone, suggesting enhancement of juglone induced suppression of Tregs. Th1 cytokines (IFN-γ), leading to enhanced NO and IL-1β enabled the development of M1

splenic macrophages, which we also observed in juglone treated mice immunized with BCG vaccine. This indicates protective immunomodulatory responses like M1 polarisation in BCG-vaccinated mice. It is known that BCG specific $CD4^+$ Tcm and Tem are modulated by Treg cells (Li, Qiao et al. 2011). We established that juglone develops enhanced Tcm ($CD44^{hi}$) in BCG-vaccinated mice as compared to BCG alone, though a decline in CD62L may be due to the simultaneous generation of Tem. Finally, as a measure of effector function, we demonstrated juglone induced modulation of BCG vaccine by BCG challenge test. We observed that juglone significantly reduces the cfu count of challenge dose-BCG in mice ear.

Juglone has previously shown a direct inhibitory effect on human lymphocyte proliferation due to its K^+ ion channel blocking (Varga, Bene et al. 1996). Further studies are required to understand the mechanisms of juglone on various immune cell types. Although we have observed dose-dependent toxicity in RAW264.7 cell line treated with juglone, there was no toxicity induced in animals treated with juglone at metronomic doses, and this was confirmed by biochemical analysis of the mice serum. Histology data suggests congruent recruitment of immune cells in various tissues in BCG-vaccinated mice. Here in Figure 28, we schematically represent a series of experiments and their outcome, which helped us conclude the favourable immunomodulatory effect of juglone on BCG vaccine efficacy.

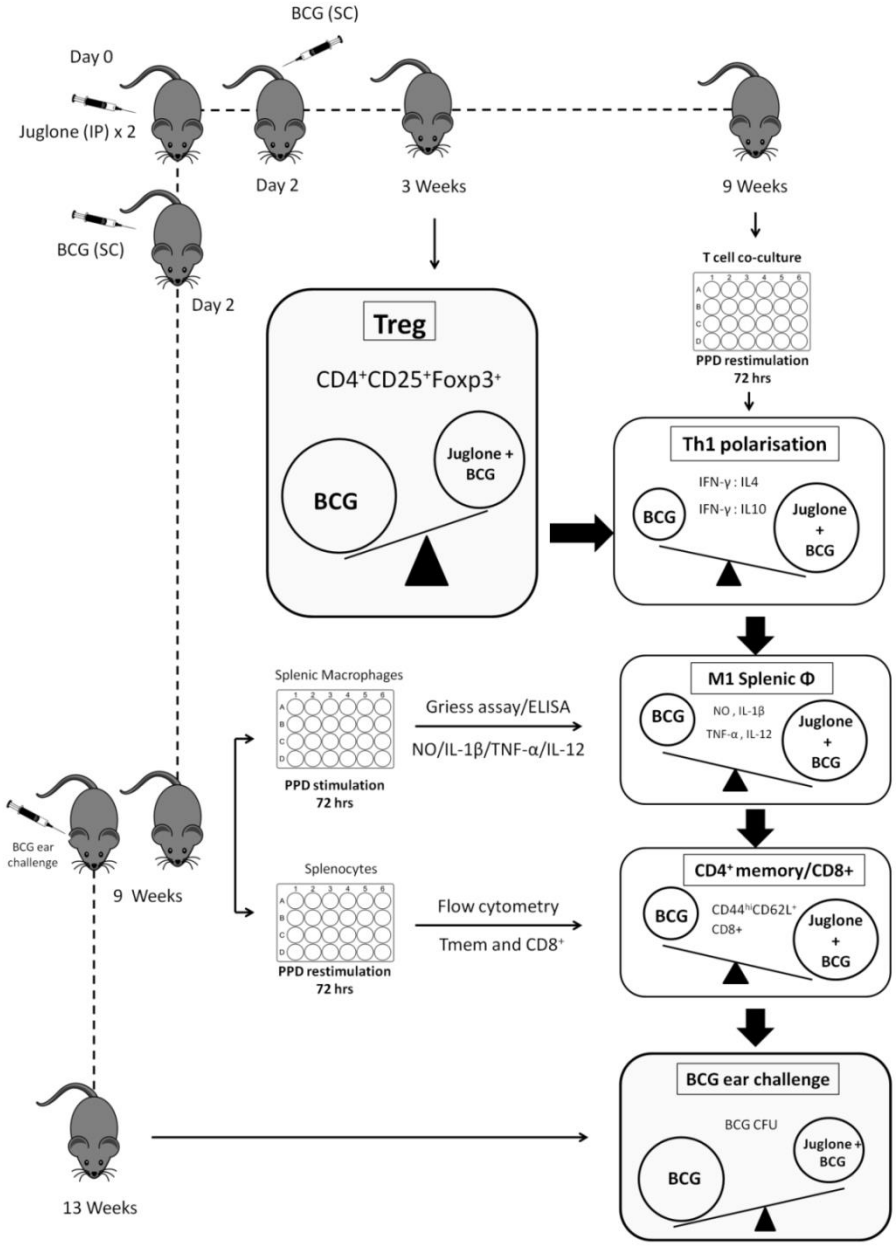


Figure 28: Schematic representation of the potential mechanism involved in the juglone-induced immunomodulation in BCG-vaccinated mice*:*

Juglone induces higher Th1 responses in BCG-vaccinated mice, thereby polarizing macrophages to M1 phenotype. This is also evident from the switching of the antibody isotype switch from IgG1 to IgG2a. Reduction of the Treg population in juglone treated mice vaccinated with BCG might be the reason for the Th1 polarisation. Further, juglone also shows a better protective response, thereby leading to increased clearance of BCG in mice ear when injected as a challenge dose in BCG-vaccinated mice as compared with BCG-vaccinated mice alone.

Chapter 4. Effect of Mtb Lipid Immunomodulation on the BCG vaccine efficacy

4.1 Introduction

The extraordinary molecular intricacy of the mycobacterial cell wall is a principally unique characteristic that separates *Mycobacterium* species from the common prokaryotic organism. The mycobacterium is classified as gram-positive organisms, and their envelopes share remarkable features with the gram-negative cell wall, such as an outer permeability barrier which acts as a pseudo-outer membrane (Minnikin, Ratledge et al. 1982). The cell wall of *Mtb* is a lipid-rich dynamic arrangement involved in regulating the entry of nutrients, toxic host-cell effector molecules release, and entry to anti-TB drugs (Queiroz and Riley 2017). The cell wall of mycobacterium is therefore considered to increase the bacterial survival in the infected host. Lipids are the most malleable biomolecules that adapt to changes resulting from multidrug resistance in *Mtb,* virulence for host evasion, and to circumvent host immunity. The precise molecular mechanism of this adaptation is achievable by *Mtb* using multiple pathways not yet studied in details. The current TB-related mortality scenario could be reduced by translating the knowledge of the diverse *Mtb* strains, existing in the endemic population pool into therapeutic or vaccine guide.

Mtb lipids predominantly contribute to strain diversity. These lipids are amongst the first biochemicals to interact with specialised phagocytic receptors (complement receptor, scavenger receptor, DC-SIGN, mincle) (Jackson 2014), TLRs and other PRRs (Neyrolles and Guilhot 2011) of the host cells like macrophage,

dendritic cells, neutrophils and monocytes which plays a pivotal role in defining bacterial persistence or evasion post-infection. Many *Mtb* lipids interfere in building up of adaptive immune response by developing inconsistent Th1-promoting cytokines (Salgame 2005) while lesser polymorphic CD1 complexes of the host present self as well as mycobacterial lipid antigens to T or NKT cells which can proliferate and produce Th1-derived inflammatory cytokines (Siddiqui, Visvabharathy et al. 2015; Van Kaer, Wu et al. 2016). Further, the activated T or NKT cells can modulate several other cell types, including NK cells, conventional $CD4^+$ and $CD8^+$ T cells, macrophages, neutrophils and B cells as well as recruiting and activating dendritic cells leading to protective Th1 polarized immunity in response to BCG vaccine (Venkataswamy, Baena et al. 2009).

Group I CD1 (CD1a, b and c) restricted T cells recognize several lipid antigens from *Mtb* in humans (Van Rhijn, Ly et al. 2013; Siddiqui, Visvabharathy et al. 2015) while Group II CD1 (CD1d) restricted NKT cells present lipid antigen in mice, which lack the genes encoding CD1a, b and c (Dascher and Brenner 2003). CD1d presents various *Mtb* antigens to an NKT cell type I or invariant (iNKT) and type II NKT cells (Skold and Behar 2003). Mycobacterial phosphatidylinositol mannides (PIM_4) are presented to iNKT cells (Fischer, Scotet et al. 2004) while phosphatidylglycerol (PG), diphosphatidylglycerol (DPG/cardiolipin) and phosphatidylinositol (PI) from both microbial and mammalian sources can stimulate type II NKT cells (Tatituri, Watts et al. 2013). The CD1d expression is upregulated in response to the virus, which could lead to higher iNKT cell response (Raftery, Winau et al. 2008; Chung, Tsai et al. 2013). Many microorganisms modulate surface CD1d, including *Mtb*. Several viruses, including vaccinia, herpes simplex, hepatitis, and lymphocytic choriomeningitis

viruses decrease CD1d expression in myeloid cells (Lin, Roberts et al. 2005; Sanchez, Gumperz et al. 2005) *Mtb* infection or mycobacterial lipids upregulate CD1d on bone-marrow-derived macrophages (Skold and Behar 2003) while *Mtb* infection prevents the upregulation of CD1d in monocytes that differentiate to DC (Mariotti, Teloni et al. 2004; Gagliardi, Lemassu et al. 2007). In vitro *Mtb* infection studies have demonstrated that NKT cells suppress intracellular bacterial growth when co-cultured with *Mtb*-infected macrophages by generating IFN-γ. Further, it has been shown in vivo that transferring these NKT cells significantly reduces bacterial burden. However, research in CD1d-deficient mice suggests that NKT cells may play a limited role in protection against *Mtb* (Behar, Dascher et al. 1999). Many contrasting reports showing instrumental role of CD1d-restricted NKT cells in mycobacterial protection in host and improve BCG vaccine responses are also available (Guidry, Olsen et al. 2004; Venkataswamy, Baena et al. 2009; Sada-Ovalle, Skold et al. 2010; Rothchild, Jayaraman et al. 2014; Zhao, Siddiqui et al. 2015) .

Different strains and clades of *Mtb* vary genotypically and phenotypically along with geographic distribution which shows a difference in lipid profiles (Coscolla and Gagneux 2010; Jackson 2014). *Mtb* bacillus is classified under six major lineages, each strongly associated with strain predominance in different geographical locations specific to the human population (Gagneux and Small 2007). Different lineages of *Mtb* have reportedly shown differential virulence pattern in an animal model and their behavioural pattern in human disease, however, strain-specific host responses largely remains uncertain (Bishai, Dannenberg et al. 1999; Tsenova, Ellison et al. 2005). Further, there is not enough evidence regarding diverse CD1-specific lipid presentation due to strain or lineage difference between various *Mtb*

strains. More recently, it has been established that differences in the genotypes of the *Mtb* strain are responsible for the differences in virulence, antibiotic susceptibility, relapse and prevalence (Coscolla and Gagneux 2010; Reiling, Homolka et al. 2013; Coscolla and Gagneux 2014). The precise pathogenesis of TB and the factors determining the highly variable outcome of infection are only partly understood. Besides, a majority of the studies related to the pathogenesis of TB used only the laboratory strains which can suffer from artefacts because of their adaptation to the laboratory (Devasundaram and Raja 2016; Chiner-Oms, Gonzalez-Candelas et al. 2018). A study using clinical isolates may be advantageous as they may represent the behaviour of the pathogen in the human population. In view of this, in the present study, clinical isolates of specific genotypes (EAI-5, LAM-6 and Beijing) and laboratory strains (H37Rv and BCG) both were selected for evaluating the host-pathogen interactions *ex vivo*. The modern *Mtb* strains have been described as more virulent with MDR strains inducing lesser pro-inflammatory and apoptotic responses compared to primitive strains (Romero, Balboa et al. 2012; Chakraborty, Kulkarni et al. 2013; Chen, Chang et al. 2014). The previous finding in our lab attested manifestation of different *Mtb* lineages in host, human monocytic cell line, exhibiting responses to ancient East African Indian (EAI-5) and modern Beijing and Latin American Mediterranean (LAM) clinical strains (Chakraborty, Kulkarni et al. 2013).

Lipid composition of the *Mtb* changes with change in microenvironment of the bacterium, especially the CD1d lipids. Recently mouse primary lung epithelial cells have been described to present lipid antigen by CD1d pathway and their up-regulation was shown in mice post *Mycobacterium bovis* BCG infection (Rizvi, Puri et al. 2018). Therefore determination of Cd1d putative lipid in mice infected lungs can determine

any correlation with immunopathology. We also compared Cd1d putative lipid profiles between different modern and ancestral strain by LC-MS and compared their association with inflammatory responses induced by live bacteria and free lipids in the RAW264.7 cell line. We also determined the adjuvant effect of the free lipids from different clinical isolates in BCG vaccinated mice when used as a booster. LC-MS comparative analysis generates a consensus between biological response and differential lipid expression. This lipid fingerprint is a statement for its utility in designing vaccine and drugs as well as it helps in understanding the surface lipid evolution of an *Mtb* strain from particular lineage. Although lipids of *Mtb* have been characterized in details to some extent, a comparable resource does not exist between drug-sensitive, resistant, various strains or growth parameters like in culture, in the diseased host or in latency in mycobacterium. This can be achieved by employing a high-throughput mass spectrometry-based lipidomic approach to understand the dynamics of differential lipidome profile of *Mtb* in above-mentioned fates of the bacterium. Considering the fact that 30% of the *Mtb* genome codes for lipid, this comprehensive lipidomic approach unravels extensive lipid alterations which would serve as a resource for identifying lipid biomarkers aimed at improving BCG vaccine by identifying immunogenic lipids as adjuvants.

4.2 Study objectives*:*

I. To isolate lipids fractions from various *Mtb* isolates.

II. Evaluating *Mtb* lipids by 2D thin layer chromatography and LC-MS.

III. To determine if isolated lipids induce a differential immune response in a murine macrophage cell line.

IV. To determine whether in vitro cytokines generated by lipids of various strains correlate to the lipid composition in various states, e.g. Culture state and Infection state.

V. To determine the strain-specific behaviour of lipids isolated from various clinical isolates when used as a booster to the BCG vaccine.

4.3 Material and Methods

4.3.1 Mycobacterium tuberculosis isolates and culture maintenance.

Mtb clinical isolates were kindly provided by the department of microbiology, KEM hospital, Parel, Mumbai (later identified as Beijing, EAI-5, and LAM-6). These strains were identified by their lineage by spoligotyping and MIRU-VNTR typing, and are shown in table 6 (Chakraborty, Kulkarni et al. 2013). *Mycobacterium bovis* BCG (Strain Moscow) was obtained from Serum Institute (India). H37Rv was used as a reference laboratory strain. All these strains were isolated on Lowenstein-Jensen (LJ) media (Himedia, Mumbai) and propagated in Middlebrook 7H9 broth (Himedia, Mumbai) supplemented with oleic acid–dextrose catalase (Himedia, Mumbai) and 0.05% Tween 80 (Sigma-Aldrich, St. Louis, MO) medium at 37°C for 14 days as

previously described (Gouzy, Larrouy-Maumus et al.). Cells were stored in glycerol at -70°C at a mid-log phase as stocks.

	Spoligotype	MIRU	TbD1	RD1	Resistant	Sensitive
EAI5	777700777413700	254326223513	+	+	Ethambutol, Streptomycin, Isoniazide, Pyrazinzmide, Cycloserin, Ethionamide, Kanamycin, Clarithromycin	Rifampicin, Ciprofloxacin, Amikacin, P-Amino salicylic acid, Rifabutin
LAM6	777777607560771	123326153328	-	+	Rifampicin, Ethambutol, Streptomycin, Kanamycin, Pyrazinzmide,, Ethionamide	Isoniazide, Rifampicin, Clarithromycin, Ciprofloxacin, Cycloserine, Amikacin, PAS, Rifabutin
Beijing	000000000003771	222325153533	-	+	Ethambutol, Streptomycin, Isoniazide, Pyrazinzmide, Rifampicin, Cycloserin, Ethionamide, Kanamycin, Clarithromycin, Rifabutin	Amikacin, P-Amino salicylic acid, Ciprofloxacin

Table 6 : Drug sensitivity of the clinical isolates used in the current study
(Chakraborty, Kulkarni et al. 2013)

4.3.2 Ethics statement and animal grouping

BALB/c mice, free of common pathogens were purchased from the National Institute of Nutrition (Hyderabad) while C57BL/6 mice were issued by BARC animal house and the mice were housed individually in polycarbonate cages with stainless-steel feeders and tap water. They were maintained in a temperature and humidity controlled environment and exposed to a 12hr light-dark cycle. Each animal was randomly assigned to a vaccination treatment group. All procedures were reviewed and approved by the BARC Animal Ethics Committee, Mumbai, India (BAEC, 18/17) and the protocol for mice lung infection was approved by the animal ethics committee of the International Centre for Genetic Engineering and Biotechnology, India (ICGEB)

4.3.3 Mice lung infection

Mice groups were infected by *M. bovis* BCG and each strain of *M tuberculosis*, such as H37Rv, EAI-5, LAM-6 and Beijing. Mice were exposed to the aerosol with the various *Mtb* strains (bacterial count of 10^6 bacilli/ml) for 15 min using a Lovelace nebulizer (In-Tox Products, Albuquerque, NM) and aerosol chamber (U.V. Madison College of Engineering Shops, Cambridge Square Inc.). This aerosol infection implants approximately 150 organisms into the lungs of each mouse which was confirmed by plating lung homogenates next day after infection. Another group of mice were left uninfected, undisturbed and were sacrificed after 1 month post-infection along with uninfected mice which served as a negative control. Mice lungs were used for histopathological study and lipid isolation. The animal aerosol challenging experiments were performed in BSL-III at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi under strict supervision.

4.3.4 Extraction and analysis of Mtb clinical isolate derived free lipids

Mtb strains were propagated in Middlebrook 7H9 broth till log phase growth was achieved. The mycobacterial cells were heat-killed in a water bath at 80°C to 90°C for between 1 and 2 hrs, and the cell-suspensions were harvested cells by centrifugation. Cells were washed in PBS and freeze-dried by vacuum freeze dryer (Christ, Germany). Lipids were extracted as polar and non-polar by using organic liquid separation methods mentioned elsewhere (Besra 1998). Further, Two dimensional (2D) TLC analysis of apolar and polar lipid fractions. Aluminium backed

silica gel 60 F254TLC plates (Merck, Darmstadt, Germany) were cut into approximately 6 cm squares and 100µg of lipid extract was spotted onto the plates using glass microcapillary pipettes. Plates were dried thoroughly before being placed in a TLC tank containing appropriate solvent mixtures (systems A-D). Staining was performed by using a 5% solution of molybdophosphoric acid (MPA) (Sigma Aldrich) in 95% ethanol.

4.3.5 Lipid extraction for liquid chromatography-mass spectrometry (LC/MS)

Cultured *Mtb* cells or homogenized infected mice tissue slurry were harvested by centrifugation (3000g for 10 min) and washed three times with phosphate-buffered saline pH 7.4 (PBS) before inactivating by heat. To prepare crude lipid extracts, a modified Bligh and Dyer method (Singh, Sinha et al. 2014) was used. Briefly, 200 mg of wet bacterial pellets cells or 600 mg of mice tissue were lyophilised, and the cells were extracted with 6 ml of chloroform/methanol/water (10:10:3 v/v/v) to obtain an approximate lipid concentration of 4 µg/µl (bacterial culture) and 10 µg/µl (mice lung tissue). The dilution series was prepared by homogenising cells by bead–beating and serially diluting it two-fold with water in glass tubes. The diluted homogenates were lyophilised and reconstituted before use. Based on the starting wet weight of the cell pellet, the dry lipid weights were estimated from routinely observed lipid extraction yields. The cell pellets were harvested and washed three times with PBS (pH 7.4). These crude lipid extracts were prepared as described above and randomised for LC/MS analyses.

4.3.6 Liquid Chromatography

An Agilent 1290 HPLC (Agilent Technologies, Palo Alto, CA) with 2.1 inner diameters (i.d) x 100 mm, 1.8µm Agilent Zorbax SB-C18 was placed in series in front of the analytical column. Solvent A was maintained at 100% of solvent A (5mM ammonium acetate in methanol/water (99:1 v/v)], and an aliquot of the lipid extract (5µl = 20 µg dried extract) was applied to the column. Solvent A was maintained at 100% for 2.0 min, followed by a 30.0- min linear gradient to 100% solvent B (5mM ammonium acetate in n-propanol/ hexane/water (79:20:1 v/v/v)], and held at 100% solvent B for 3.0 min (Flow rate 0.3ml/min). All solvents and chemicals purchased were MS or HPLC grade (Sartain, Dick et al. 2011).

Solvent System Runs	Run Direction	Components	Run	Lipids Resolved
A	1	Petroleum ether / ethyl acetate 98:2	3	Phthiocerol dimycocerosate family, Menaquinone, Triacylglycerols.
	2	Petroleum ether / acetone 98:2	1	
B	1	Petroleum ether/acetone 92:8	3	Free fatty acid; Apolar mycolipenates of trehalose
	2	Toluene/acetone 95.5	1	
C	1	Chloroform / Methanol 96:4		Polar lipids.
	2	Toluene/acetone 80:20		
D	1	Chloroform/ Methanol/ Water 100:14:0.8		
	2	Chloroform/Acetone/Methanol/ Water 50:60:25:3		

Table 7: Solvent systems used for the 2D-TLC analysis of mycobacterium lipids

4.3.7 Mass Spectrometry

An Agilent 6550 series q (TOF) mass spectrometer equipped with an Agilent ESI source was used for accurate mass analysis of the LC eluent. Positive (+) and Negative (-) ion data were generated by operation of the mass spectrometer in a mixed ESI/APCI mode with a capillary voltage of 2000 V, Nebulizer of 45 PSIG, drying gas of 8 L/min, gas temperature of 300°C, vaporizer temperature of 200°C, Corona of 2uA, fragmentor of 120 V, charging voltage of 2000 V, skimmer of 60V, and OCT RF of 250V. Mass spectra were acquired in 4 GHz high-resolution mode at a rate of 1.02 spectra/s and 9700 transient/spectrum, and data was collected as profiled spectra over a mass range of 250 to 3200 Da. Data was collected with the Agilent Mass Hunter Work station Agilent Mass Hunter Qualitative Analysis B.06.00 (Sartain, Dick et al. 2011). Positive ion data were used for analysis.

4.3.8 Mtb Lipid identification

The *Mtb* lipid database was in the form of searchable ion peak entries corresponding to a single *Mtb* lipid group in positive as well as negative ion mode as a database (MtbLipidDB); named "+MH_MtbLipid.csv" and "-MH_MtbLipid.csv" were obtained from previously published data (Sartain, Dick et al. 2011). Data for ion peaks of 5 strains were compared with this database using Agilent Masshunter software as with interface which allows for searching of MS data against the *Mtb* LipidDB. The software provides the option to identify multiple molecular ions and adducts into a single feature. The molecular ions were established in each by an iterative process based on empirically observed ionization properties of each lipid subclass.

4.3.9 MPP analysis of lipids

The Molecular Feature Extractor (MFE) algorithm in the Mass Hunter Qualitative analysis software was used to extract molecular features of unidentified, untargeted compounds in each of the data with the provided database as mentioned before. The MFE algorithm scans for mass signals (ions) that are covariant in time and considers possible chemical associations (adducts, isotopes, multiple charge states, dimers) and generates an extracted compound chromatogram and compound mass spectrum for each molecular feature. The extracted compound list for each file was exported as Compound Exchange Format (.cef) file for further Mass Profiler Professional (version B.2.00, Agilent) statistical analysis. The resulting feature files for each sample were processed by ANOVA and PCA analysis utilizing the MPP software, which aligned, normalised, visualised and filtered the molecular features (MFs), for further processing. Subsequently, hierarchical clustering (condition tree) was applied to the data files. Hierarchical cluster analysis is a statistical method to group samples unsupervised in different clusters or branches of the hierarchical tree. In this way, the relationships between the different groups were shown. The condition tree was displayed as a heat map. ID Browser feature of MPP determined the identity of biomarkers with significant changes in the groups. *Mtb* database was modified by retaining only Cd1d putative lipids for CD1d putative lipid analysis.

4.3.10 Preparation of clinical isolate derived free lipids (CIDFL) and macrophage stimulation

Of all lipid antigens, polar and apolar were prepared in an aqueous phase for use in cell culture experiments after first removing any $CHCl_3$:CH_3OH by evaporation

using an N_2 gas stream. Cell culture medium was added to the lyophilised lipid, and the mixture was subjected to 2 cycles of heating at 80°C and then sonication for 5 min. (Pirson, Jones et al.). Murine macrophage cell line RAW264.7 was obtained from the NCCS Pune and was grown in 25 ml culture flask in RPMI medium supplemented with 10% heated-inactivated FCS, 2 mmol/L. L-glutamine, and 100 U/mL penicillin/streptomycin (GIBCO) at 37ºC in a humidified incubator of 5% CO_2. The cells were gently scrapped, enumerated and seeded in 24 well plates ($3x10^5$) cells per well and incubated with polar or apolar lipid with LPS (1mg/ml) as control used to stimulate macrophage cells in vitro at 80 μg/mL for 12 to 16 hrs. The supernatant was collected, and TNF-α and IL-1β cytokine secretion were estimated by cytokine ELISA kits (BD, Biosciences, USA).

4.3.11 BCG vaccination

BCG was reconstituted in 0.9% physiological saline, and 0.1 ml ($1x10^6$/ml) was administered to the experimental animals (N=6 per group) subcutaneously into the left inguinal region. The animals were rested for 6 to 8 weeks postvaccination before being sacrificed for splenocyte isolation as described earlier (Young, O'Donnell et al. 2002)

4.3.12 Preparation of cell cultures for restimulation studies

Spleens of C57BL/6 mice were removed aseptically, and cell suspensions were made using sterile cell strainer (40μm, BD, Biosciences) and RBCs in it were lysed subsequently. Non-adherent splenocytes passed through nylon wool column

(Polysciences) were used as T-cells and were adjusted at the concentration of 1×10^6 cell per well. Mitomycin-C treated syngenic mice splenocytes were used as antigen-presenting cells (APCs) which were previously incubated with strain-specific lipids and were co-cultured with T-cells at a concentration of 2.5×10^6 cells per well in 24-well plates (BD Biosciences) (Hsieh, Hsu et al. 2009). Cells were stimulated in triplicates with protein purified derivate (PPD) (50µg/ml). After 72 hrs, the supernatant was collected, and ELISA estimated IFN-γ according to the manufacturer's instructions (BD Biosciences). As protein-free lipids were used to stimulate the nylon passed column, the IFN-γ secreted by them was considered to be from NKT cells.

4.3.13 Cytokine estimation

T cell co-cultures or RAW264.7 macrophages were incubated with either culture medium, BCG-PPD (50µg/ml) or Strain-specific lipids (µg/ml) for a period of 72h in 24 well plates. Supernatants were removed, pooled and analysed for the presence of IFN-γ, TNF-α, IL-1β wherever necessary, using sandwich ELISA. The ELISA was performed according to the BD Biosciences manufacturer's instructions.

4.3.14 Statistical analysis

All experimental data were obtained from at least three independent experiments. Statistical analyses were performed using Sigma Stat 3.5. Data for each experiment was expressed as a mean ±SD. Significant differences between the groups were determined by analysis of variance followed by One Way ANOVA. Student T-

test determined the difference between the two groups. A value of $p<0.05$ was considered significant.

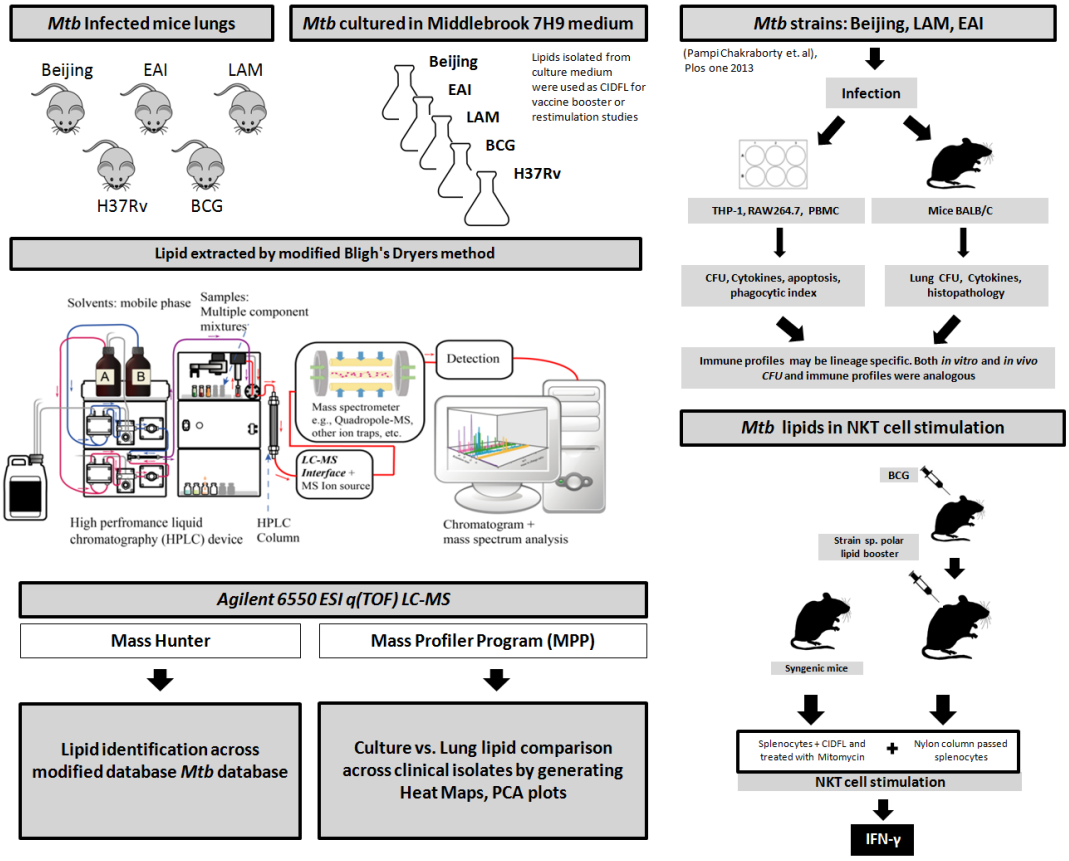


Figure 29: Summary of experiments to determine the efficacy of Mtb derived lipids in improving BCG vaccine. *Mtb clinical isolates (Beijing, EAI-5, and LAM) and control Mtb (H37Rv, BCG) were grown in culture medium or in mice lungs. LC-MS was performed on the lipids isolated from Mtb culture and mouse infected lungs. CD1d putative analysis was performed on all the strains in the culture state and infection state. Mtb strains grown in culture were used to isolate polar and apolar lipid fractions by biphasic separation methods and were used to stimulate RAW264.7 and compared with live strains infections by evaluating culture supernatant IFN-γ by ELISA. Further lipids from various Mtb strains were used as BCG vaccine adjuvant. The vaccine response in terms of secreted IFN-γ was evaluated by restimulating splenocyte co-culture with strain-specific lipids. A comparative evaluation was made from the observations from all the experiments.*

4.4 Results

4.4.1 Analysis of Mtb strain lipids by TLC

To establish any association of the *Mtb* cell wall lipids to their role, their identification is essential. The cell wall was lipids from Beijing, LAM, EAI-5 strains were isolated into polar and apolar fractions and were compared with H37Rv (reference strain). These lipids were subjected to 2D-TLC using organic liquids to separate the lipids on the basis of their polarity. Figure 30A represents mycolic acids from various clinical isolates. We identified phthiocerol dimycocerosates (PDIMs), menaquinone (MK) and triacylglycerol in the least polar fraction. Pentacyl trehalose (PAT), mycolipenates of trehalose and free fatty acids were identified in apolar fraction (Fig. 30B). Discrete spots for polar lipid fraction in TLC were not observed due to lesser lipid load. The presence of mycolipenates of trehalose was observed in H37Rv, Beijing and LAM while it was absent in EAI-5 strain. Mycolipenic acids, the major acyl residue found in PAT are present only in the virulent species of *Mtb* but absent in BCG its role in leucotoxicity, leucocyte migration and T-cell activation is already reported (Draper, Payne et al. 1983; Daffe, Papa et al. 1989; Rousseau, Neyrolles et al. 2003). This shows that EAI-5, on the contrary to BCG being of the ancient lineage might be less pathogenic. The deficiency in polyacylated trehaloses has biological consequences relevant to the interaction of the bacterial cells with the host. PDIM was identified in LAM and EAI-5 strains while it was absent in H37Rv and Beijing**.** PDIM is surface-exposed polyketides lipids which constitute the most-abundant free lipids found in the cell wall. The H37Rv is highly prone to losing the ability to synthesize the cell wall lipid phthiocerol dimycocerosate (PDIM) during extended periods of *in vitro* culture**.** At least independent studies have confirmed that

Mtb mutants deficient in PDIM synthesis or translocation are severely attenuated *in vivo* (Domenech and Reed 2009; Gomez-Velasco, Bach et al. 2013). The DIM production contributes to the initial growth of *Mtb* by protecting it from the nitric oxide-dependent killing of macrophages and modulating the early immune response to infection. (Rousseau, Winter et al. 2004). The TLC methods allow only macro-analysis of lipids while the lipids of the cell envelope, which are diverse and lesser produced by the cell wall are not suitable for analysis by using method is not suitable. A, therefore, finer analysis of lipids was performed by LC-MS and is described in later text.

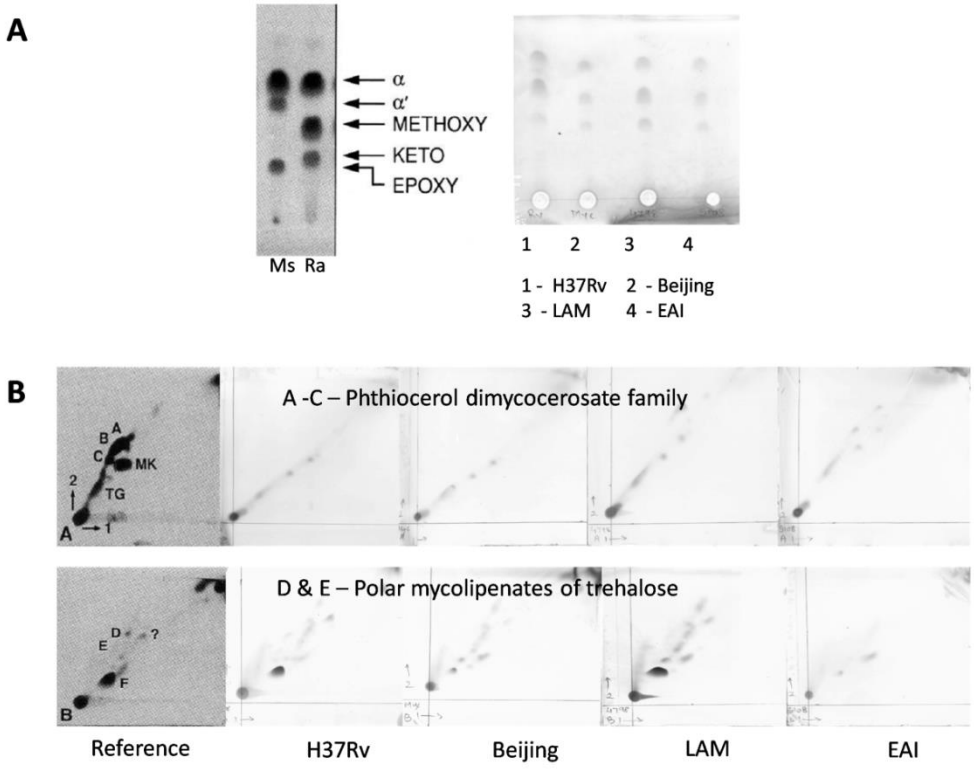


Figure 30: Two-dimensional TLC analysis of the organic soluble cellular-envelope lipids of Mtb strains using solvent systems A-E (Table no.7). *Fig. 30A represents TLC of mycolic acid esters from various clinical isolate and compared with published standard M. smegmatis mc2155 (lane 1 left) and Mtb H37Ra (lane 2 right). Fig. 30B represents 2D TLC for lipid spots labelled as A-C, phthiocerol dimycocerosate family; CF, "cord-factor"; D and E, apolar mycolipenates of trehalose ; F, free fatty acid; MK, menaquinone*

4.4.2 In vitro and in vivo lipid expression by various Mtb strains studied by LC-MS

LC-MS was performed on the lipid samples isolated from the *Mtb* strains (clinical isolates) grown in Middlebrook 7H9 culture medium or from mice lung infected with the same strains. Fig. 31A represents the frequency plot of the lipids from *Mtb* strains grown in culture medium and the PCA plot which shows the difference between strains based on their difference in lipid composition when grown in culture medium while Fig. 31B represents frequency plot of the combined lipids from both *Mtb* strains and mice lung infected with different *Mtb* strains and the PCA plot which shows the difference between strains based on the difference in lipid composition in infected lung. We observed that Beijing, LAM-6, EAI-5, H37Rv and BCG strains share multiple common lipids while many are strain-specific in both culture and infected state. Strain Beijing showed a lower abundance of lipids in culture or infected lung. Lipids from H37Rv and LAM-6 grown in the culture medium showed similar abundance. EAI-5 showed the highest abundance of lipids in culture and infection state as compared to other strains. Fig. 32 shows a heat map and PCA plot of lipid of mycobacterial origin from all *Mtb* strains clubbed together when isolated from bacteria growing in culture or from infected mice lung. PCA plot shows lipid composition of H37Rv, similar to LAM-6 in culture state while in infected mice lung lipid composition shows Beijing and H37Rv to be closely associated with respect to other strains.

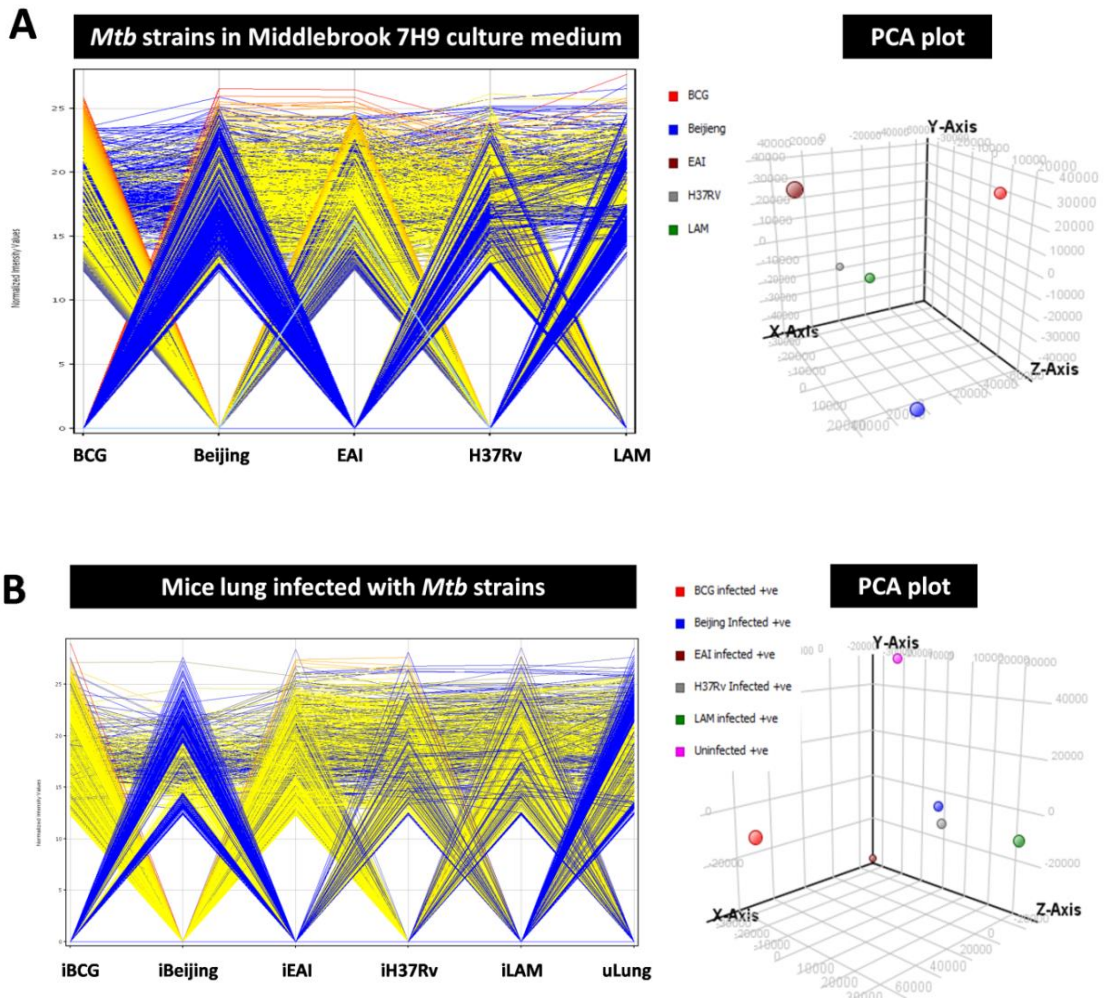


Figure 31: Lipids expressed by various Mtb strains grown in Middlebrook 7H9 culture medium shows diversity in expression. *LC-MS was performed on the lipid samples isolated from the Mtb strains (clinical isolates) grown in Middlebrook 7H9 culture medium or from mice lung infected with the same strains. Fig. 31A and 31B represent a frequency plot of the lipids from Mtb strains grown in culture medium and from infected lungs, respectively. PCA plot which shows the difference between strains based on their difference in lipid composition when grown in culture medium or infected mice lungs.*

A heat map was generated to demonstrate the relative abundance of lipids upregulated or down-regulated lipids in culture state or mice lungs for all strains in the study was shown together. It was observed that most of the lipids are down-regulated in infected stages as compared to culture stage. There are some unique lipids upregulated in infection state. Table 8 and 9 shows *up* or down-regulated *Mtb* lipids in

the clinical isolates grown in Middlebrook culture 7H9 medium or infected mice lungs.

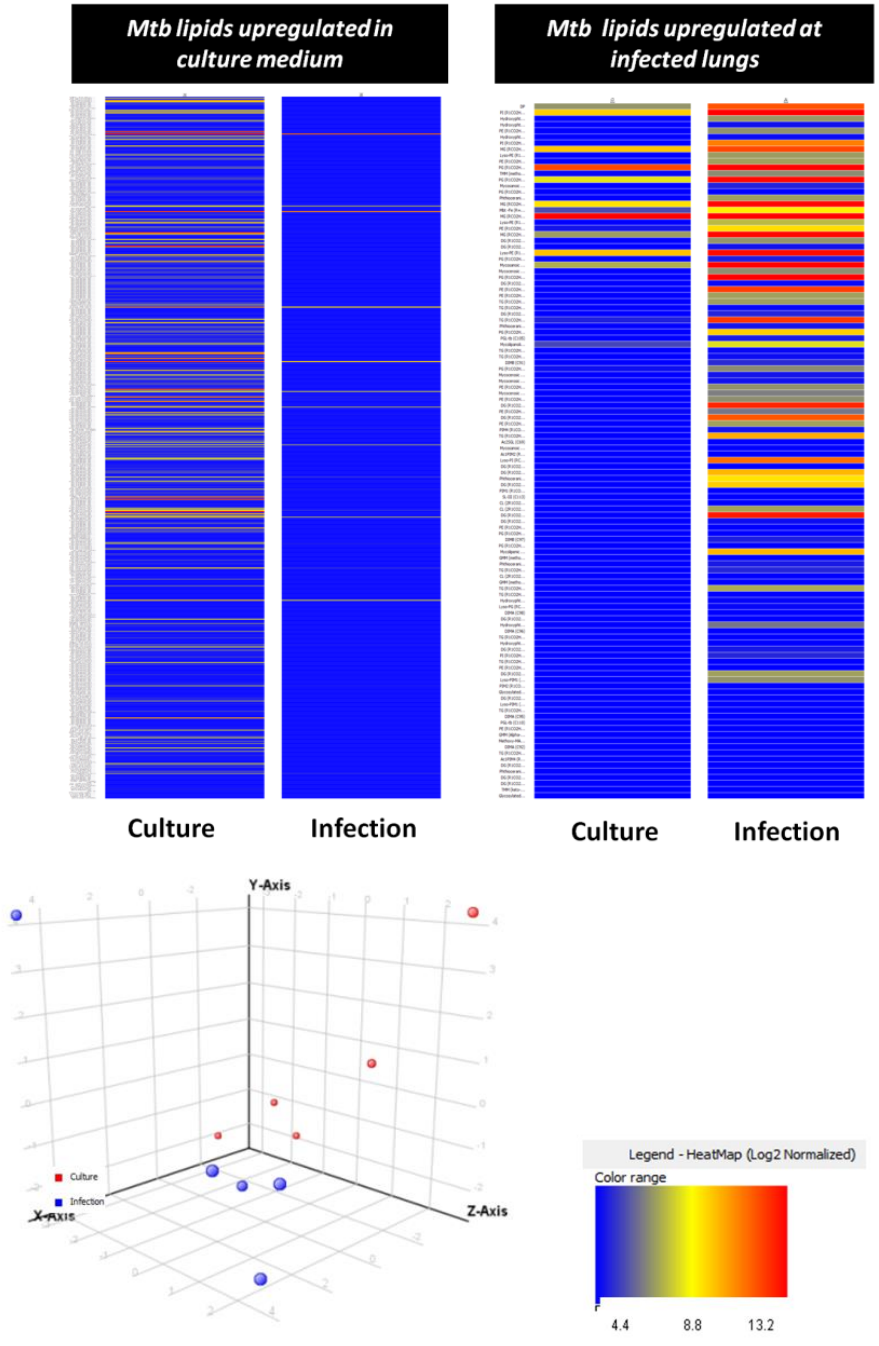


Figure 32: Lipids expressed by various Mtb strains in infected mice lungs shows diversity in expression. *Fig. 3C shows a heat map and PCA plot of lipid of mycobacterial origin from all Mtb strains clubbed together when isolated from bacteria growing in culture or from infected mice lung. Blue to red shades indicates the relative abundance of lipids in increasing order, respectively.*

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Mbt -Fe (R=19:1)	7.906385	0	1733.059	97.6
PI (R1CO2H+R2CO2H=34:4)	11.49714	0	1682.977	72
TG (R1CO2H+R2CO2H+R3CO2H=58:2)	6.850408	0	959.887	1105.1
MG (RCO2H=21:0)	16.14377	0	422.3364	79.475006
PI (R1CO2H+R2CO2H=37:1)	15.06843	3.3514934	1779.162	48.997402
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:1)	8.041407	0	1964.225	188.84999
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=60:0)	7.40141	0	1739.066	47.850002
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:4)	7.854472	0	1661.008	70.55
PIM4 (R1CO2H+R2CO2H=37:1)	6.82484	0	1548.781	39.849995
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:2)	3.648507	0	1755.182	145.4
Ac2SGL (C78)	3.795135	0	1435.025	66.5
Mycocerosic or Phthioceranic acid (C34)	7.768794	6.644211	552.4873	56.472
PG (R1CO2H+R2CO2H=35:2)	14.34359	0	777.5552	84.87501
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=49:2,R4=H)	8.59844	0	1561.928	77.6
PI (R1CO2H+R2CO2H=37:0)	14.64463	0	1783.187	147.925
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=54:5,R4=H)	7.802474	0	1463.902	77.80001
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:0)	3.72714	0	1966.245	181.10002
PI (R1CO2H+R2CO2H=36:1)	7.46349	0	1751.125	59.100002
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:5)	3.405879	0	2174.277	66.6
DAT1 (C56)	6.48729	0	1003.787	76
DG (R1CO2H+R2CO2H=39:1)	7.009342	0	686.585	167.64998
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=44:0)	11.43866	0	1198.71	738.76666
CL (2R1CO2H+2R2CO2H=73:1)	8.431321	4.194434	1521.066	45.90767

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
DG (R1CO2H+R2CO2H=46:1)	3.331229	0	784.6897	599.5
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:6)	3.121686	0	1847.061	39.8
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=46:1)	3.733971	0	1202.742	58.400005
PIM1 (R1CO2H+R2CO2H=36:2)	3.825609	0	2071.209	52.8
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:4)	8.087332	0	1723.123	88.15
PIM1 (R1CO2H+R2CO2H=34:2)	3.385456	0	1018.559	39.5
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=51:5)	3.23368	0	1259.799	245.7
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=47:0,R4=H)	7.337336	0	1375.869	64.200005
PI (R1CO2H+R2CO2H=36:0)	19.2504	0	1755.154	116.98003
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:4)	8.424084	0	1751.156	112.45001
PI (R1CO2H+R2CO2H=34:1)	20.98078	18.110233	853.5643	23.509897
SL-III (C103)	3.787708	0	1815.392	246.8
Mycocerosic acid (C27)	11.43247	3.9796896	427.4398	160.19724
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=53:2,R4=H)	8.60493	0	1617.99	76.85
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:2)	8.111341	0	1707.079	65.850006
PIM1 (R1CO2H+R2CO2H=36:1)	12.35228	0	1048.61	80.00001
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:5)	3.887928	0	1822.142	51.5
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=54:0,R4=H)	3.781904	0	1803.039	54.400005
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=47:0,R4=H)	7.661297	0	1704.932	58.850002
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=54:0,R4=H)	4.175333	0	1500.913	52
CL (2R1CO2H+2R2CO2H=90:1)	4.513143	0	1759.335	138.2
PIM1 (R1CO2H+R2CO2H=34:1)	14.8066	0	1015.623	52.399998

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=53:3,R4=H)	3.193148	0	1615.968	90.80001
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:5)	7.019798	0	1749.141	107.9
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:6)	3.986404	0	1658.074	185
CL (2R1CO2H+2R2CO2H=86:2)	7.456804	0	1701.255	86.5
CL (2R1CO2H+2R2CO2H=66:2)	3.975342	0	1420.93	140.9
CL (2R1CO2H+2R2CO2H=69:2)	3.261916	0	1462.982	74.9
CL (2R1CO2H+2R2CO2H=68:2)	8.403818	0	1426.978	98.75
TG (R1CO2H+R2CO2H+R3CO2H=43:0)	11.13381	3.8252854	758.6372	293.23904
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=50:0)	3.713718	0	1260.819	107.4
Lyso-PG (RCO2H=19:0)	3.492714	0	543.3519	75.5
CL (2R1CO2H+2R2CO2H=77:2)	9.283474	0	1575.114	77.6
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=51:2,R4=H)	8.577392	0	1589.959	98.24999
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:5)	7.385047	0	1947.105	126.35
PIM2 (R1CO2H+R2CO2H=36:2)	3.186277	0	1208.651	39.1
TMM (keto-MA,C93)	3.222411	0	1709.499	872.3
Ac2SGL (C64)	7.092215	0	1238.808	67.600006
CL (2R1CO2H+2R2CO2H=69:1)	4.189397	0	1465	84.1
TG (R1CO2H+R2CO2H+R3CO2H=69:1)	7.525044	0	1121.032	244.55
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=55:1,R4=H)	8.853841	0	1810.088	97.15
CL (2R1CO2H+2R2CO2H=83:2)	9.069911	0	1659.207	173.70001
CL (2R1CO2H+2R2CO2H=66:3)	3.545456	0	1418.914	115.19999
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=60:1)	4.370635	0	1570.044	58.8

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
PIM2 (R1CO2H+R2CO2H=34:2)	3.445243	0	1175.664	52.2
PG (R1CO2H+R2CO2H=43:1)	6.705852	3.2371144	891.6951	207.68498
Lyso-PG (RCO2H=19:1)	3.814488	0	1070.601	52.400005
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:0)	3.724807	0	2069.295	81.8
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=50:1)	7.172356	0	1258.808	121.00001
CL (2R1CO2H+2R2CO2H=87:2)	7.60635	0	1715.266	306.65
SL-III (C93)	8.658705	0	1675.232	225.44997
CL (2R1CO2H+2R2CO2H=69:4)	8.172891	0	1432.017	77.899994
GMM (Alpha-MA,C90)	3.32932	0	1489.4	1333.2
CL (2R1CO2H+2R2CO2H=60:2)	4.203689	0	1336.839	68.6
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:3)	3.967222	0	1965.156	137.3
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=49:0,R4=H)	7.70105	0	1403.902	77.5
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=50:3)	7.601667	0	1254.78	47.349995
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:2)	7.216981	0	2060.309	232.15002
PIM2 (R1CO2H+R2CO2H=36:0)	6.723271	0	1207.718	63.2
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=50:3,R4=H)	3.565911	0	1438.81	52.3
CL (2R1CO2H+2R2CO2H=67:4)	3.733063	0	1403.978	58.5
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=51:3,R4=H)	3.290203	0	1587.941	75.5
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:1)	8.714276	0	1752.234	127.29999
Ac2SGL (C62)	7.949333	0	1210.778	55.349995
GMM (Alpha-MA,C91)	3.667	0	1503.416	1493.9
TMM (keto-MA,C91)	7.746409	3.5693197	1681.468	541.379

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM6 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:6)	3.078886	0	2306.29	501.6
CL (2R1CO2H+2R2CO2H=82:1)	3.354073	0	1647.197	63.7
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=53:2)	3.957779	0	1298.834	54
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=51:5,R4=H)	3.368384	0	1583.907	39.8
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=55:4,R4=H)	7.878705	0	1506.879	76.35
CL (2R1CO2H+2R2CO2H=60:1)	3.796448	0	1316.87	50.400005
Hydroxyphthioceranic acid (C39)	3.38568	0	638.5587	143.8
PIM3 (R1CO2H+R2CO2H=36:0)	3.698142	0	1374.726	45.2
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=51:0,R4=H)	7.753459	0	1431.932	97.700005
Lyso-PG (RCO2H=20:0)	13.74389	11.201896	562.3235	32.148994
DP-PP	7.808266	0	1739.117	156.5
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=61:1)	3.349767	0	2097.152	224.7
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=56:0,R4=H)	3.950612	0	1528.955	51.7
CL (2R1CO2H+2R2CO2H=59:1)	6.950074	0	1302.856	92.299995
MG (RCO2H=15:0)	19.13599	17.296247	654.5059	30.558104
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=46:0,R4=H)	4.07514	0	1550.846	40.2
CL (2R1CO2H+2R2CO2H=67:1)	7.504865	0	1436.967	90.700005
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=47:2,R4=H)	8.311593	0	1538.846	54.249996
TG (R1CO2H+R2CO2H+R3CO2H=76:2)	3.42307	0	1217.12	339.7
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=56:0)	6.840415	0	1366.9	391.34998
PIM1 (R1CO2H+R2CO2H=33:0)	6.834857	0	1030.559	41.2
PE (R1CO2H+R2CO2H=33:0)	11.8591	4.3344355	705.5302	43.191006

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:1)	7.019104	0	2196.345	95.299995
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=56:1,R4=H)	8.49246	0	1688.99	77.200005
PE (R1CO2H+R2CO2H=31:1)	14.82833	3.7114248	719.4471	101.886406
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:5)	7.963777	0	1646.063	47.55
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=61:1)	3.449404	0	1746.11	248.10002
CL (2R1CO2H+2R2CO2H=62:2)	4.235777	0	1364.87	84.4
PG (R1CO2H+R2CO2H=39:0)	10.86233	3.1593802	837.6465	702.6052
GMM (Alpha-MA,C89)	3.400941	0	1475.385	1469.3002
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=63:0)	8.164039	0	1943.164	85.00001
TMM (methoxy-MA,C85)	3.467416	0	1599.386	747.8
PIM3 (R1CO2H+R2CO2H=36:3)	3.407254	0	1368.689	77.5
MG (RCO2H=17:0)	19.4304	7.059783	366.2748	41.941708
DG (R1CO2H+R2CO2H=30:1)	14.6678	3.5232396	560.4442	102.91758
CL (2R1CO2H+2R2CO2H=59:3)	7.79027	0	1298.825	41.2
CL (2R1CO2H+2R2CO2H=64:3)	3.22739	0	1390.884	100.9
PIM1 (R1CO2H+R2CO2H=30:0)	4.45776	3.4048073	961.5766	27.82
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=52:3,R4=H)	4.153456	0	1601.954	51.3
MPM (C34)	14.51296	0	753.5898	126.575005
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=52:0,R4=H)	3.36945	0	1472.885	114.9
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:6)	3.616871	0	1671	62.3
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:2)	12.01967	0	1629.043	81.700005
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=57:1,R4=H)	8.445735	0	1518.963	37.3

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=46:1,R4=H)	4.186057	0	1364.795	51.099995
MG (RCO2H=18:0)	20.51424	6.889982	380.2898	46.49043
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=52:0)	10.54852	0	1288.851	1412.2333
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:6)	6.771101	0	2093.201	57.45
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:3)	3.739712	0	1655.057	75.3
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=55:5,R4=H)	4.06381	0	1639.967	51.599995
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:3)	3.773153	0	1649.005	113.4
TG (R1CO2H+R2CO2H+R3CO2H=77:1)	3.632788	0	1233.153	399.9
GMM (Alpha-MA,C86)	3.070077	0	1433.34	1440.3002
Mbt -Fe (R=18:1)	15.27185	7.0104156	841.5221	39.565006
DAT2 (C52)	8.671965	0	963.7243	74.85
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=57:1,R4=H)	8.635532	0	1838.119	126.9
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=57:2,R4=H)	3.985067	0	1863.038	52.2
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:3)	7.961035	0	1840.181	235.7
TG (R1CO2H+R2CO2H+R3CO2H=73:1)	7.964666	0	1177.092	315.15
MG (RCO2H=17:1)	15.67553	0	706.5371	63.99999
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:1)	4.187982	0	1645.074	52.2
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=56:4)	8.150504	0	1358.832	41.449997
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=52:1)	6.891034	0	1286.836	100.9
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=49:3,R4=H)	7.327624	0	1397.852	89.299995
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=49:0,R4=H)	4.233818	0	1732.971	60.2
PIM1 (R1CO2H+R2CO2H=34:3)	9.989978	0	1011.594	81.43333

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:4)	7.034485	0	1675.025	141.70001
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:4)	7.652567	0	1620.049	321.95
CL (2R1CO2H+2R2CO2H=73:2)	7.619185	0	1519.052	86.799995
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=62:3)	8.851005	0	1756.091	97.149994
TG (R1CO2H+R2CO2H+R3CO2H=75:2)	3.368745	0	1203.109	333.3
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:2)	3.272522	0	2250.4	60
SL-III (C101)	8.315212	0	1787.364	235.09998
CL (2R1CO2H+2R2CO2H=75:2)	8.424097	0	1547.073	64.4
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=53:1,R4=H)	8.719279	0	1782.057	77.1
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:1)	3.566735	0	2224.377	103.6
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=62:1)	3.622039	0	1603.019	185.8
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:4)	7.527431	0	2070.31	110.35
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:6)	8.35393	0	1904.223	76.65
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=61:0)	8.402479	0	1910.174	171.05
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=55:3,R4=H)	7.279752	0	1644.01	77.799995
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:6)	3.476106	0	1895.156	76.8
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=63:3)	3.852378	0	1613.017	139.3
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=44:0,R4=H)	8.293643	0	1500.827	40.850002
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:1)	8.681952	0	1844.226	76.9
Ac2PIM6 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:0)	3.534872	0	2387.348	557.4
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:5)	3.940691	0	1878.198	182.89998
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=48:3,R4=H)	8.248644	0	1550.849	41.750004

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:5)	3.488924	0	1933.178	186.39998
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=47:1,R4=H)	3.221909	0	1373.862	221.7
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:2)	8.126313	0	1657.066	82.049995
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=56:2)	3.217281	0	1340.882	100.69999
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:0)	3.364887	0	1979.161	52.7
CL (2R1CO2H+2R2CO2H=73:4)	8.181974	0	1488.074	128.99998
DG (R1CO2H+R2CO2H=37:1)	3.791205	0	653.5989	122.80001
GMM (Alpha-MA,C85)	3.24294	0	1419.323	1274.2
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=62:2)	8.603909	0	1601.006	55.399994
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:6)	7.908064	0	1903.121	136.7
DG (R1CO2H+R2CO2H=31:1)	11.10002	3.6415064	569.5047	198.25923
PIM1 (R1CO2H+R2CO2H=37:2)	6.99372	0	1060.61	52.749996
Lyso-PE (R1CO2H=18:0)	16.4942	14.479581	503.3002	20.173502
PG (R1CO2H+R2CO2H=30:1)	4.060865	0	709.492	54.8
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=55:0)	3.377099	0	1325.948	79.9
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:3)	3.628914	0	1868.21	247.2
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=50:5,R4=H)	3.906077	0	1736.906	45.5
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=54:1,R4=H)	3.771967	0	1471.971	60.2
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:1)	7.362353	0	2053.277	214.09999
Mbt -Fe (R=17:1)	4.24885	0	849.4836	67.3
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:2)	3.495141	0	2088.351	179
CL (2R1CO2H+2R2CO2H=65:4)	7.072889	0	1375.948	55.550003

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=54:2,R4=H)	7.810609	0	1474.904	79.35
TG (R1CO2H+R2CO2H+R3CO2H=50:1)	12.27084	0	854.7319	878.69995
PG (R1CO2H+R2CO2H=30:0)	6.997702	0	711.5016	266.6
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:2)	6.979726	0	1875.185	85.75
PIM1 (R1CO2H+R2CO2H=37:1)	14.76553	3.2025642	1057.671	148.59479
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=54:4)	7.523922	0	1308.828	45.2
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:1)	3.525464	0	2117.302	54.8
CL (2R1CO2H+2R2CO2H=65:3)	7.693704	0	1377.969	65.450005
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:3)	9.190068	0	1761.13	77.200005
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:3)	7.790176	0	1733.096	91.700005
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=50:4,R4=H)	7.511787	0	1409.856	65.4
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=54:3,R4=H)	8.126605	0	1629.985	65.69999
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:3)	9.26813	0	1789.161	97.24999
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=52:2,R4=H)	8.256845	0	1446.876	46.399998
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=63:0)	3.890144	0	1619.05	60.2
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:1)	8.982374	0	1877.212	96.799995
CL (2R1CO2H+2R2CO2H=65:1)	3.716225	0	1408.934	58.400005
CL (2R1CO2H+2R2CO2H=61:0)	7.526802	0	1332.913	99.25001
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:0)	7.250939	0	2041.266	68.299995
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:2)	8.401674	0	1741.172	236.49998
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=52:3)	3.762974	0	1282.81	50.900005
Mbt +Fe (R=18:1)	7.688606	0	894.429	52.949997

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
CL (2R1CO2H+2R2CO2H=72:2)	8.14707	0	1483.048	176
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=75:4)	12.42484	0	1779.189	97.399994
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:0)	7.790152	0	1921.263	76.3
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=48:3,R4=H)	7.423133	0	1870.004	41.4
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=48:1,R4=H)	4.188441	0	1392.826	59.8
CL (2R1CO2H+2R2CO2H=65:0)	3.56883	0	1388.967	138.8
DG (R1CO2H+R2CO2H=29:1)	6.494341	3.8180645	546.4284	59.10001
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:5)	4.152117	0	1729.078	105.80001
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:3)	7.111822	0	2086.337	134.09999
PI (R1CO2H+R2CO2H=32:0)	16.04466	3.3316116	832.5118	44.4114
PIM1 (R1CO2H+R2CO2H=31:0)	17.51477	0	980.5416	46.819996
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=53:0,R4=H)	3.889141	0	1951.081	58.900005
CL (2R1CO2H+2R2CO2H=79:2)	9.380883	0	1603.146	97.99999
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:0)	4.058078	0	1795.215	254.89998
PG (R1CO2H+R2CO2H=40:0)	17.53062	0	851.6613	659.8251
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=48:1)	7.743871	0	1230.778	93.200005
PIM2 (R1CO2H+R2CO2H=37:3)	4.133745	0	1215.69	50.7
MG (RCO2H=20:0)	18.89045	15.05422	408.3232	36.42433
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:5)	3.573331	0	2040.246	111.30001
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=62:1)	8.123094	0	1765.074	55.399998
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=50:0,R4=H)	4.091809	0	1606.91	51.2
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:4)	7.657399	0	2014.243	96

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
CL (2R1CO2H+2R2CO2H=85:0)	4.277943	0	1669.288	113.4
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:5)	8.219484	0	1850.175	171.65001
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:0)	3.046821	0	1739.148	146
DG (R1CO2H+R2CO2H=28:0)	14.88879	6.565533	534.4237	88.94116
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=48:0)	3.644238	0	1254.77	45.400005
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=75:2)	8.029517	0	1805.191	110.50001
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:4)	7.352894	0	2042.27	125.70001
PG (R1CO2H+R2CO2H=38:0)	12.05589	0	823.6306	389.80002
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:1)	8.745324	0	1933.271	171.84999
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=55:0,R4=H)	3.564233	0	1650.05	75.1
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:3)	8.46626	0	1784.121	74.4
DG (R1CO2H+R2CO2H=32:2)	10.50512	4.595548	581.5024	111.66952
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:6)	3.94488	0	1848.161	112.19999
PIM2 (R1CO2H+R2CO2H=35:4)	7.471802	0	1185.641	45.099995
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:0)	7.645998	0	2097.341	95.75
PG (R1CO2H+R2CO2H=36:0)	9.061674	3.6891596	795.5991	324.51062
Ac2SGL (C58)	7.957583	0	1132.725	60.2
CL (2R1CO2H+2R2CO2H=78:1)	3.517577	0	1591.139	113.69999
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=48:3)	7.595659	0	1226.748	45.149998
PIM3 (R1CO2H+R2CO2H=37:2)	7.465991	0	1384.716	44.5
PI (R1CO2H+R2CO2H=31:0)	18.81716	0	818.4944	63.919994
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=75:1)	9.17167	0	1807.217	127.350006

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
PG (R1CO2H+R2CO2H=44:0)	13.20449	11.515578	907.7212	499.10413
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:2)	8.147874	0	1931.256	136.05
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:2)	3.529973	0	2045.206	111
PIM2 (R1CO2H+R2CO2H=37:1)	7.217914	0	1246.658	49.249996
MK S881	8.072594	0	904.5689	92.75
MG (RCO2H=26:0)	17.6732	7.503344	487.4577	360.79926
CL (2R1CO2H+2R2CO2H=78:0)	4.110952	0	1593.153	51.3
PG (R1CO2H+R2CO2H=38:1)	8.236313	7.179293	826.5703	53.719498
CL (2R1CO2H+2R2CO2H=85:2)	8.383302	0	1687.236	237.2
PG (R1CO2H+R2CO2H=42:0)	17.19608	3.635792	879.6924	965.81537
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:2)	3.778655	0	1769.199	248.60002
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:0)	3.976157	0	1795.125	59.7
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:6)	7.427241	0	1728.142	57.800007
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:2)	9.102418	0	1713.143	173.29999
MPM (C30)	15.08721	10.783032	702.4846	40.707573
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:6)	3.738923	0	2023.13	66
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=48:2)	3.656726	0	1228.756	70.1
PG (R1CO2H+R2CO2H=44:1)	7.031642	3.8802123	932.6428	176.87701
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:5)	3.540324	0	1730.156	46
PG (R1CO2H+R2CO2H=36:2)	15.17984	3.635151	796.5264	65.711205
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:1)	3.284928	0	1715.156	101.5
PG (R1CO2H+R2CO2H=46:0)	12.82781	7.1260595	935.7538	643.27466

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:1)	7.377428	0	1788.161	123.4
PG (R1CO2H+R2CO2H=42:1)	7.031547	0	882.6353	118.25
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=53:5,R4=H)	7.821546	0	1611.948	47.55
PG (R1CO2H+R2CO2H=37:0)	7.996279	0	809.6157	514.3
TG (R1CO2H+R2CO2H+R3CO2H=71:2)	7.019581	0	1147.047	252.55
PI (R1CO2H+R2CO2H=33:2)	7.868605	0	1663.009	61.000004
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=54:0,R4=H)	3.723957	0	1636.03	52.900005
CL (2R1CO2H+2R2CO2H=71:1)	4.14296	0	1493.03	51.099995
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:6)	3.668328	0	2051.155	82
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:1)	7.730632	0	2019.192	290.05002
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=50:2,R4=H)	3.912869	0	1418.841	58.5
PG (R1CO2H+R2CO2H=34:0)	12.50309	7.2431545	767.5679	259.56317
Ac2SGL (C60)	7.542459	0	1160.764	68.549995
Ac2SGL (C56)	8.036778	0	1104.693	50.95
PE (R1CO2H+R2CO2H=34:1)	15.35342	7.1473475	717.5282	155.57098
TG (R1CO2H+R2CO2H+R3CO2H=52:0)	9.426742	0	884.7783	999.85004
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:6)	6.815677	0	1713.034	343.44998
DG (R1CO2H+R2CO2H=36:2)	10.57825	0	637.5617	262.53333
PIM1 (R1CO2H+R2CO2H=32:2)	3.137967	0	990.5322	41.7
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:2)	4.183163	0	1861.164	107.1
CL (2R1CO2H+2R2CO2H=74:0)	7.987806	4.042585	1537.085	67.21166
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:1)	7.461141	0	2047.223	287.85

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:3)	8.631411	0	1812.151	172.65001
TG (R1CO2H+R2CO2H+R3CO2H=52:1)	9.264084	0	882.7627	941.35004
Lyso-PIM1 (RCO2H=18:0)	10.774	0	779.4105	115.100006
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=51:1,R4=H)	6.349074	0	1780.955	53.699997
PE (R1CO2H+R2CO2H=34:0)	12.25967	10.328354	741.5259	81.31866
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=50:0,R4=H)	3.974604	0	1742.02	51.7
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=57:2)	10.85859	0	1354.901	84.26667
CL (2R1CO2H+2R2CO2H=58:2)	3.34275	0	1308.806	67.6
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=48:2,R4=H)	7.604619	0	1390.809	45.149998
PIM1 (R1CO2H+R2CO2H=32:1)	8.218679	0	987.5913	63.350006
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=62:2)	3.240872	0	2082.208	81.1
Lyso-PE (R1CO2H=19:1)	7.994683	3.4753184	1008.614	31.039001
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:4)	7.904879	0	1810.14	138.65001
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=53:0,R4=H)	7.118938	0	1464.925	254.15001
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=55:2,R4=H)	8.449762	0	1646.019	174.15001
CL (2R1CO2H+2R2CO2H=63:3)	7.027293	0	1349.933	54.550003
Mycolipanolic acid (C27)	13.17797	6.884021	874.7942	576.4203
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:4)	11.21202	0	1717.064	161.26668
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:2)	7.906447	0	1805.118	318.55
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:2)	3.396338	0	2003.151	60.7
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=75:5)	3.59562	0	2285.318	60.900005
CL (2R1CO2H+2R2CO2H=74:2)	3.731881	0	1511.073	186.2

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Mbt +Fe (R=17:0)	7.200608	0	904.409	51.45
PIM3 (R1CO2H+R2CO2H=34:0)	7.479255	0	1346.696	40.600002
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=49:2)	6.550245	0	1237.821	128.14998
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:4)	7.899256	0	1689.039	98.149994
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=51:3,R4=H)	3.71298	0	1425.885	140.3
PI (R1CO2H+R2CO2H=33:0)	8.024082	0	841.5705	78.950005
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:2)	8.251514	0	2004.249	126.05001
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:1)	8.768627	0	1905.242	126.35
PI (R1CO2H+R2CO2H=30:0)	7.370153	0	799.524	111.850006
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:6)	3.646428	0	2079.19	104.19999
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:1)	3.237806	0	2215.323	73.2
DG (R1CO2H+R2CO2H=34:0)	11.89831	7.8248453	618.5174	149.46721
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=52:4,R4=H)	3.359375	0	1929.004	40.2
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:2)	7.616337	0	1898.268	347.80002
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:2)	8.023469	0	2032.279	170.05
PG (R1CO2H+R2CO2H=32:0)	12.11962	7.058953	739.5365	208.06438
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=54:1)	6.775201	0	1314.865	132.34999
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=56:2,R4=H)	7.993751	0	1502.934	89.25
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=54:4,R4=H)	4.252832	0	1627.976	51.7
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=55:4)	3.491866	0	1322.841	88.69999
DG (R1CO2H+R2CO2H=38:1)	7.776551	3.523664	672.5654	143.2113
CL (2R1CO2H+2R2CO2H=63:1)	3.82848	0	1380.903	51.900005

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
PG (R1CO2H+R2CO2H=29:1)	11.39348	7.2938843	722.4083	43.715397
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:4)	7.477975	0	1986.214	75.700005
PI (R1CO2H+R2CO2H=32:2)	4.756709	3.3204384	828.4803	24.305502
CL (2R1CO2H+2R2CO2H=71:4)	8.337012	0	1460.044	98.399994
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:3)	7.746296	0	1705.068	89.5
CL (2R1CO2H+2R2CO2H=63:0)	3.77877	0	1360.934	140.7
Mbt -Fe (R=20:0)	8.342106	0	888.5954	52.3
Mbt +Fe (R=19:0)	17.71268	0	910.4599	87.799995
Ac2PIM6 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:6)	3.150126	0	2348.335	557.2
PI (R1CO2H+R2CO2H=35:1)	18.95789	0	872.5348	49.960007
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:4)	7.626319	0	1894.229	311.6
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=55:0,R4=H)	3.94149	0	1487.993	140
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=49:4,R4=H)	4.057496	0	1422.778	51.900005
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=51:2)	7.219802	0	1265.856	139.85
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=75:2)	3.469412	0	2129.31	81.2
CL (2R1CO2H+2R2CO2H=61:4)	3.320835	0	1346.83	45.900005
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=54:3)	7.258608	0	1310.839	65.50001
PG (R1CO2H+R2CO2H=32:2)	11.38009	0	740.4587	310.4
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=55:2)	10.55223	0	1348.856	83.46667
PE (R1CO2H+R2CO2H=35:1)	13.30412	7.75786	748.5723	139.56764
PG (R1CO2H+R2CO2H=34:2)	20.53831	3.561004	790.4718	52.180164
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:0)	7.179023	0	1865.212	86.25

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Mbt +Fe (R=19:1)	16.04073	3.536784	908.4432	93.93839
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=54:1,R4=H)	8.287925	0	1660.95	58.5
PE (R1CO2H+R2CO2H=32:0)	11.26681	3.350408	691.5124	111.14276
PI (R1CO2H+R2CO2H=35:0)	15.3934	10.871913	869.6023	42.05957
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:4)	7.639718	0	1866.198	170.5
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=63:2)	7.877056	0	1777.083	97.399994
Lyso-PG (RCO2H=17:0)	3.416548	0	1018.573	43.900005
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=61:0)	4.308416	0	1591.027	43.599995
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=57:2,R4=H)	7.859653	0	1679.007	189.24998
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:1)	8.801566	0	1696.173	77.25
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=55:0,R4=H)	3.553314	0	1974.16	51.900005
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=52:1,R4=H)	3.837214	0	1610.937	51.8
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:5)	4.121563	0	1674.104	58.900005
PE (R1CO2H+R2CO2H=35:2)	15.02925	3.3198981	773.4918	207.6068
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=55:1,R4=H)	3.095172	0	1490.935	476.29996
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:5)	8.260609	0	1794.116	70.399994
CL (2R1CO2H+2R2CO2H=81:2)	9.225982	0	1631.177	128.14998
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:2)	7.581659	0	1833.142	171.89998
TG (R1CO2H+R2CO2H+R3CO2H=54:2)	4.230218	0	908.7771	1033.7
Ac2PIM6 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:4)	3.630195	0	2351.269	39.8
GMM (keto-MA,C75)	3.65725	0	1290.207	475.79996
Ac2PIM6 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:5)	3.516865	0	2313.264	40.7

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
GMM (Alpha-MA,C83)	3.294823	0	1386.332	847.2
TG (R1CO2H+R2CO2H+R3CO2H=54:3)	4.27067	0	906.7616	959.5
TG (R1CO2H+R2CO2H+R3CO2H=55:2)	4.183188	0	917.8416	1263.6
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=75:3)	3.890584	0	1943.25	70
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=52:2,R4=H)	10.88051	0	1766.019	38.899998
PG (R1CO2H+R2CO2H=35:1)	10.77781	7.427519	784.5266	53.730995
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:2)	3.902332	0	1953.147	85.69999
TG (R1CO2H+R2CO2H+R3CO2H=53:0)	4.388784	0	898.793	1056
TG (R1CO2H+R2CO2H+R3CO2H=57:2)	4.198755	0	945.868	1318.7
TG (R1CO2H+R2CO2H+R3CO2H=54:0)	8.954547	3.568485	912.8093	618.0613
PI (R1CO2H+R2CO2H=34:2)	10.22291	3.7208583	856.505	163.484
SL-III (C104)	3.284374	0	1807.422	618.9
Ac2PIM6 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:3)	3.206913	0	2396.424	72.7
TG (R1CO2H+R2CO2H+R3CO2H=55:3)	4.007728	0	915.8276	1283
TG (R1CO2H+R2CO2H+R3CO2H=58:0)	7.814331	0	968.8701	1191.05
PG (R1CO2H+R2CO2H=35:0)	7.833375	4.425589	786.5409	277.38928
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:4)	3.552781	0	2181.253	40.9
Hydroxyphthioceranic acid (C46)	3.890281	0	714.683	562.3
PI (R1CO2H+R2CO2H=35:4)	4.29163	0	861.5363	86.30001
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:0)	3.429954	0	2254.437	73.1
TG (R1CO2H+R2CO2H+R3CO2H=54:1)	8.532398	0	910.7944	862.89996
TG (R1CO2H+R2CO2H+R3CO2H=57:0)	3.645648	0	954.8544	1322.9

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2SGL (C66)	3.580563	0	1266.839	57.900005
TG (R1CO2H+R2CO2H+R3CO2H=58:1)	7.592837	0	966.8546	1093.5502
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=46:0)	3.488103	0	1226.736	48.900005
TG (R1CO2H+R2CO2H+R3CO2H=53:2)	4.394796	0	889.8086	1194.7
CL (2R1CO2H+2R2CO2H=66:0)	3.425395	0	1424.961	103.30001
PE (R1CO2H+R2CO2H=36:1)	14.50871	7.802781	762.5902	108.816505
CL (2R1CO2H+2R2CO2H=88:2)	3.883628	0	1729.282	123.6
Glycosylated phthiodiolone dimycocerosate (C120)	3.399844	0	1968.679	475.5
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:5)	3.305865	0	1956.164	79.2
Phthioceranic acid (C42)	3.978831	0	642.6266	448.20004
CL (2R1CO2H+2R2CO2H=62:0)	3.429959	0	1346.918	103.30001
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:1)	3.474351	0	2209.271	41.1
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:0)	3.776701	0	1893.244	122.30001
DAT1 (C52)	3.455808	0	947.7241	1078.4
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:4)	3.748961	0	1958.181	70
TG (R1CO2H+R2CO2H+R3CO2H=52:3)	8.97606	0	878.7314	809.95
CL (2R1CO2H+2R2CO2H=82:2)	3.3788	0	1645.188	80.5
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=60:0)	3.340229	0	1598.992	80.9
CL (2R1CO2H+2R2CO2H=68:0)	3.393119	0	1453	137.6
SL-I (C125)	3.254664	0	2110.771	529.7
Phthioceranic acid (C40)	3.674784	0	636.5827	285.5
TG (R1CO2H+R2CO2H+R3CO2H=52:2)	9.349339	0	880.7471	917.74994

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=51:0,R4=H)	3.289982	0	1598.948	62.900005
PIM1 (R1CO2H+R2CO2H=33:1)	3.286329	0	1991.13	146.9
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=54:5,R4=H)	3.199842	0	1792.964	34.1
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:3)	3.578169	0	1909.164	110.30001
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=50:5,R4=H)	3.754107	0	1407.847	58.2
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=51:1,R4=H)	3.64642	0	1921.038	44.3
MPM (C32)	6.5981	0	730.5158	128.75
GMM (Alpha-MA (monoenoic or monocyclopropanoic),C80)	3.52383	0	1351.26	625.5
CL (2R1CO2H+2R2CO2H=67:2)	3.412194	0	1434.945	102.80001
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:0)	3.704384	0	2083.325	625.6
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=52:0,R4=H)	3.953332	0	1775.007	58.5
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=45:0,R4=H)	3.666331	0	1347.838	59.7
MG (RCO2H=19:0)	15.01263	12.215149	394.3039	33.219437
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:2)	3.518214	0	1889.203	58.400005
TG (R1CO2H+R2CO2H+R3CO2H=46:2)	4.772519	0	796.6517	874.9001
TG (R1CO2H+R2CO2H+R3CO2H=47:2)	4.851869	0	805.712	938.4001
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=63:1)	3.512663	0	1941.144	42.7
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:4)	3.867042	0	1838.171	226.7
TG (R1CO2H+R2CO2H+R3CO2H=62:1)	6.80616	0	1022.92	673.60004
DG (R1CO2H+R2CO2H=42:2)	7.167954	3.4570622	726.6141	195.90298
TMM (methoxy-MA,C83)	3.339184	0	1571.355	1204.7
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=65:0)	3.735612	0	1642.129	71.8

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=52:1,R4=H)	4.118379	0	1794.973	42.8
PIM1 (R1CO2H+R2CO2H=34:4)	3.480569	0	1014.532	39.7
TG (R1CO2H+R2CO2H+R3CO2H=60:2)	6.795455	0	992.8716	1155.0502
SL-III (C102)	3.610308	0	1779.383	510.29996
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:3)	3.2301	0	2248.376	686.3
Keto-MA (C85)	3.250363	0	2524.545	638.6
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=52:4,R4=H)	3.321171	0	1604.896	43.599995
TG (R1CO2H+R2CO2H+R3CO2H=42:0)	12.19592	6.8096976	744.6223	177.79184
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:3)	3.654344	0	1937.2	222.60002
TG (R1CO2H+R2CO2H+R3CO2H=60:0)	3.779692	0	996.9011	1389
GMM (Alpha-MA (monoenoic or monocyclopropanoic),C93)	3.556339	0	1533.452	1205
PIM1 (R1CO2H+R2CO2H=37:3)	10.00744	0	1080.578	60.666668
PIM1 (R1CO2H+R2CO2H=33:2)	3.531342	0	1004.545	45.5
TG (R1CO2H+R2CO2H+R3CO2H=60:1)	7.683955	0	994.8857	1088.15
TG (R1CO2H+R2CO2H+R3CO2H=55:0)	3.926402	0	926.828	1270.3
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:1)	4.386188	0	1724.205	111.69999
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=52:1,R4=H)	3.084623	0	1935.044	40.8
DG (R1CO2H+R2CO2H=44:2)	4.557253	0	754.6467	479.9
Methoxy-MA (C86)	10.09904	0	1311.273	1387.4332
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=48:0,R4=H)	4.157999	0	1556.89	57.3
PIM1 (R1CO2H+R2CO2H=35:0)	6.718097	0	1036.603	52.850002
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=56:1,R4=H)	7.297285	0	1829.054	44.25

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=56:1,R4=H)	3.769762	0	1991.106	43.099995
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:4)	3.559802	0	1759.113	102.9
TG (R1CO2H+R2CO2H+R3CO2H=56:1)	8.12174	0	938.8253	1100.9501
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=73:1)	3.329627	0	1941.232	80.2
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=53:5,R4=H)	3.980565	0	1476.832	42.3
CL (2R1CO2H+2R2CO2H=61:3)	3.478406	0	1326.859	49.900005
Mbt -Fe (R=17:0)	3.520976	0	1681.024	517.7
PIM4 (R1CO2H+R2CO2H=31:0)	3.725785	0	1466.703	39.8
TG (R1CO2H+R2CO2H+R3CO2H=50:0)	9.494863	7.1225314	856.7472	466.5055
PIM2 (R1CO2H+R2CO2H=37:0)	3.729502	0	1226.69	43
TG (R1CO2H+R2CO2H+R3CO2H=56:0)	12.60566	0	940.8405	1038.9
CL (2R1CO2H+2R2CO2H=72:3)	3.627993	0	1481.034	167.5
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=55:2,R4=H)	3.168033	0	1510.9	62.5
Methoxy-MA (C88)	10.0866	0	1339.304	1419.9666
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=74:4)	3.521155	0	2089.279	41.4
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:6)	7.24248	0	1700.11	325.50003
PI (R1CO2H+R2CO2H=32:1)	7.202376	0	830.49	52.149998
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:0)	3.329604	0	1901.197	79.2
CL (2R1CO2H+2R2CO2H=61:1)	3.668887	0	1330.893	139.3
TG (R1CO2H+R2CO2H+R3CO2H=50:2)	8.542331	0	852.7141	964.39996
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=56:3,R4=H)	3.379247	0	1847.016	85.80001
CL (2R1CO2H+2R2CO2H=78:3)	3.960621	0	1565.131	49.599995

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
TG (R1CO2H+R2CO2H+R3CO2H=49:1)	4.919276	0	835.7589	1109.3
TDM (C191H368O16)	3.497669	0	2940.769	1389.5
DG (R1CO2H+R2CO2H=32:1)	12.01319	3.8610454	588.4704	168.64952
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:2)	3.483744	0	1813.07	41.9
TG (R1CO2H+R2CO2H+R3CO2H=48:1)	7.898981	0	826.6998	795.74994
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=62:0)	3.727106	0	1767.1	58.3
TG (R1CO2H+R2CO2H+R3CO2H=49:2)	4.683694	3.4931977	838.6987	522.93604
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=54:2,R4=H)	3.643196	0	1636.958	64.8
TG (R1CO2H+R2CO2H+R3CO2H=48:0)	4.83076	0	828.7165	1010.8
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:5)	3.347956	0	1998.202	50.400005
TG (R1CO2H+R2CO2H+R3CO2H=56:3)	4.773822	0	934.7941	958.7
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=52:2,R4=H)	3.296527	0	1608.929	63.7
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=55:4,R4=H)	3.886687	0	1809.001	43.2
PG (R1CO2H+R2CO2H=37:1)	7.78705	3.4851532	812.5556	46.656998
PIM2 (R1CO2H+R2CO2H=31:0)	3.665814	0	2263.219	40.4
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=71:2)	3.141164	0	2046.296	120.30001
TG (R1CO2H+R2CO2H+R3CO2H=56:2)	4.889888	0	936.8097	1033.6
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:2)	3.473358	0	1814.179	80.2
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:1)	3.768272	0	1885.163	42.6
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=57:4,R4=H)	3.744294	0	1832.076	49.900005
TG (R1CO2H+R2CO2H+R3CO2H=69:0)	3.239121	0	1118.095	40.9
TG (R1CO2H+R2CO2H+R3CO2H=51:3)	7.621069	3.3140807	864.7217	739.17395

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac2PIM5 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:1)	3.210059	0	2168.316	84.6
PE (R1CO2H+R2CO2H=35:0)	11.10726	7.09716	733.5596	164.18062
TG (R1CO2H+R2CO2H+R3CO2H=66:1)	3.259247	0	1078.98	1386.6
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=64:4)	3.574718	0	1809.053	48.900005
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=69:3)	3.352495	0	1854.193	122.6
CL (2R1CO2H+2R2CO2H=80:1)	3.886793	0	1597.182	232.39998
Alpha-MA (C84)	3.560669	0	1238.295	491.6
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=49:1)	3.484283	0	1266.77	71.3
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=60:0)	3.440962	0	1901.107	41.4
CL (2R1CO2H+2R2CO2H=79:0)	3.502724	0	1585.188	103.30001
PAT (C136H260O20)	3.643934	0	2230.954	572.6
Lyso-PIM6 (RCO2H=18:1)	3.909586	0	1614.596	458.9
DG (R1CO2H+R2CO2H=35:1)	7.891511	0	630.5172	191.5
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:1)	3.475529	0	1983.203	110.1
TG (R1CO2H+R2CO2H+R3CO2H=44:0)	8.675286	6.9139	772.6519	402.0165
Mycolipanolic acid (C26)	9.164747	0	846.7629	923.7
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:5)	3.793487	0	1989.155	42.3
TG (R1CO2H+R2CO2H+R3CO2H=51:0)	7.327856	3.3681433	870.7627	667.80865
CL (2R1CO2H+2R2CO2H=80:3)	3.971522	0	1593.162	58.2
TDM (C160H310O15)	3.285999	0	2489.366	40.5
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=53:0,R4=H)	3.424191	0	1622.025	101.5
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:0)	3.150147	0	1767.176	531

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=57:3,R4=H)	4.164688	0	1698.964	86.6
TG (R1CO2H+R2CO2H+R3CO2H=64:2)	3.493658	0	1048.933	1307.9
Mycolipanolic acid (C24)	8.437789	0	790.701	554.39996
Ac2SGL (C53)	3.740537	0	1057.699	145.9
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=56:2,R4=H)	3.581482	0	1822.087	58.099995
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:0)	3.419392	0	2008.288	58.8
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:0)	3.409659	0	1818.193	148.9
Alpha-MA (C80)	3.375057	0	2352.4	72.7
Hydroxyphthioceranic acid (C31)	16.56227	0	986.9169	1133.9
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:6)	4.064939	0	1677.045	58.400005
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=67:1)	3.727731	0	1673.11	266.7
Ac2PIM3 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=66:1)	4.04533	0	1816.194	70.3
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=72:3)	3.627458	0	2058.304	217.3
Ac2PIM4 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=70:3)	3.729425	0	2030.268	159.6
TG (R1CO2H+R2CO2H+R3CO2H=57:1)	3.630229	0	947.8911	1121.8
MPM (C33)	3.604118	0	744.5271	63
DG (R1CO2H+R2CO2H=35:2)	7.111024	3.6668327	628.5069	108.39168
DG (R1CO2H+R2CO2H=41:2)	3.651759	0	712.5951	175.7
PI (R1CO2H+R2CO2H=35:2)	7.386049	0	865.5713	69.15
Lyso-PE (R1CO2H=16:0)	11.6859	3.3010566	928.554	33.276005
Mbt -Fe (R=19:0)	3.244578	0	857.5485	170.6
Mycocerosic acid (C32)	3.547119	0	502.4706	142.3

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
CL (2R1CO2H+2R2CO2H=63:4)	3.305901	0	1347.924	34.3
DG (R1CO2H+R2CO2H=39:2)	7.383705	0	684.5645	282.65
SL-II or SL-II' (C156)	3.20945	0	2561.25	46.7
PIM1 (R1CO2H+R2CO2H=35:2)	9.962211	0	1032.576	51.033333
MK-8 (H2)	10.52193	0	718.5714	61.26667
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=49:3,R4=H)	3.326207	0	1888.975	41.8
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=45:0,R4=H)	6.983938	0	1860.943	41.400005
Mbt +Fe (R=20:1)	8.855244	0	922.4583	82.049995
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=50:1,R4=H)	3.464121	0	1420.852	162.1
DG (R1CO2H+R2CO2H=29:0)	10.0341	3.8832362	543.4886	92.47701
TG (R1CO2H+R2CO2H+R3CO2H=45:2)	3.600019	0	777.6869	2042.4
Mycolipenic acid (C29)	7.164625	4.2649674	480.3897	62.171997
Ac1PIM1 (R1CO2H+R2CO2H+R3CO2H=47:2)	2.943952	0	1214.748	59
MG (RCO2H=22:0)	12.41762	3.0507548	436.3509	55.850742
TG (R1CO2H+R2CO2H+R3CO2H=61:1)	3.443042	0	1003.951	1122.5
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=57:3,R4=H)	3.530088	0	1536.92	51
MG (RCO2H=15:1)	10.64695	0	331.2726	66.73332
Hydroxyphthioceranic acid (C33)	6.682408	0	554.463	48.3
TG (R1CO2H+R2CO2H+R3CO2H=59:2)	3.615836	0	978.861	1128.8
SL-I (C156)	3.146306	0	2572.191	42.4
Mbt +Fe (R=18:0)	7.709443	0	896.4429	73.799995
CL (2R1CO2H+2R2CO2H=70:3)	3.116046	0	1453.002	37.8

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
PG (R1CO2H+R2CO2H=41:1)	3.345339	0	868.6212	149.9
Mycolipenic acid (C27)	3.794726	0	430.3776	93.69999
Lyso-PIM4 (RCO2H=19:0)	3.624385	0	1279.58	46.3
MG (RCO2H=16:1)	6.826003	0	350.245	51.8
SL-I (C157)	3.26646	0	2564.239	46
MG (RCO2H=20:1)	11.66597	4.1946363	406.306	54.309002
Mbt +Fe (R=20:0)	9.003401	3.714089	924.474	59.123337
Lyso-PG (RCO2H=18:1)	3.589274	0	1042.57	45
DG (R1CO2H+R2CO2H=34:2)	8.424379	3.882378	614.4857	133.07
TG (R1CO2H+R2CO2H+R3CO2H=64:1)	3.244596	0	1050.949	1144.2
Lyso-PIM2 (RCO2H=19:0)	3.157315	0	982.4112	150.5
Ac1PIM5 (R1CO2H+R2CO2H+R3CO2H=46:1,R4=H)	3.389607	0	1872.936	48.599995
DG (R1CO2H+R2CO2H=34:1)	8.954279	3.5516694	616.5012	163.98132
Lyso-PIM1 (RCO2H=17:0)	7.01443	0	765.393	151.65001
MPM (C31)	10.51486	7.4834304	716.4951	78.886185
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=50:3,R4=H)	3.393707	0	1600.863	41.2
Lyso-PE (R1CO2H=17:0)	7.018779	0	956.5846	45.7
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=50:0,R4=H)	3.192769	0	1422.87	151.1
Ac2PIM2 (R1CO2H+R2CO2H+R3CO2H+R4CO2H=68:1)	3.308969	0	1687.128	78.5
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=56:3,R4=H)	3.392981	0	1495.962	35.8
TMM (Alpha-MA,C82)	3.335888	0	1534.369	719.8
PGL-tb (C108)	3.461831	0	1807.462	868.9001

Table 8 : *Mtb* lipids upregulated in Middlebrook 7H9 culture medium

Compound	Culture	Infection	Mass	Retention Time
MK-9	4.027456	0	784.6165	621
CL (2R1CO2H+2R2CO2H=77:3)	3.71479	0	1573.094	66.5
PE (R1CO2H+R2CO2H=30:1)	3.597482	0	661.4695	47.099995
DAT2 (C59)	3.539955	0	1066.782	50
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=54:3,R4=H)	3.268116	0	1472.896	38.5
Lyso-PI (RCO2H=19:1)	3.563266	0	629.3546	83.9
DG (R1CO2H+R2CO2H=33:1)	7.421041	3.8499198	602.4856	81.820335
Ac1PIM3 (R1CO2H+R2CO2H+R3CO2H=51:1,R4=H)	3.246218	0	1591.963	38.2
DP-P	3.346473	0	800.5811	328.9
Mycocerosic or Phthioceranic acid (C33)	3.217028	0	538.4719	156.4
Lyso-PG (RCO2H=15:1)	3.480636	0	490.2307	39.8
Lyso-PE (R1CO2H=17:1)	3.343736	0	952.5485	51.099995
Lyso-PI (RCO2H=18:0)	3.289297	0	617.3538	33.7
Mbt +Fe (R=17:1)	4.931845	0	880.4118	68.4
Ac2SGL (C55)	3.40374	0	1090.676	48.7

Table 9 *: Mtb* lipids upregulated in infected mice lungs				
Compound	**Culture**	**Infection**	**Mass**	**Retention Time**
DP	10.898151	18.357443	715.6621	108.54089
PI (R1CO2H+R2CO2H=36:2)	15.062289	21.067333	884.5375	32.733994
Hydroxyphthioceranic acid (C36)	3.2582123	11.007929	596.5135	62.599747
Hydroxyphthioceranic acid (C37)	3.3343165	7.3919644	588.5474	42.249676
PE (R1CO2H+R2CO2H=30:0)	3.1633868	10.836023	680.5137	34.8295
Hydroxyphthioceranic acid (C34)	4.537926	7.1729517	568.4821	37.082664
PI (R1CO2H+R2CO2H=34:0)	6.768061	17.205696	860.5364	22.249165
MG (RCO2H=24:0)	15.277082	18.70645	464.3826	53.925125
Lyso-PE (R1CO2H=20:0)	6.9095254	11.114929	526.3771	22.707201
PE (R1CO2H+R2CO2H=37:0)	3.7070694	11.200405	783.5726	39.12799
PG (R1CO2H+R2CO2H=31:0)	18.650696	21.782614	730.4781	40.614185
TMM (methoxy-MA,C93)	7.1008244	10.693952	1711.503	352.9896
PG (R1CO2H+R2CO2H=31:2)	13.110202	20.389347	1430.911	17.04963
Mycosanoic or Mycocersoci acid (C26)	3.5959687	7.8312693	418.3795	44.3
PG (R1CO2H+R2CO2H=39:1)	3.5230064	6.542798	840.583	146.27298
Phthioceranic acid (C37)	3.3209453	11.228821	594.5309	29.660246
MG (RCO2H=18:1)	14.4899645	20.47865	378.2727	51.84499
Mbt -Fe (R=18:0)	8.913935	14.120067	860.5638	15.792498
MG (RCO2H=16:0)	22.167057	27.442219	682.5352	36.7422
Lyso-PE (R1CO2H=18:1)	3.7938066	11.886383	496.326	10.491749
PE (R1CO2H+R2CO2H=36:2)	7.4173613	14.5982065	743.5498	34.487663
MG (RCO2H=14:0)	11.095751	20.4281	626.4744	19.371874

Table 9 *: Mtb* lipids upregulated in infected mice lungs				
Compound	**Culture**	**Infection**	**Mass**	**Retention Time**
DG (R1CO2H+R2CO2H=36:1)	3.4435668	10.869938	644.5328	62.544765
DG (R1CO2H+R2CO2H=33:2)	2.973079	7.363146	600.4759	71.89333
Lyso-PE (R1CO2H=19:0)	15.446181	20.331121	512.359	22.51211
PG (R1CO2H+R2CO2H=32:1)	4.066819	7.6511407	764.4617	22.901335
Mycosanoic or Mycocerosic acid (C25)	11.654317	23.516537	426.3429	59.90375
Mycocerosic acid (C28)	3.5303771	10.721376	441.4558	26.92399
PG (R1CO2H+R2CO2H=33:0)	4.0184216	21.965448	758.5092	105.56886
DG (R1CO2H+R2CO2H=47:0)	3.96807	7.088621	795.7674	105.22299
PE (R1CO2H+R2CO2H=37:1)	3.869491	18.974707	776.6027	24.80783
PE (R1CO2H+R2CO2H=39:0)	3.0955641	11.191116	806.6536	36.535004
TG (R1CO2H+R2CO2H+R3CO2H=47:0)	4.7350087	11.151707	809.7435	275.83078
TG (R1CO2H+R2CO2H+R3CO2H=47:1)	3.9881554	7.019514	812.69	386.0127
DG (R1CO2H+R2CO2H=40:2)	4.750948	7.849866	698.5847	160.9367
TG (R1CO2H+R2CO2H+R3CO2H=45:0)	7.733519	19.091167	781.7138	159.21162
Phthioceranic acid (C48)	4.1272073	7.45165	1431.458	328.2984
PG (R1CO2H+R2CO2H=45:0)	4.191921	15.02708	921.7384	256.40283
PGL-tb (C105)	3.685157	7.5852633	1760.461	188.73737
Mycolipanolic acid (C28)	8.7171955	12.936813	462.4071	427.99414
TG (R1CO2H+R2CO2H+R3CO2H=49:0)	4.4767466	7.098328	842.7315	355.58072
TG (R1CO2H+R2CO2H+R3CO2H=46:0)	4.7507496	7.231092	800.6854	304.24338
DIMB (C91)	3.788059	8.026406	1368.392	334.97934
PG (R1CO2H+R2CO2H=33:1)	7.267164	10.6502905	756.4912	33.500404
Mycocerosic acid (C35)	3.6296408	7.4147816	566.5036	64.154686
Mycocerosic acid (C29)	3.9273686	7.3166547	460.4241	37.024334

Table 9 *: Mtb* lipids upregulated in infected mice lungs				
Compound	**Culture**	**Infection**	**Mass**	**Retention Time**
PE (R1CO2H+R2CO2H=33:2)	3.5225823	10.854535	701.503	55.156994
Mycocerosic acid (C30)	3.6535788	10.285045	474.4394	34.422245
PE (R1CO2H+R2CO2H=37:2)	3.26661	10.706936	757.5644	17.231005
DG (R1CO2H+R2CO2H=43:1)	4.140826	19.564018	737.6895	54.578667
PE (R1CO2H+R2CO2H=32:2)	7.0712566	10.070138	704.5073	50.3764
DG (R1CO2H+R2CO2H=45:1)	3.1912205	18.29477	765.7198	16.110664
PE (R1CO2H+R2CO2H=34:2)	3.7578878	11.240248	715.5129	22.511002
PIM4 (R1CO2H+R2CO2H=37:0)	3.8345098	7.351711	1572.777	14.283666
TG (R1CO2H+R2CO2H+R3CO2H=48:3)	3.2943094	15.96558	817.7138	410.5505
Ac2SGL (C69)	0	3.3556437	1281.939	7.587
Mycosanoic or Mycocerosic acid (C24)	0	3.1876173	390.3482	0.707
Ac1PIM2 (R1CO2H+R2CO2H+R3CO2H=47:2,R4=H)	0	3.3322332	1371.837	9.149
Lyso-PI (RCO2H=18:1)	0	17.6226	620.2959	0.91074985
DG (R1CO2H+R2CO2H=42:0)	0	6.8394437	725.6879	3.7974997
DG (R1CO2H+R2CO2H=41:0)	0	15.613461	711.6739	4.92575
Phthioceranic acid (C39)	0	14.4233885	622.5653	8.17525
DG (R1CO2H+R2CO2H=45:0)	0	15.03894	767.7359	7.3159995
PIM1 (R1CO2H+R2CO2H=36:0)	0	3.5625758	1072.609	0.586
SL-III (C113)	0	3.4950721	1933.558	17.247
CL (2R1CO2H+2R2CO2H=72:0)	0	3.6818147	1487.073	5.078
CL (2R1CO2H+2R2CO2H=82:3)	0	11.36636	1621.197	0.7186666
DG (R1CO2H+R2CO2H=43:0)	0	19.93831	739.7042	5.9068007
DG (R1CO2H+R2CO2H=33:0)	0	7.2278028	604.5059	1.1485001
PE (R1CO2H+R2CO2H=39:1)	0	3.8123038	804.6379	8.115

Table 9 *: Mtb* lipids upregulated in infected mice lungs				
Compound	**Culture**	**Infection**	**Mass**	**Retention Time**
PG (R1CO2H+R2CO2H=45:1)	0	6.4357157	924.6793	2.7909997
DIMB (C97)	0	7.7000575	1457.444	14.176499
PG (R1CO2H+R2CO2H=36:1)	0	3.416105	798.5358	1.364
Mycolipenic acid (C25)	3.5255895	15.708979	402.3463	19.278997
GMM (methoxy-MA,C94)	0	3.9426765	1563.469	13.711001
Phthioceranic acid (C36)	0	7.564459	558.5372	5.94
TG (R1CO2H+R2CO2H+R3CO2H=45:1)	0	7.8629727	779.6986	9.692
CL (2R1CO2H+2R2CO2H=74:1)	0	3.7457986	1513.089	5.143
GMM (methoxy-MA,C92)	0	3.6743164	1530.477	16.173
TG (R1CO2H+R2CO2H+R3CO2H=44:1)	0	11.517232	765.6812	10.296667
TG (R1CO2H+R2CO2H+R3CO2H=47:3)	0	3.8207219	803.6973	4.281
Hydroxyphthioceranic acid (C45)	0	6.9850974	722.6527	2.985
Lyso-PG (RCO2H=16:0)	0	7.0300193	990.5468	0.572
DIMA (C98)	0	4.0189342	1473.477	12.84
DG (R1CO2H+R2CO2H=44:1)	0	3.7902405	751.7066	8.023
Hydroxyphthioceranic acid (C42)	0	10.049769	680.6039	9.1293335
DIMA (C96)	0	3.6519873	1440.487	15.402
TG (R1CO2H+R2CO2H+R3CO2H=55:1)	0	3.618949	919.8559	19.372
Hydroxyphthioceranic acid (C40)	0	3.9964454	625.6373	7.365
DG (R1CO2H+R2CO2H=35:0)	0	7.5109854	627.5827	6.008
PI (R1CO2H+R2CO2H=37:3)	0	7.860017	896.5385	0.75600004
TG (R1CO2H+R2CO2H+R3CO2H=53:1)	0	3.493	896.7772	11.166
PE (R1CO2H+R2CO2H=40:0)	0	3.41119	803.6422	2.342
DG (R1CO2H+R2CO2H=39:0)	0	11.2055	688.5971	3.2136667

Table 9 *: Mtb* lipids upregulated in infected mice lungs				
Compound	**Culture**	**Infection**	**Mass**	**Retention Time**
Lyso-PIM1 (RCO2H=19:0)	0	10.858263	820.3613	0.5293333
PIM2 (R1CO2H+R2CO2H=35:1)	0	6.325038	1218.628	0.799
Glycosylated phthiodiolone dimycocerosate (C115)	0	3.1435726	1903.551	18.353
DG (R1CO2H+R2CO2H=51:0)	0	3.7934072	856.7897	17.524
Lyso-PIM1 (RCO2H=18:1)	0	3.2083445	804.3299	0.511
TG (R1CO2H+R2CO2H+R3CO2H=68:1)	0	3.1600535	1102.058	17.673
DIMA (C95)	0	3.6207168	1431.434	14.172
PGL-tb (C110)	0	3.5497355	1835.48	17.178
PE (R1CO2H+R2CO2H=38:1)	0	3.3590918	790.6239	4.713
GMM (Alpha-MA (monoenoic or monocyclopropanoic),C89)	0	3.0394893	1477.401	14.169
Methoxy-MA (C91)	0	3.5478175	1354.416	12.037
DIMA (C92)	0	3.5109339	1384.426	12.457
TG (R1CO2H+R2CO2H+R3CO2H=49:3)	0	3.9566283	831.7286	15.382
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=52:4,R4=H)	0	3.0674825	1766.957	37.858
DG (R1CO2H+R2CO2H=37:0)	0	3.373651	660.5699	1.663
Phthioceranic acid (C45)	0	3.839469	706.6591	10.428
DG (R1CO2H+R2CO2H=53:0)	0	3.847838	879.8647	12.735
DG (R1CO2H+R2CO2H=47:1)	0	3.7621922	798.7097	7.926
TMM (keto-MA,C94)	0	3.431935	1723.508	12.799
Glycosylated phthiodiolone dimycocerosate (C109)	0	3.2480094	1814.5	13.207

4.4.3 Differential TNF-α and IL-1β production by stimulating RAW264.7 cells by Mtb clinical isolate or CIDFL

To understand inflammatory response induced by live *Mtb* bacilli or free lipids separated as a polar and apolar, RAW264.7 cells were infected with Beijing, LAM-6, EAI-5, H37Rv and BCG or stimulated with polar and apolar CIDFL from the same strains respectively. Supernatant collected post-treatment were analysed for pro-inflammatory cytokines TNF-α and IL-1β. The results obtained are shown in figure 33. Differential secretions of pro-inflammatory cytokines were observed in the RAW264.7 cells infected with live strain or treated with CIDFL from all respective *Mtb* strains. *Mtb* infected cells induced higher IL-1β and lower TNF-α as compared to the cells treated with CIDFL, respectively. RAW264.7 cells infected with live BCG and EAI-5 or treated with respective CIDFL stimulated higher secretion of TNF-α and IL-1β as compared to a cell infected or treated with H37Rv or LAM-6 strain or CIDFL respectively. RAW264.7 cells treated with polar CIDFL stimulated higher levels of TNF-α and IL-1β as compared to apolar CIDFL from all the *Mtb* strains. Polar CIDFL of H37Rv and LAM-6 induced lower levels of the TNF-α and IL-1β cytokines compared to other strains. RAW264.7 cells infected with Beijing strain-induced lesser cytokine stimulation with respect to other strains while Beijing polar CIDFL treated RAW264.7 cells stimulate TNF-α and IL-1β secretion as compared with cells treated with polar CIDFL from rest of the *Mtb* strains.

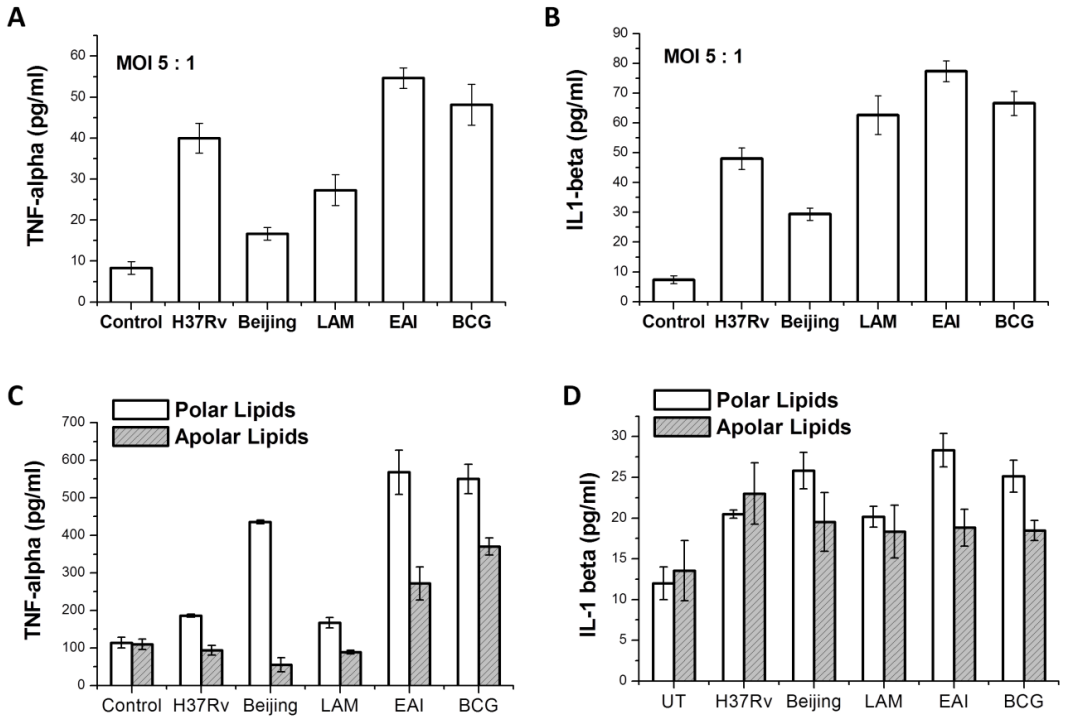


Figure 33: Evaluation of culture supernatant for TNF-α and IL-1β generated by stimulating RAW264.7 cells by infecting with individual clinical isolate and CIDFL, respectively. *To determine the immunogenicity of the live bacteria vs extracted lipids, RAW264.7 cells were infected and treated with lipids derived from them separately. It was observed that clinical isolates differentially induce TNF-α (Fig. 33A) and IL-1β (Fig. 33B) production after infection compared to uninfected control. Further extracted polar lipid fraction shows a higher stimulation of RAW264.7 cells than an apolar fraction from all the strains (Fig. 33C and 33D). The cytokines were measured, and the results were expressed as the mean ratio ± S.D (N=3).*

4.4.4 CD1d putative lipid analysis of Mtb clinical isolates grown in Middlebrook 7H9 culture medium and from infected mice lungs, respectively.

LC-MS was performed on the total lipids isolated from *Mtb* clinical isolates grown in Middlebrook 7H9 culture medium and infected mice lungs, respectively. The lipids were identified against a modified *Mtb* database which determined only CD1d putative lipids in the samples. Mass profiler software generated a comparative heat map of these lipids across various *Mtb* strains grown in culture and infected mice

lung. Heat-map enlisted a relative abundance of lipids across various experimental groups. The comparison was made between each strain grown in culture medium and in the infected mice lung as well as between the strains based on the lipid expression, as shown in figure 34. We observed that CD1d putative lipids were down-regulated by clinical isolates in their order of descending virulence (Beijing > LAM-6 > H37Rv > EAI-5 > BCG) in the infected mice lungs as compared to those expressed in the culture medium. The number of diverse CD1d lipid moieties (PI, PG and Ac-PIM_4) were analysed, and it was found that they were increased by Beijing strain in the culture medium as compared to lipids from Beijing strain-infected mice lung. We observed an increase of PIM_4 in LAM-6, H37Rv, EAI-5 and BCG but not in Beijing infected mice lung as compared to these strains growing in the culture medium. We also observed that numbers of diverse PG moieties were decreased mostly in Beijing, followed by EAI-5 and BCG infected mice lungs as compared to the same strains growing in the culture medium, respectively. No significant change in number was seen in H37Rv and LAM-6 infected mice lungs as compared to these strains grown in the culture medium. We also observed that numbers of diverse PI moieties were decreased in mostly in Beijing and followed by H37Rv and LAM-6 infected mice lungs as compared to the same strains growing in culture medium respectively. No significant change in number was seen in BCG and EAI-5 infected mice lungs as compared to these strains grown in the culture medium. Table 10 and 11 shows CD1d putative lipids from *Mtb* grown in Middlebrook culture 7H9 medium and mice

infected lungs respectively.

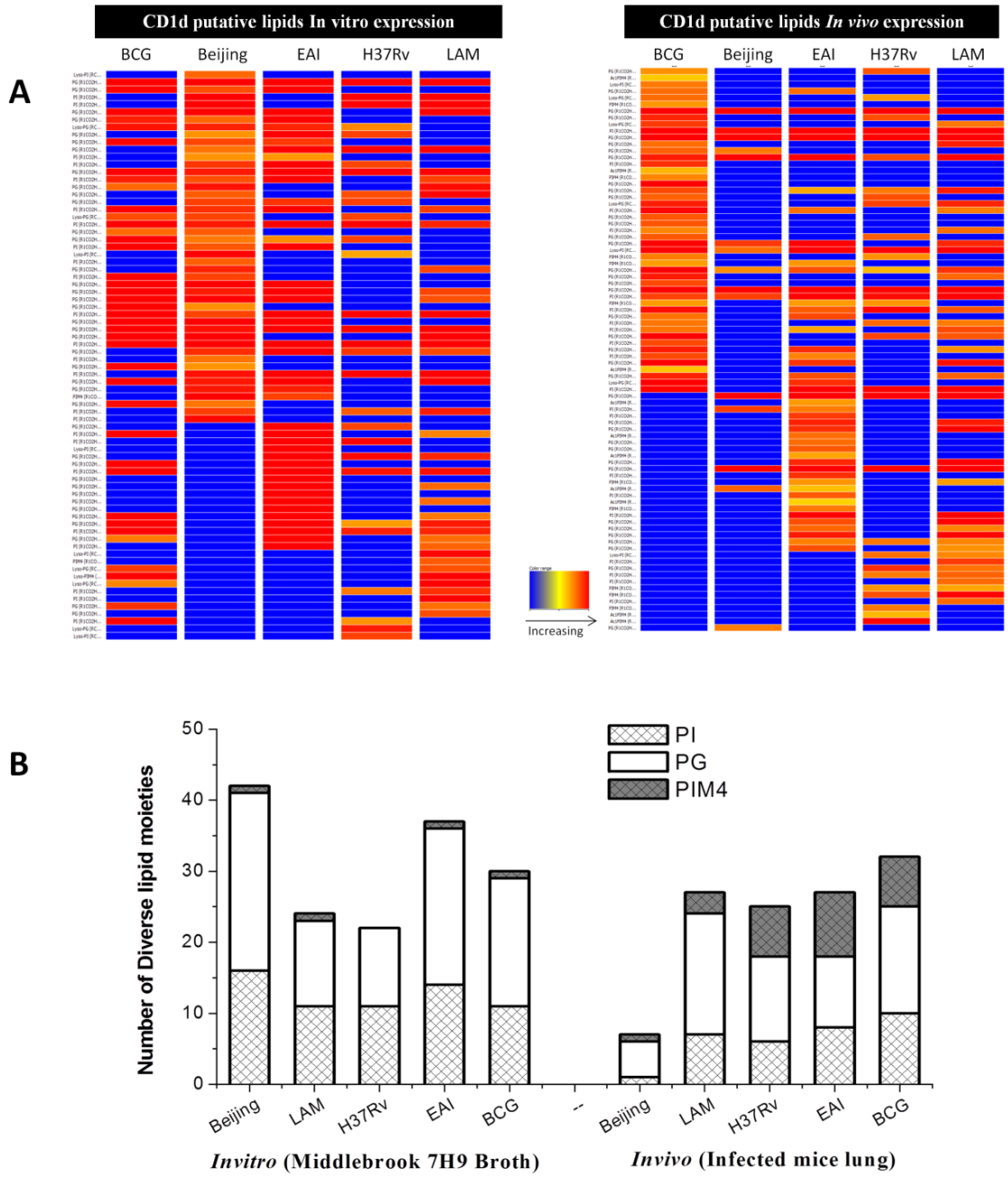


Figure 34: CD1d putative lipid analysis of the Mtb clinical isolates grown in Middlebrook 7H9 culture medium and infected mice lungs, respectively. *Fig. 34A represents a heat map generated by mass profiler software (MPP) from LC-MS data of CD1d putative lipids from Mtb strains grown in Middlebrook 7H9 culture medium and infected mice lungs. Blue to red shades indicates the relative abundance of lipids in increasing order, respectively. Fig. 34B represents a number of diverse moieties of PI, PG and PIM$_4$.*

Table 10: CD1d putative lipids from *Mtb* clinical isolates grown in Middlebrook 7H9 culture medium

Compound	BCG	Beijing	EAI	H37Rv	LAM	Mass	Retention Time
Lyso-PI (RCO2H=16:0)	0	15.725846	0	0	0	589.3082	0.523
PG (R1CO2H+R2CO2H=33:2)	20.331793	21.965837	21.932364	19.219933	21.427038	749.5211	2.5438
PG (R1CO2H+R2CO2H=32:0)	18.958426	16.29516	20.574026	0	0	739.5361	1.057
PI (R1CO2H+R2CO2H=36:2)	0	18.239542	0	18.88303	18.289862	884.5417	0.80100006
PI (R1CO2H+R2CO2H=35:2)	0	18.26558	0	18.32021	18.470314	865.5714	1.3440001
PG (R1CO2H+R2CO2H=31:0)	19.24098	19.011198	20.079268	0	19.476389	725.5185	1.3107499
PG (R1CO2H+R2CO2H=30:0)	17.459991	15.63518	21.196302	0	0	716.4604	1.084
Lyso-PG (RCO2H=18:0)	17.708141	17.334997	17.276562	14.885506	0	1046.6005	1.40175
PG (R1CO2H+R2CO2H=45:0)	0	14.591756	18.929453	16.592136	0	948.6757	2.152
PG (R1CO2H+R2CO2H=33:1)	19.58658	16.45959	16.80787	0	0	756.4912	1.3633333
PG (R1CO2H+R2CO2H=29:1)	0	15.3590975	18.096437	18.137997	19.410202	722.4093	1.0645
PI (R1CO2H+R2CO2H=34:3)	0	14.618156	14.53065	0	0	876.4721	3.2839997
PI (R1CO2H+R2CO2H=32:2)	0	16.853687	23.81085	16.45738	0	828.4803	1.0183332
PG (R1CO2H+R2CO2H=35:2)	19.882845	17.534113	21.086285	18.805264	18.31107	782.5081	1.1754
PI (R1CO2H+R2CO2H=37:0)	17.411983	16.216537	19.407682	0	16.578402	924.5701	3.004
PG (R1CO2H+R2CO2H=34:1)	15.67593	17.7364	0	0	17.872698	770.504	2.1993332
PG (R1CO2H+R2CO2H=35:1)	0	15.925345	0	16.269657	18.027761	806.5024	1.6340001
PG (R1CO2H+R2CO2H=43:1)	0	16.595665	16.94695	16.663502	0	918.6319	3.8259997
PI (R1CO2H+R2CO2H=36:1)	22.236443	16.966055	22.369232	0	16.71635	881.5962	1.4167501
Lyso-PG (RCO2H=19:1)	16.331547	16.52295	0	16.560751	0	546.2933	0.7570001
PI (R1CO2H+R2CO2H=35:1)	22.763678	17.565802	22.707468	18.515665	17.324682	867.5813	1.1204
PG (R1CO2H+R2CO2H=45:1)	15.611918	16.008165	0	0	0	946.666	4.9585
PG (R1CO2H+R2CO2H=39:0)	19.388527	15.228593	14.732007	16.57236	0	864.587	2.011

Table 10: CD1d putative lipids from *Mtb* clinical isolates grown in Middlebrook 7H9 culture medium

Compound	BCG	Beijing	EAI	H37Rv	LAM	Mass	Retention Time
PI (R1CO2H+R2CO2H=31:0)	21.124662	16.08949	22.459122	0	0	818.4944	0.84166664
Lyso-PI (RCO2H=18:1)	0	18.312674	0	14.204188	0	615.3356	0.606
PI (R1CO2H+R2CO2H=37:3)	0	15.904682	0	0	0	918.5203	0.654
PG (R1CO2H+R2CO2H=35:0)	0	17.29944	0	0	16.480505	786.5413	0.895
PI (R1CO2H+R2CO2H=32:1)	19.910633	16.558361	0	0	0	825.5346	0.8665001
PG (R1CO2H+R2CO2H=32:2)	20.903688	18.994589	21.166374	0	0	735.5025	1.353
PG (R1CO2H+R2CO2H=38:1)	20.554035	17.49737	19.13916	0	16.49118	826.5662	2.0270002
PG (R1CO2H+R2CO2H=42:0)	21.24706	18.096413	21.673073	0	16.211166	884.6504	1.3645
PG (R1CO2H+R2CO2H=36:1)	19.081715	14.730311	0	0	0	820.5209	1.1325
PI (R1CO2H+R2CO2H=36:0)	18.304283	16.496744	19.593758	19.7381	19.047318	910.5492	1.3933998
PG (R1CO2H+R2CO2H=31:2)	20.518751	21.59643	21.167091	0	0	726.4483	1.1096668
PG (R1CO2H+R2CO2H=34:2)	20.658281	17.73477	22.88038	18.2717	18.31107	790.4718	0.87419987
PG (R1CO2H+R2CO2H=43:0)	19.967823	18.206722	0	0	18.86005	920.6461	1.9516667
PI (R1CO2H+R2CO2H=34:1)	22.470512	20.071459	22.59432	21.931404	19.317928	853.5651	1.0116
PG (R1CO2H+R2CO2H=39:1)	0	17.105263	18.304823	16.900406	16.439554	840.5848	2.65975
PI (R1CO2H+R2CO2H=37:2)	0	15.374734	0	0	0	898.5529	3.738
PG (R1CO2H+R2CO2H=44:1)	19.857893	14.597295	0	0	0	932.6431	0.9615
PI (R1CO2H+R2CO2H=32:0)	0	17.74138	24.14051	18.275097	19.959782	832.5118	0.9234999
PG (R1CO2H+R2CO2H=36:2)	21.270107	17.592005	20.901033	0	18.881893	791.5654	1.17675
PG (R1CO2H+R2CO2H=31:1)	0	17.604177	17.40247	0	0	723.5079	1.203
PIM4 (R1CO2H+R2CO2H=37:0)	0	21.754715	16.538052	0	0	1572.7867	0.69449997
PG (R1CO2H+R2CO2H=30:2)	17.901703	15.323372	0	0	0	707.4745	0.84999996

Table 10: CD1d putative lipids from *Mtb* clinical isolates grown in Middlebrook 7H9 culture medium

Compound	BCG	Beijing	EAI	H37Rv	LAM	Mass	Retention Time
PI (R1CO2H+R2CO2H=34:0)	0	16.641699	0	16.001299	17.508963	882.5218	0.82900006
PI (R1CO2H+R2CO2H=30:0)	0	17.988724	0	0	0	826.4605	2.518
PG (R1CO2H+R2CO2H=30:1)	0	0	20.304323	16.570032	0	709.492	1.2185
PI (R1CO2H+R2CO2H=33:2)	18.866358	0	21.297207	0	14.972262	864.462	0.8233334
PI (R1CO2H+R2CO2H=34:4)	0	0	18.84237	20.862165	0	874.4607	0.96950006
Lyso-PI (RCO2H=17:0)	0	0	18.480423	0	0	603.3409	0.474
PG (R1CO2H+R2CO2H=40:0)	0	0	19.942188	17.931713	17.265715	851.6627	1.5006666
PG (R1CO2H+R2CO2H=34:0)	18.50741	0	20.664997	0	0	794.5013	1.041
PI (R1CO2H+R2CO2H=37:1)	21.870756	0	21.890314	17.688066	17.844296	895.6111	1.8329998
PG (R1CO2H+R2CO2H=41:1)	0	0	21.417135	0	0	868.6212	2.308
PG (R1CO2H+R2CO2H=32:1)	0	0	20.37009	0	15.661723	737.5239	0.8235
PG (R1CO2H+R2CO2H=37:2)	0	0	20.58294	0	0	810.5388	2.326
PG (R1CO2H+R2CO2H=38:0)	0	0	20.830149	0	15.608544	823.6334	1.4914999
PG (R1CO2H+R2CO2H=36:0)	0	0	23.08718	0	0	822.5372	0.911
PG (R1CO2H+R2CO2H=44:0)	19.608982	0	22.228918	0	15.273614	934.6569	2.165
PG (R1CO2H+R2CO2H=37:1)	21.408295	0	19.935936	14.439246	17.564268	812.5556	1.08525
PI (R1CO2H+R2CO2H=35:0)	21.22431	0	23.596617	17.430233	17.289316	874.5572	1.21925
PG (R1CO2H+R2CO2H=42:1)	15.233058	0	19.928051	0	15.502925	882.6361	2.8006666
PI (R1CO2H+R2CO2H=33:0)	0	0	23.033594	0	15.745884	846.5259	1.0999999
Lyso-PI (RCO2H=19:1)	0	0	0	0	17.976072	1246.642	0.769
PIM4 (R1CO2H+R2CO2H=37:1)	0	0	0	0	16.012144	1543.8223	0.704
Lyso-PG (RCO2H=19:0)	16.815557	0	0	0	16.234106	1074.6115	0.619

Table 10: CD1d putative lipids from *Mtb* clinical isolates grown in Middlebrook 7H9 culture medium

Compound	BCG	Beijing	EAI	H37Rv	LAM	Mass	Retention Time
Lyso-PIM4 (RCO2H=19:0)	18.12128	0	0	0	18.282604	1279.5804	4.353
Lyso-PG (RCO2H=18:1)	14.939487	0	0	0	18.463308	1042.5702	0.7145
PI (R1CO2H+R2CO2H=34:2)	0	0	0	15.268688	16.988918	856.5042	0.764
PI (R1CO2H+R2CO2H=35:4)	0	0	0	0	20.82178	888.4781	1.44
PG (R1CO2H+R2CO2H=46:0)	16.998514	0	0	0	15.45815	940.7132	10.683999
PG (R1CO2H+R2CO2H=37:0)	0	0	0	0	16.304583	814.5678	1.059
PI (R1CO2H+R2CO2H=33:1)	20.115328	0	0	14.682502	0	839.5488	0.91099995
Lyso-PG (RCO2H=15:1)	0	0	0	17.486897	0	490.2307	0.664
Lyso-PI (RCO2H=18:0)	0	0	0	16.569885	0	617.3567	0.563

Table 11: CD1d putative lipids from *Mtb* clinical isolates grown in mice infected lungs

Compound	iBCG	iBeijing	iEAI	iH37Rv	iLAM	Mass	Retention Time
PG (R1CO2H+R2CO2H=30:1)	15.327728	0	0	16.78013	0	736.4278	0.603
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=46:1,R4=H)	13.880731	0	0	0	0	1710.8776	11.284
Lyso-PI (RCO2H=16:0)	15.88293	0	0	0	0	1166.5731	0.684
PG (R1CO2H+R2CO2H=40:0)	16.194103	0	16.124685	0	0	851.6638	3.036
Lyso-PG (RCO2H=15:1)	16.220795	0	0	14.991078	0	958.4776	0.6505
PIM4 (R1CO2H+R2CO2H=36:3)	15.086759	0	0	0	0	1552.7079	0.392
PG (R1CO2H+R2CO2H=34:0)	19.253914	22.032793	20.541016	19.494516	20.375824	772.5243	3.2390006
PG (R1CO2H+R2CO2H=32:0)	17.913216	0	0	16.869318	0	744.4926	1.1669999
Lyso-PG (RCO2H=16:1)	17.41781	0	0	0	15.957736	986.5104	0.64150006
PI (R1CO2H+R2CO2H=36:2)	22.894989	18.642344	21.93543	20.538918	21.302078	884.5375	0.81999993
PG (R1CO2H+R2CO2H=31:0)	25.124802	19.129309	24.0846	22.522459	23.44615	730.4785	0.82900006
PG (R1CO2H+R2CO2H=41:1)	16.124607	0	0	0	18.111635	868.6144	3.427
PG (R1CO2H+R2CO2H=30:0)	16.470707	15.875989	0	0	0	716.4613	0.81899995
PG (R1CO2H+R2CO2H=45:0)	18.237335	18.853216	18.94149	17.062826	19.019327	921.7387	4.7451997
PI (R1CO2H+R2CO2H=34:3)	17.666578	0	0	0	0	854.4948	0.536
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=47:2,R4=H)	14.352871	0	0	0	0	1700.899	4.272
PIM4 (R1CO2H+R2CO2H=34:0)	15.572995	0	0	0	0	1503.8002	0.694
PG (R1CO2H+R2CO2H=32:1)	20.380919	0	0	0	0	764.4617	0.792
PG (R1CO2H+R2CO2H=34:1)	17.052526	0	14.705147	15.862758	18.202679	770.5043	6.810751
PG (R1CO2H+R2CO2H=43:1)	16.518515	0	0	16.959799	0	918.6321	2.1765
Lyso-PG (RCO2H=16:0)	18.110983	0	0	17.358358	17.964256	990.5468	0.554
PI (R1CO2H+R2CO2H=34:2)	18.758875	0	16.088871	0	15.773861	856.505	1.5603334

Table 11: CD1d putative lipids from *Mtb* clinical isolates grown in mice infected lungs

Compound	iBCG	iBeijing	iEAI	iH37Rv	iLAM	Mass	Retention Time
PG (R1CO2H+R2CO2H=36:0)	16.728333	0	0	0	0	800.5513	3.9140003
PG (R1CO2H+R2CO2H=45:1)	16.786512	0	0	0	0	924.6795	4.902
PI (R1CO2H+R2CO2H=35:1)	15.555099	0	0	0	16.203444	867.5795	2.2770002
PG (R1CO2H+R2CO2H=39:0)	16.697021	0	0	16.56345	0	837.647	1.2215
PG (R1CO2H+R2CO2H=44:0)	20.297707	17.473183	19.1296	0	17.926638	907.7217	4.30825
Lyso-PI (RCO2H=18:1)	21.572607	16.143723	21.946085	22.52971	22.42639	620.2959	0.8454
PIM4 (R1CO2H+R2CO2H=33:0)	15.769993	0	0	15.237098	0	1489.7839	0.536
PIM4 (R1CO2H+R2CO2H=35:3)	14.941093	0	15.143064	0	0	1538.6998	0.39450002
PG (R1CO2H+R2CO2H=35:0)	22.118395	15.370244	16.944277	14.299781	17.651451	786.5409	13.313
PG (R1CO2H+R2CO2H=42:0)	18.202866	0	0	0	16.309227	879.6895	9.858001
PG (R1CO2H+R2CO2H=36:1)	17.110147	0	0	0	0	798.5371	1.3650001
PG (R1CO2H+R2CO2H=31:2)	19.307127	19.668425	21.063517	20.471796	21.790522	721.4898	0.73939997
PI (R1CO2H+R2CO2H=34:1)	17.28069	17.337473	18.812855	18.555485	19.302324	853.5643	0.74
PIM4 (R1CO2H+R2CO2H=36:0)	15.027776	0	15.012143	15.143543	0	1536.778	0.39
PI (R1CO2H+R2CO2H=35:0)	18.564342	0	16.664156	19.450674	16.790094	896.5376	0.81500006
PG (R1CO2H+R2CO2H=39:1)	16.05943	0	17.066404	0	0	835.6325	1.9185
PI (R1CO2H+R2CO2H=36:3)	15.938569	0	0	16.325218	15.814481	882.524	0.6016666
PI (R1CO2H+R2CO2H=37:2)	16.067099	0	14.807757	0	17.040558	893.6011	2.1693335
PG (R1CO2H+R2CO2H=44:1)	19.514585	0	0	17.259918	16.76627	905.7055	9.887333
PI (R1CO2H+R2CO2H=32:0)	17.087183	0	0	0	0	832.5109	0.557
PG (R1CO2H+R2CO2H=46:0)	18.16731	0	17.757313	0	15.26459	935.7516	10.123001
PI (R1CO2H+R2CO2H=33:0)	17.080246	0	15.670297	0	0	841.572	2.09

Table 11: CD1d putative lipids from *Mtb* clinical isolates grown in mice infected lungs

Compound	iBCG	iBeijing	iEAI	iH37Rv	iLAM	Mass	Retention Time
PG (R1CO2H+R2CO2H=31:1)	19.752106	0	17.640747	0	18.542072	723.5044	3.4169998
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=48:1,R4=H)	14.012799	0	0	0	0	1738.9155	22.432
PG (R1CO2H+R2CO2H=30:2)	18.00504	0	16.921179	0	16.518007	734.4138	0.7546667
Lyso-PG (RCO2H=20:0)	19.069105	0	17.731989	0	0	562.3239	0.66400003
PI (R1CO2H+R2CO2H=34:0)	21.421728	0	22.524773	20.774687	21.362967	860.5364	1.3985
PG (R1CO2H+R2CO2H=33:2)	0	20.279745	20.036339	20.415556	20.672483	749.5212	3.32775
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=50:5,R4=H)	0	0	15.074642	0	0	1758.8757	0.37099996
PI (R1CO2H+R2CO2H=34:4)	0	17.321999	15.875149	0	0	852.4778	2.9124997
PI (R1CO2H+R2CO2H=33:1)	0	0	17.423231	0	0	866.4906	0.659
PG (R1CO2H+R2CO2H=33:1)	0	0	18.072126	0	18.152359	751.5368	4.602999
PG (R1CO2H+R2CO2H=29:1)	0	0	17.722033	0	18.984137	700.427	1.0884999
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=49:0,R4=H)	0	0	16.141687	0	0	1754.936	0.318
PG (R1CO2H+R2CO2H=37:2)	0	0	16.189535	0	0	810.5393	4.73
PG (R1CO2H+R2CO2H=38:0)	0	0	16.705795	0	0	828.5824	4.563
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=44:0,R4=H)	0	0	14.82774	0	0	1684.8636	0.38399997
PG (R1CO2H+R2CO2H=35:1)	0	0	17.856054	0	19.213888	784.5215	2.0765002
PG (R1CO2H+R2CO2H=33:0)	0	19.90187	22.834068	20.979933	22.029272	758.5092	1.2805
PI (R1CO2H+R2CO2H=36:1)	0	0	17.670248	0	0	881.5989	1.278
PIM4 (R1CO2H+R2CO2H=37:2)	0	0	15.142226	0	15.026868	1546.7681	0.39
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=49:3,R4=H)	0	16.504663	14.1062355	0	0	1726.9186	0.851
PI (R1CO2H+R2CO2H=35:3)	0	0	16.64941	0	0	863.5536	1.487
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=48:4,R4=H)	0	0	13.650828	0	0	1732.8744	11.281001

Table 11: CD1d putative lipids from *Mtb* clinical isolates grown in mice infected lungs

Compound	iBCG	iBeijing	iEAI	iH37Rv	iLAM	Mass	Retention Time
PIM4 (R1CO2H+R2CO2H=32:1)	0	0	15.882309	0	0	1473.7405	0.391
PI (R1CO2H+R2CO2H=37:3)	0	0	19.876722	0	19.626469	896.5385	0.75600004
PG (R1CO2H+R2CO2H=38:1)	0	0	16.94146	0	19.09001	826.5681	3.0894997
PG (R1CO2H+R2CO2H=37:1)	0	0	16.359734	0	15.58889	834.5362	1.016
PG (R1CO2H+R2CO2H=36:2)	0	0	18.200846	0	18.756401	791.5648	1.7789999
PG (R1CO2H+R2CO2H=42:1)	0	0	15.685379	15.946084	15.621536	882.6313	1.0556667
PG (R1CO2H+R2CO2H=37:0)	0	0	16.937086	0	15.249187	814.5718	1.0120001
Lyso-PI (RCO2H=19:1)	0	0	0	16.138773	15.486426	1246.6312	0.78099996
PI (R1CO2H+R2CO2H=37:1)	0	0	0	0	17.428549	900.5731	2.788
PG (R1CO2H+R2CO2H=35:2)	0	0	0	17.770329	16.029028	777.5547	0.8875
PI (R1CO2H+R2CO2H=36:0)	0	0	0	15.803197	16.422968	910.5496	0.6485
PI (R1CO2H+R2CO2H=35:4)	0	0	0	0	16.265213	888.4777	0.628
PIM4 (R1CO2H+R2CO2H=32:0)	0	0	0	15.135669	14.827938	1502.7009	0.38900003
PIM4 (R1CO2H+R2CO2H=37:0)	0	0	0	16.95607	20.336931	1572.7769	0.678
PI (R1CO2H+R2CO2H=30:0)	0	0	0	0	16.246183	826.462	0.621
PIM4 (R1CO2H+R2CO2H=37:1)	0	0	0	15.830391	0	1548.7798	0.38900003
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=47:1,R4=H)	0	0	0	14.335809	0	1724.8928	1.284
Ac1PIM4 (R1CO2H+R2CO2H+R3CO2H=51:4,R4=H)	0	0	0	18.748352	0	1752.9352	0.21999998
PG (R1CO2H+R2CO2H=32:2)	0	15.750785	0	0	0	740.4616	0.602

4.4.5 Diversity in responses induced by Mtb strain-specific polar CIDFL, in splenocyte co-culture from the mice vaccinated with BCG vaccine and boosted with polar Mtb CIDFL as adjuvants.

IFN-γ from splenocyte co-culture was used as a measure of the immune response, which is determined by the production of Th-1 cytokines when exposed to polar CIDFL from a respective clinical isolate. As the cells were restimulated with CIDFL in mice, the T cells restricted to these lipids in the co-cultures were considered as NKT cells. We observed that BCG derived lipids significantly stimulated IFN-γ production as compared to the co-cultures stimulated by non-BCG polar lipids from the rest of the clinical isolates. This diverse response by non-BCG strains may be due to the differential lipid composition of the strain in comparison to BCG, and thereby IFN-y produced in response to these lipids is diverse (Fig. 35A).

In the other experiment, we determined if the response to CIDFL is strain-specific, BCG vaccinated mice were given booster with polar CIDFL from various strains, and co-cultures were stimulated with the strain-specific CIDFL for evaluating IFN-γ. It was observed that BCG vaccinated mice boosted with polar CIDFL from EAI-5, LAM-6 and Beijing have shown better IFN-y response as compared to polar lipids from BCG and H37Rv. This shows that clinical isolate lipids are immunogenic, and they add up to the repertoire of lipid antigens from BCG, which could enhance BCG vaccine efficacy.

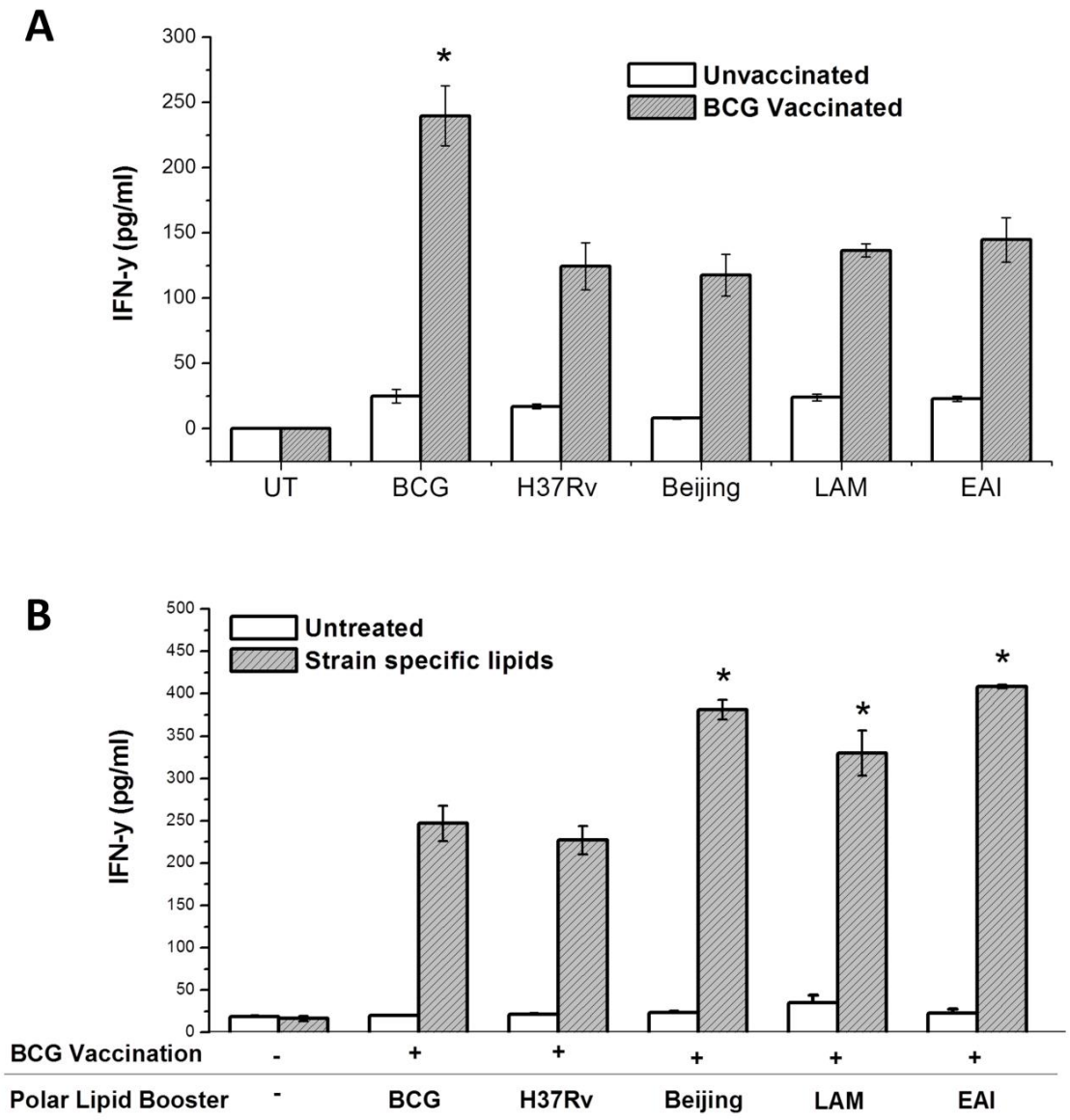


Figure 35: Levels of IFN-γ in response to lipid stimulation of splenocyte co-culture from the mice vaccinated with the BCG vaccine and boosted with polar Mtb lipids. *Fig. 35A and 35B represent levels of IFN-γ in splenocyte co-cultures from BCG vaccinated mice. As shown in Fig. 35A BCG vaccinated, and unvaccinated mice splenocyte co-culture was restimulated with CIDFL Fig. 35B the co-cultures were derived from mice boosted with polar lipid boosters from various clinical isolates after BCG vaccination. Results were expressed as mean ratio ± S.D (N=3) *p <0.001 when compared with controls.*

4.5 Discussions

Strain diversity, differential virulence and pathogenesis of *Mtb* bacilli are associated with the complex genetic makeup and virulence determinants. *Mtb* lipids are the major virulence factors, and their diversity across lineages determine immunogenicity or virulence between the strains (Krishnan, Malaga et al. 2011). Strain virulence is reflected by the ability of a strain to survive host immunity (Wang, Peyron et al.), and such host-pathogen interactions lead to inflammatory host responses in a lineage-specific manner though no firm evidence of mechanism has been established (Krishnan, Malaga et al. 2011). Lineage 1 (EAI-5) was shown to induce higher Th1 response compared to Lineage-2 (Beijing) in the PBMC of the infected person (Rakotosamimanana, Raharimanga et al. 2010). In our previous study (Chakraborty, Kulkarni et al. 2013), EAI-5 consistently elicited a stronger pro-inflammatory response (TNF-α, IL-12 and IL-1β) and apoptosis compared to Beijing strain in human PBMCs as well as in THP-1 cells. In the BALB/c infection model, EAI-5 showed significantly low lung CFU after 30 days of infection compared to Beijing and LAM-6 (lineage 4) strain while CFU were highest in Beijing compared to EAI, BCG and H37Ra. LAM-6 showed significantly high lung CFU after 30 days of infection compared to H37Rv proving its higher virulence than laboratory virulent strain (data not published). Lineage 4 strains grow more rapidly in liquid culture and generally induce high levels of TNF-α and IL-12 in the host as compared to lineage 2 (Sarkar, Lenders et al. 2012). LAM-6 induced similar proinflammatory response and apoptosis to THP-1 compared to H37Rv, and they both belong to lineage 4. Few studies have shown that lineage 2 induce lower levels of proinflammatory cytokines than H37Rv (Sohn, Lee et al. 2009; Tanveer, Hasan et al. 2009). In our previous study,

the Beijing genotype which is known to be highly virulent induced the lowest proinflammatory cytokine, reactive nitrogen and reactive oxygen species in the host without affecting the expression of immunosuppressive cytokines (Chakraborty, Kulkarni et al. 2013). These findings are consistent with previous reports with other members of the W/Beijing family of strains (Portevin, Gagneux et al. 2011). Thus, it will not be irrelevant to hypothesise that EAI-5 strain is less virulence compared to Beijing and LAM-6 or H37Rv with the order of virulence as Beijing > LAM-6 > H37Rv > EAI-5 > BCG.

CD1 putative *Mtb* lipid immunization was shown to induce protection in guinea pigs challenged with *Mtb where* purified mycobacterial lipid antigens, the diacylated sulfoglycolipids (Ac_2SGL) and the phosphatidyl-*myo*-inositol dimannosides (PIM_2) were formulated in liposomes made of dimethyl-dioctadecyl-ammonium (DDA) and synthetic trehalose 6,6′-dibehenate (TDB) (Larrouy-Maumus, Layre et al. 2017). Similarly, it was shown that CD1d putative based vaccine could additionally involve CD1d restricted NKT cell immunity (Larrouy-Maumus, Layre et al. 2017). CD1d restricted lipids like PI, PG and PIM_4 are known to induce an immune response (De Libero and Mori 2014). In this study, the total numbers of diverse lipids were lowest in Beijing while highest in BCG in infection state. The mice lung infection and survival pattern was coherent (unpublished data) with the lipid composition (CD1d putative lipids) of the strains. Phosphatidylinositol down-regulation was evident in all the strains but was more prominent in Beijing than BCG in infection state. All strains up-regulate PIM_4 in the infected state except Beijing. This indicates that these lipids are available or accessible to immune cells which may be important in the generation of the immune response in animals infected with a

clinical isolate. All strains down-regulate PG in the infected state except LAM-6 and H37Rv. LAM-6 and H37Rv show similar CD1d lipid profile. BCG and EAI-5 which are known to be less virulent in animals might show lesser pathology as compared to Beijing as various PI, PG and especially PIM_4, were accessible to the immune system in animals. This gives a co-relate of the responses induced by these strains in mice lungs and vaccination using these lipid adjuvants or booster might improve challenge-response. Interestingly the same lipid molecules PI or PG are present also in mammalian host cells, and thus they represent self-antigens. Thereby, the increased availability of these self-antigens within APC might also contribute to the activation of CD1d-restricted T-cells cross-reactive with microbial antigens (De Libero and Mori 2014).

Venturing beyond immunopathogenesis of TB into *Mtb* lipids may further represent therapeutic applications, using targets for future preventive interventions, as illustrated by the current study, which aimed at investigating the vaccine potential of CD1-restricted lipid antigens. CD1d putative lipid-based adjuvant vaccines complementing conventional BCG could help to achieve this consolation. The analysis of CD1 restricted lipid present in strain empower us with the choice of lipid dominant strain to be used as an adjuvant could provide a promising avenue with this context and such analysis could help understand lipid expression pattern of the bacilli in its immediate niche. Such a study needs bacterial lipid analysis in various stages of culture, infection or dormancy. Lipid-specific CD1d restricted NKT cells determine the immune discourse, and therefore they are a good candidate for vaccine adjuvant. CD1d based adjuvants can also be useful in HIV-infected patients from co-infection with *Mtb* due to depletion of $CD4^+$ T cell. Thus, targeting group 1 or 2 CD1-restricted

NKT cells by vaccination with CD1d putative lipid vaccines or adjuvant could be particularly beneficial for HIV-infected patients. This study shows the important role of CD1d lipids and NKT cells in the modulation of BCG vaccine response. Also "pooled lipid" from CD1d lipid-rich strains could be used as an adjuvant, which can take into account the lipid profile of all geographically dominant strains. Our results with pooled lipids from all the clinical isolates showed similar restimulation response as that of EAI. Such LC-MS analysis would not only be helpful to select the strains for adjuvant but can also cater to the diagnostic or epidemiological evaluation of clinical isolates.

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