TLR4 MEDIATED IMMUNOMODULATION IN TUBERCULOSIS

By

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Pramod Kumar Gupta

DECLARATION

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.

Pramod Kumar Gupta

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2. **Pramod Kumar Gupta**, M.G.R. Rajan, Savita Kulkarni. Activation of murine macrophages by G1-4A, a polysaccharide from *Tinospora cordifolia, in* TLR4/MyD88 dependent manner**.** International.Immunopharmacology, **2017**, 50, 168-177.

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3. **Pramod Kumar Gupta**, Savita Kulkarni, M.G.R. Rajan. Immunomodulatory effects of G14A (a polysaccharide from Indian medicinal plant, *Tinospora cordifolia)* in *M.tuberculosis* H37Rv infected THP-1 cells. (2011) 37th Conference of Indian Immunology Society .

Pramod Kumar Gupta

Dedicated to my Parents

&

Wife

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CONTENTS

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SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student: Pramod Kumar Gupta**
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SYNOPSIS

Introduction

The ability of *Mycobacterium tuberculosis* (MTB), a causative agent of tuberculosis (TB) to successfully parasitize the macrophages, is a result of its capacity to adapt to the changing host environment by utilizing the resources available to it within host and inhibiting the host responses directed against it [1]. Host processes inhibited by pathogenic species of MTB include fusion of phagosome and lysosome [2,3], antigen processing [4], responsiveness to IFN-γ [5], production of cytokines, reactive oxygen intermediates, reactive nitrogen intermediates [6] and host cell apoptosis [7-9]. It has also been reported that MTB up-regulates the expression of anti-inflammatory cytokine IL-10 to suppress macrophage activation [10] and host cell apoptosis [8].

Despite the availability of effective treatment of TB, rapid emergence and spread of drug resistant MTB strains, along with need for extended period of current drug

regimens, are worsening the burden of disease [11] and thus making it compulsory to explore the novel strategies of anti-tubercular therapies. One alternative approach involves selective modulation of host immune responses to enhance the therapeutic potential [12]. Modulation of immune responses has been proven to work as useful therapeutic approach in many cases such as prevention and treatment of various infections, augmentation of anti-tumor immunity in cancer and suppression of autoimmune and inflammatory conditions [13,14]. This approach is largely used as adjunct therapy to support and enhance the efficacy of antibiotics and antivirals [12]. Innate immune responses in tuberculosis are suboptimal and hence immunomodulation offers the potential to skew the equilibrium back in favor of host either by enhancing or suppressing the selective elements of innate immune system as well as exploiting the strong and intricate effector mechanisms that have evolved over time for pathogen clearance. The discovery of the pathogen recognition receptors of innate immunity, particularly the Toll-like receptors (TLRs), has opened new possibilities for modulation of the innate immune system. Different TLR agonists are being investigated as potential therapeutic agents for the treatment of various diseases, for example, TLR3, 7, 8 and 9 agonists for cancer, TLR4 and 9 agonists for allergies and TLR3, 7 and 9 agonists for viral infections. Further, agonists of TLR 4/5/7/8/9 are being developed as prophylactic and therapeutic vaccines and vaccine adjuvants for the treatment of cancer and viral infectious diseases [15]. CpG DNA, a TLR9 agonist has ability to control the infection by boosting the innate immune responses which indicates the potential to use such compounds as antimicrobials by activating or enhancing innate responses [16].

Crude aqueous extracts of stem of the Indian medicinal plant *Tinospora cordifolia* have been shown to enhance the intracellular bactericidal activity of macrophages and

neutrophills in *E. coli* induced peritonitis [17,18]. It was shown to be mitogenic to B cells and its activity based purification resulted in isolation of a polysaccharide, known as G1-4A, an acidic arabinogalactan of m.w. 2.2 x 10^6 Da [18-20]. Macrophages were the principal target cells of G1-4A whereas it also augmented the DC maturation [18,19,21].

In the present study, we wish to study the immunomodulatory role of this G1-4A in treatment of TB and mechanism underlying it. Immunomodulators offering limited protection against MTB infection may be useful as adjunctive therapy with a potential to reduce the duration and increase the effectiveness of conventional drug regimen. Unlike antibiotics, immunomodulators target the host rather than the pathogen, thus immunomodulation remains impervious to the evolution of any drug resistance by MTB.

ORGANISATION OF THE THESIS

The work reported in the thesis is organized into four chapters: Chapter 1 deals with general introduction about the scientific information available related to the present work and its objectives. Methodology is discussed in chapter 2. Chapter 3 deals with the results of the study. Chapter 3 is divided into 3 subchapters. Subchapter 1 includes the results involving the study in macrophages. Results obtained in study involving BMDCs are summarized in subchapter 2. Subchapter 3 includes *in vivo* results. Chapter 4 deals with discussion of the whole study.

Chapter 1: General Introduction

This chapter deals with the general introduction of tuberculosis disease, its pathology, associated immune responses, their subversion by MTB strains and modulation of such responses by immunomodulators to achieve the reduced bacterial survival inside the host. Further it includes the details of anti-TB drugs, resistance of MTB against

these drugs, the mechanism of acquiring the drug resistance by MTB strains, various in vitro and in vivo models to study tuberculosis. This chapter also describes background literature and current scenario of immunomodulation in tuberculosis, the basis of the present study, hypothesis and objectives of the present study.

Chapter 2: Methodology

G1-4A was extracted from the stem of *Tinospora cordifolia*. All MTB strains were grown in Middelbrook 7H9 medium till in their mid log phase, harvested and single cell suspension was prepared. RAW 264.7 cells maintained in DMEM, THP-1 cells maintained in RPMI or murine peritoneal macrophages were infected with MTB strains for four hours at a multiplicity of infection (MOI) of 5. After the initial period of co-culture, the extracellular bacteria were washed off thoroughly and fresh medium containing amikacin was added for 1 h to kill the extracellular bacteria. Cytokine expression was monitored by ELISA. Nitric oxide in the supernatants was estimated by Griess' reagent and concomitant NOS2 expression was evaluated by western blot. Effect of G1-4A on the MAPK activation was monitored by western blot. Expression of MHC-II and CD-86 was determined by flow cytometry. The intracellular growth of the bacterium was monitored by counting Colony Forming Units (CFU). Phagocytic index was investigated by confocal microscopy using GFP-MTB. Apoptosis in infected host cells was assessed by flowcytometric analysis (PI and Annexin V staining). Anti-TLR4 antibody and siRNA specific to TLR4 and MyD88 were used to delineate the role of TLR4 and MyD88 in G1-4A mediated effects. BALB/c mice were infected by aerosolization using different strains of MTB, treated with G1-4A and intra-pulmonary bacterial growth was monitored as CFU in lungs. Bone marrow derived dendritic cells (BMDCs) were generated from BALB/c mice and infected with MTB strains and their differential activation is measured by ELISA and Flow

cytometry. In all the experiments, uninfected cells were considered as negative control.

Chapter 3: Results

This chapter is divided in to three sub chapters.

Sub-chapter 1: Effect of G1-4A in MTB infected and/or uninfected macrophages

G1-4A was extracted from the stem of Indian medicinal plant *Tinospora cordifolia.* Effect of G1-4A on the macrophage activation was monitored by determining the expression of proinflammatory cytokines, nitric oxide levels in cell supernatant and surface expression of MHC-II and CD86 after G1-4A treatment. Our data demonstrated that G1-4A treatment up-regulated the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ and IL-6), nitric oxide with concomitant up regulation of NOS2. G1-4A treatment also up-regulated the expression of CD-86 and MHC-II in RAW cells as well as murine peritoneal macrophages. Involvement of TLR4 and MyD88 in G1-4A mediated macrophage activation was determined by measurement of proinflammatory cytokines, nitric oxide, CD86 and MHC-II in the presence of anti-TLR4 Ab and siRNA against TLR4 and MyD88. Our data demonstrated that G1-4A treatment activated macrophages in TLR4-MyD88 dependent manner. Further, it was observed that G1-4A treatment phosphorylated all the three MAPKs and phosphorylation started after 15 min of treatment, reached to maximum 30 min post treatment and declined gradually at further time points. With the help of pharmacological inhibitors of MAPK, their involvement in macrophage activation mediated by G1-4A was monitored. It was observed that there was a marked decrease in the levels of IL-6, TNF-α, IL-1β and IL-12 ($p<0.05$) in the presence of U0126 and SB203580 but it remained unchanged in presence of SP600125 which suggests the involvement of p-38 and ERK1/2 in G1-4A mediated

induction of above cytokines. Further, we observed a significant decrease in the level of nitric oxide ($p<0.01$) in presence of SB203580 and SP600125 whereas, there was no change in presence of U0126 which indicated the involvement p-38 and JNK1/2 in G1-4A mediated NO induction. Similar pattern was observed in G1-4A mediated NOS2 expression which was down regulated in presence of p-38 and JNK1/2 inhibitors confirming the role of these two MAPKs in G1-4A mediated NOS2 expression in RAW cells. Therefore, it is logical to infer that G1-4A activates all the three MAPKS which in turn plays a role in macrophage activation. Using pharmacological inhibitor of NF-kB, PDTC (Pyrrolidine dithiocarbamate,) we demonstrated the involvement of NF-kB in G1-4A mediated up regulation in the expression of MHC-II in RAW 264.7 cells**.**

It is reported that MTB strains subvert the macrophage activation to ensure its survival within the macrophages. Hence, in the next step we decided to find out the role of G1-4A in the activation of MTB infected macrophages. RAW cells infected with MTB H37RV and other two MDR strains (LAM & Beijing), were treated with G1-4A and expression of proinflammatory cytokines, nitric oxide, CD86 and MHC-II was investigated. We observed that G-14A treatment significantly enhanced the expression of IFN-γ, IL-1β, IL-6 and TNF-α, though all the three strains modulated the level of these cytokines which was characteristic of their genotype, but the common trend that we observed here was increase in the level of all these cytokines irrespective of the strain or their genotype. Further, we observed that all the three strains exhibited differential NO response where H37Rv and LAM were comparable but Beijing displayed minimum NO levels. However, when macrophages harboring these MTB strains were incubated in the presence of G1-4A, level of NO was enhanced significantly and even in Beijing strain the level was almost similar to two

other strains. Similar pattern was obtained during the quantitative RNA expression analysis of NOS2 by Q-PCR. This was yet again confirmed by the monitoring the protein level of NOS2 by western blotting. Further, to investigate the activation of infected macrophages by G1-4A, we studied the expression of CD-86 and MHC-II by G1-4A. Data suggested that MHC-II expression was low in case of infection by all three strains in absence of G1-4A but it dramatically increased by several folds in presence of G1-4A. In case of CD-86, though infection with different MTB strains exhibited differential expression pattern in absence of G1-4A, in presence of G1-4A the expression of CD-86 was up-regulated for all the strains. Phagocytosis assay results demonstrated that prior treatment of G-14A enhanced the phagocytosis of MTB by macrophages. Phagocytic index of G1-4A treated cells was comparable to LPS treated cells and it was significantly higher than untreated control.

Further, our data demonstrated that G1-4A decreases the intracellular survival of MTB strains in RAW 264.7 cells, murine peritoneal macrophages and human PBMCs. It was proved with the help of Ressazurin Microtitre Plate Assay (REMA) that G1-4A did not act directly against MTB but it exerted its anti-TB effects through modulation of host immune responses.

Final isolation of G1-4A requires gel filtration chromatography of crude extract [PRE (Polysaccharide Rich Extract), which is a cost intensive procedure if we have to go for production of G1-4A on a large scale for further clinical studies. Hence, we decided to evaluate the efficacy of PRE on macrophage activation and intracellular survival of MTB in RAW cells. Our data suggested that like G1-4A, treatment of PRE also activated macrophages through classical pathway. It up-regulated the expression of cytokines such as IL-6, IL-1β, IFN-γ, IL-12, TNF-α and NO in cell supernatants. Flow cytometry data revealed a significant increase in MHC-II+ and CD-86+

population which indicated the activation of macrophages due to PRE treatment. It was observed that PRE treatment phosphorylated all the three MAPKs and phosphorylation started after 15 min of treatment, reached to maximum 30 min post treatment and declined gradually at further time points. Further, our CFU data in RAW cells demonstrated a significant decrease in counts in PRE treated groups irrespective of the infecting MTB strain type. It was observed that bacterial burden was decreased by approx 50% after 48 hours of treatment and 65-70% by 72 hours of treatment. Thus, we conclude that PRE exhibited effects similar to G1-4A in MTB infected macrophages.

By virtue of the immunomodulatory effects of G1-4A to inhibit the intracellular survival of MTB in RAW cells and mice lungs, we decided to investigate the effects of G1-4A in MTB infected human monocytic cell line THP-1 and PBMCs. It was observed that G1-4A modulates cytokine levels in MTB infected THP-1 cells. It also induced apoptosis in MTB and BCG infected cells. Further, CFU data revealed that G1-4A inhibited the intracellular survival of MTB strains in THP-1 and human PBMCs.

Sub-chapter 2: Differential activation of BMDCs by MTB strains and effect of G1-4A on the intracellular survival of MTB in BMDCs

Although macrophages are primary hosts for MTB it infects DCs as well. Owing to their ability to prime naive and memory T cells, Dendritic cells (DCs) play a key role in the host defense against MTB infections. Dendritic cells being the part of the innate immune system trigger T-cells to polarize towards Th-1 or Th-2 type of immune response depending upon their maturation status. It has been reported that H37Rv partially inhibits the maturation of human monocyte derived DCs but the impact of drug resistant clinical isolates on the maturation of DCs is poorly understood. Hence,

we investigated effect of sensitive and MDR strains of MTB on the activation and maturation of BMDCs. Expression of cytokines TNFα, IL-6 & IL-12 and surface expression of MHC-II, CD86 & CD40 were monitored in MTB infected BMDCs and it was observed that infection with virulent MTB strains down regulated the expression of above molecules compared to one observed in infection with BCG or immature BMDCs. Further, MTB infected BMDCs were treated with G1-4A and CFU assay was performed which demonstrated reduction in CFU counts by 25% at 48 h and 33% at 72h ($p<0.05$) after G1-4A treatment. Though G1-4A inhibited intracellular survival of MTB in BMDCs but it was not comparable to the reduction in CFU counts observed in macrophages after G1-4A treatment. DCs are mostly APCs and their intrinsic antimicrobial properties are lower than macrophages. This could be the reason for the above observations.

Sub-chapter 3: Evaluation of *in vivo* efficacy of anti-TB effects of G1-4A in BALB/c mice.

After establishment of inhibitory effect of G1-4A on intracellular survival of MTB strains *in vitro*, we determined the in *vivo* efficacy. Our CFU data suggested a significant reduction in bacilli count of each MTB strain after G1-4A treatment thus reducing the bacterial burden of all three strains in the lung of animals irrespective of their drug resistance status, indicating its *in vivo* efficacy. Further, we evaluated the adjunctive role of G1-4Aby including Isoniazid (INH, 50mg/kg of body wt) Anti-TB drug along with G1-4A. Animals were given daily dose of INH through drinking water along with G1-4A injection. Since LAM and Beijing were resistant to INH only H37Rv was included in this study. .It was observed that G1-4A caused significant reduction in CFU counts and bacillary load, when given in combination with INH as compared to INH or G1-4A alone (*$p<0.01$,#$p<0.001$ compared to control).

Therefore, it was concluded that G1-4A exhibited better efficacy in combination with INH and therefore it may be potential therapeutic candidate in adjunctive therapy for tuberculosis.

Further treatment of G1-4A in MTB infected mice up regulated the expression of cytokines like IL-1β, TNF-α, IL-12 & IFN-γ and down regulated the expression of IL-10 and IL-4 in the serum of infected mice. Further, G1-4A treatment up-regulated the RNA expression of TNF-α, IFN-γ and NOS2 in the lung of infected mice. Restimulation assay demonstrated an increase in IFNγ/IL-4 ratio in MTB infected BALB/c mice after G1-4A treatment indicating activation of Th1 response. Hence, it was concluded that induction of Th1 response by G1-4A treatment was one of the major mechanisms responsible for its anti-TB effects in BALB/c mice.

Chapter 4: Discussion & Conclusions

Present study provides an insight into an alternative therapy for drug resistant tuberculosis. G1-4A exerts its effects mainly by modulating the host responses in both *in vitro* and *in vivo* models. Also it shows its adjunctive effects along with Isoniazid. This study also proves that G1-4A was able to inhibit the intracellular survival of MTB strains independent of their drug resistance and genotype status in murine macrophage cell line RAW 264.7, peritoneal macrophages, and human monocytic cell line THP-1, human PBMCs and in BALB/c mice. Present study provides a proof of concept of host targeted therapy in tuberculosis by natural immunomodulator compound, G1-4A. Further studies may determine the efficacy of G1-4A in TB patients which can be helpful in the management of MDR and XDR TB cases.

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iii) Pramod Kumar Gupta, Savita Kulkarni, M.G.R. Rajan. Immunomodulatory effects of G14A (a polysaccharide from Indian medicinal plant, *Tinospora cordifolia)* in *M.tuberculosis* H37Rv infected THP-1 cells. (2011) 37th Conference of Indian Immunology Society .

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CHAPTER 1

1. *General Introduction*

1.1. Background of Tuberculosis: TB, a global emergency

The battle of man and tuberculosis (TB) has been dated back to ancient times. The DNA of *Mycobacterium tuberculosis* (MTB) has been detected in Egyptian mummies from 2000 B.C. [1] and Hippocrates described TB as early as 400 B.C. [2]. Based on its clinical manifestations it was identified as the disease of "consumption", "wasting away", "king's evil", "lupus vulgaris", "the white plague" or "phthisis" in ancient times [2,3]. Transmission of TB is caused by aerosols containing MTB, released through coughing from the lungs of an infected individual and subsequently infecting a new host by the inhalation through airways. Industrial revolution during 18th and 19th centuries in Europe resulted into crowded living conditions in urbanized areas, which provided optimal conditions for spread of TB and it became one of the deadliest infectious diseases in Europe. Due to improved socio-economic conditions in post industrialization era, Europe witnessed the decline in incidence of TB, even before the discovery of antibiotic, signifying the importance of living conditions and socio-economic factors in the control of TB, and pointing out some of the difficulties that low income developing countries still face today [4,5].

TB became a medically treatable disease with the discovery of streptomycin in 1943, and its incidence continued to fall in industrialized countries throughout the 20th century, but TB continued to take its toll on poor and vulnerable populations in low-income courtiers, as well as marginalized populations in high-income countries [4]. However, with the advent of antibiotic resistance and the HIV epidemic, infectious diseases have again become a global threat. The increased susceptibility of HIV-positive persons to TB, the emergence of extensively drug-resistant TB, the relative

ineffectiveness of the Bacillus Calmette Guerin (BCG) vaccine and increased mobilization of people due to globalization have put TB back on the agenda during the past twenty years [6,7]. Since early 20th century, BCG vaccine, a live attenuated variant of *M. bovis* has been used. However, it has failed to provide protection against pulmonary tuberculosis in adults; hence, a more effective vaccine is urgently needed [7]. With nearly two million deaths per year, TB is truly a global emergency. In addition to HIV infection and socio-economic factors that facilitate the development of TB, unavailability of optimal diagnostic tools, lack of cheap and effective new therapeutic modalities and the lack of an effective vaccine, are other major contributors to the advancement of this global threat. However, a better understanding of basic mechanisms by which MTB successfully develops active TB and how the host can overcome it, can facilitate the development of new immunodiagnostic tools, evaluation of new therapeutic interventions and effective vaccine [4,7].

1.2. TB epidemiology

Every year, approximately 10 million new TB cases are registered in the world; of which, India and China constitute 30% and 20-25 highest burden countries mainly in Africa, South America, and Asia constitute 80 % of total no of TB cases [5]. It is estimated that MTB infects one third of the world's population and TB causes about two million deaths annually [4,7]. Only 5-10% of exposed individuals develop active TB where as remaining population either resolve the infection or remain latently infected. It is believed that approximately half of the individuals exposed to MTB, neutralize the infection by innate immune response. Such individuals do not exhibit any symptom of the disease and lack immunological memory against the pathogen and yield negative results in diagnostic tests based on the presence of memory T cells. In remaining 50% of the individuals, either exposure is cleared through adaptive

immune response or persists as latent infection leaving primed T cells which then give positive results in the diagnostic tests based on presence of memory T cells. Therefore, it is very difficult to discriminate a resolved infection from latent TB infection by a diagnostic test based on immunological memory.

Further, development of active TB occurs in only 5% of latently infected individuals within 2-5 years whereas, in remaining 95% latent TB exists throughout their life time and develop active disease only when there is any immune suppression through HIV infection or any other disease pathology and/or by use of immunosuppressive drugs, old age, or re-infection [7]. The fact that significant number of MTB infected individuals clear the infection through a robust innate immune response, suggests a critical role of innate immunity in achieving an eradication of infection and it also provides a hint that how boosting innate immune system can clear MTB infection [7]. The fact that many individuals are able to clear the infection through an effective innate immune response indicates an important role for this part of the immune system in achieving sterilization of infection, and may provide a hint as to how one can boost the immune response to clear MTB.

1.3. *Mycobacterium tuberculosis:* The bacterium

By culturing crushed granulomas, Robert Koch identified the pathogen responsible for TB in 1881. MTB is rod-shaped and non-sporulating bacterium, although recent studies reported the formation of spores in aged mycobacterial cultures [2,8,9]. MTB lacks flagellum or capsule and its complex waxy cell wall makes the bacillus acid fast i.e. that it is resistant to decolourization by acids during staining procedures. Though MTB is classified as a gram positive bacterium, it stains poorly with crystal violet due to its unique cell wall composition. MTB is a slow grower which replicates slowly

with doubling time of about 24 h. The bacillus is an aerobic intracellular pathogen which measures around 0.5 μm in diameter and 1-4 μm in length [10].

1.3.1. Cell wall

The cell wall of MTB is a lipid rich structure and largely comprise of long-chain fatty acids termed mycolic acids linked to arabinogalactan, which is attached to the peptidoglycan. Additionally, several lipoglycans including lipoarabinomannan (LAM), its precursors lipomannan (LM), and phosphatidyl-myo-inositol mannosides (PIM) are present in cell wall and these components are non-covalently attached to the plasma membrane through their GPI anchors extending to the exterior of the cell wall [11]. LAM consists of a phosphatidyl-myo-inositol anchor, a D-mannan polymer attached to the inositol ring, D-arabinose chains, and capping motifs at the end of the arabinose residues [12]. LAM is one of the most important virulence factor of MTB, involved in the inhibition of critical macrophage functions required for killing the pathogen, like inhibition of phagosomal maturation, alteration of cell signaling and shifting the cytokine response from pro to anti-inflammatory [11,13,14]. LAM exhibits differential capping pattern among bacterial strains which is very important for the virulence. Virulent, slow-growing mycobacteria like MTB possess mannose-capped LAM (Man-LAM) in their cell wall, while non-capped AraLAM or phospho-myo-inositol-capped LAM (PILAM) is present in rapidly growing non-virulent species of mycobacteria such as *M. smegmatis* [15]. The cell wall of MTB also contains a lipoprotein 19-kDa of unknown function which has been implicated in virulence through a role in host cell death and manipulation of bactericidal mechanisms [16].

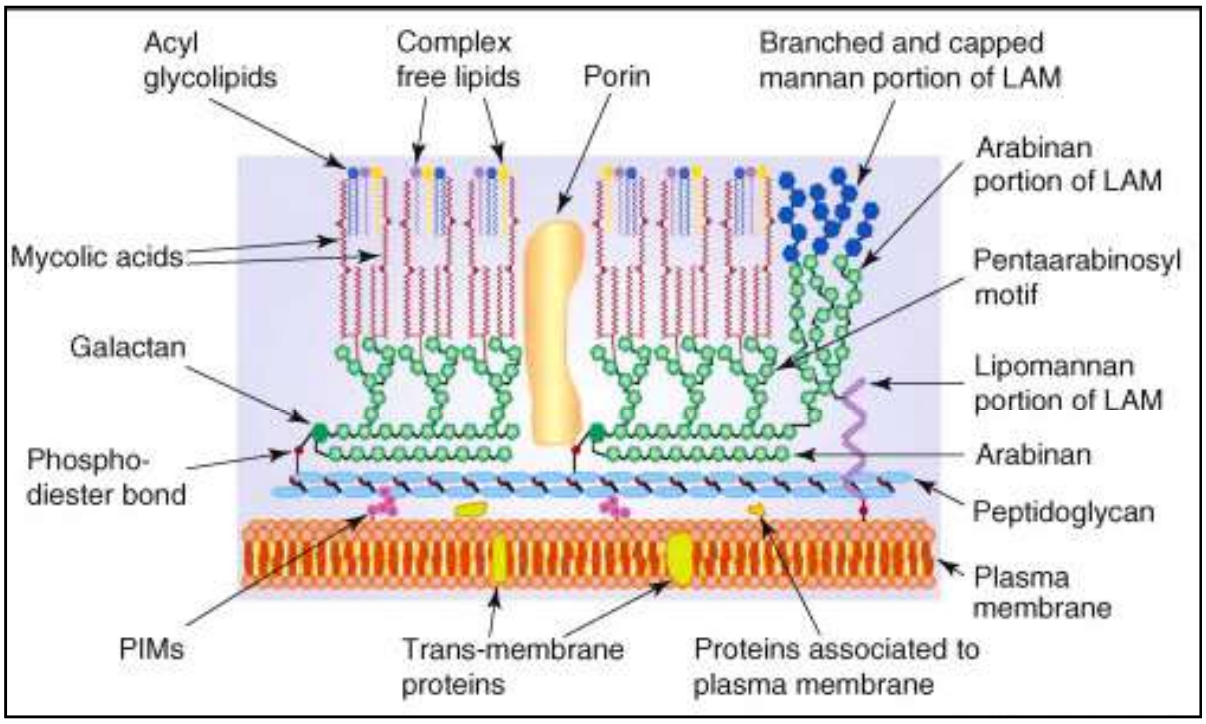


Figure 1.1. Schematic representation of the MTB cell wall [17] .

1.3.2. Mycobacterial strains

The genus *Mycobacterium* includes mainly soil dwelling saprophytes, with only few members of the genus having the ability to cause diseases of diverse nature and varying severity [18]. Tuberculosis is caused by members of the *M. tuberculosis* complex that consists of *M. tuberculosis, M. bovis, M. africanum, M. canettii* and *M. microti*. The members of this complex exhibit 99.9% similarity at the nucleotide level and identical 16S rRNA sequences [19,20], but they differ widely in terms of phenotypes, pathogenicity and their host tropisms. Some members such as *M. tuberculosis, M. africanum, M. canettii* are exclusively human pathogens, whereas *M. bovis* has a wide host spectrum [18]. All members of the MTB complex are slow-growing, with generation time ranging from 12 to 24 hrs depending on environmental and microbial variables. MTB exhibits diversity in the genome and using large sequence polymorphisms (LSPs) Comas and Gagneux, grouped MTB into six

lineages and 15 sub-lineages on the basis geographical distribution [21]. Ancient strains (e.g. EAI-5) were grouped in lineages 1, 5 and 6 and modern strains (e.g. Beijing and LAM-6) were grouped in lineage 2, 3 and 4 on the basis of TbD1 analysis [22]. In the present study, the clinical isolates used, belong to Beijing and LAM-6 lineages. MTB strains exhibit variability in their phenotype and virulence e.g Beijing strain is more virulent and have ability to develop drug resistance extra-pulmonary TB more often compared to other strains, whereas LAM lineage causes cavitary disease [23]. Main laboratory strain of MTB is H37 which was isolated from a 19 year old pulmonary TB patient in 1905, and later on the basis of its virulence in guinea pigs it was divided into two strains; a virulent strain (H37Rv) and an avirulent strain (H37Ra) [24]. Both strains can be grown in suitable culture medium in the laboratory however, only H37Rv strain has ability to replicate inside human macrophages [25]. Recent reports have suggested that H37Rv and H37Ra exhibit genetic and phenotypic differences, and the major difference being the mutation in the *PhoP* gene, necessary for adaptation to the intracellular environment [26-28]. *PhoP* along with *PhoR* constitutes a two-component regulatory signal transduction system important for adaptation to the environmental stimuli. *PhoP* acts as a transcriptional regulator in two component system [29]. Mutations in the *PhoP* gene cause a deficiency in the secretion of ESAT-6, which can be synthesized but not released from the bacillus [30,31]. The H37 strains, BCG and different clinical isolates, are the most common strains of MTB to be used to study the pathogenesis of mycobacteria in different *in vivo* and *in vitro* systems. Further, *M. marinum* shares many features of MTB and avirulent to humans, is also commonly used to infect zebrafish and the amoeba *Dictyostelium* [32-34]. However, data obtained with mycobacteria that are not pathogenic to humans should be extrapolated with great

care, as many mechanisms specific to MTB are dependent on various genetic elements like a functional ESX-1 region and *PhoP/PhoR* regulatory system.

1.4. Pathogenesis of TB

MTB is an obligate, aerobic, intracellular pathogen, with predilection for oxygen rich lung tissue. TB transmission occurs almost exclusively through the inhalation of aerosol droplet containing viable MTB expelled by coughing, spitting or sneezing of an individual with active pulmonary TB. Post inhalation, aerosol droplets containing viable MTB are deposited in the alveolar spaces, where the phagocytic cells, mainly alveolar macrophages engulf the bacteria [35], an event which leads to a rapid inflammatory response and migration of various immune cells to the site of infection.

1.4.1. Mycobacterial infections

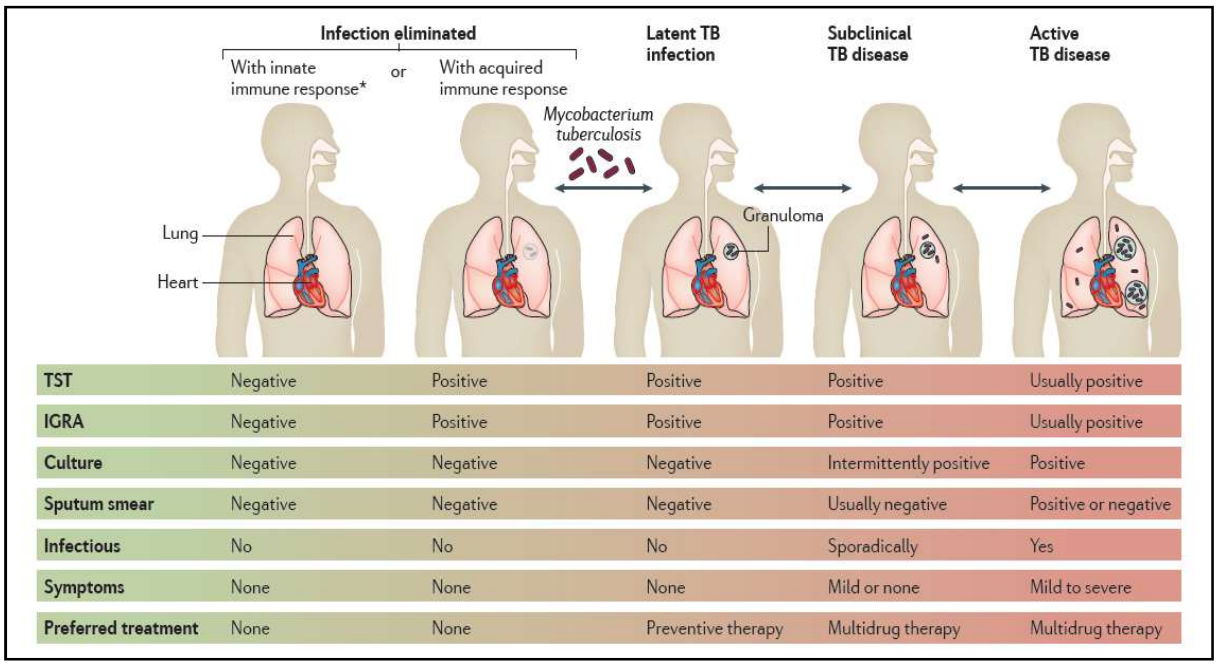
Infection with MTB may exhibit several clinical manifestations [36]:

Primary TB: It is characterized by development of clinical symptoms within the first 1–2 years of infection. On the other hand, infection can also be characterized by chronic and slowly progressive TB in which development of clinical symptoms takes place after more than 2 years of infection. TB infection remains latent and totally asymptomatic in 90% of the cases. The latter two groups of infected individuals constitute the reservoir of MTB.

Secondary TB: Observed mainly in adults due to reinfection or reactivation of previous infection (latent TB), especially when health status of such individuals decline. Generally, the upper lung lobes are most affected, and cavitations can occur.

Isolated occurrence of tuberculosis outside the lungs is termed as extra-pulmonary TB (EPTB) which is more common in children and HIV infected individuals [37]. This often leads to the manifestation of a number of rare findings with characteristic patterns. Skeletal TB also known as Pott's disease, involves mainly the thoracic and

lumbar vertebrae; fallopian tube, prostate and epididymis are involved in genital TB and lymph node TB involves lymph node. Other forms of EPTB are: urinary tract TB, TB of the central nervous system and cardiac TB, etc [38].



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 1.2. Spectrum of TB: From MTB infection to active pulmonary TB disease [39].

1.5. Diagnosis of TB

Prompt diagnosis is the most powerful tool in the control of any disease. Current diagnostic methods available for the detection of TB are given in the Table 1.1 [39].

Test	Assay principle	Use	Sensitivity (%)	Specificity (%)	TAT
		Imaging techniques			
Chest X-ray	Imaging of the lungs	Active TB disease screening	87 (using TB abnormality as a threshold)	89 (using TB abnormality as a threshold)	Same day
		Microscopy			
Conventional sputum smear microscopy	Direct visualization of mycobacteria using light microscopy	Active TB disease diagnosis	32–94	50–99	Same day
LED fluorescence smear microscopy	Direct visualization of mycobacteria using fluorescence microscopy	Active TB disease diagnosis	52–97	94–100	Same day
		Culture-based techniques			
Liquid culture with DST	Mycobacterial culture on liquid media	1. Active TB disease diagnosis 2. Drug resistance	89 (among smear-positive and culture-positive) 73 (among smear-negative and culture-positive)	>99	10–21 days
		Antigen detection techniques			
LAM lateral flow assay	Antigen detection	Active TB disease diagnosis in HIV-positive individuals	44 (all) 1.54 (in HIV-positive individuals)	92 (all) 90 (in HIV-positive individuals)	Same day
		Molecular techniques (nucleic acid amplification tests)			
Xpert MTB/RIF	NAAT (qPCR)	Active TB disease diagnosis Drug resistance (rifampicin)	98 (smear-positive and culture-positive) 67 (smear-negative and culture-positive) 95 (rifampicin resistance)	99 (smear-negative and culture-negative) 98 (rifampicin resistance)	Same day
First-lineLPA (GenoType MTBDR plus and NIPRO)	NAAT (LPA)	Active TB disease diagnosis Drug resistance (isoniazid and rifampicin)	98 (rifampicin resistance) 84 (isoniazid resistance)	99 (rifampicin resistance) >99 (isoniazid resistance)	1–2 days
Second-line LPA (GenoType MTBDRs)	NAAT (LPA)	Drug resistance (fluoroquinolones and second-line injectable drugs)	86 (fluoroquinolone resistance) 87 (second-line injectable drugs)	98 (fluoroquinolone resistace) 99 (second-line injectable drugs)	1–2 days
Loopamp *Mycobacterium tuberculosis* complex assay	NAAT (LAMP)	Active TB disease diagnosis	76–80	97–98	Same day

Table 1.1. Diagnostic modalities reviewed by the WHO for the detection of active TB disease and the drug resistance [39].

1.6. Treatment of TB

As per WHO statistics, 80% of TB patients diagnosed with disease each year are infected with fully sensitive MTB strains and the remaining 20% with drug-resistant strains (13.3% isoniazid mono-resistant and 5.3% MDR) [39]. Treatment regimens according to the WHO recommendations for the management of drug sensitive TB cases are discussed in Table 1.2 and for MDR TB are discussed in Table 1.3

Intensive phase		**Continuation phase**		
Drugs	**Interval and dose**	**Drugs**	**Interval and dose**	**Total doses**
1. Isoniazid 2. Rifampicin 3. Pyrazinamide 4. Ethambutol	Daily for 8 weeks or 5 days per week for 8 weeks	1. Isoniazid 2. Rifampicin	Daily for 18 weeks or 5 days per week for 18 weeks	182 or 130
1. Isoniazid 2. Rifampicin 3. Pyrazinamide 4. Ethambutol	Daily for 8 weeks or 5 days per week for 8 weeks	1. Isoniazid 2. Rifampicin	3 days per week for 18 weeks	110 or 94
1. Isoniazid 2. Rifampicin 3. Pyrazinamide 4. Ethambutol	3 days per week for 8 weeks	1. Isoniazid 2. Rifampicin	3 days per week for 18 weeks	78
1. Isoniazid 2. Rifampicin 3. Pyrazinamide 4. Ethambutol	Daily for 2 weeks, then 2 days per week for 6 weeks	1. Isoniazid 2. Rifampicin	2 days per week for 18 weeks	62

Table 1.2. **Drug regimens for drug-sensitive pulmonary TB.**

Class	Mechanism of action	Drugs	Key adverse events
Group A: fluoroquinolones			
Fluoroquinolones	Inhibition of DNA gyrase	Levofloxacin Moxifloxacin Gatifloxacin*	QTc prolongation (levofloxacin less so than moxifloxacin)
Group B: second-line injectable anti-TB drugs			
Aminoglycosides	Inhibition of protein synthesis	Kanamycin Amikacin Capreomycin (Streptomycin)‡	Nephrotoxicity (all) Ototoxicity (all) Electrolyte derangement (all)
Group C: core second-line agents			
Thioamides	Inhibition of cell wall synthesis	Ethionamide Prothionamide	Nausea and vomiting (all) Hypothyroidism (all)
Oxazolidinones	Inhibition of protein synthesis	Cycloserine Terizidone Linezolid Clofazimine	CNS effects, including psychosis, confusion and depression (terizidone and cycloserine) Peripheral neuropathy (linezolid) Myelosuppression (linezolid) Ocular toxicity (linezolid) QTc prolongation (clofazimine) Skin and conjunctival pigmentation (clofazimine)
Group D: add-on agents			
D1, various classes: isonicotinic acid hydrazide (high-dose isoniazid);	Inhibition of mycolic acid synthesis	High-dose isoniazid	Hepatotoxicity Peripheral neuropathy CNS toxicity
nicotinamide analogue (pyrazinamide);	Disruption of plasma membranes	Pyrazinamide	Hepatotoxicity Gout
aminoalcohols (ethambutol	Inhibition of cell wall synthesis	Ethambutol	Ocular toxicity
D2, various classes: diarylquinoline (bedaquiline);	Inhibition of mitochondrial ATP synthase	Bedaquiline	QTc prolongation Arthralgia Hepatitis Headache
nitro-dihydro-imidazooxazole (delamanid)	Inhibition of mycolic acid synthesis	Delamanid	Nausea Vomiting Dizziness QTc prolongation
D3, various classes: amino-phenol (para-aminosalicylic acid);	Inhibition of DNA precursor synthesis	Para-aminosalicylic acid	Gastrointestinal toxicity
carbapenems	Inhibition of peptidoglycan synthesis	Imipenem plus cilastatin or meropenem plus clavulanate (available orally with amoxicillin)	Seizures
thiosemicarbazone (thiocetazone)	Inhibition of mycolic acid synthesis	Thiocetazone	Severe skin reactions (for example, Stevens–Johnson syndrome and toxic epidermal necrolysis), especially in patients with HIV infection

Table 1.3. First-line and second-line drugs used for the treatment of drug-resistant TB (WHO classification)

1.7. Immunity to mycobacterial infections

1.7.1. Innate immunity

In case of intracellular pathogens like MTB, activation of the host innate immune responses is critical to the initial resistance to infections before the adaptive cell mediated immunity fully develops. Innate immune system provides the first line of defense to any invading pathogen and if activated correctly, it can clear the invading pathogen in many cases. Since MTB has developed several strategies to subvert the innate immune responses to ensure its survival, innate immunity plays a critical role in mycobacterial pathogenesis. Macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells and γδT cells constitute the major cell types of the innate immunity and soluble mediators such as cytokines and chemokines, released by these cells serve as a linker to cell-mediated immunity. During the initial phase of infection, MTB is phagocytosed by resident alveolar macrophages. However, alveolar epithelial type II pneumocytes which outnumber macrophages in the lung alveoli can also ingest MTB [40].

Overall, phagocytic cells play a crucial role in restricting the multiplication and dissemination of intracellular pathogens, as well as initiation and propagation of the adaptive immune response. Additionally, DCs, known to have much better Ag presentation ability than macrophages [41,42], present specific mycobacterial Ag to T cells and thus play an important role in early stages of infection [43]. Various receptors are important for MTB detection and uptake by phagocytes.

1.7.1.1. Receptor mediated detection of MTB

Phagocytosis of MTB can be mediated by several receptors present on the surface of phagocytic cells. In human macrophages, the mannose receptors (MR) and complement receptors 3 (CR3) [44,45] are the primary receptors for MTB recognition

and uptake. Other receptors that interact with MTB are: surfactant protein A and its receptors, scavenger receptor class A, mannose binding lectin, and possibly dectin-1 [46,47]. The mode of entry into macrophages is considered as an important predetermining factor for the subsequent intracellular fate of mycobacteria. Contrary to the human macrophages, human DCs primarily use DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) for mycobacterial detection and uptake, with no significant role for complement or mannose receptors [48]. However, it has been reported that blocking individual receptors does not have significant effect on the intracellular trafficking of MTB [47].

1.7.1.2. Innate defenses to MTB in the air ways: the respiratory mucosa

Once inhaled, MTB reaches to the alveoli in the lung through the trachea, bronchus, and bronchioles. Respiratory mucosa present along the airways forms the first anatomical barrier against MTB [49]. It contains (i) the epithelium, a layer of airway epithelial cells (AECs); (ii) the lamina propria, a layer of connective tissue and immune cells, including lymphocytes and macrophages; and (iii) a coating of a airway surface liquid (ASL) containing mucus, immunoglobulin A and other innate immune factors. Bronchial or nasal associated lymphoid tissues critical to MTB antigen (Ag) sampling, are also present along the airways [50]. MTB is recognized by AECs through their interaction to the pathogen-associated molecular patterns (PAMPs), which induces the expression of cytokines and other chemical mediators leading to the generation of an effective immune response. AECs present the MTB Ag to mucosal-associated invariant T cells (MAITs) [51] which then express interferon (IFN-γ), tumor necrosis factor (TNF-α) and granzyme helping in the eradication of MTB. ASL consist of anti-microbial peptides, such as β-defensin 2 [52], cathelicidin

(LL-37) [53] and hepcidin [54], as well as different cytokines and chemokines secreted by AECs for the recruitment and activation of phagocytes [55].

1.7.1.3. Innate defenses to MTB in the airways: the alveoli

After passing through the upper airways, MTB is delivered to the alveoli which contain type I and type II epithelial cells as well as alveolar macrophages (AMs), DC and neutrophills. In alveoli AM phagocytose MTB however, type II epithelial cells that outnumber the AMs in alveoli may also take up the MTB [56]. Like AECs, type II pneumocytes also produce antimicrobial molecules [52]. Further these cells produce and secrete hydrolases, hydrolytic enzymes and pulmonary surfactant in the extracellular surface of the lung. Surfactant proteins cause agglutination of MTB and increase the phagocytosis by macrophages. Hydrolases can alter the cell wall of MTB and may affect interactions with macrophages and host immune responses [50].

1.7.1.4. Innate defenses to MTB in the airways: resident defenders

Though the number of AMs are relatively low (per alveolus around 10), they have longer life approximately 3 months in humans. AMs are armed with whole range of antimicrobial mechanisms. On the contrary, MTB is also equipped with various mechanisms to evade host anti microbial mechanisms and the outcome of the initial standoff depends on the host microbicidal capacity and the virulence factors of the MTB. If MTB is killed by host then infection is controlled, but in case of ineffective immune response MTB survives, replicates and the infection spreads [50]. Apart from AMs, DCs are also the first cell type to encounter MTB [57]. After engulfing MTB in alveoli, DCs migrate to draining lymph nodes where they mature and present MTB Ag in conjunction with MHC class I and II to T cells [42] and act as a link between the innate and adaptive immune system. Some reports suggest that MTB alters the DC

function and impairs their ability to control infection [58] whereas other reports indicate that DCs are beneficial to bolster the cellular immune response [48]

1.7.1.5. Innate defenses to MTB in the airways: recruited defenders

In active TB patients, neutrophils are predominately infected in the lung airways [59]. These cells play a conflicting role in disease pathology; human neutrophils can either favor or restrict MTB growth. Post MTB infection, neutrophils produce ROI and RNI through respiratory burst and secrete pro-inflammatory cytokines and chemokines which lead to the recruitment and activation of other immune cells [50]. Neutrophils may undergo apoptosis after infection with MTB and these apoptotic neutrophils can be phagocytosed by MTB infected macrophages, leading to the fusion of anti microbial contents of neutrophil granules with MTB containing phagosomes in macrophages, which results into improved killing [60].

Natural killer (NK) cells are active components of innate immune system. During early phase of MTB infection, NK cells are recruited to the site of infection and help in amplifying the innate defense to TB. These cells recognize infected macrophages through receptor molecules such as NKp44, NKp46 and NKG2D [61] and secrete IFN-γ to further activate macrophages, secrete cytokines that expand NKT cell (NKTs) populations and CD8+ T cells [62] and can lyse infected macrophages [63].

Lipid Ag of MTB are presented by CD1a molecules, that are recognized by NKTs and its deficiency is often associated with active TB [64]. Other T cell subsets such as γδT cells are also present in the alveoli and recognize MTB phospho Ag [65] and participate in the killing of infected macrophages through cytotoxic granules.

The details of anti-microbial mechanism implied by the host and the mechanism of subversion of these mechanisms by MTB virulence factors are discussed in next section.

1.7.1.6. Macrophages: Function and activation

Macrophages are large mononuclear cells of the innate immune system. They are capable of engulfing particles larger than 0.5μm, including microbes, thus called as professional phagocytes. In a resting state, the role of the macrophage is to internalize debris and apoptotic cells in a non inflammatory manner [66]. During infection, they ingest and kill pathogens, recruit other cells of the immune system, and present microbial Ag to T cells. Resident macrophages are terminally differentiated and placed at fixed location in the body, where infection can occur, *e.g.* alveolar macrophages are localized in the lungs, kupffer cells in the liver and microglia in the nervous system. Monocytes are the precursor of macrophages which circulate in the blood stream and recruited to the sites of infection or tissue damage when stimulated. Later they are differentiated into a macrophage which can become activated upon microbial or inflammatory stimulation. Activated macrophages, following the stimulation by microbial products, such as LPS, acquire the anti microbial properties required for the elimination of the invader, although some microbes, including MTB, have evolved various mechanisms to circumvent this response [67,68].

1.7.1.6.1. Macrophage polarization

After the stimulation, macrophages are functionally polarized and can adopt a pro or anti-inflammatory profile depending on the nature of stimuli. Functional polarization of macrophages is illustrated in Figure 1.3.

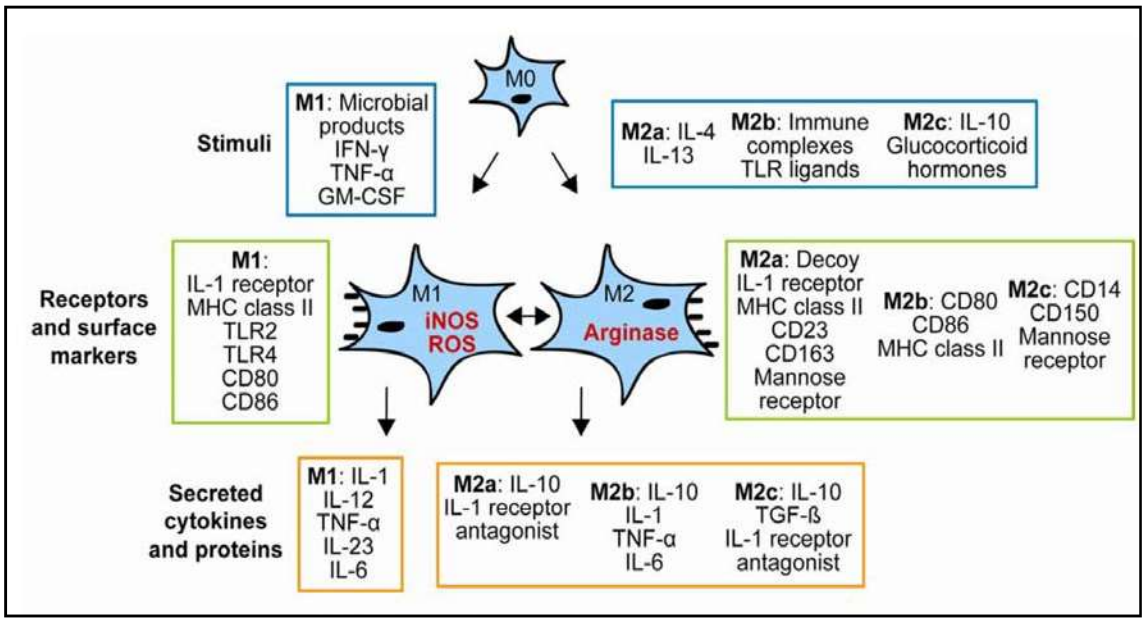


Figure1. 3. Functional polarization of macrophages [67].

Broadly, macrophages are functionally divided in two types: M1, or classically activated macrophages, and M2, or alternatively activated macrophages.

M1 activation is caused by pro-inflammatory or Th1 cytokines (IFN-γ, TNF-α, GM-CSF) and microbial products like LPS. M1 macrophages exhibit microbicidal properties and secrete inflammatory cytokines like IL-1, IL-12, TNF-α, IL-23, IL-6. Further, they have enhanced expression of MHC class II, inducible nitric oxide synthase (iNOS or NOS2) and produce ROS. On the other hand, M2 polarization is induced by anti-inflammatory or Th2 cytokines (IL-4, IL-13, M-CSF, IL-10). M2 macrophages have regulatory properties and are not microbicidal. It has been reported that macrophage polarization process is highly plastic and reversible, the same macrophage can participate in both induction and resolution of inflammation [69]. Furthermore, the M2 macrophages have been classified into three subsets, having immunoregulatory properties, as well as roles in angiogenesis, tissue remodeling and repair. M2a macrophages are induced by IL-4 or IL-13, M2b by immune complexes and TLR agonists and M2c by IL-10 and glucocorticoid hormones. M2a and M2c

macrophages secrete anti-inflammatory cytokines such as IL-10 or TGF-β, as well as different chemokines, and IL-1 receptor antagonist. M2a also express decoy IL-1 receptor and MHC class II. M2b, on the other hand, display an intermediate phenotype producing IL-10, IL-1, TNF-α, and IL-6, but contribute in immunoregulation despite production of pro-inflammatory cytokines [67,70-72]. It is becoming evident that these subtypes represent a continuum of differently activated macrophage phenotypes [67]. Thus, the macrophage is a dynamic cell with multiple complex roles in different immune processes, and the type of activation can have a great impact on the outcome of infection.

1.7.1.7. Dendritic cells

Steinman and Cole discovered Dendritic cells in 1973, from cells of mouse peripheral lymphoid organs [73]. The term dendritic cell is attributed to the characteristic long cytoplasmic processes, or dendrites of these cells. Dendritic cells are differentiated from mouse bone marrow cells and human monocytes, using the cytokine GM-CSF during *in vitro* studies [74,75]. In the lungs, 80% of dendritic cells are found in the sub epithelial regions while the remaining 20% are located in the interstitial spaces [76,77]. One to five percent of intraepithelial dendritic cells are found to extend their dendrites between the epithelial cells, without disturbing the epithelial barrier, in order to sample the Ag in the airway lumen [77,78]. Exposure to Ag with or without inflammation leads to the migration of dendritic cells to the lymph nodes where they can relay innate signals to the adaptive immune cells [77,79]. Though macrophages also migrate to the lymph node after Ag exposure, they appear to be less efficient than dendritic cells at transporting particles [79]. After exposure to the pathogens, dendritic cells undergo morphological and functional differentiation from immature to mature phenotype followed by migration to the lymph nodes. Characteristics of immature

dendritic cells include their high proliferation rate, low motility with high expression of CCR1, CCR2 and CCR5, high phagocytic potential and Ag processing and low surface expression of the costimulatory molecules CD80, CD86, MHC II and CD40 [75,80,81]. After stimulation with inflammatory mediators, such as TNF-α, uptake of particles and binding of PAMPs leads to the maturation of dendritic cells, resulting into lower proliferation rates, high motility with down regulated expression of CCR1, CCR2 and CCR5, and up-regulated expression of CCR7, lowered phagocytosis and Ag processing and up-regulated expression of costimulatory molecules CD80, CD86, MHC II and CD40 [75,80-82]. Expression of IL-12, the critical cytokine involved in promoting Th1 polarization, is also up-regulated, but only in those dendritic cells that come into direct contact with PAMPs [83]. It is speculated that the dendritic cells activated indirectly by exposure to inflammatory mediators or particle uptake are likely to present self Ag for induction of self tolerance, an important feature of the immune system that prevents destruction of self tissues [83]. *In vitro* studies suggested that dendritic cells secrete the cytokines TNF-α, IL-1 and IL-10, chemokines MCP-1 and RANTES in response to MTB infection [57,84-86]. Clinical strains of MTB impair the functions of dendritic cells like maturation, cytokine secretion and Ag presentation [87]. An in-depth analysis of the tissue localization after aerosol infection of mice with MTB expressing green fluorescent protein (GFP), demonstrated that dendritic cells are the predominantly infected cells in the lungs, even though they represent only a small percentage (6.8%) of the total cells in the lungs [88]. In addition, dendritic cells are the predominant infected cell type in the lymph node at days 14 to 28 post infection, with a peak at day 21, corresponding to activation of the adaptive immune response, that is followed by a drastic decline in the number of infected lymph node dendritic cells. The number of infected dendritic

cells in the lungs, however, continues to increase, demonstrating that dendritic cell migration to the lymph node is only transient. Infected macrophages are present in the lung and the lymph node as well, but at significantly lower numbers than dendritic cells. Comparison of the number of bacteria per cell in macrophages versus dendritic cells, demonstrates that approximately 60% of the macrophages as against 32% of dendritic cells have five or more intracellular bacteria. These data coincide with other studies showing that MTB is unable to replicate in dendritic cells [48,82]. It has been suggested that the dissimilar intracellular behavior of MTB in macrophages and dendritic cells may be due to differences in the receptors involved in bacterial uptake; macrophages predominantly use MRs and CRs, whereas DC-SIGN has been found to be the major MTB receptor on dendritic cells, recognizing mannose-rich molecules within the mycobacterial envelope, such as Man-LAM [48,89].

1.7.1.8. Toll-like receptors

When the body is invaded with any pathogen, recognition of specific molecular patterns conserved among the microorganism known as pathogen associated molecular patterns (PAMPs) by germ line encoded receptors, the pattern recognition receptors (PRRs), is critical to the initiation of innate immune response [90]. Once the PRR recognizes PAMP, it initiates a series of signaling cascade leading to the initiation of first line of host defensive immune responses responsible for neutralization of infectious microbes. Further, PRR signaling mediates simultaneous maturation of dendritic cells (DCs), which connects the innate signals to adaptive immune system and induces second line of host defense immune responses.

Among PRRs, Toll-like receptors (TLRs) were the first to be identified. TLRs are the most studied and well characterized PRRs with a wide range of PAMPs [91-94]. TLRs are type I transmembrane proteins containing an ectodomain, which mediates

the recognition of PAMPs through leucine-rich repeats, a transmembrane region, and cytosolic Toll-IL-1 receptor (TIR) domains involved in the activation of downstream signaling pathways. TLRs are expressed on the cell surface as well as in the intracellular vesicles. Till date, 10 and 12 types of TLRs have been identified in human and mouse, respectively. Each TLR recognizes specific PAMPs derived from infectious agents like viruses, bacteria, mycobacteria, fungi, and parasites. TLR1, TLR2, and TLR6 recognize lipoproteins, TLR3 recognizes double-stranded (ds) RNA. Lipopolysaccharide (LPS) is detected by TLR4, flagellin by TLR5, single-stranded (ss) RNA by TLR7 and TLR8, and CpG DNA is detected by TLR9 (Table 1) [91]**.** After recognition of respective PAMPs, TLRs initiate a signaling cascade for the generation of appropriate immune response. TLRs recruit adaptor molecules containing TIR domain, such as MyD88 and TRIF which initiate downstream signaling events leading to the secretion of type I IFN, chemokines, inflammatory cytokines, and antimicrobial peptides [95]. TLR mediated immune responses induce the activation of macrophages recruitment of neutrophils, and induction of IFN-stimulated genes, leading to the direct killing of the infected pathogens. Moreover activation of TLR activation causes maturation of DCs thus helps in the induction of adaptive immunity.

Although TLRs, the most studied PRR are required for the initiation of immune responses, other PRRs such as membrane-bound C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are also involved in PAMP recognition and induction of innate immune responses [96-98]. Microbial pathogens consist of several kinds of PAMPs which are recognized by multiple PRRs. Further same PAMP can be recognized by different PRRs.

Species	PAMPs	TLR Usage	PRRs Involved in Recognition
Bacteria, mycobacteria	LPS	TLR4	
	lipoproteins, LTA, PGN, lipoarabinomannan	TLR2/1, TLR2/6	NOD1, NOD2, NALP3, NALP1
	flagellin	TLR5	IPAF, NAIP5
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI, IFI16
	RNA	TLR3, TLR7, TLR8	RIG-I, MDA5, NALP3
	structural protein	TLR2, TLR4	
Fungus	zymosan, β-glucan	TLR2, TLR6	Dectin-1, NALP3
	Mannan	TLR2, TLR4	
	DNA	TLR9	
	RNA	TLR7	
Parasites	tGPI-mutin (*Trypanosoma*)	TLR2	
	glycoinositolphospholipids (*Trypanosoma*)	TLR4	
	DNA	TLR9	
	hemozoin (*Plasmodium*)	TLR9	NALP3
	profilin-like molecule (*Toxoplasma gondii*)	TLR11	

Table 1.4. Detection of PAMPs with TLRs and PRRs [90].

1.7.1.8.1. Cellular Localization of TLRs

TLRs are expressed on the cell surface as well as within the intracellular vesicles.

TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the cell surface and involved in the recognition of microbial membrane components whereas expression of TLR3, TLR7, TLR8, and TLR9 is localized within intracellular vesicles and they recognize nucleic acids [99]. TLR11 is also expressed in intracellular compartments [100]. Expression of TLR13 is also localized in the intracellular vesicles although its cognate PAMP is yet to be identified [99]. Intracellular vesicles within innate immune cells,

harboring TLR3, TLR7, TLR8, and TLR9 include the endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes [99]. Nucleases rapidly degrade the nucleic acid present in the extracellular environment. After the uptake of viruses and other pathogens by immune cells, their nucleic acid is delivered to the intracellular compartments where they are recognized by vesicular TLRs. Therefore, intracellular localization of TLRs avoid the risk of the initiation of autoimmune diseases due to the recognition of ‘‘self’’ nucleic acids.

1.7.1.8.2. TLR Signaling Pathways

Individual TLRs present in various cell types such as macrophages (MP), conventional DC (cDC), plasmacytoid DC (pDC), lamina propria DC (LPDC), and inflammatory monocytes (iMO) initiate overlapping and distinct signaling pathways [90]. PAMP recognition by TLRs leads to the conformational changes in them resulting into homo or heterophilic interactions of TLRs and recruitment of various adaptor proteins like MyD88, TIRAP, TRIF, and TRAM [95]. TLR5 uses MyD88 adaptor protein for downstream signaling and activates NF-kB through IRAKs, TRAF6, TAK1, and IKK complex leading to the induction of inflammatory cytokines. In macrophages and cDCs, TLR1-TLR2 and TLR2-TLR6 form heterodimers culminating into the NF-kB activation through recruitment of adaptor protein TIRAP and MyD88. In iMO, TLR2 expression is localized within the endosome and through recruitment of IRF3 and IRF7 it induces type I IFN in response to viruses [90]. TLR4, which is expressed on the cell surface, initially recruits TIRAP and MyD88 which leads to the early phase activation of NF-kB. Later TLR4 is transported into Rab11a positive, bacteria containing phagosomes, followed by recruitment of TRAM and TRIF [90,101]. This leads to the late-phase NF-kB activation as well as activation of TRAF3-TBK1-IRF3 axis for the induction of type I IFN. TLR4 mediated induction of

inflammatory cytokines requires both early and late phase activation of NF-kB. All TLRs except TLR3 utilize MyD88 adaptor protein for signal transduction which leads to the induction of inflammatory cytokines by activation of NF-kB and MAP kinase. TLR3, TLR7, and TLR9 are expressed in ER and later trafficked to the endosomal compartment where they engage with their ligands. TLR3 engagement leads to the activation of the TRIF-dependent pathway leading to the induction of type I IFN and inflammatory cytokines in macrophages and cDCs. TLR7 and TLR9 activate NF-kB and IRF7 through MyD88 dependent manner in pDCs to induce inflammatory cytokines and type I interferon, respectively [102,103]. TLR7 and TLR9 induce inflammatory responses in cDCs and macrophages by activating NF-kB in MyD88 dependent manner but fail to activate IRF7.

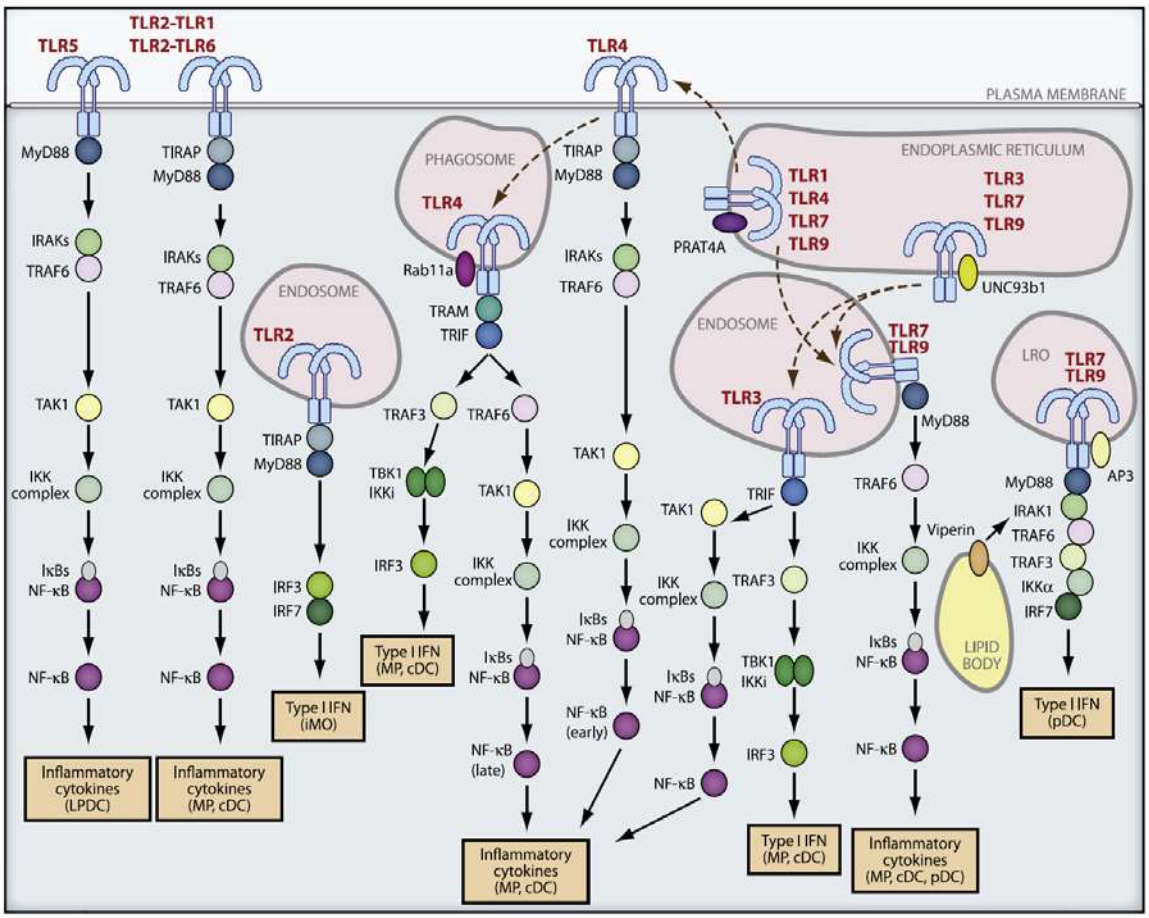


Figure 1.4. TLR trafficking and signaling [90].

1.7.1.8.3. Interaction of MTB and TLR

TLRs represent key receptors for the detection of mycobacterial Ag and subsequent stimulation and activation of various cells of innate immunity including macrophages and dendritic cells [89,104,105]. Engagement of TLRs with their respective mycobacterial ligands facilitates production of anti mycobacterial effector agents such as microbicidal peptides, e.g. defensins, ROI, and RNI. TLR2 after binding to the p19 lipoprotein induces cathelicidin (LL37), an anti mycobacterial peptide, by modulating the vitamin D signaling [106]. Polymorphism in TLR2 is associated with enhanced susceptibility to tuberculosis suggesting the importance of TLR2 in immune response against MTB [107]. Various mycobacterial components are detected by TLR2 in combination with either TLR1 or TLR6, and also by TLR4 and TLR9 [108-110]. Studies involving TLR2 knockout mice have exhibited defective granuloma formation and elevated vulnerability to high-dose MTB infection [107,111]. TLR2 agonists present in the MTB include arabinose capped lipoarabinomannan (AraLAM), mannosylated lipoarabinomannan (Man-LAM), 19-kDa lipoprotein Ag of MTB, mycobacterial glycolipid, phosphatidylinositol dimannoside (PIM), lipomannan (LM) and trehalose 6,60-dimycolate (TDM and a heat-stable and protease-resistant culture filtrate component of MTB [107]. TLR4 agonists of MTB include heat shock protein 60, 65, and 70 and 38-kDa lipoprotein (PhoS1) [107]. TLR signaling after engagement with mycobacterial TLR agonist occurs via the MyD88 as a common adapter. It recruits IL-1 receptor associated kinases (IRAK), TNF receptor associated factor-6, transforming growth factor β (TGF-β)-activated protein kinase 1(TAK1) that activates mitogen activated protein kinases (MAPKs) and transcription factors like activator protein 1 (AP-1) and nuclear factor kB [112].This signaling cascade results into the expression of proinflammatory cytokines such as IL-1, IL-6, IL-8, IL-12,

TNF-α, chemokines, NO and induction of costimulatory molecules [113]. These cytokine along with MTB Ag help in the priming of CD4 T cells which secrete interferon-γ (IFN-γ) responsible for enhanced Ag presentation on APCs by up-regulation of major histocompatibility complex (MHC) class II expression [114]. Further TIRAP/MyD88 adapter like (Mal) another adapter protein, has also been involved in MyD88 dependent activation in the TLR2, TLR4 and TLR9 signaling pathways [115]. A MyD88 independent pathway which uses TRIF related adapter molecule (TRAM) and TIR domain containing adapter inducing interferon-β (TRIF) has also been reported to induce type I IFNs in the TLR4 signaling pathway [115,116]. It has also been shown that TLR9 detects CpG DNA from mycobacteria and induces type 1 IFNs that enhance MHC class I Ag cross processing [117]. Specific functions of individual TLRs and various signaling systems involved in detection of agonists of MTB origin are summarized below.

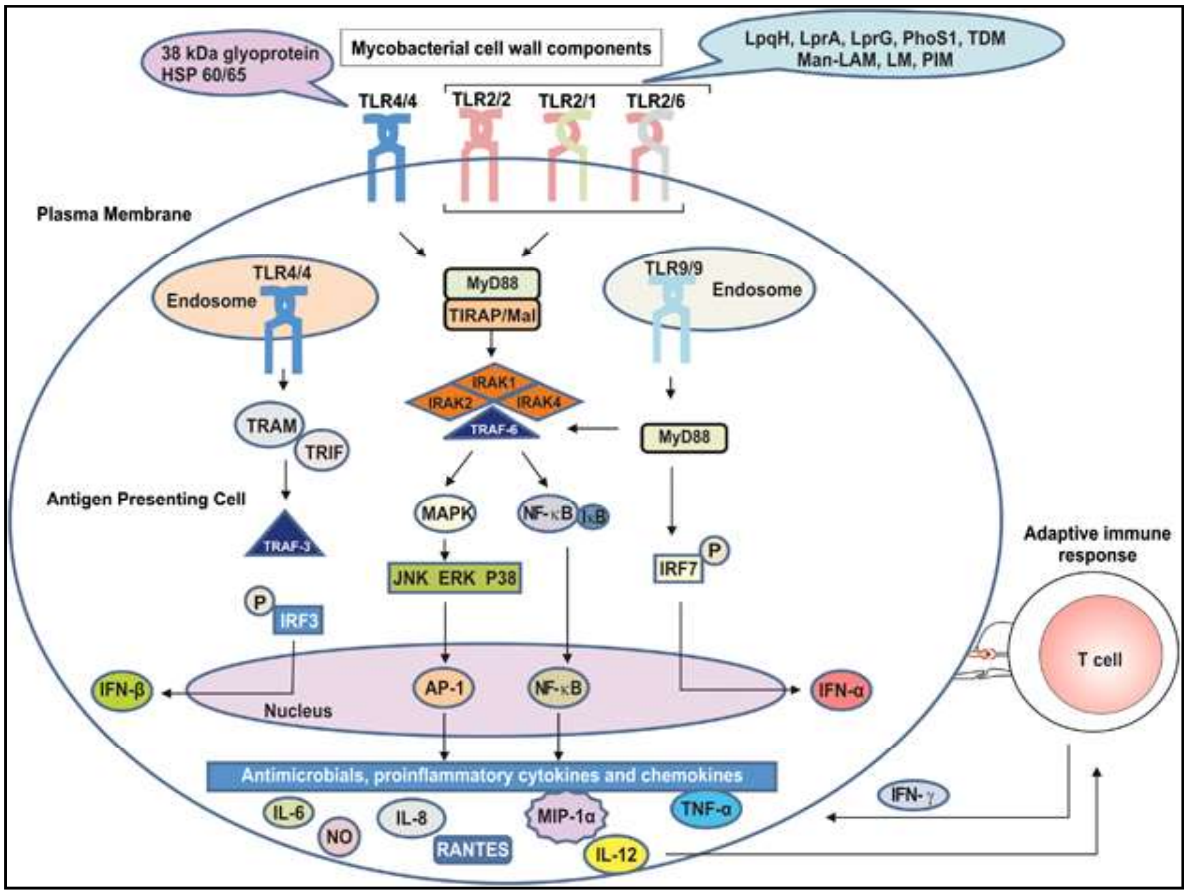


Figure 1.5. Schematic representation of MTB and TLR interaction [107].

1.7.2. Adaptive immunity to MTB

Role of T lymphocytes rather than B cells and antibodies in adaptive immunity of tuberculosis have been emphasized in scientific investigations. Compared to other pathogens, the time required in the initiation of MTB specific CD4T cell response is delayed in humans and mouse which is attributed to the long lag between time of infection and the migration of MTB infected DCs from the lungs to the local lymph node, where they are recognized by naive CD4 T cell. This delay gives the bacteria a survival advantage over the host by allowing the growth of MTB population in lungs. CD4 and CD8 T cells constitute the adaptive immune response in tuberculosis.

1.7.2.1. CD4 T Cells

CD4 T cells, essential part of adaptive immunity to TB in humans and mice, are activated by the recognition of peptide Ag presented with MHC/HLA class II molecules. Importance of CD4 T cells in TB is evident from the fact that in HIV infected individuals the rate of progression from latent to active TB increases by 5 to 10 fold and only modest reductions in CD4 T cell counts are associated with increase in the incidence of TB [118]. However, treatment of HIV with antiretroviral therapy results into decrease in the incidence of TB [118]. In mice, depletion of CD4 T cells leads to higher bacterial burden, poor control of infection and accelerated mortality [114]. Among CD4 T cells, Th1 cells are the best characterized cells and their development and maintenance is dependent on the expression of cytokine IL-12 and the transcription factor T-bet [118,119]. Th1 cells secrete mainly IFN-γ, however TNF, IL-2, lymphotoxin, and certain CCL chemokines are other soluble mediators secreted by Th1. IFN-γ helps in the macrophage activation resulting into an enhanced antimicrobial activity of macrophages. Further, macrophages activated by IFN-γ

express MHC class II, act on non hematopoietic cells to regulate IL-17-mediated inflammation at the site of infection [118]. Despite the immense importance of IFN-γ in immunity to TB, its secretion by CD4 T cells is a poor correlate of protection in humans infected with MTB [120]. Th17 cells which secrete IL-17, have recently gained importance in TB immunity. Th17 cells play a critical role in the establishment of mature granuloma in BCG inoculated mice and it augments responses to a protein subunit TB vaccine in mice [121]. Regulatory T cells (Tregs) are also emerging as important constituents of TB immunity and expression of cytokine TGF β and the transcription factor FoxP3 is crucial for their development. Tregs are found at high frequencies in humans with active TB disease [122] and are more frequent in tissue sites in miliary TB than in pleural TB [123], although it is not known whether this greater frequency is the cause or the consequence of widespread infection in miliary TB.

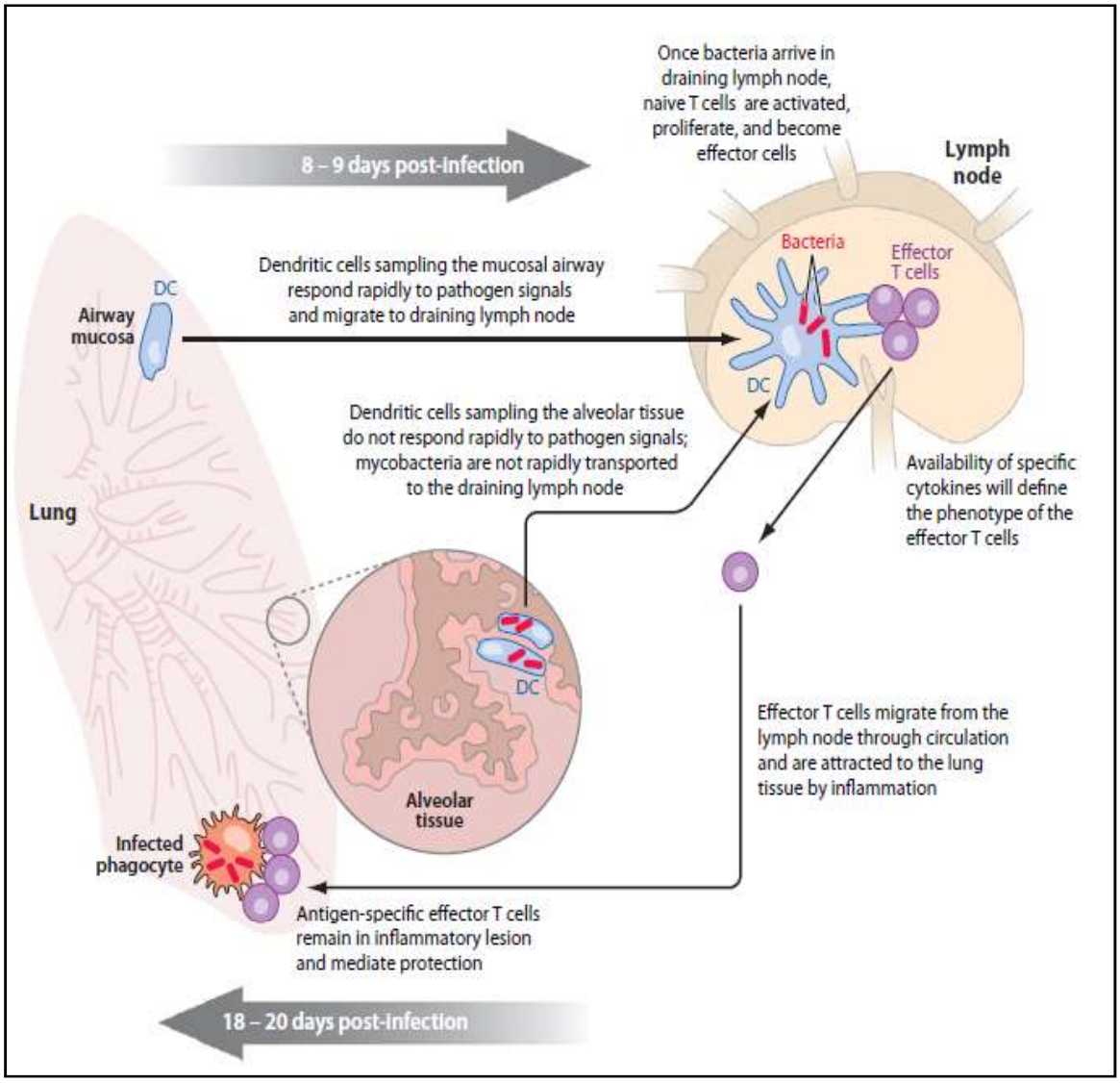


Figure 1.6. Schematic diagram of initiation of effector immune response after MTB infection [124].

Low-dose aerosol infection, which deposits low numbers of MTB (red) in the lower airways. After 9 days post infection MTB is detected in draining lymph. The dissemination of MTB and activation of naïve T cells (purple) occur at the same time The delayed dissemination of MTB suggests that either dendritic cells migration is inhibited by MTB or the infected macrophages can't migrate rapidly to the lymph node.Migration of the effector cells to the lung occurs due to the inflammation and by activating infected phagocytes (pale red) it protects them. The initial response reaches to effective level in 18-20 days [124].

1.7.2.2. CD8 T Cells

CD8 T cells recognize MTB peptide Ag presented with MHC I/HLA class Ia molecules (HLA A, B, or C in humans). MTB Ag specific CD8 T cells secrete cytokines such as IFN-γ after stimulation and can lyse MTB infected Ag-presenting cells [125]. Human CD8 T cells can also recognize MTB peptide Ag presented by the nonpolymorphic class Ib molecule HLA-E [126,127], and the aforementioned MAITs can express CD8. Recent reports have suggested that apoptosis and subsequent cross-presentation pathways in which uninfected DCs phagocytose the apoptotic vesicles containing MTB Ag and present them to CD8 T cells on MHC/HLA class I. It has been reported that in mice, enhancement of apoptosis of MTB infected cells *in vivo* leads to more robust expansion and stimulation of CD8 T cells, which lends strong support to a role for apoptosis and cross-presentation in immunity to TB. Further MTB specific CD8 T cells cause the death of MTB infected cells by induction of apoptosis or through granulysin and perforin mediated pathways. Hence, vaccines with potential to evoke strong CD8 T cell response are desirable for the efficient protection against TB.

1.7.2.3. Granulomas

After successful MTB infection, innate and adaptive cells of immune system are recruited to the site of infection under the influence of cytokines. This marks the initiation of granuloma which represents the intersection of innate and adaptive immunity. Studies suggest that granulomas may be beneficial for both host and pathogen [118].

1.7.2.3.1. Cellular Composition of Granulomas

Macrophages, the sine qua non of granulomas, comprise of various phenotypes such as mature macrophages, foamy macrophages, differentiated or epithelioid

macrophages, and multinucleated (or Langhans) giant cells (MGCs) macrophages [128,129]. Zebrafish is a well established model to study the granuloma formation and transparent zebrafish embryos infected with *M. marinum* have revealed that macrophages are sufficient for the initiation of granulomas [130]. Similarly, an *in vitro* system involving human peripheral blood cells and *M. tuberculosis* has also confirmed the indispensible role of macrophages in the early stages of granuloma and MGC formation [131]. Foamy macrophages, which contain large amounts of lipids, are present in granulomas in human and murine TB [132]. MGCs are another phenotypic variant of macrophage present in the granuloma induced by virulent MTB but not avirulent mycobacteria (e.g. *M. smegmatis*). Formation of MGCs is promoted by mycobacterial glycolipids: PIMs, lipomannan (LM), and TDM whereas phosphatidylinositol and Man-LAM do not promote MGC formation. MGCs are formed by the homotypic fusion of macrophages mediated by fusion-regulatory protein 1 (also known as CD98). Like mature DCs, MGCs also exhibit lower phagocytosis with higher expression of MHC class II molecules [133]. Apart from macrophages, DCs are also one of the critical components of granulomas. They resemble macrophages but owing to their ability to prime naïve T cells, DCs present the Ag to T cells. T lymphocytes are also present in mature granulomas and play a critical role in the maintenance of organized granulomas and the control of mycobacterial progression after the acute phase of infection. In human TB granulomas, T lymphocytes, typically 60–70% CD4+ [134] are either diffusely dispersed among other cells or may be present at the periphery of granulomas as a discrete band of cells [135]. TB granulomas also contain B cells although its role is poorly understood [135], and recent reports have suggested their immunoregulatory role during chronic infection with MTB [136].

Due to the ability of granulomas to contain the infection and accumulation of immune cells, they represent equilibrium between host and the pathogen by providing benefit to both. Recent studies reported that early granuloma formation is beneficial for the pathogen as macrophages recruited at the site serve as additional sanctuaries for MTB growth [137]. Granulomas provide sites for interaction between MTB infected macrophages and DCs, and effector T lymphocytes which allows the immune control of mycobacterial infection.

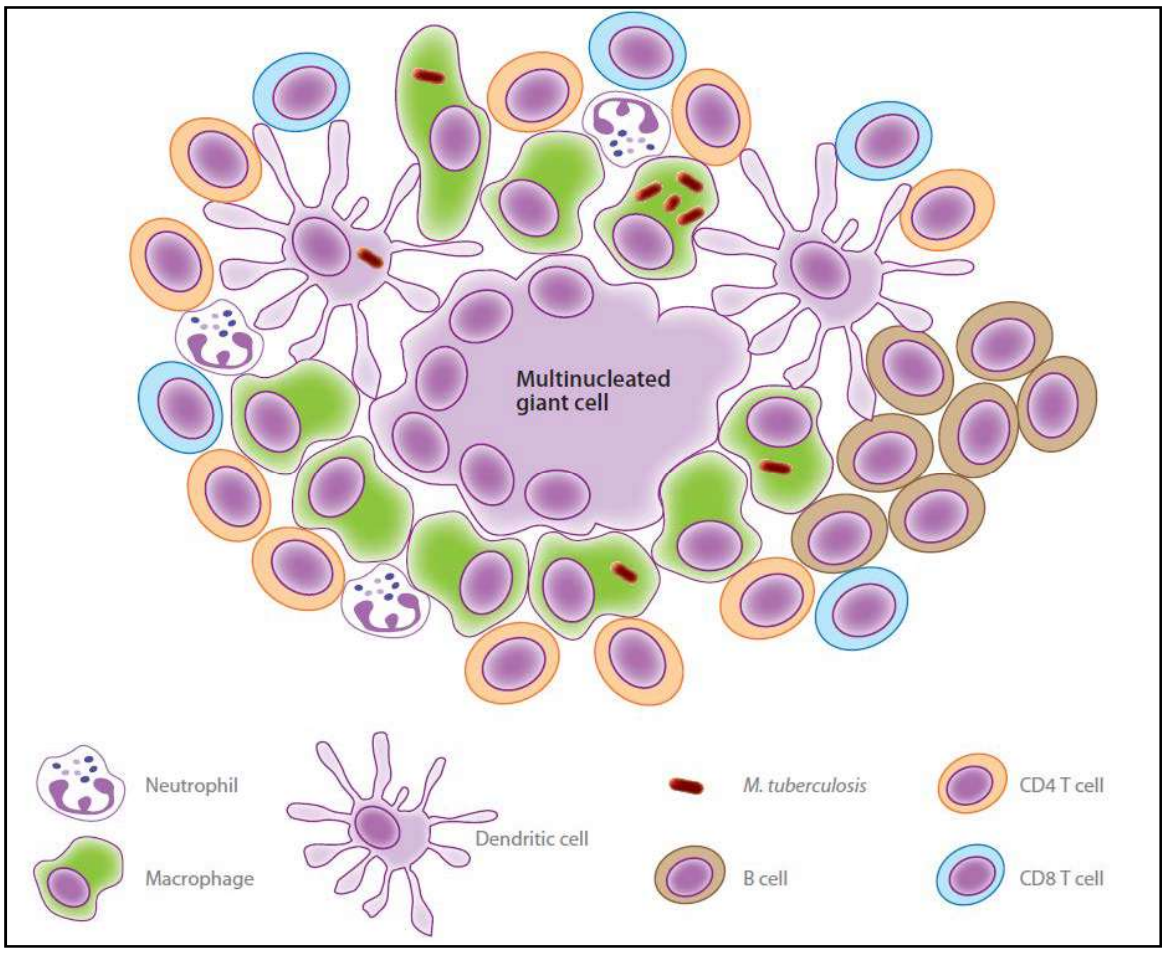


Figure 1.7. The cellular composition of a representative MTB granuloma [118].

1.8. Immune evasive mechanisms

MTB invades, resides and replicates in macrophages, the main cells of innate immune system with the primary function to neutralize the pathogenic microorganisms, through perturbation of several of immune strategies [138]. MTB uses non-activating complement receptors (CR) to gain entry into macrophages, since engagement of these receptors does not induce the release of cytotoxic reactive oxygen intermediates (ROI) [139]. The adaptability of virulent mycobacteria to the hostile intracellular environment of macrophages by perturbing its microbicidal function has been the key to its success as a pathogen. The pathogenic mycobacteria subverts several host processes for its survival inside the host including fusion of phagosomes with lysosome, Ag presentation, apoptosis and the stimulation of bactericidal responses due to the activation of pathways involving mitogen-activated protein kinases (MAPKs), IFN-γ and calcium (Ca2+) signaling [138,140]. The modulation of host signaling mechanisms by bacterial pathogens is a dynamic process requiring several virulence mediators that interfere with these pathways. Mycobacteria release a heterogeneous mixture of lipids and glycolipids into the cytoplasm of the host in a vesicle-bound form where they accumulate in late endosomal/lysosomal organelles [141]. Lipoarabinomannan (LAM), a cell wall glycolipid is the best studied mycobacterial virulence factor, which is anchored to the cell wall by its phosphatidylinositol moiety [142]. LAM is usually present in two variants: man LAM which is abundant in slow-growing pathogenic mycobacteria, such as MTB, and Ara LAM which is abundant in non-pathogenic mycobacteria. Other virulence factors produced by Mycobacteria include eukaryotic-like kinases and protein tyrosine phosphatases [138].

1.8.1. Inhibition of phagosome maturation by mycobacteria

Phagosome maturation is associated with a series of sequential fusion events with various vesicles from the endocytic pathway, which helps the nascent phagosomes attain microbicidal properties and convert into phagolysosomes. Phagolysosomes are acidic in nature and contain many hydrolytic enzymes which help in digestion of engulfed bacteria and other ingested particles [138]. During the initial events of phagocytosis, phagosome acquires Rab5 (a small GTPase) and EEA1 (early endosomal Ag 1) which mediate the fusion of phagosomes with early endocytic vesicles [143]. Late phagosomes are characterized by the acquisition of vacuolar proton ATPase molecules which result into the acidification of phagolysosmes [144]. Further it acquires other lysosmal markers including lysosome-associated membrane protein 1 (LAMP1), and acid hydrolysases, such as cathepsin D.

The mycobacteria containing phagosome, while connected to the endocytic pathway, does not fuse with lysosomes or mature into phagolysosomes [145,146]. MTB harboring phagosomes, inhibit the incorporation of proton ATPase in the vacuolar membrane resulting into the reduced level of acidification which ensures the intracellular survival and growth of mycobacteria [146]. Mycobacteria retain the important host protein termed tryptophan aspartate containing Coat protein (TACO), also known as coronin 1 on the phagosome, which behaves as self Ag. TACO imparts non fusogenic properties to the phagosomes and thus retention of TACO prevents phagosomes from fusing with lysosomes, thereby ensures long-term survival of bacilli within the phagosome [146].

Other mechanism implied by mycobacterium to inhibit the phagosome lysosome fusion involves alterations in the expression of early endosomal markers, such as Rab5 and EEA1, which are normally lost from the intermediate phagosome followed

by their fusion with late endosomes which results into the acquisition of a second GTPase, Rab7. Later late phagosomes fuse with lysosomes to form phagolysosomes, which are characterized by an acidic pH and the presence of hydrolytic proteases, such as cathepsin D. The whole process takes less than one hour. In phagosomes harboring mycobacteria Rab5 is retained and Rab7 is selectively excluded from the phagosomal membrane [147]. Furthermore, EEA1 recruitment in the phagosomal membrane which is essential for the fusion of phagosomes with lysosomes, is blocked thereby inhibiting the overall phagosome lysososme fusion [148].

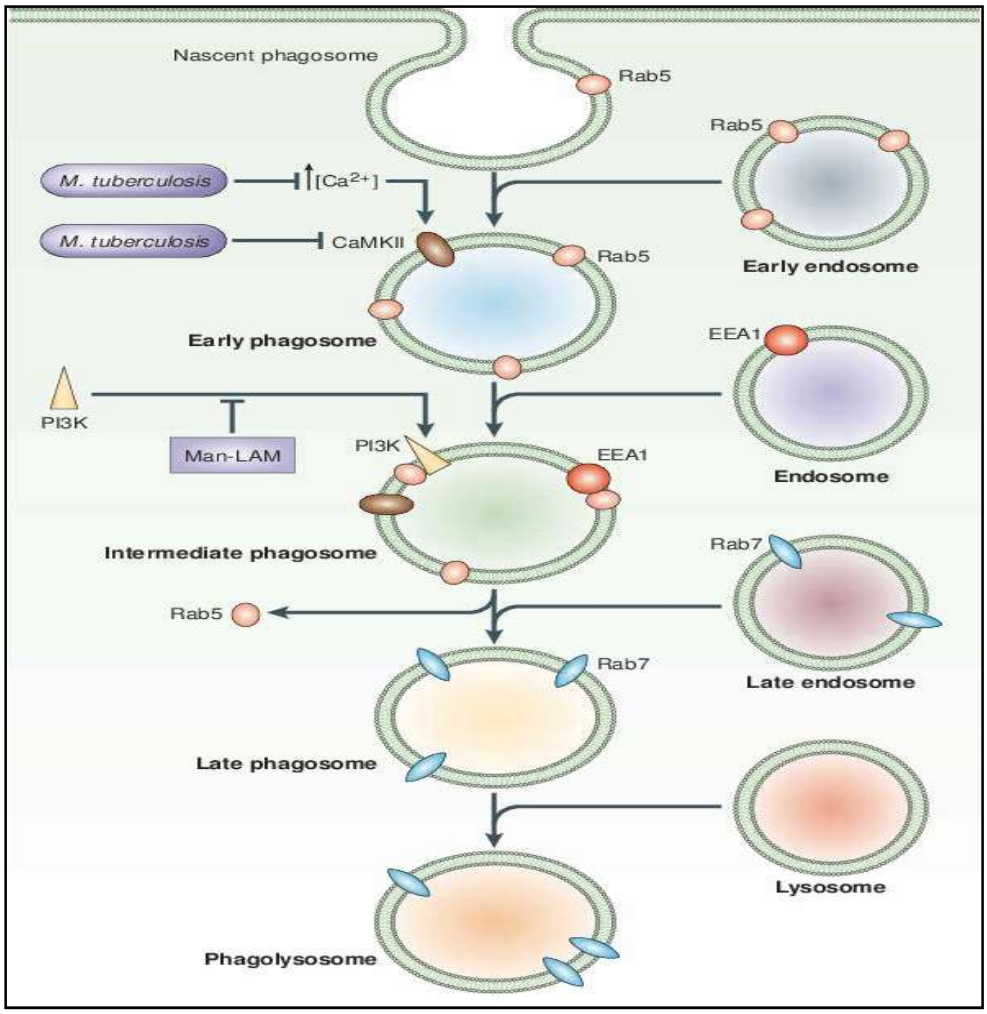


Figure 1.8. Inhibition of phagosome maturation by MTB [138].

1.8.2. Alteration of Ca^{2+} signaling

Ca^{2+} plays an important role in phagosome maturation as it recruits and activates PI3K by activation of calmodulin and the calmodulin-dependent protein kinase CaMKII. Man-LAM (mannose-capped lipoarabinomannan) from MTB blocks the rise in cellular Ca^{2+} concentration [Ca^{2+}] thus inhibits the Ca2+/calmodulin mediated recruitment of PI3K to the phagosomes and thereby further hampers phagosomal maturation. By blocking the delivery of MTB containing phagosomes to lysosomes, the mycobacterium escapes the acidic proteases of lysosomes; avoids exposure to the antibacterial mechanisms within lysosomes; prevents its degradation in the lysosome and hence subverts processing and presentation of mycobacterial Ag to the immune system, leading to suboptimal or delayed immune response[149] .

1.8.3. Modulation of Ag presentation

MTB infected macrophages, process and present the mycobacterial Ag to CD4+ T cells. This phenomenon depends on expression of major histocompatibility complex (MHC) class II on macrophages and its expression is up-regulated upon activation with IFN-γ. MTB inhibits the presentation of mycobacterial Ag to T-helper cells through the inhibition of expression of MHC-II expression, and thus avoids its immune elimination and ensures its survival inside macrophages [150-153]. Viable MTB bacilli are not required for the down regulation of MHC II expression or Ag processing and can be achieved by exposure to bacterial lysate [150,151,154]. MTB expresses several proteins which are attributed to such immune inhibitory effects, one of them is 19-kDa lipoprotein (19-kDa), which has been reported as the predominant ligand involved in inhibiting MHC II expression and Ag processing in TLR2 dependent manner [155]. Several authors have reported that MHC-II dependent Ag presentation as well as the expression of several interferon gamma (IFN-γ) responsive

genes, including MHC class II transactivator (CIITA) and MHC II are inhibited by 19-kDa in a TLR2 dependent manner [156-158].

1.8.4. Alteration of host apoptotic pathways by MTB

Macrophages when infected with pathogenic bacteria activate their apoptotic programme to control the infection. However, many bacterial pathogens including MTB alter host apoptotic pathways to prolong their survival. Virulent strains of MTB exhibit lower level of apoptosis compared to attenuated strains of MTB [159,160]. Mycobacterial virulence factors modulate macrophage apoptosis through several mechanisms. Man-LAM subverts the macrophage apoptosis by preventing the rise of cellular Ca^{2+} which facilitates apoptosis by altering the mitochondrial membrane permeability and thereby promoting the release of pro-apoptic factors such as cytochrome *c* [138]. Man-LAM also stimulates the phosphorylation of the apoptotic protein Bad, which interferes with its binding to the anti-apoptotic proteins Bcl-2 and Bcl-XL [161]. MTB also interferes with macrophage apoptosis through the induction of IL-10 which blocks the production of TNF-α, an inducer of apoptosis in infected macrophages. IL-10 inhibits TNF-α activity by inducing the release of the soluble TNF receptor type 2 protein (TNFR2), which forms an inactive complex with TNF-α that prevents the induction of TNF-α mediated apoptosis [162]. Mycobacterial lipid molecules also modulate host cells death and the outcome of this determines the antimycocterial effects of cell. Attenuated MTB strains enhance the production of prostaglandin E2 (PGE2). PGE2 helps in the maintenance of mitochondrial potential by protecting the inner mitochondrial damage. This process prevents the induction of necrosis of infected cells and induces apoptosis which is an innate defense mechanism. On the contrary, virulent MTB strains inhibit biosynthesis of PGE2. In the absence of PGE2, the infected macrophage has higher probability to undergo

necrosis, a form of cell death that helps the bacterium to evade innate immunity and adaptive immunity [160,163].

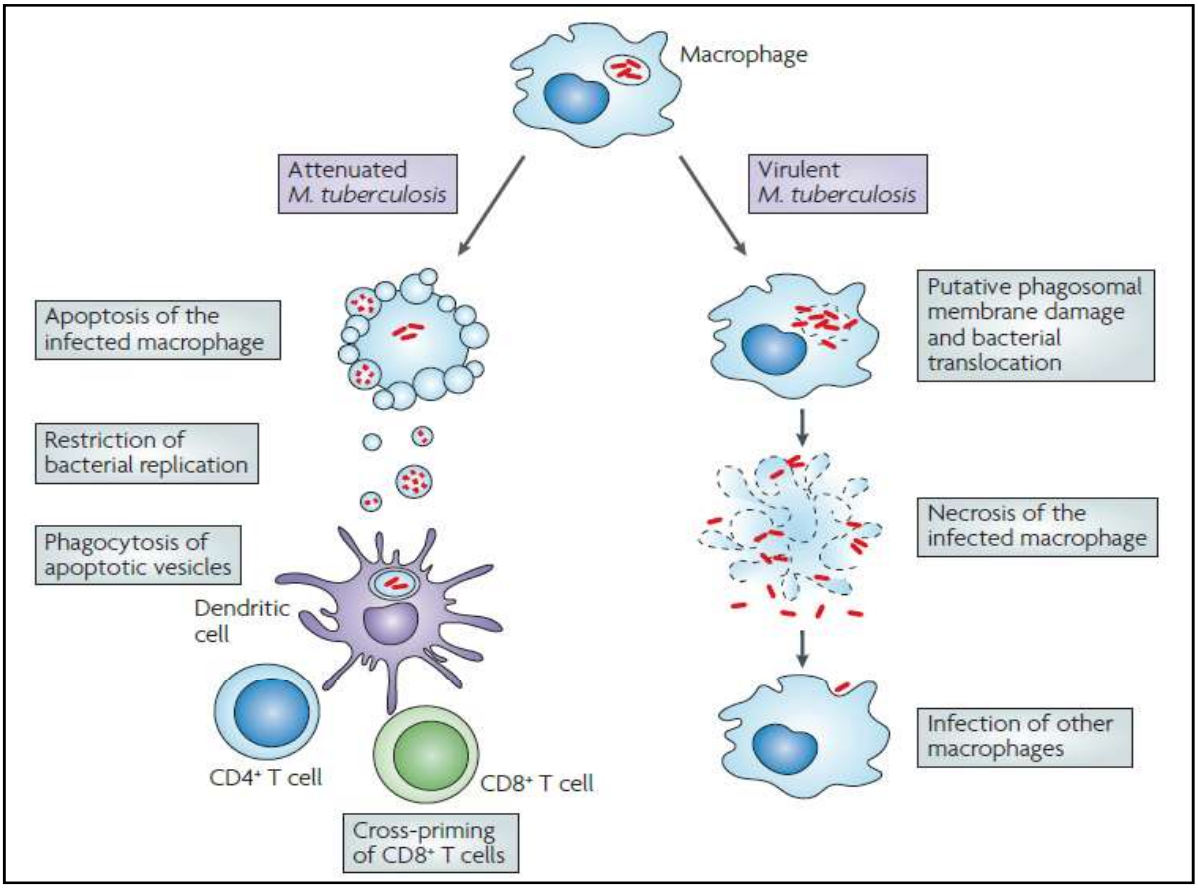


Figure 1.9. Modulation of cell death in macrophages infected with virulent and attenuated strains of MTB[163].
Attenuated strains of MTB induce apoptosis in infected macrophages which impairs bacterial replication. Apoptotic vesicles containing bacterial Ag are phagocytosed by dendritice cells which then present Ag to naïve T cells thus providing an important link to adaptive immunity. In the contrary virulent strains of MTB inhibit apoptosis and induce necrosis which helps in the dissemination of MTB.

1.8.5. Prevention of autophagy by MTB

Autophagy is a cellular homeostatic process by which the cells can eradicate damaged organelles or other cellular structures. The process leads to the formation of double membrane autophagosome which is later fused with lysosome and the cellular contents delivered in this process are digested [164,165]. It has been reported that MTB can be localized to autophagic vacuoles and induction of autophagy can add to

the clearance of bacteria [166,167]. It has been reported that IFN-γ, vitamin D and LPS treatment or nutrient starvation can induce autophagy in MTB infected cells resulting into localization of MTB to autophagosomes in infected macrophages. Autophagy induction has been shown to decrease the survival of MTB in infected cells [168-170]. Recent studies have suggested that MTB inhibits autophagy to ensure its prolonged survival [171]. Further, several reports have suggested that the enhanced intracellular survival (eis) gene of MTB that encodes a temperature-stable hexameric protein, inhibits phagosome maturation, reactive oxygen species (ROS) production, and autophagy through the direct acetylation of a specific c-Jun N-terminal kinase (JNK) specific phosphatase [172-174].

1.8.6. Perturbation of dendritic cell maturation by MTB

Dendritic cells (DCs) are the most important Ag presenting cells (APCs) of immune system and play an important role in development of adaptive immune response during bacterial infections. Immature DCs undergo several changes after phagocytosis of bacteria and convert into mature DCs and lead to activation of T cells. Further T cells differentiate into two phenotypes T helper 1 (Th1) cells, which secrete IFN-γ, or Th2 cells, which secrete IL-4. IFN-γ secretion is associated with the killing of intracellular pathogens, whereas IL-4 plays an important role in killing extracellular pathogens [138].

DC-SIGN and TLRs are expressed on the surface of DCs. When MTB binds with TLRs, it activates NF-κB, leading to the DC maturation and production of T cell activating cytokines which mediate killing of pathogenic mycobacteria. Man-LAM of pathogenic MTB binds to DC-SIGN which has antagonistic effect on TLR signaling. This leads to the blockage of DC maturation and enhanced expression of the

immunosuppressive cytokine IL-10 which causes impairment of T cell activation [48,175].

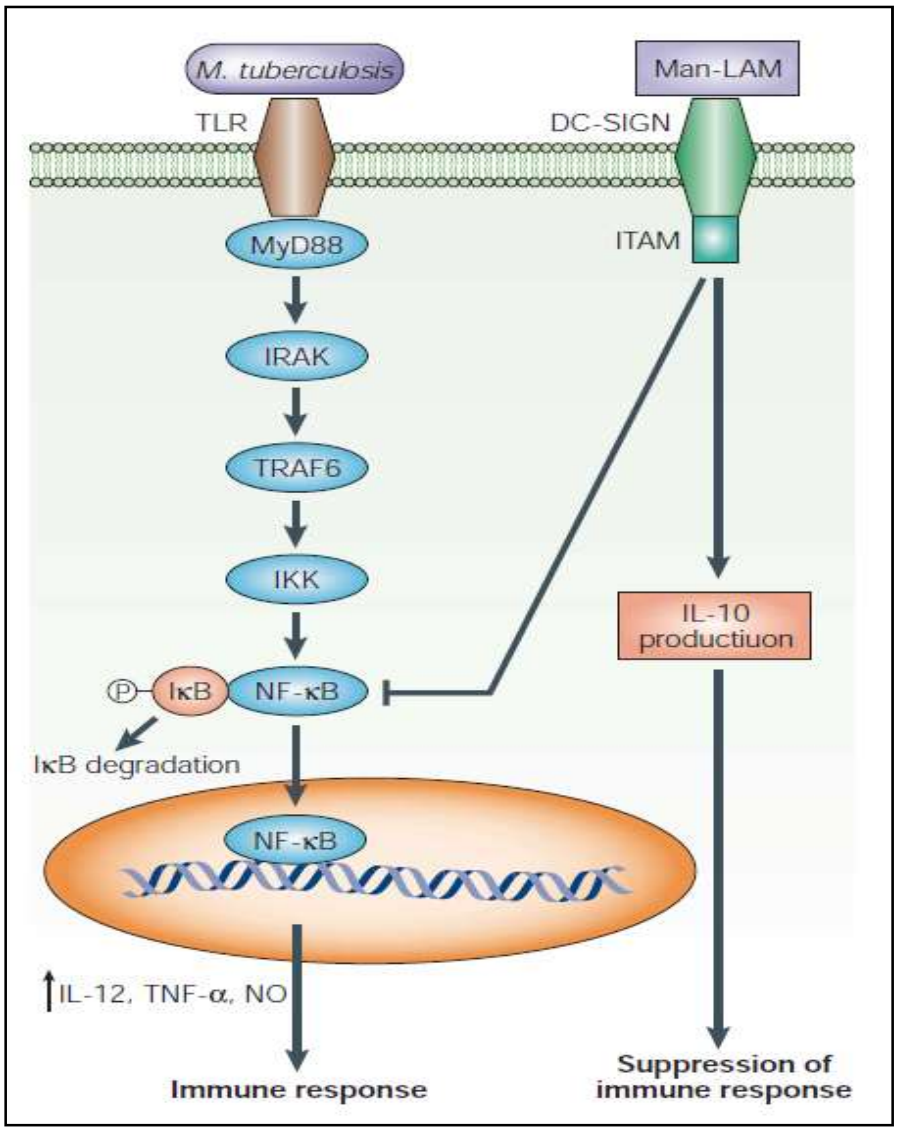


Figure 1.10. Modulation of dendritic cell maturation by MTB [138].

Other immune evasive mechanisms include the secretion of enzymes such as superoxide dismutase or catalases by MTB which are antagonistic to ROI [176]. Furthermore, macrophages infected with MTB produce inhibitory cytokines, such as transforming growth factor TGF-β and IL-10, which reduce macrophage activation, thereby leading to decreased clearance of bacteria [177,178].

1.8. *Tinospora cordifolia*

Tinospora cordifolia a member of family Menispermaceae, is a large, deciduous, glabrous, climbing shrub with weak and fleshy stem. Geographical distribution of this plant is spread throughout the Indian subcontinent and China. It is commonly known as Guduchi, Giloy and Amrita [179]. Up to 223 chemical compounds including terpenoids, steroids, alkaloids and polysaccharides etc have been isolated from the genus *Tinospora* [180]. Pure compounds and crude extracts from *Tinospora* have shown several medicinal properties such as antioxidant, antidiabetic, anti-inflammatory, anti-tumor, antiosteoporosis, immunostimulation and antimicrobial properties [180].

1.9. Present study

Crude aqueous extracts from the stem of the Indian medicinal plant *Tinospora cordifolia* have been shown to enhance the intracellular bactericidal activity of macrophages and neutrophills in *E. coli* induced peritonitis [181,182]. It was shown to be mitogenic to B cells and its activity based purification resulted in isolation of a polysaccharide, known as G1-4A, an acidic arabinogalactan of m.w. 2.2 x 10^6 Da [182-184]. Macrophages were the principal target cells of G1-4A whereas it also augmented the DC maturation [182,183,185].

In the present study, we wish to study the immunomodulatory role of this G14A in tuberculosis and mechanism underlying it. Immunomodulators offering limited protection against MTB infection may be useful as adjunctive therapy with a potential to reduce the duration and increase the effectiveness of conventional drug regimen. Unlike antibiotics, immunomodulators target the host rather than the pathogen, thus immunomodulation remains impervious to the evolution of any drug resistance by MTB.

Chapter 2

2. *Methodology*

2.1. Cell culture

Chemicals Reagents	Source
DMEM, RPMI-1640	Gibco/Invitrogen, USA; Hi media, India
Foetal Bovine Serum (FBS)	Gibco/Invitrogen, USA; Hi media, India
Penicillin-Streptomycin	Hi media, India
100x antifungal and antibacterial solution	Hi media, India
Trypsin-EDTA	Hi media, India

Standard aseptic cell culture procedures were followed. Different macrophage cell lines and respective media used in this study are listed below:

Cell line	Origin	Source	Culture Medium
RAW 264.7	Murine macrophage cell line	NCCS, Pune	DMEM
THP-1	Human monocytic leukemic cell line	NCCS, Pune	RPMI-1640

These cell lines were sub-cultured and maintained in their respective media supplemented with 10% FBS, 100U/ml penicillin and 100 µg/ml streptomycin solutions and incubated at 37°C in environment containing 5% CO2.

2.1.1. Sub culturing and maintenance of cells

- Cell culture media from culture flasks were aspirated and cells were gently washed twice with 1X sterile PBS.

- For 25 cm^2 culture flasks, 1ml of trypsin-EDTA was added to the cells and incubated at 37° C for 4-5 min (till cells started to get detached from the substratum).
- Later, 5ml of complete medium (complete medium contains 10% serum) was added to neutralize the proteolytic action of trypsin.
- Cells clumps were dissolved to make single cell suspension by gentle pipetting, and centrifuged at 1500 rpm for 5min.
- Supernatant was discarded and the cells were resuspended in 1ml complete medium followed by trypan blue staining to estimate viable cell count. Briefly, viable cells excluded the dye and appeared bright, and counted from 10µl, 1:1 mixture of cell suspension and trypan blue dye using haemocytometer.
- Following formula was used to calculate number of cells per ml:

  No of cells/ml= average number of cells per WBC chamber x 10^4 .
- Later cells were either sub-cultured with 1:4 split ratio into new culture vessel(s) or seeded as per the experimental requirements.

2.1.2. Cryopreservation

- Cryopreservation is used for storage of cell cultures for longer duration. Cryopreservation media contains basic cell culture media, cryo-protective agent, and a protein source. Cryoprotective agents avoid death due to the ice crystal formation, by reducing the freezing point of the media.
- Single cell suspensions of trypsinized cells were used to for the cryopreservation.
- Media used for freezing the cells was prepared (70% serum + 5%-8% of DMSO + Rest media) and 1 x 10^6 cells were gently resuspended in 1ml of chilled freezing medium and immediately transferred to a cryo vial.

- Cryo vials were then transferred to chiller containing isopropanol where temperature reduces 1-2° C /hr and stored in -80° C freezer overnight and later transferred to liquid nitrogen for cryopreservation.

2.1.3. Revival of cryo preserved cells

- Cryo vial containing cells was thawed at room temperature for 20-30 minutes and immediately added to 15 ml conical tube containing 9 ml of media.
- Cells were centrifuged at 1000 rpm for 5 min, supernatant was discarded, cell pellet was resuspended in complete media and suspension was transferred to a new culture flask and incubated at 37°C in 5% CO2

2.2. Animals

- Six-week old female BALB/c mice free of common pathogens were used for all experiments.
- Mice were housed under specific-pathogen-free conditions in our departmental animal facility.
- For *in vivo* infection studies, mice were housed under specific-pathogen-free conditions in bio-safety level III facilities at the Tuberculosis Aerosol Challenge Facility (International Centre for Genetic Engineering and Biotechnology, New Delhi, India). Experimental groups of BALB/c mice were matched for age (within 1to 2 weeks) and gender both, for each experiment.

2.2.1. Ethics statement

Use of animals was approved by BARC animal ethics committee (Project no-BAEC/16/11) and standard protocols approved and created by BARC animal ethics committee and ICGEB animal ethics committee were followed.

2.3. Bacterial Cultures

Chemicals Reagents	Source
Middlebrook 7H9 medium	Difco, Becton Dickinson, USA and Himedia India
Middlebrook 7H11 medium	Difco, Becton Dickinson, USA and Himedia India
OADC (oleate-albumin-dextrose-catalase) and ADC (albumin-dextrose-catalase)	Difco, Becton Dickinson, USA and Himedia India
Tween 80	Sigma-Aldrich, USA
Lowenstein–Jensen (LJ) medium	Himedia India

- MTB clinical isolates Strain -1 (Bejing), and strain-2 (LAM); both multi drug resistant strains were recovered from patients in Tata memorial hospital and KEM hospital, Mumbai (INDIA), and characterized as described [22].
- The laboratory strain H37Rv was also included in the study.
- The MTB strains were plated on Lowenstein–Jensen (LJ) medium and single colony was added to Middlebrook 7H9 medium supplemented with 10% ADC and 0.05% Tween 80 at 37°C with daily agitation and grown until the mid log phase.
- The cells were harvested at this point and stored in glycerol at -70° C.

2.4. Preparation of single cell suspension of MTB strains

- For *in vitro* infection studies, single cell suspension of MTB was prepared.
- Cells growing in the mid log phase were harvested and the cell pellets were washed twice with 1xPBS.
- Cells were resuspended in 1xPBS or suitable media like DMEM or RPMI-1640 and transferred to glass test tube containing around 20-25 glass beads (3mm diameter).

- Clumps of the MTB were dissolved by vigorous vortexing for 5 minutes and the suspension was kept undisturbed for half an hour.
- The upper half of the bacterial suspension was collected in 50 ml conical tube and suspension was passed through 26G niddle for 8-10 times to remove cell clumps and prepare single cell suspension.
- The cell count was monitored by taking optical density (OD) of the cell suspension at 600nm.
- Suspension containing 10^8 bacilli/ml was dispensed in microfuge tubes and stored at -80°C and finally adjusted as required for infection experiments.

2.5. Extraction of polysaccharide G1-4A from *T. Cordifolia*

Chemical Reagents	Source
Methanol	SDFCL, India
Acetone	SDFCL, India
TCA	SDFCL, India
Sephacryl-S400	Sigma-Aldrich, USA

- G1-4A was isolated and purified from *Tinospora cordifolia,* an Indian medicinal plant, as described in the Indian Patent no. 56/Bom/98 [184].
- Powdered dry stems of *T. Cordifolia* was dissolved in methanol (2 litres per kg) and kept at room temperature for overnight to remove low molecular weight constituents.
- Next day methanol was removed and replaced with acetone (2 litres per kg) and kept at room temperature for overnight to obtain polysaccharide rich fraction.
- Later acetone was removed and equal volume of D/W was added and the solution was boiled for 30 minutes.

- The whole solution was filtered with muslin cloth, solid fraction was discarded and liquid containing polysaccharide rich fraction was mixed with acetone in 1:3 ratio.
- Polysaccharide rich fraction was precipitated and centrifuged at 2500 rpm for 5 minutes.
- Supernatant was discarded and pellet was resuspended in equal volume of D/W and mixed with trichloroacetic acid (TCA) to remove protein impurities.
- In next step the solution was centrifuged at 2500 rpm for 5 minutes, pellet was discarded and supernatant was mixed with equal volume of acetone.
- Mixture was centrifuged at 2500 rpm for 5 minutes and pellet was dissolved in equal volume of D/W.
- Later, the whole mixture was subjected to dialysis in D/W at 4° C for overnight to get rid of salts dissolved in the preparation.
- After dialysis, the mixture was centrifuged at 2500 rpm for 5 minutes and supernatant containing polysaccharide fraction was collected and subjected to column chromatography on a Sephacryl S-400 gel for further purification.
- Purified fraction was freeze dried and used for further experiments.
- The biological activity of the purified fraction was determined by its TNF-α producing properties in RAW 264.7 cells and primary murine macrophages.
- The G1-4A used in the present study was devoid of endotoxin contamination as estimated by the limulus amebocyte lysate (LAL) assay using the E-TOXATE kit.

2.6. Isolation of peritoneal macrophages

Chemical Reagents	**Source**
FITC conjugated CD11b (clone M1/70)	Biolegend, USA
Brewer thioglycollate	Sigma-Aldrich, USA

- For isolation of peritoneal macrophages 1 ml of 3% brewer thioglycollate medium was injected into the peritoneal cavity of BALB/c mice.
- After 4 days, 10 ml of media was injected into the peritoneal cavity and peritoneal exudate cells (PEC) were aspirated, added to 6 well plates and kept in 5% CO2 incubator at 37°C for overnight.
- Next day, non adherent cells were washed out and adherent macrophages were used in different experiments.
- This method yielded $8x10^6$ cells per mice.
- Cells were stained with FITC conjugated CD11b antibody and analyzed with flow cytometer to obtain the purity of the cells.

2.7. Western blot

Chemical Reagents	**Source**
Phosphatase inhibitor cocktail	Sigma-Aldrich, USA
Protease inhibitor cocktail	Sigma-Aldrich, USA
Nitrocellulose membrane	Pall Corporation, Mexico
Enhanced Chemiluminisence kit	Roche, Germany
Anti-NOS2 (clone C-11)	Santacruz Biotechnology, USA
Anti-MyD88 (clone E-11)	Santacruz Biotechnology, USA
Anti-TLR4 (clone 76B357.1	Santacruz Biotechnology, USA
Anti-β-actin (clone 8H10D10)	Cell Signaling Technology, USA
MAPK/phospho MAPK antibody sampler kits	Cell Signaling Technology, USA
Apotosis Sampler kit	Cell Signaling Technology, USA

- Western blot is used to monitor the expression of various proteins under different conditions. Western blot was performed as described [186].

- Briefly, RAW 264.7 or THP-1 cells cultured under different conditions were lysed in RIPA buffer containing 1% protease inhibitor cocktail and 3% phosphatase inhibitor cocktail.
- The lysate containing equal amounts of protein (60 µg/well) were subjected to SDS PAGE and electroblotted to nitrocellulose membrane.
- Membranes were incubated with 5% w/v skimmed milk in 1X TBST for 30 minutes to block the non-specific binding sites and further incubated with required primary antibody for 4 hours at room temperature.
- Membranes were later incubated with appropriate HRP conjugated secondary antibody for 1 h and protein bands were visualized using Enhanced Chemiluminisence kit according to the manufacturer's protocol.
- Densitometric analysis was performed by Image J software.

2.8. Propidium iodide staining

Chemical Reagents	**Source**
Propidium Iodide (PI)	Sigma, USA
RNase A	Sigma, USA
DAPI	Sigma, USA

- Composition of PI buffer (for 500µl) : 1×PBS (470µl) + RNAse A 1µg/µl (5µl) + PI stain 1 µg/µl (25µl) The detailed procedure of PI-staining is given as below.
- The cells (1 x 10^6) were harvested and centrifuged at 1200 rpm for 5 minutes and supernatant was discarded.
- Pellet was resuspended in 1×PBS and centrifuged at 1200 rpm for 5 minutes and supernatant was discarded.
- Ice cold 70% alcohol was added (1ml/10^6 cells) to the cells in drop wise manner with gentle tapping and cells pellet was resuspended.

- The cells were stored at -20ºC for at least 1 h or long for fixation.
- Fixed cells were collected in another centrifuge tube and were pelleted by spinning at 1200 rpm for 5 minutes and alcohol as supernatant was discarded.
- The cells were washed thrice with 1×PBS, centrifuged and supernatant discarded.
- For staining with PI, 500 µl of PI buffer was added in each centrifuge tube, pipetted gently and collected in dark condition.
- The cells in PI buffer were incubated in pre-warmed incubator/water-bath at 37ºC for 1h.
- The FACS-tubes were tapped every 10 minutes to mix the cell and buffer properly.
- The cells were passed through a 26 gauge needle followed by PI fluorescence acquisition using CyFlowSpace flow cytometer. (Note: PI is light sensitive. So, PI buffer was made fresh before use).
- PI buffer in tube were stored in amber coloured bottle to minimize light exposure.
- For staining with DAPI: 500µl of DAPI buffer (0.5µg/ ml) along with RNase A (0.1 µg/ ml) was poured in each centrifuge tube and transferred to FACS-tubes.
- The FACS-tubes were tapped every 10 minutes to mix the cell and buffer properly.
- The cells were passed through 26 gauge needle followed by DAPI fluorescence acquisition using CyFlow Space flow cytometer.

2.9. Reverse Transcription (RT) PCR

Chemical Reagents	**Source**
RNeasy Mini Kit	QIAGEN USA
cDNA synthesis kit.	Thermo, USA
SYBR Green Mastermix kit	Agilent,USA

Table 2.1. Primer Sequence

Primer	Base sequence
IL-10 (F)	5'-CGGGAAGACAATAACTG-3'
IL-10 (R)	5'-CATTTCCGATAAGGCTTGG-3'
IL-12 (F)	5'-CAACATCAAGAGCAGTAGCAG-3'
IL-12 (R)	5'-TACTCCCAGCTGACCTCCAC-3'
TNF-α (F)	5'-GGCAGGTCTACTTTGGAGTCATTGC-3'
TNF-α (R)	5'-ACATTCGAGGCTCCAGTGAATTCGG-3'
IFN-γ (F)	5'-GGATATCTGGAGGAACTGGC-3'
IFN-γ (R)	5'-CGACTCCTTTTTCCGCTTCCT-3'
NOS2 (F)	5'-CCCTTCCGAAGTTTCTGGCAGCAGC3'
NOS2 (R)	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
GAPDH (F)	5'-CAAGGCTGTGGGCAAGGTCA-3'
GAPDH (R)	5'-AGGTGGAAGAGTGGGAGTTGCTG-3'

- RAW 264.7 cells or single suspension of the lung from three mice per group were used to isolate total RNA using RNeasy Mini Kit according to manufacturer's protocol.
- cDNA was prepared from total RNA using RevertAid M-MuLV Reverse Transcriptase supplied with cDNA synthesis kit.
- Real-time PCR was performed using the Mx3000P real-time PCR system (Stratagene) and SYBR Green Mastermix kit.
- The cDNA was amplified using specific primers (Table 2.1) deduced from the published sequence [187].
- GAPDH was used as internal control.
- The thermal cycling profile for PCR amplifications were as follows: (denaturation for 30 s at 94°C, annealing for 30 s at 58°C and then extension for 1 min at 72°C) X 40 cycles followed by melt curve analysis.
- Relative quantitation of gene expression was performed using $2^{-\Delta\Delta Ct}$ method [188].

2.10. Measurement of cytokines by ELISA

- The levels of cytokines such as TNF-α, IL-12, IL-6, IL-4, IL-10, IL-1β and IFN-γ from the culture supernatants of macrophages infected with MTB strains or serum of infected animals were measured using BD OptEIA mouse ELISA sets (BD Biosciences) according to the manufacturer's instructions.
- Briefly, monoclonal capture Ab were coated in 96 well ELISA plates for overnight at 4°C.
- Next day, blocking buffer (200µl) was added to the wells to block non specific binding sites and incubated at room temperature for 1 h.
- Diluted standards and unknown samples were added to respective wells and incubated for 2 h at room temperature.
- Subsequently biotinylated monoclonal detection antibody and streptavidin POD were added for 1 h at RT followed by addition of chromogenic substrate (3,3',5,5'-Tetramethylbenzidine, TMB) to give a colored product.
- Stop solution was added and absorbance was measured at 450nm and 570nm.
- Subsequently concentrations of cytokines in samples were extrapolated from standard curve.

2.11. Nitric oxide assay

- RAW 264.7 cells were treated with G1-4A for 8h prior to infection with MTB strains followed by treatment with G1-4A for 48 h.
- The concentration of nitrite, which is the stable end product of NO, was determined by Griess' reaction in the supernatants [189].

2.12. siRNA transfection and Blocking of TLR4 by anti-TLR4 blocking antibodies

Chemical Reagents	Source
siRNA (TLR4, MyD88 or Scrambled)	Santacruz Biotechnology, USA
Xtreme GENE transfection reagent	Roche, Germany
TLR4 (clone 76B357.1)	Santacruz Biotechnology, USA
MyD88 (clone E-11)	Santacruz Biotechnology, USA
β-actin (clone 8H10D10)	Cell Signaling Technology, USA
Anti-TLR4 antibody (clone MTS510)	Biolegend, USA
Isotopes control antibody (clone RTK2758)	Biolegend, USA

- Transfection is the process of deliberately introducing nucleic acids in eukaryotic cells by non-viral methods.
- Transfection was done using Xtreme Gene transfection reagent as per manufacturer's instructions. In brief, the following procedure was followed for transient/stable transfection of cell lines :
- RAW 264.7 cells were seeded in 6 well plates at density of $5x10^5$ cells/well in DMEM and incubated at 37°C with 5% CO2 overnight.
- Working solutions of siRNA (TLR4/MyD88/scrambled) and transfection reagent were prepared in 100ul of SFM separately and mixed with each other in such a way that the final concentrations of siRNA provided to each well were 100nM in a total volume of 20µl of transfection Reagent.
- Above mixture was further diluted by adding 600ul of SFM media (total volume 800µl) and incubated for 10 minutes at room temperature.
- Transfection reagent- siRNA mix was added to the cells and plate was swirled gently to ensure uniform distribution of the mix.

- Plates were incubated for 4-5 h at 37° C and at 5% CO2.
- In next step, 800µl medium containing 20% FCS was added to the cells at the end of incubation and further incubated for 48 hrs.
- After 48 h, cells were treated with G1-4A, the supernatants were collected for estimation of cytokines and cells were used for western blot and flow cytometric analysis.
- Cells were incubated with siRNAs (TLR4, MyD88 and scrambled) for 48, 96 and 144 h followed by detection of TLR4 and MyD88 proteins by immunoblotting to establish effective knock down period.
- Similarly, in other set of experiments, cells were incubated with anti-TLR4 blocking antibody or isotype control antibodies for 1 h followed by treatment with G1-4A. Supernatants were collected for estimation of cytokines and cells were used for western blot and flow cytometric analysis.
- During infection studies, cells were first infected with MTB, followed by transfection with siRNAs or treatment with blocking antibodies. This was done to avoid any interference in MTB uptake by the cells.

2.13. Phagocytosis Assays

Chemical Reagents	**Source**
Trypan blue	Sigma, USA
DAPI	Sigma, USA
Prolong gold antifade	Invitrogen, USA
Amikacin	Sigma, USA

- RAW 264.7 cells were grown on cover slips in six well plates, and either treated with G1-4A, LPS or left untreated 8 h prior to the infection with GFP expressing MTB at MOI 5.

- Cells were incubated with GFP-MTB H37Rv at 37°C in a 5% CO2 environment for two hours.
- After infection, macrophages were washed thrice with 1XPBS and treated with 10µg/ml amikacin for 1 h to get rid of extracellular bacteria.
- Cells were again washed with 1XPBS, fixed with 4% para formaldehyde. Cover slips were mounted onto slides with prolong gold antifade (Invitrogen) and analyzed on a Nikon TE 2000E laser scanning confocal microscope.
- For evaluation of phagocytosis, 300 macrophages per well were counted at 63X magnification under oil immersion and serial optical sections with Z-stack spanning were taken to monitor colocalization and images were deconvolved.
- Cells containing at least one ingested bacterium were counted to estimate the per cent of infected macrophages, and the mean number of internalized bacteria was multiplied to generate phagocytic index. Trypan blue (final concentration, 0.025%) was used to quench extracellular bacterial fluorescence.
- Phagocytic index = (percentage of macrophages containing at least one bacterium) X (mean number of bacteria per positive cell).
- In another set of experiments cells either pre treated with G1-4A for 8 h or untreated were infected with GFP-MTB for 4 h.
- Cells were harvested and their intracellular fluorescence was analyzed by flow cytometer.

2.14. MTB infection and CFU assay

Chemical Reagents	**Source**
Trypan blue	Sigma, USA
DAPI	Sigma, USA
Prolong gold antifade	Invitrogen, USA
Amikacin	Sigma, USA

- RAW 264.7, THP-1, murine peritoneal macrophages or human PBMCs were seeded in 24 well tissue culture plates at a density of 2 x10^5 cells/well and treated with G1-4A, 8-12 hrs prior to infection with MTB strains at MOI of 5.
- Frozen stocks of the MTB H37Rv, clinical isolates Beijing and LAM were thawed, followed by passage through 26G needle for 8-10 times to remove cell clumps and prepare single cell suspension.
- Cells were washed twice with 1XPBS to remove remaining FBS as it interferes with the phagocytosis process.
- Later, single cell suspension of MTB diluted in suitable media without FBS was added to the cells at MOI of 5 and plates were incubated at 37° C in 5% CO2 environment.
- Four hours post infection; cells were washed thrice with pre-warmed media or 1xPBS and incubated with medium containing 10 µg/ml of amikacin for 1 h to kill extracellular bacteria.
- Cells were again treated with G1-4A (1mg/ml) for 24, 48 and 72 hrs.
- Supernatants were stored at -80°C at each time point for estimation of cytokines and NO levels. For CFU assay, infected cells were washed thrice with sterile PBS and lysed with 0.01% SDS in PBS at different time points.
- Serial dilutions were prepared in PBS and plated on 7H11 medium supplemented with OADC.
- Colonies were observed after 3 weeks and values were represented as CFU counts or bacterial burden.

2.15. Aerosol Infection and drug treatment

Chemical Reagents and Instruments	Source
Isoniazid (INH)	Sigma, USA
RNAlater	Ambion, USA
MB7H11	Difco, USA
Aerosol exposure chamber	U.V. Madison College of Engineering Shops, Cambridge Square Inc.

- For each experiment, frozen stocks of bacteria were thawed and passed through 26G needle to disperse the clumps. Aerosol exposure chamber was used to generate the aerosol.
- For each experiment BALB/c mice (6 per group), either injected with G1-4A (12.5mg/kg of body weight) through tail vein twice within the interval of 48 h or untreated, were exposed for 15 min to the aerosol, resulting in implantation of 150-200 organisms into the lungs of each mouse, as determined by the culture of lung homogenates of one mouse from each group, after 24 hours.
- Seven days post infection, G1-4A (12.5mg/kg of body weight) was injected into the tail vein of mice again.
- G1-4A treatment was repeated after every 72 hours till 15, 30 or 60 days. Mice were sacrificed by carbon dioxide narcosis after 15, 30 and 60 days of infection.
- The lungs were removed aseptically and divided in two parts. One part was homogenized and appropriate dilutions of lung homogenates were placed on Middlebrook 7H11 agar plates for subsequent enumeration of the CFU after 2, 4 and 8 weeks.
- Other half of the lung was stored in RNAlater and used for RNA isolation

2.16. *In vitro* restimulation Assay

Chemical Reagents	Source
Concanavalin A (ConA)	Sigma, USA
ELISA sets for cytokines	BD Biosciences, USA
anti MHC-II (IA^d) (clone M5/114.15.2)	Biolegend, USA
FITC conjugated anti mouse CD-86 (clone PO3)	Biolegend, USA

- Spleen cells from mice were dissociated by squeezing the spleen through a sterile nylon mesh in a petri plate containing RPMI medium.
- Red blood cells were lysed by treatment with 0.83% ammonium chloride for 5 min.
- Splenocytes were washed thrice with 1X sterile PBS and resuspended in RPMI 1640 .
- Splenocytes ($2.5x10^6$cells/ml) were incubated with 2 ml of complete media containing 5µg/ml concanavalin A (ConA), or PPD of MTB (3µg/ml) or medium alone for 74 h at 37°C.
- Levels of IFN-γ and IL-4 were assayed in culture supernatants by ELISA as per manufacturer's protocol by using antibody pairs.
- To ensure that IFN-γ and IL-4 are specifically produced by T helper cells, splenocytes were divided in two groups, one group being incubated with anti MHC-II (IA^d) antibodies for 1 h prior to the treatment with ConA or PPD, while other group was not incubated with anti-IA^d antibodies and IFN-γ and IL-4 levels were analysed in each culture supernatant.

2.17. Isolation of bone marrow derived dendritic cells (BMDC)

Chemical Reagents	Source
Recombinant mouse GM-CSF	Sigma-Aldrich USA

Day 0:

- BALB/c mice (4-6 week old) were sacrificed and the femurs and tibias were removed.
- Bones were placed in a petri dish containing 1X PBS and the tissues surrounding the femurs and tibias were removed.
- Bones were placed in petri plate containing 70% alcohol for approximately 1 minute for surface sterilization followed by washing with 1X PBS
- The bone marrow was flushed in RPMI-1640 media with the help of needle (26 G) and syringe (2ml) into sterile petri plates.
- Cells were centrifuged at 1500 rpm for 5 min and pellet was resuspended in 1 ml chilled DW for 10 seconds to lyse the RBCs followed by the addition of equal volume of 2X PBS.
- Cells were centrifuged at 1500rpm for 5 min, supernatant was discarded and pellet was resuspended in 10 ml of RPMI 1640 medium.
- Cells were mixed thoroughly and counted by heamocytometer.
- Cells were seeded at the density of 2 X 10^6 cells/10 ml in 10 cm sterile culture plates containing 10 ml of RPMI-1640, 10% FBS and recombinant mouse GM-CSF (20ng/ml) and incubated at 37°C with 5% CO2.
- Control plate was prepared in similar way but GMCSF was not added to it.

DAY 3

- On day 3, fresh RPMI-1640 (10ml), 10% FBS and recombinant mouse GM-CSF (20ng/ml) was added to the plates and incubated at 37°c with 5% CO2

DAY 6

- Non adherent and loosely adherent cells were harvested by gentle pipetting.
- Cells were counted and used for various experiments.

2.18. Infection of BMDCs with MTB strains

- BMDCs were harvested at day 6 and pellet was resuspended in RPMI 1640 and the cells were counted.
- Cells resuspended in RPMI 1640 containing mouse GM-CSF (20ng/ml) were seeded at density of 1 x 10^6 cells in each well of the 6 well plates.
- Single cell suspension of MTB strains growing in the mid log phase were added to respective wells at MOI of 5 and incubated for 12 h in CO_2 incubator at 37ºC with 5% CO2.
- Cells from each well were collected in 1.5 ml microfuge tubes and centrifuged at 3000 rpm for 5 min.
- Supernatant containing extracellular MTB was discarded, pellet was resuspended in RPMI 1640 containing 10% FBS and mouse GM-CSF (20ng/ml) and incubated for 24 and 48 h in CO_2 incubator at 37ºC with 5% CO_2.
- Cells were harvested at different time points, labeled with FITC labeled antibodies and analyzed using Flow cytometry.
- During CFU assay, infected cells were treated with G1-4A for 48 and 96 h and CFU assay was performed as described earlier.

2.19. Flow cytometry

Chemical Reagents and Instruments	**Source**
CD16/CD32 Fc block antibodies	Sigma, USA
FITC anti mouse CD-86 (clone PO3)	Biolegend, USA
FITC anti mouse MHC-II (IA^d) (clone M5/114.15.2)	Biolegend, USA
FITC anti mouse CD80 (clone 16-10A1)	Biolegend, USA
FITC anti mouse CD40 (clone HM40-3)	Biolegend, USA
Para formaldehyde	SDFCL, India

- Macrophages (5 X 10^5 cells/well in 24-well plates) either uninfected or infected with MTB strains were incubated in the presence or absence of G1-4A for 24 h and 48 h.
- In case of BMDCs cells were seeded at density of 1 x 10^6 cells in each well of the 6 well plates. Cells were harvested, washed thrice with 1XPBS and incubated with CD16/CD32 Fc block antibodies (Biolegend) to block FcRs.
- In next step, cells were stained with either of FITC labeled anti-mouse CD86, FITC labeled anti-mouse (IA^d) MHC-II, FITC labeled anti-mouse CD80 and FITC labeled CD40.
- FITC labeled appropriate isotype control antibody were used as labeling controls.
- Cells were washed and fixed with 4% para formaldehyde and analyzed with CyFlow Space flowcytometer.
- Cells were gated according to their forward versus side scatter.
- Positively stained cells were gated using appropriate FITC labelled isotype antibodies as negative controls using FlowJo software (Treestar).

2.20. Statistical analysis

- Data obtained from independent experiments were presented as mean ±SD and analyzed by either paired Student's t-test or one way analysis of variance (for multiple comparisons) by using Sigma Stat software (version 3.5). Differences were considered statistically significant at p value <0.05.

CHAPTER 3

3. *Results*

This chapter deals with the results obtained in the present study and divided into three sub-chapters on the basis of the host system in which MTB infection studies were carried out.

Sub-chapter 3.1. Effects of G1-4A in MTB infected and/or uninfected macrophages

This sub-chapter encompasses results obtained in MTB infection studies using macrophages (RAW264.7 cell line, peritoneal macrophages and THP-1 cells).

Sub-chapter 3.2. Differential activation of BMDCs by MTB strains and effect of G1-4A on the intracellular survival of MTB in BMDCs

This sub-chapter includes the findings of MTB infection studies in bone marrow derived dendritic cells.

Sub-chapter 3.3: Evaluation of *in vivo* efficacy of anti-TB immunomodulatory properties of G1-4A in BALB/c mice

This sub-chapter contains results of MTB infection studies using BALB/c mice.

Sub-chapter 3.1. Effect of G1-4A in MTB infected and/or uninfected macrophages

3.1.1. Effect of G1-4A in uninfected murine macrophages

3.1.1.1. Effects of G1-4A on the expression of cytokines in murine macrophages

The first objective of our study was to determine the effect of G1-4A on activation of macrophages. Hence, RAW 264.7 cells were treated with media alone and supplemented with G1-4A or LPS. After 24 and/or 48 h of treatment, the supernatants were removed and stored for the detection of cytokines and nitric oxide. Level of cytokines in the supernatants was determined by ELISA. Our data demonstrated that

G1-4A treatment for 24 h, enhanced the level of cytokines IL-1β, IL-6, IL-12, IL-10 and IFN-γ significantly in RAW cells compared to untreated controls (Fig 3.1). Levels of IL-6 and IL-12 were further enhanced after 48 h whereas levels of IL-1β, IFN-γ were decreased at later time point. For estimation of TNF-α, cells were treated with G1-4A for 6, 12 and 24 h. Expression of TNF-α was maximum at 6 h time point and it decreased at further time points. Levels of IL-4 in supernatants remained undetected by ELISA. Simultaneously we investigated the effects of G1-4A on expression in primary macrophages; hence peritoneal macrophages from BALB/c mice were isolated and treated with G1-4A as discussed above. Levels of cytokines were monitored in supernatants. Our data demonstrated an up-regulation of these cytokines in peritoneal macrophages of BALB/c (Fig 3.2).

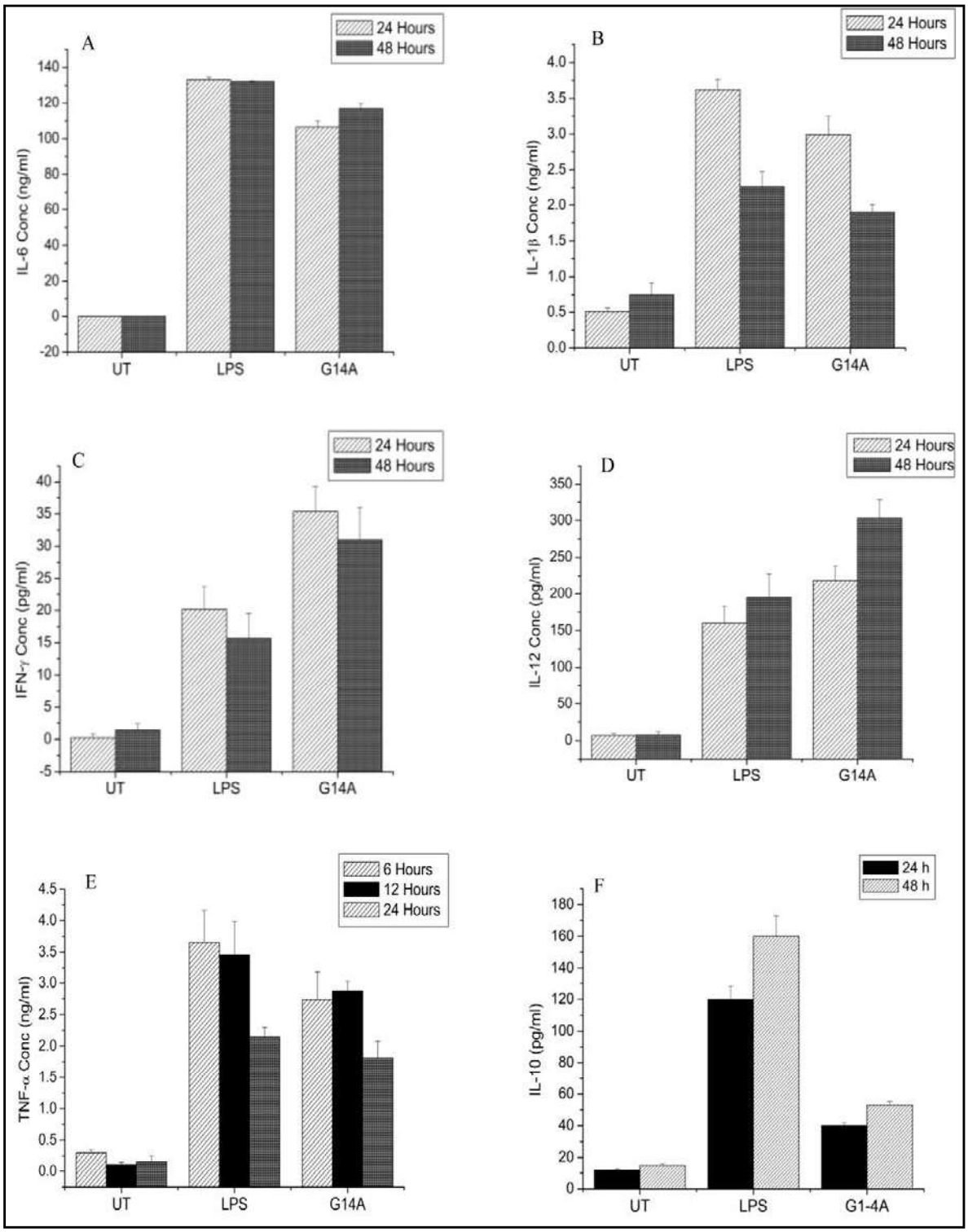


Figure 3.1. Effect of G1-4A on cytokine expression in RAW 264.7 cells.
RAW 264.7 cells were either treated with G1-4A (1mg/ml), LPS (500ng/ml) or left untreated for 6, 12, 24 and 48 h. Supernatants were collected at mentioned time points. Levels of cytokines (IL-6, IL-1β, IFN-γ, IL-12, TNF-α and IL-10) in supernatants were detected by ELISA (BD-Pharmingen) according to manufacturer's protocol. (A) IL-6 (B) IL-1β(C) IFN-γ (D) IL-12 (E) TNF-α (F) IL-10. Results represent three independent experiments and presented here as mean ± SD.

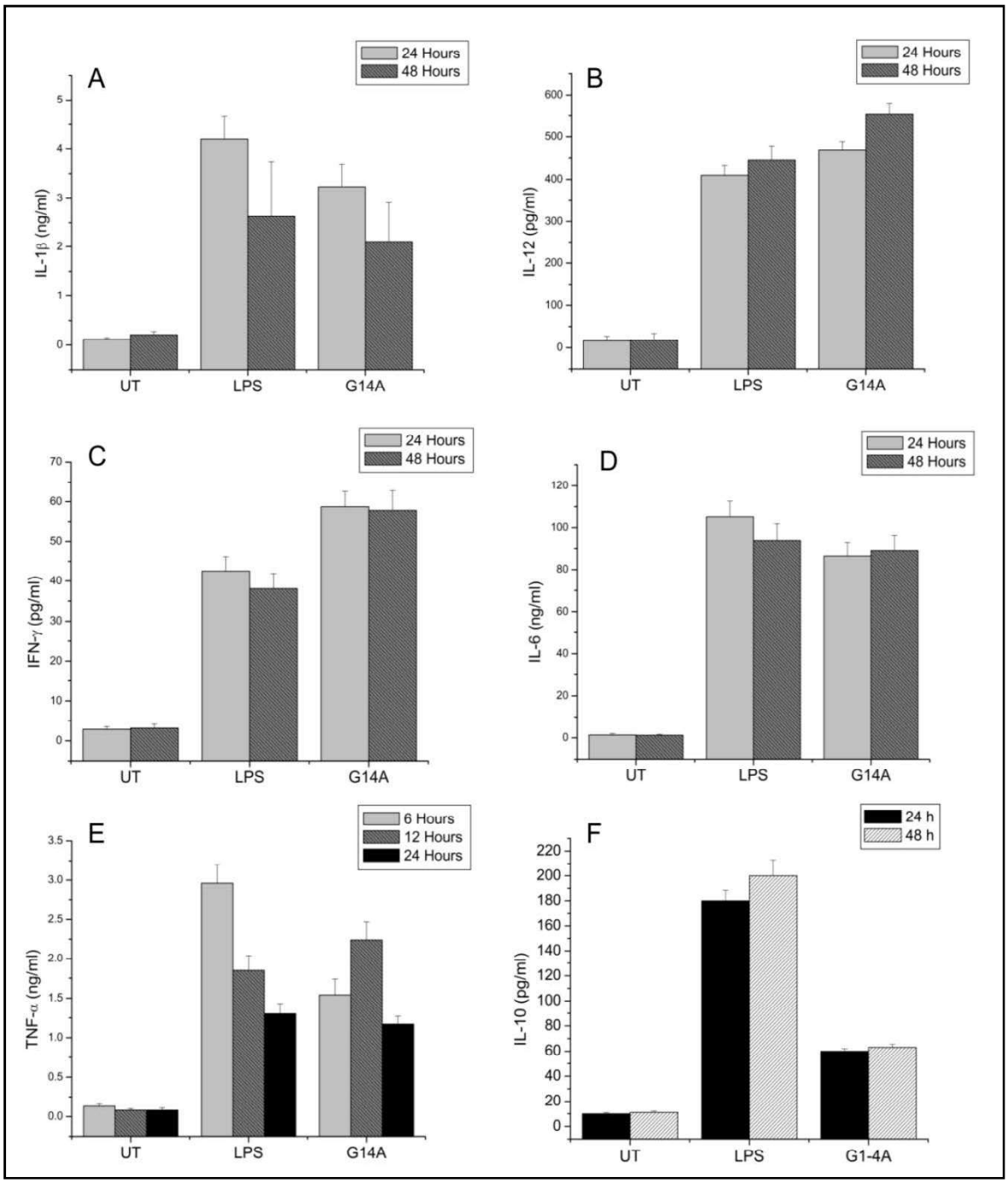


Figure 3.2. Effect of G1-4A on expression of cytokines in murine peritoneal macrophages.

Peritoneal macrophages were either treated with G1-4A (1mg/ml), LPS (100ng/ml) or left untreated. Supernatants were collected after 6, 12 and 24 h and used for detection of cytokine levels by ELISA. (A) IL-1β (B) IL-12 (C) IFN-γ (D) IL-6 (E) TNF-α (F) IL-10. Data shown here are from three independent experiments and presented as mean ± SD.

3.1.1.2. Effect of G1-4A on nitric oxide production in murine macrophages

Nitric oxide production by macrophages suggests their microbicidal properties and is a hallmark of the classically activated macrophages. In present study levels of nitric oxide were measured in supernatants of RAW cells and PEMs both, either untreated or treated with G1-4A or LPS for 24, 48 and 72 h, using Griess' reagent. Data revealed significant increase in NO levels ($p<0.05$) at all the three time points after G1-4A treatment and it was comparable to NO levels in LPS treated cells. We performed immunoblotting studies to determine the effect of G1-4A on the expression of NOS2 protein. Densitometric analysis of western blot results revealed 17 fold up-regulation in NOS2 in G1-4A treated RAW cells (Fig 3.3). Similar results were obtained when murine peritoneal macrophages were treated with G1-4A (Fig 3.4).

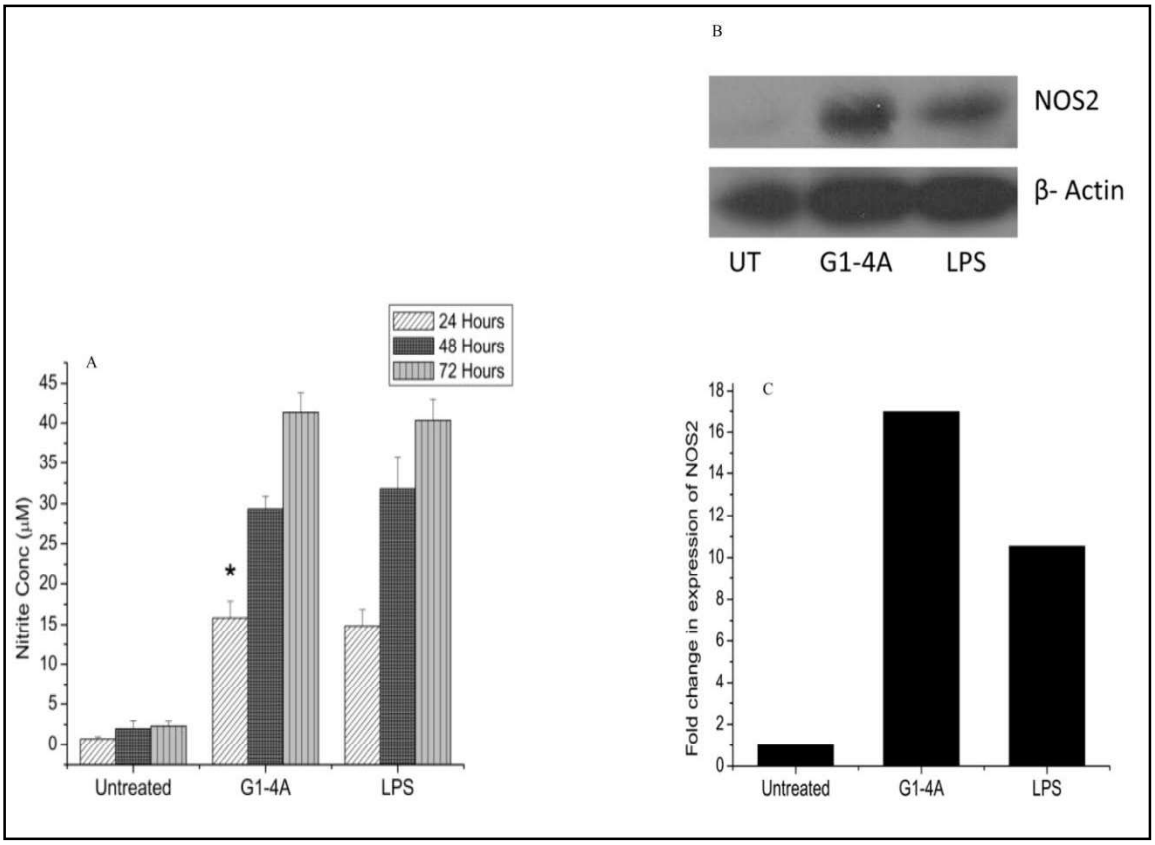


Figure 3.3. Up-regulation of NOS2 expression after G1-4A treatment.
RAW 264.7 cells were either treated with, G1-4A (1mg/ml), LPS (500ng/ml) or left untreated. (A) Nitric oxide levels were measured as nitrite concentration in supernatants by using Griess' reagent. G1-4A treatment enhanced nitric oxide level

*significantly (*p<0.05, student's t-test). (B) Cells were treated with G1-4A and LPS for 24 h and level of NOS2 protein was detected in cell lysate by immunoblotting using anti-NOS2 antibodies. (C) Fold change in NSO2 levels compared to β-Actin using densitometric analysis by Image J software. Results represent three independent experiments and presented here as mean ± SD.*

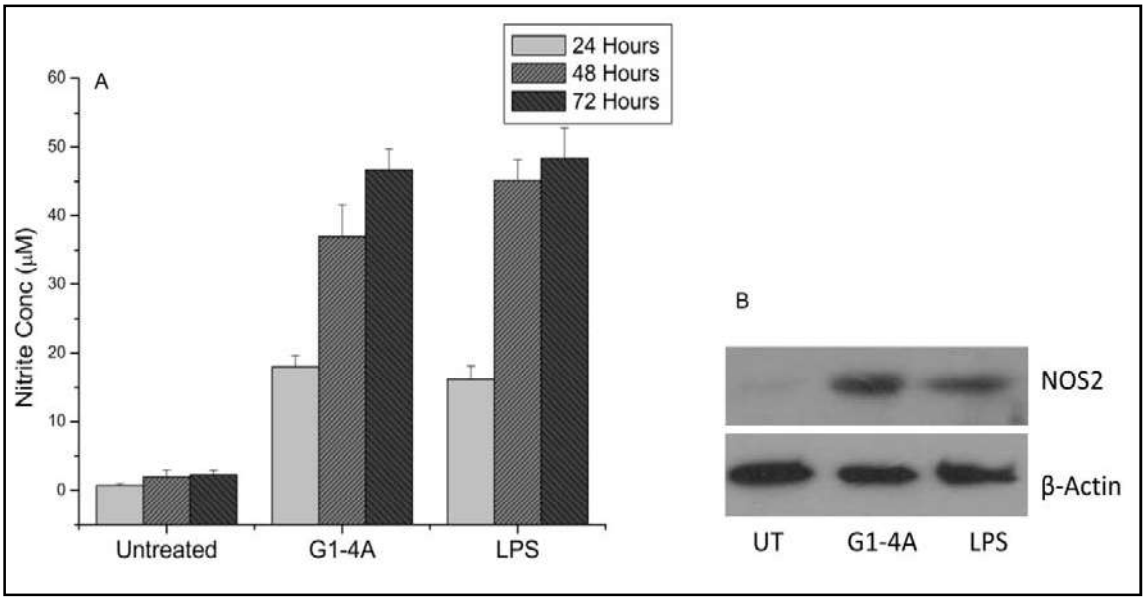


Figure 3.4. Effect of G1-4A on expression of NOS2 and NO production in murine peritoneal macrophages. *Peritoneal macrophages were either treated with G1-4A (1mg/ml), LPS (100ng/ml) or left untreated for 24, 48 and 72 h. (A) NO levels were determined by Griess' reagent. (B) Cells after 24 h treatment were lysed and lysate used for western blot of NOS2. Data shown here are from three independent experiments and presented as mean±SD.*

3.1.1.3. G1-4A treatment up-regulates the surface expression of MHC-II and CD-86 in murine macrophages

As classically activated macrophages with M1 phenotype exhibit increase in Ag (Ag) processing and presentation activity with increased surface expression of MHC-II, we also monitored the MHC-II and CD86 surface expression in RAW cells or PEMs after G1-4A treatment for 24 and 48 h by immunophenotyping. Our data demonstrated, enhancement in MHC-II positive cells from 2.35±1.15 percent to 18.57±1.68 percent ($p<0.01$) and CD-86 positive cells from 5.6±0.43 per cent to 26.83±1.10 per cent ($p<0.01$) respectively after 24 h of G1-4A treatment (Fig. 3.5). Similarly PEMs were

treated with G1-4A and surface expression of MHC-II and CD86 was monitored with flow cytometer and our data demonstrated effects similar to RAW cells (Fig. 3.6). Thus, like LPS and IFN-γ, G1-4A treatment too activated macrophages by classical pathway and conferred on them M1 phenotype.

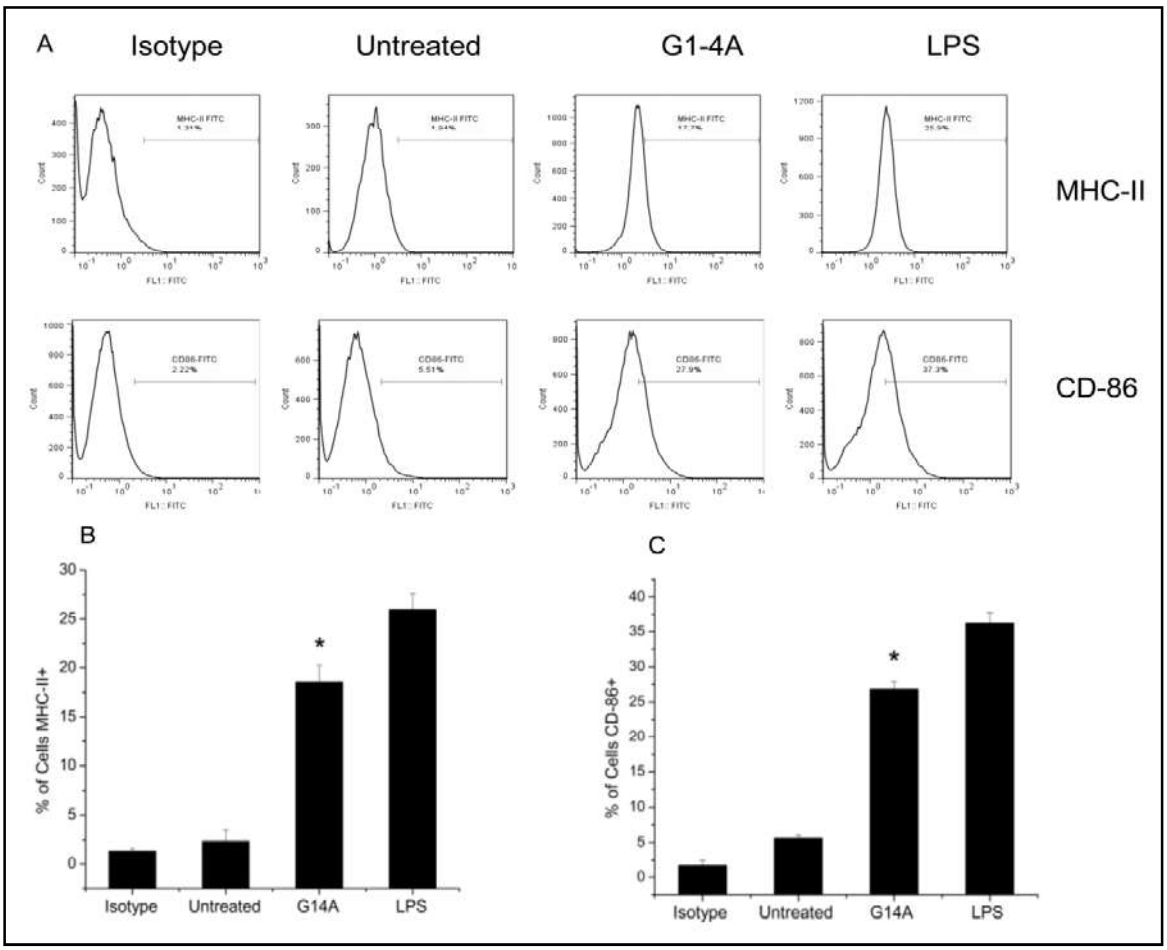


Figure 3.5. Effect of G1-4A on surface expression of MHC-II and CD86 in RAW cells.

*(A) RAW 264.7 cells were incubated in the presence or absence of G1-4A (1mg/ml) or LPS (500ng/ml) for 24 h. Cells were stained with FITC anti I-A^d, FITC anti CD86 and FITC IgG2a Isotype control Ab. Each panel is representative of three or more independent experiments. (B) and (C) are quantitative representation of percentage of cells MHC-II+ and CD-86+ (*p<0.01 as compared to untreated control, student's t-test, values presented as Mean ± SD).*

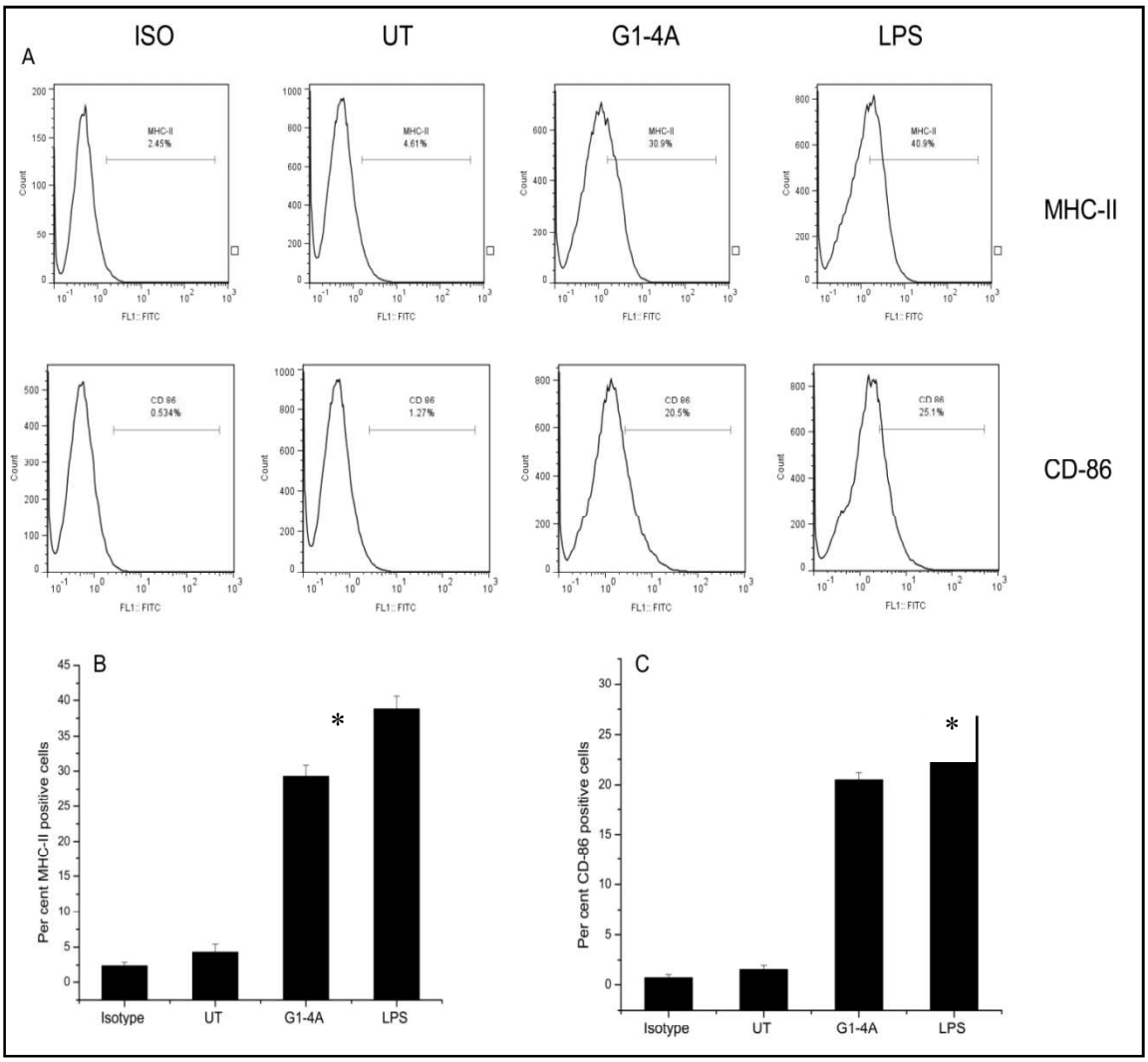


Figure 3.6. Effect of G1-4A on the surface expression of MHC-II and CD86 in murine peritoneal macrophages.
*Peritoneal macrophages were either treated with G1-4A (1mg/ml), LPS (100ng/ml) or left untreated for 48 h. (A) Cells were stained with FITC anti-MHC-II and FITC anti-CD86 monoclonal antibodies and analyzed by flow cytometry. Each panel is representative of three or more independent experiments. (B) Quantitative representation of percent MHC-II+ cells (C) Quantitative representation of percent CD86+ cells. (*p<0.01 as compared to untreated control, student's t-test, values presented as Mean ± SD).*

3.1.1.4. Activation of macrophages by G1-4A involves TLR4-MyD88 pathway

Once the effect of G1-4A on RAW macrophages was established, our next objective was to find out the mechanisms underlying the above mentioned observations therefore we used anti-TLR4 blocking antibodies and siRNAs specific for TLR4 and MyD88 to elaborate the role of TLR4 in G1-4A induced macrophage activation.

RAW cells were incubated with anti-TLR4 blocking antibodies before treatment with G1-4A. It was observed that blocking of TLR4 exhibited antagonistic effects on G1-4A induced expression of pro-inflammatory cytokines and nitric oxide. TLR4 and MyD88 specific siRNAs were used for transient knockdown of TLR4-MyD88 expression in RAW cells followed by G1-4A treatment. There was a marked decrease in expression of TNF-α, IL-1β and IL-6 (Fig 3.7A to 3.7C) in cells that were treated with both G1-4A and TLR4 siRNA or MyD88 siRNA as compared to the cells treated with either G1-4A alone or G1-4A and scrambled siRNA. Cells were incubated with siRNAs (TLR4, MyD88 and scrambled) for 48, 96 and 144 h followed by detection of TLR4 and MyD88 proteins by immunoblotting to establish how transient the knock down was. Our data demonstrated that expression of TLR4 and MyD88 at 48 and 96 h time point was substantially decreased which indicated the knockdown. At 144 h time point, expression of both the proteins was up-regulated, therefore we could conclude that up to 96 h, the expression of TLR4 and MyD88 was knocked down by their respective siRNAs. Treatment of scrambled siRNA did not cause any alteration in the expression of TLR4 and MyD88 (Fig 3.7D). Further, treatment with siRNA against TLR4 and MyD88, both abrogated G1-4A induced increase in nitric oxide levels with concomitant down regulation of NOS2 protein expression (Fig 3.8A to 3.8C). Auxiliary to this, we monitored the effect of G1-4A on surface expression of MHC-II and CD-86 in RAW cells in presence or absence of TLR4-MyD88 siRNAs, and knocked down cells exhibited down regulation of G1-4A induced MHC-II and CD-86 expression (Fig 3.9A to 3.9C, $p<0.01$). These data clearly suggested the involvement of TLR4-MyD88 axis in G1-4A mediated macrophage activation.

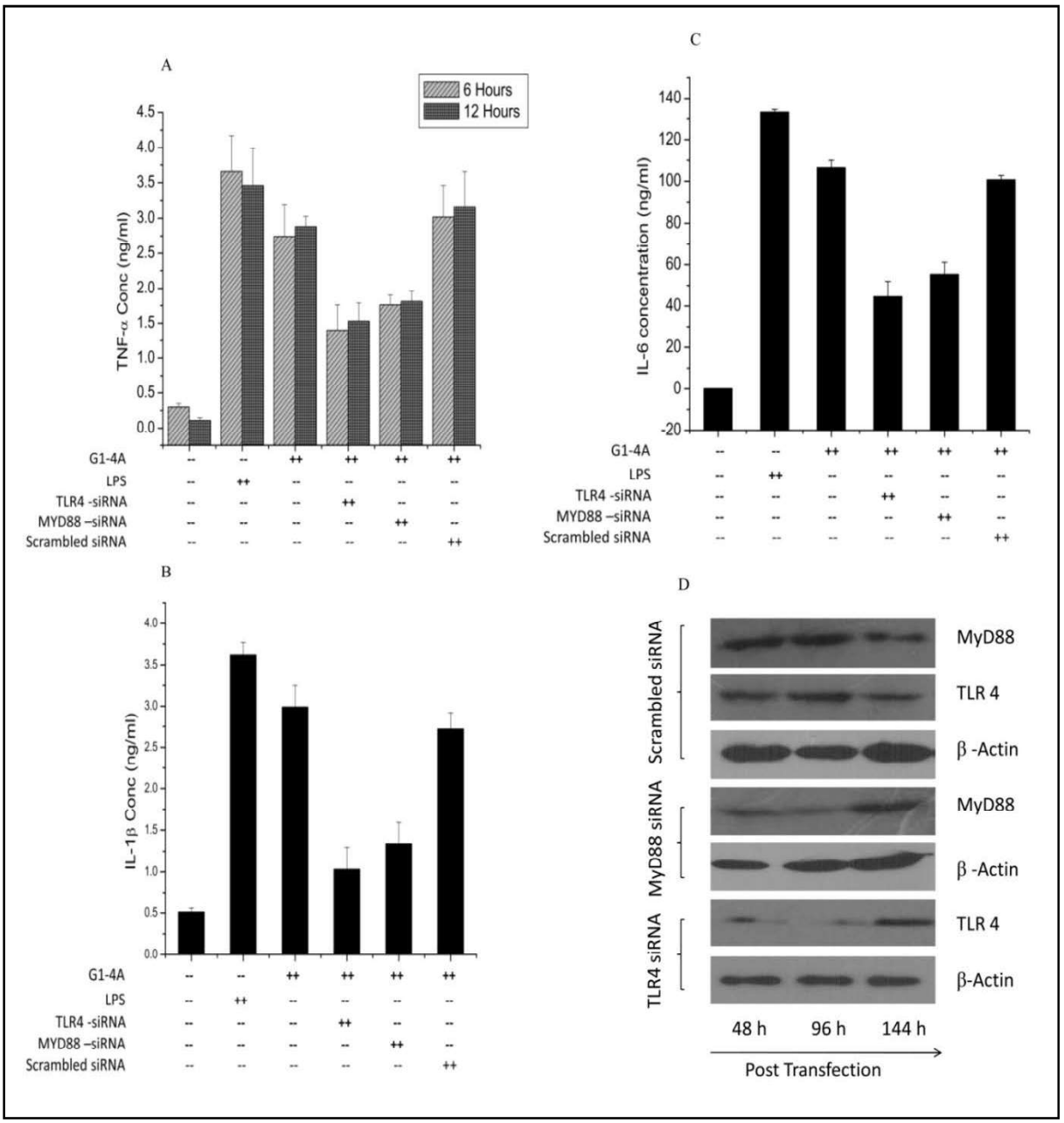


Figure 3.7. G1-4 up-regulates expression of pro-inflammatory cytokines in TLR4-MyD88 dependent manner.

RAW cells were incubated with TLR4 siRNA and MyD88 siRNA to achieve the transient knockdown of these genes, and later treated with G1-4A (1mg/ml), LPS (500ng/ml) or left untreated. Scrambled siRNA was used as control. Supernatants were collected at 6, 12 and 24 h and cytokine levels were detected by ELISA. (A) TNF-α (B) IL-1β (C) IL-6. (D) Immunoblots showing knock down of TLR4 and MyD88 after incubation with respective siRNAs for 48, 96 and 144 h to determine the effective duration of knock down of expression of both the proteins. Results represent three independent experiments and presented here as mean ± SD.

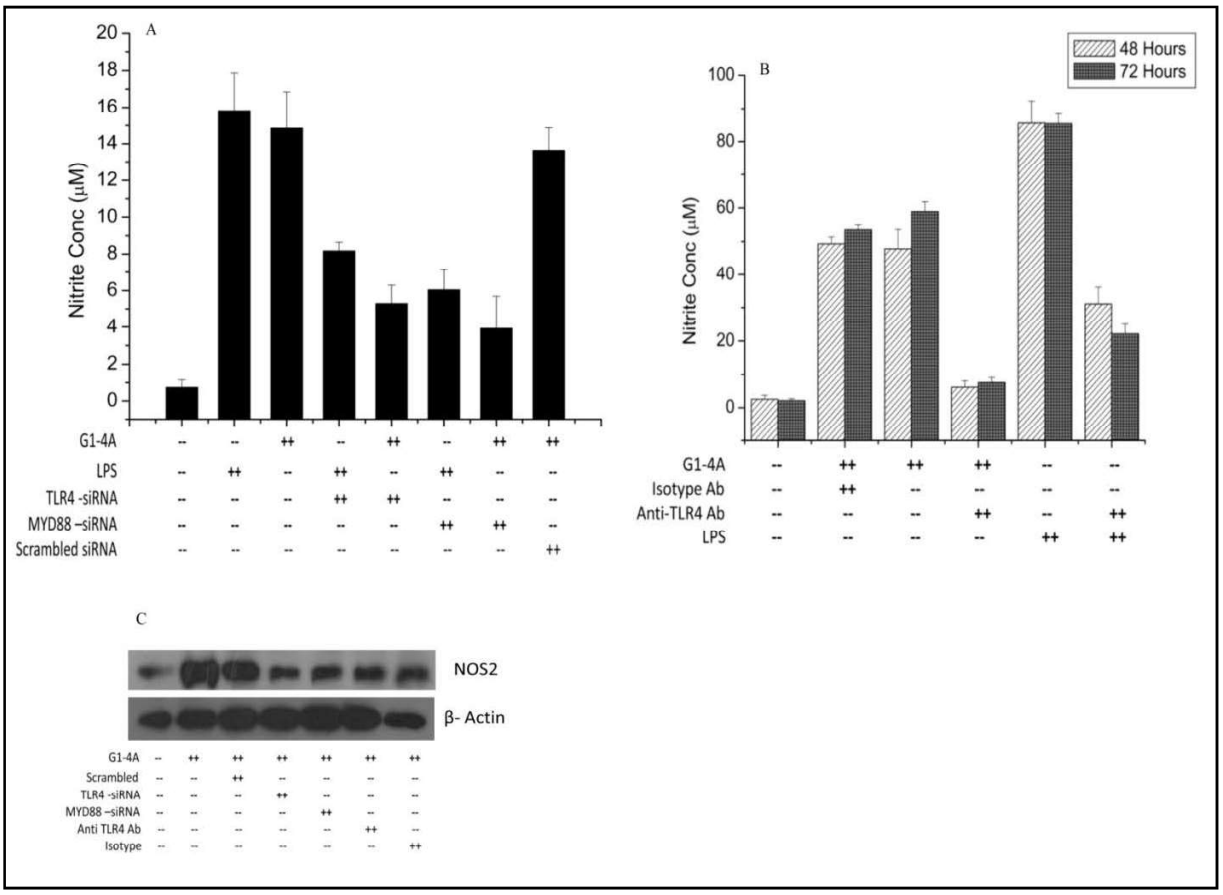


Figure 3.8. Nitric oxide production induced by G1-4A is TLR4-MyD88 dependent. *(A) RAW cells were incubated with TLR4 siRNA, MyD88 siRNA and scrambled siRNA and later treated with G1-4A or LPS. Nitrite concentration was measured in supernatants by using Griess' reagent. (B) RAW cells were treated with anti TLR4 antibody and Isotype control antibody (10μg/well), 2 h prior to the treatment with G1-4A or LPS and nitrite concentration was measured in supernatant with Griess' reagent. (C) Immunoblot showing expression of NOS2 in the presence of different siRNAs and antibodies. Results represent three independent experiments and presented here as mean ± SD.*

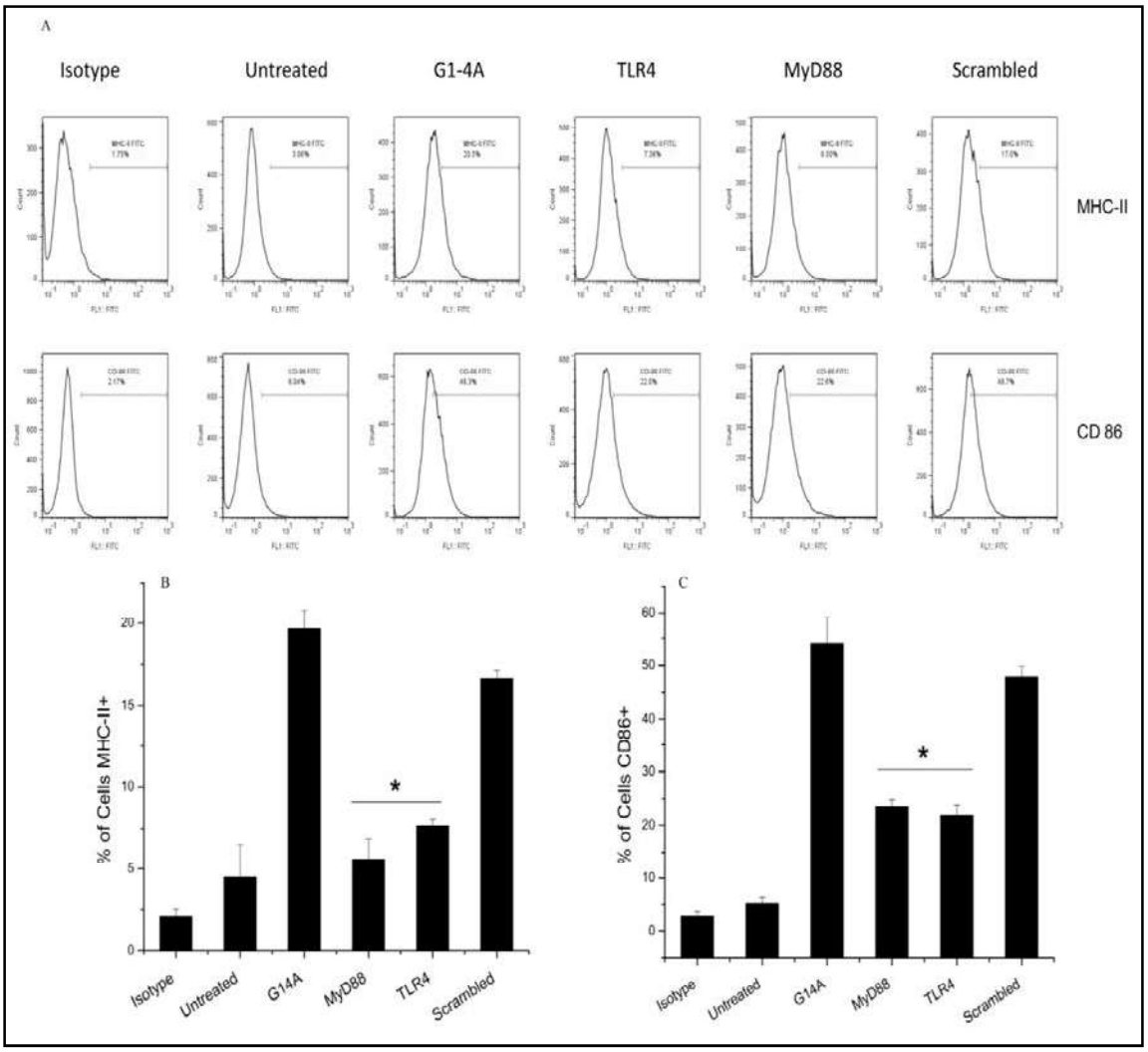


Figure 3.9. G1-4A mediated MHC-II and CD-86 surface expression is TLR4-MyD88 dependent.

*(A) RAW cells were incubated with TLR4 siRNA, MyD88 siRNA and scrambled siRNA and later either treated with G1-4A (1mg/ml) or left untreated. Cells were harvested after 48 h of treatment, washed with 1xPBS and incubated with FITC anti I-A^d, FITC anti CD86 and FITC IgG2a isotype control antibody and analyzed by flow cytometry. Each panel is representative of three or more independent experiments. (B) and (C) are quantitative representation of per cent of cells MHC-II+ and CD-86+ (*p<0.01 compared to G1-4A treated cells, student's t-test, values presented as Mean ± SD).*

3.1.1.5. Effect of G1-4A on MAP kinases (MAPK) and their involvement in macrophage activation

To delineate the mechanism of action of G1-4A mediated macrophage activation, we decided to find out the effect of G1-4A treatment on MAPK activation and involvement of MAPK in G1-4A mediated activation in RAW cells.

RAW cells were treated with G1-4A for 0, 15, 30, 60 and 120 min, and levels of phosphorylated and total MAPK i.e. p38, JNK1/2 and ERK1/2 were determined by immunoblotting. It was observed that after G1-4A treatment all the three MAPKs were phosphorylated and phosphorylation started after 15 min of treatment, reached to maximum, 30 min post treatment and declined gradually at further time points (Fig 3.10). After confirming the MAPK activation by G1-4A treatment, we further sought to investigate the role of MAPK on the G1-4A mediated production of cytokines and nitric oxide using the pharmacological inhibitors of p38 (SB203580), ERK1/2 (U0126) and JNK1/2 (SP600125). When cytokine levels were detected in the presence or absence of MAPK inhibitors after G1-4A treatment, a marked decrease in the levels of IL-6, TNF-α, IL-1β and IL-12 ($p<0.05$) was observed in the presence of U0126 and SB203580 but it remained unchanged in presence of SP600125 (Fig 3.11A to 3.11D) which suggests the involvement of p38 and ERK1/2 in G1-4A mediated induction of above cytokines. Further, we observed a significant decrease in level of nitric oxide ($p<0.01$) in presence of SB203580 and SP600125 whereas, there was no change in presence of U0126 (Fig 3.11E) which indicated the involvement p38 and JNK1/2 in G1-4A mediated NO induction. Similar pattern was observed in G1-4A mediated NOS2 expression (Fig 3.11F) which was down regulated in presence of p38 and JNK1/2 inhibitors confirming the role of these two MAPKs in G1-4A mediated NOS2

expression in RAW cells. Therefore, it is logical to infer that G1-4A activates all the three MAPKS which in turn plays a role in macrophage activation.

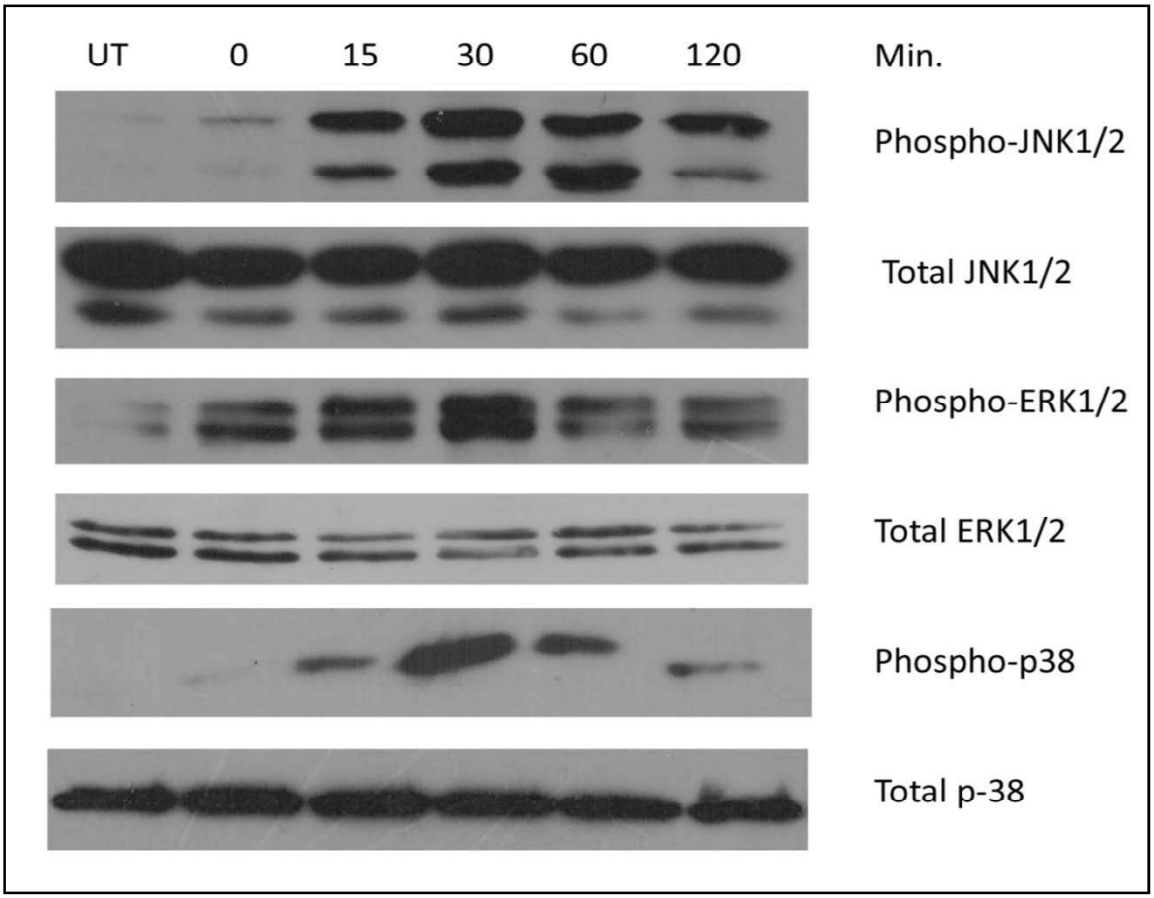


Figure 3.10. Effect of G1-4A treatment onMAPK activation.

RAW 264.7 cells were treated with G1-4A (1mg/ml) for 0, 15, 30, 60, or 120 min. Macrophages were lysed, and subjected to SDS PAGE followed by immunoblotting using monoclonal antibodies to phospho-JNK, phospho-ERK, and phospho-p38. Parallel blots were also run, using the antibodies against total JNK, ERK and p38.

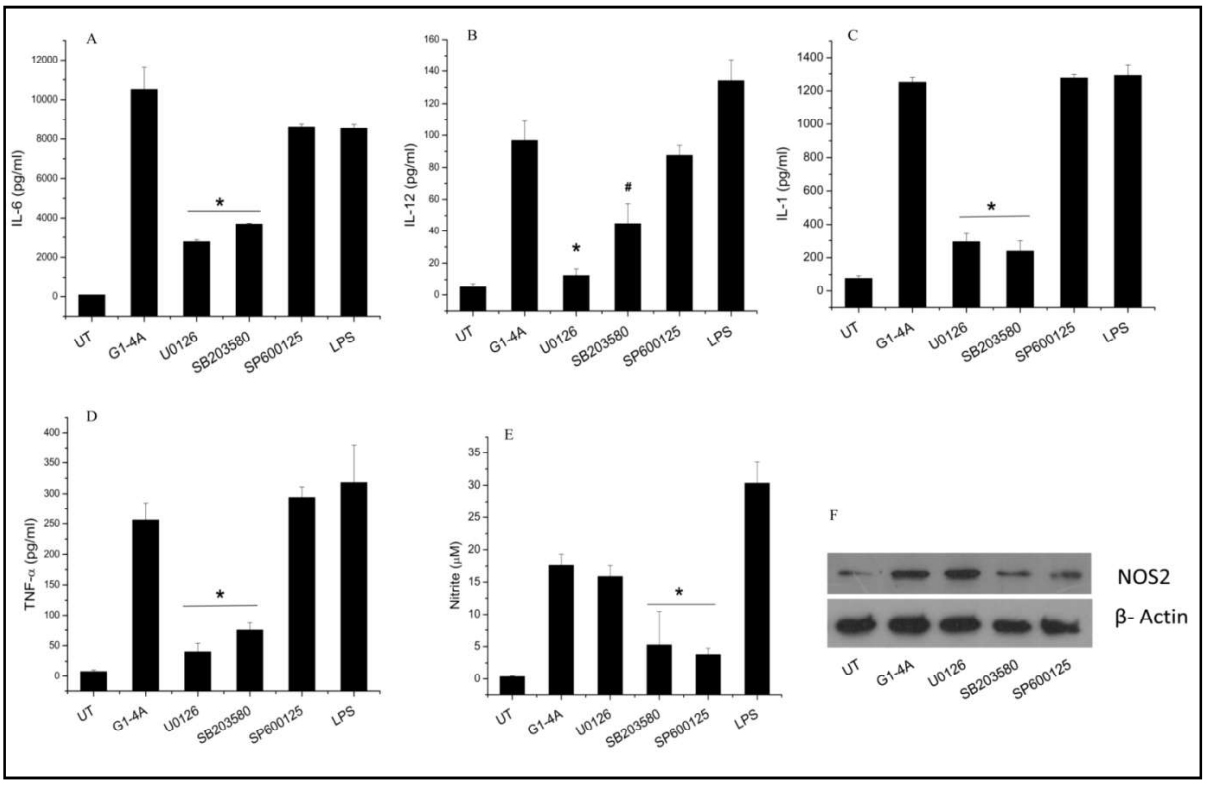


Figure 3.11. Effect of MAPK inhibitors on G1-4A mediated cytokine and NOS2 expression.

*RAW cells were treated with pharmacological inhibitors of MAP kinases i.e. UO126 (ERK), SB203580 (p-38) SP600125 (JNK) for 2 h, followed by treatment with G1-4A. Supernatants were used to determine the concentration of cytokines and nitric oxide, cells were lysed and expression of NOS2 protein was evaluated by immunoblotting. (A-D)IL-6, IL-12, IL-1β, TNF-α, (E) Nitric oxide (F) Immunoblot of NOS2. Results represent three independent experiments and presented here as mean ± SD. *P<0.01 and #P<0.05 compared to G1-4A treated samples, student's t-test, values presented as Mean ± SD.*

3.1.1.6. Involvement of NF-kB in G1-4A mediated up-regulation in the expression of MHC-II in RAW 264.7 cells

RAW cells were pre-treated with pharmaceutical inhibitor of NF-kB, PDTC (Pyrrolidine dithiocarbamate) at 25 μM, 50μM and 100 μM for 1 h, prior to treatment with G1-4A. After 24 and 48 h of the treatment with G1-4A, cells were labeled with FITC anti-MHC-II Ab and analysed by flow-cytometry. We observed a significant decrease in the expression of MHC-II in presence of PDTC (100μM), which suggested that NF-kB was involved in G1-4A, mediated MHC-II expression which is

TLR4-MyD88 dependent and hence it could be concluded that NF-kB was the link between TLR-MyD88 and MHC-II expression (Fig 3.12).

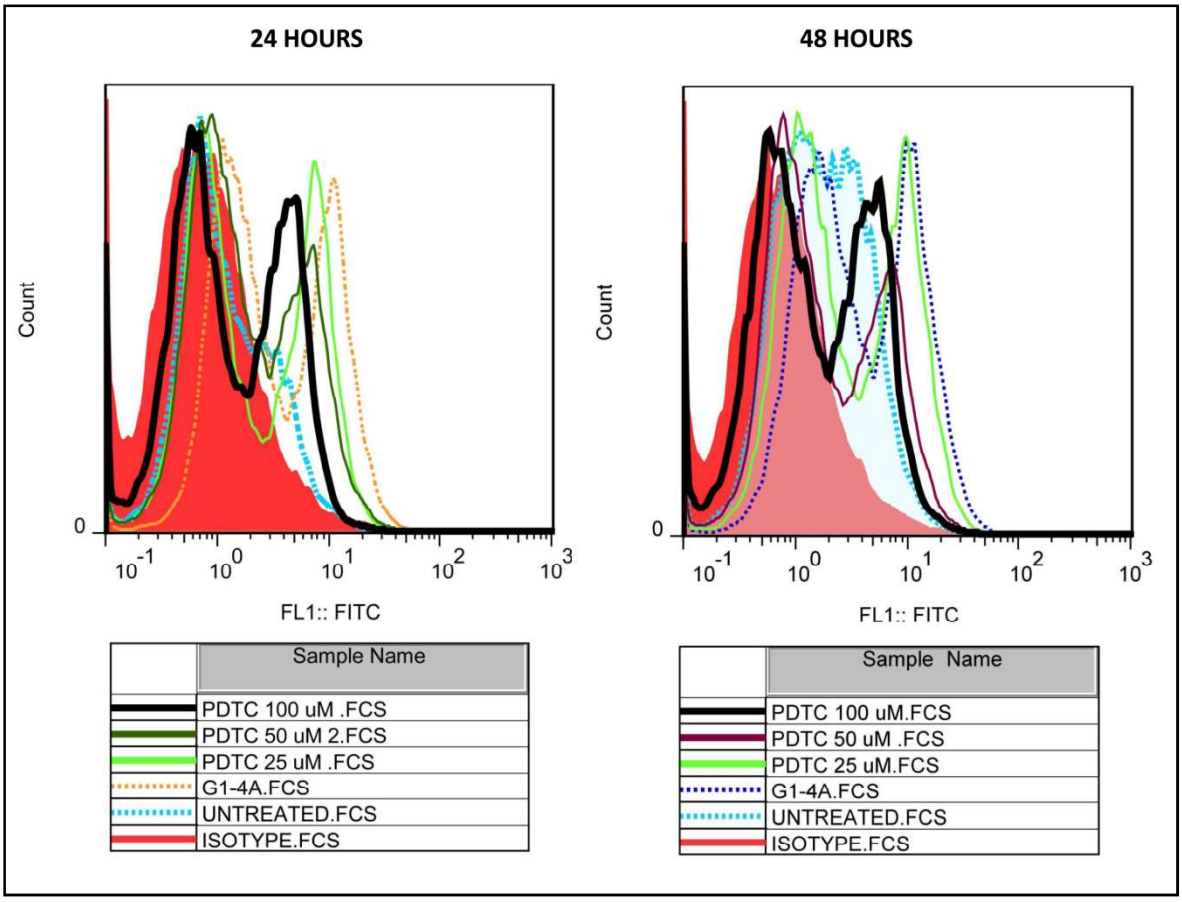


Figure 3.12. Expression of MHC-II in RAW 264.7 cells in the presence of PDTC. *RAW cells were pre-treated with pharmaceutical inhibitor of NF-kB, PDTC (Pyrrolidine dithiocarbamate,) at 25 µM, 50µM and 100 µM for 1 h prior to treatment with G1-4A and later cells were stained with FITC anti-MHC-II Ab and analyzed by flow cytometer. Panels represent representative of three or more independent experiments.*

So far we concluded that G1-4A activates macrophages through classical pathway in TLR4-MyD88 dependent pathway. Further it activated all three MAPKs and NFkB which subsequentlyleads to the expression of pro-inflammatory cytokines, NO, MHC-II and CD86. The detailed mechanism of action of G1-4A is summarized in diagram

depicted in Fig. 3.13.

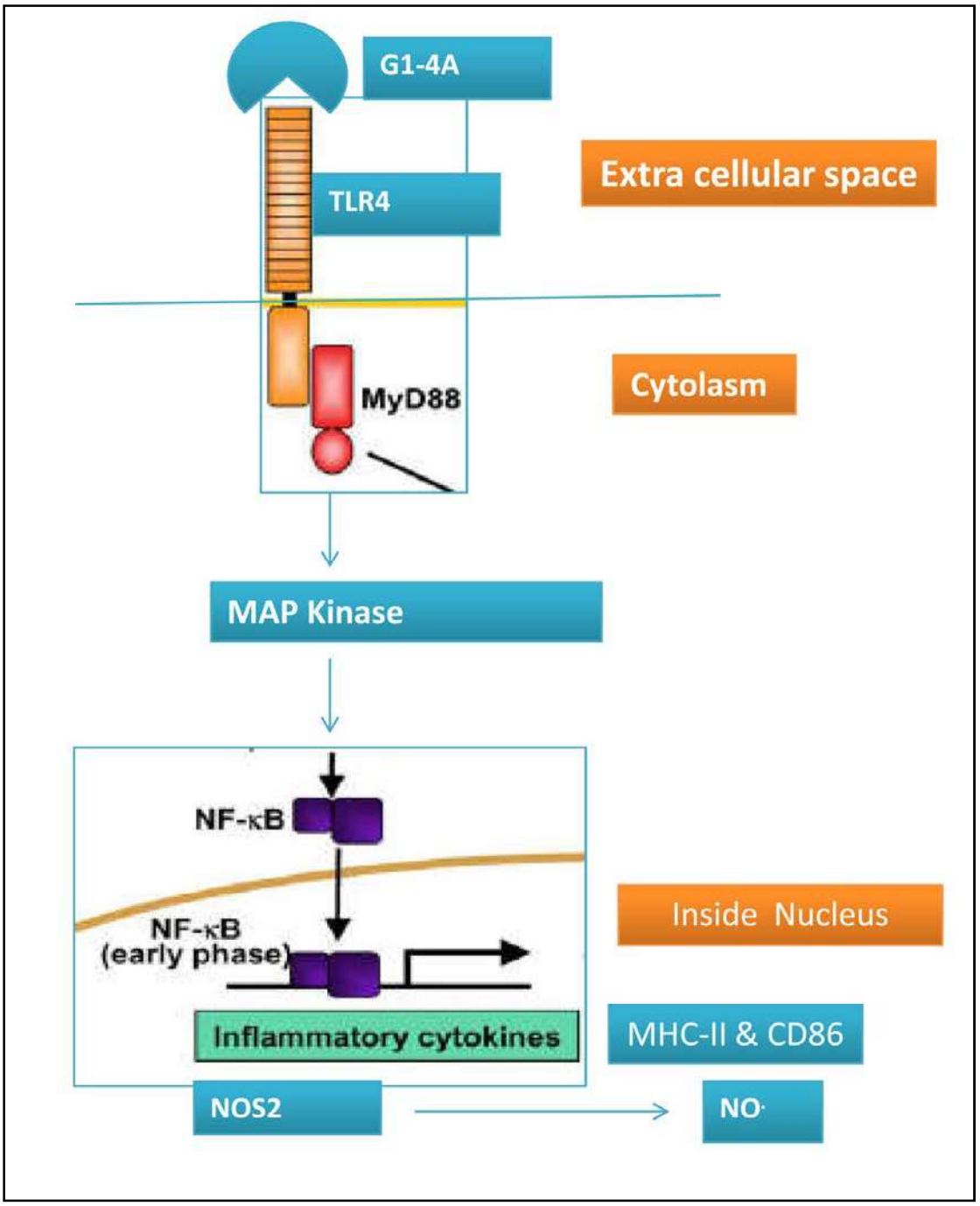


Figure 3.13. Diagrammatic representation of mechanism of action of G1-4A in murine macrophages

3.1.2. Effect of G1-4A in MTB infected macrophages

3.1.2.1. Modulation of pro-inflammatory cytokine expression by G1-4A in macrophages infected with drug-sensitive and MDR strains of MTB

To determine the effect of purified G1-4A on the expression of pro-inflammatory cytokines in MTB infected macrophages, murine macrophage cell line RAW 264.7 cells were incubated with G1-4A for 8 h followed by infection with MTB H37Rv, a drug-sensitive laboratory strain and two MDR clinical isolates of MTB belonging to two different genetic lineages viz: Beijing and LAM. The cells were subsequently treated again with G1-4A. The effects of G1-4A on cytokines in MTB infected RAW cells were then analyzed in the culture supernatants. MTB H37Rv, Beijing and LAM differentially regulated the expression of cytokines in macrophages. Our cytokine data demonstrated that IFN-γ levels were very low in supernatants of cells infected with all the MTB strains in absence of G1-4A but increased significantly after treatment with G1-4A (Fig 3.14A). In case of IL-1β, differential expression was observed in untreated cells, with H37Rv infected cells showing the highest and Beijing showing the lowest levels (Fig 3.14B). However, G1-4A treatment up-regulated the levels of IL-1β in cells infected with all the strains. Similar pattern was also observed in case of IL-6 (Fig 3.14C). In case of TNF-α, LAM induced maximum and Beijing induced minimum level, although G1-4A treatment yet again increased the production of TNF-α in cells infected with all the strains (Fig 3.14D). Therefore, it was evident that G1-4A modulated the expression of pro-inflammatory cytokines in MTB infected cells irrespective of the genotype, lineage and drug resistance status of MTB strains.

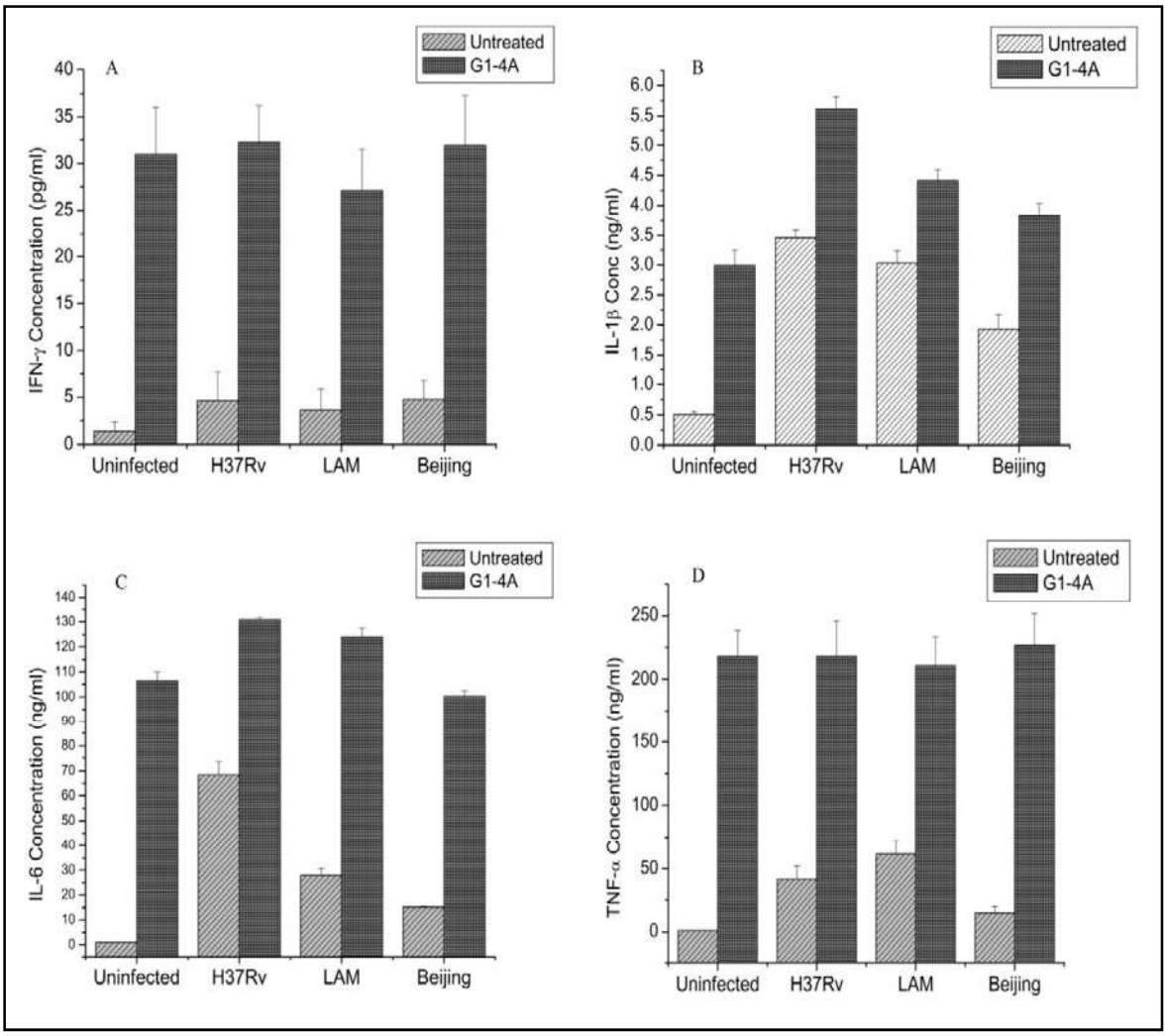


Figure 3.14. G1-4A mediated cytokine production by MTB infected RAW cells.
RAW cells were treated with G1-4A (1 mg/ml), for 8 h and infected with three strains of MTB i.e H37Rv, LAM and Beijing at MOI 5. Four h after infection cells were retreated with G1-4A for 24 h. Cytokines levels were quantified in supernatants by ELISA, (A)- IFN-γ, (B)-IL-1β, (C)-IL-6, (D)-TNF-α. Results shown here are representative of at least three independent experiments presented as mean ± SD.

3.1.2.2. Effect of G1-4A on the nitric oxide production in macrophages infected with drug-sensitive and MDR strains of MTB

Further, we investigated the effect of G1-4A on nitric oxide production in MTB infected cells and observed that Beijing induced minimum while LAM induced maximum levels of NO in the absence of G1-4A, but strikingly, levels of NO increased significantly after G1-4A treatment irrespective of MTB lineage or drug

resistance status (Fig 3.15A). Additionally, immunoblotting and real time RT PCR data also suggested G1-4A mediated up-regulation of NOS2 at protein and mRNA level in MTB infected cells (Fig 3.15B-D).

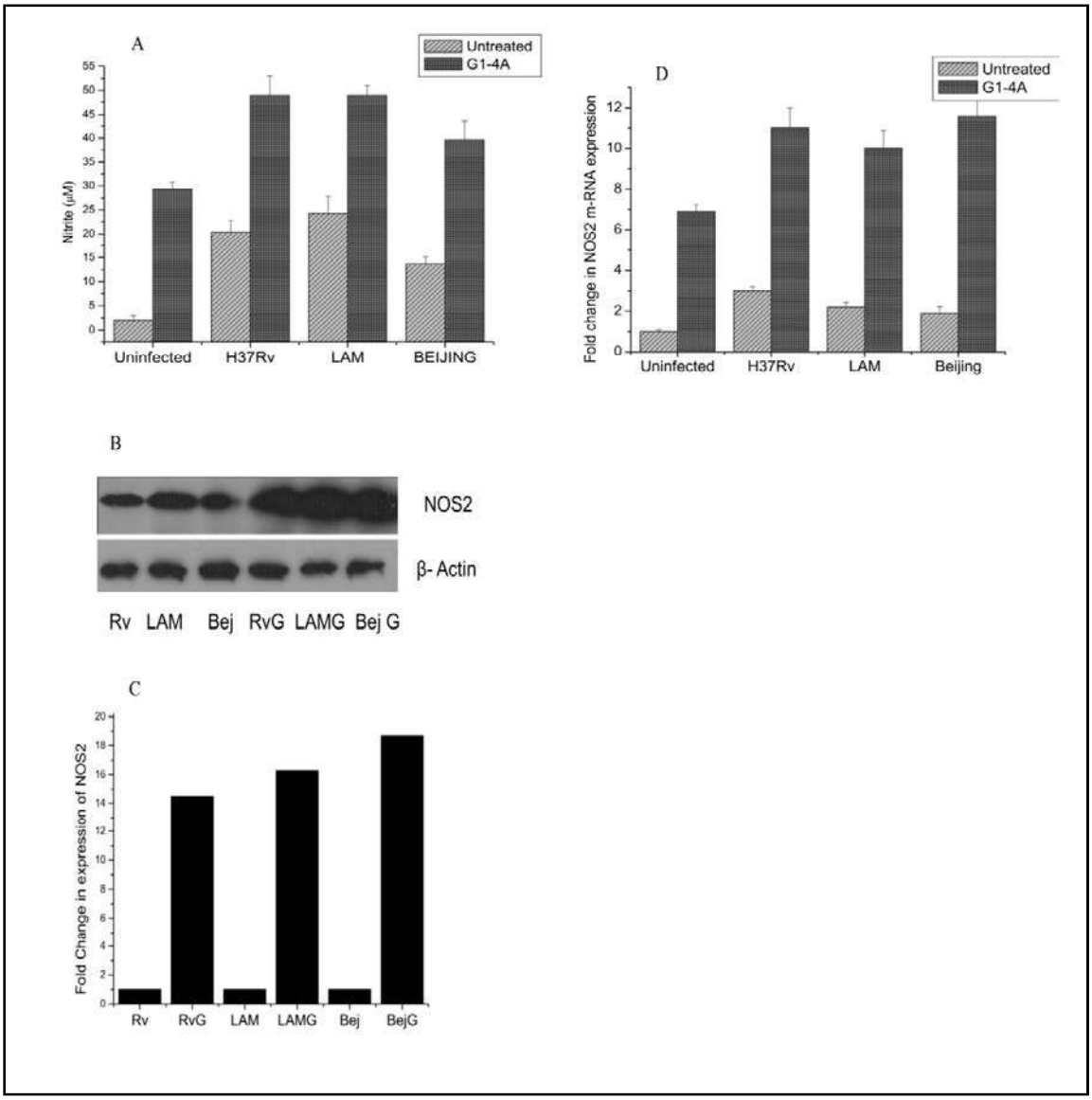


Figure 3.15. G1-4A mediated nitric oxide production by MTB infected RAW cells. *RAW cells were treated with G1-4A and infected with three strains of MTB as described earlier. (A) Nitric oxide levels were measured as nitrite concentration in supernatants by using Griess' Reagent. (B) Immunoblotting using anti-NOS2 antibodies to determine the protein level of NOS2. (RvG- cells infected with MTB H37Rv and treated with G1-4A, LAMG- cells infected with MTB LAM and treated with G1-4A, BejG- cells infected with MTB Beijing and treated with G1-4A) (C) Densitometric analysis of NOS2 protein expression by Image J (D) Relative quantification of NOS2 mRNA was performed by* $2^{-\Delta\Delta Ct}$ *method to determine the fold change in NOS2 gene expression compared to GAPDH. Results shown are representative of at least three independent experiments and presented here as mean ± SD.*

3.1.2.3. G1-4A up-regulates the MHC-II and CD86 surface expression in the macrophages infected with drug-sensitive and MDR strains of MTB

We monitored the effect of G1-4A on surface expression of MHC-II and CD86 in MTB infected cells by immunophenotyping. A significant increase in percentage of MHC-II positive cells from 13.33±2.31 percent to 66.93±5.49 percent in case of H37Rv ($p<0.01$), from 10.59±2.72 percent to 66.1±3.16 percent in case of LAM ($p<0.01$) and from 11.77±1.57 percent to 68.9±7.35 percent in case of Beijing ($p<0.01$) infected cells after 24 h of G1-4A treatment was observed (Fig 3.16A). Likewise, G1-4A treatment augmented the percentage of CD86 positive cells from 9.50±1.56 percent to 30.1±0.92 percent in case of H37Rv ($p<0.01$), from 24.53±1.91 percent to 44.0±1.25 percent in case of LAM ($p<0.01$) and from 37.73±1.53 percent to 37.7±1.55 percent in case of Beijing ($p<0.01$) infected cells (Fig 3.16B).

Above observations indicated the activation of macrophages infected with MTB by G1-4A treatment which may trigger Th1 responses *in vivo* and enhance the protective immunity.

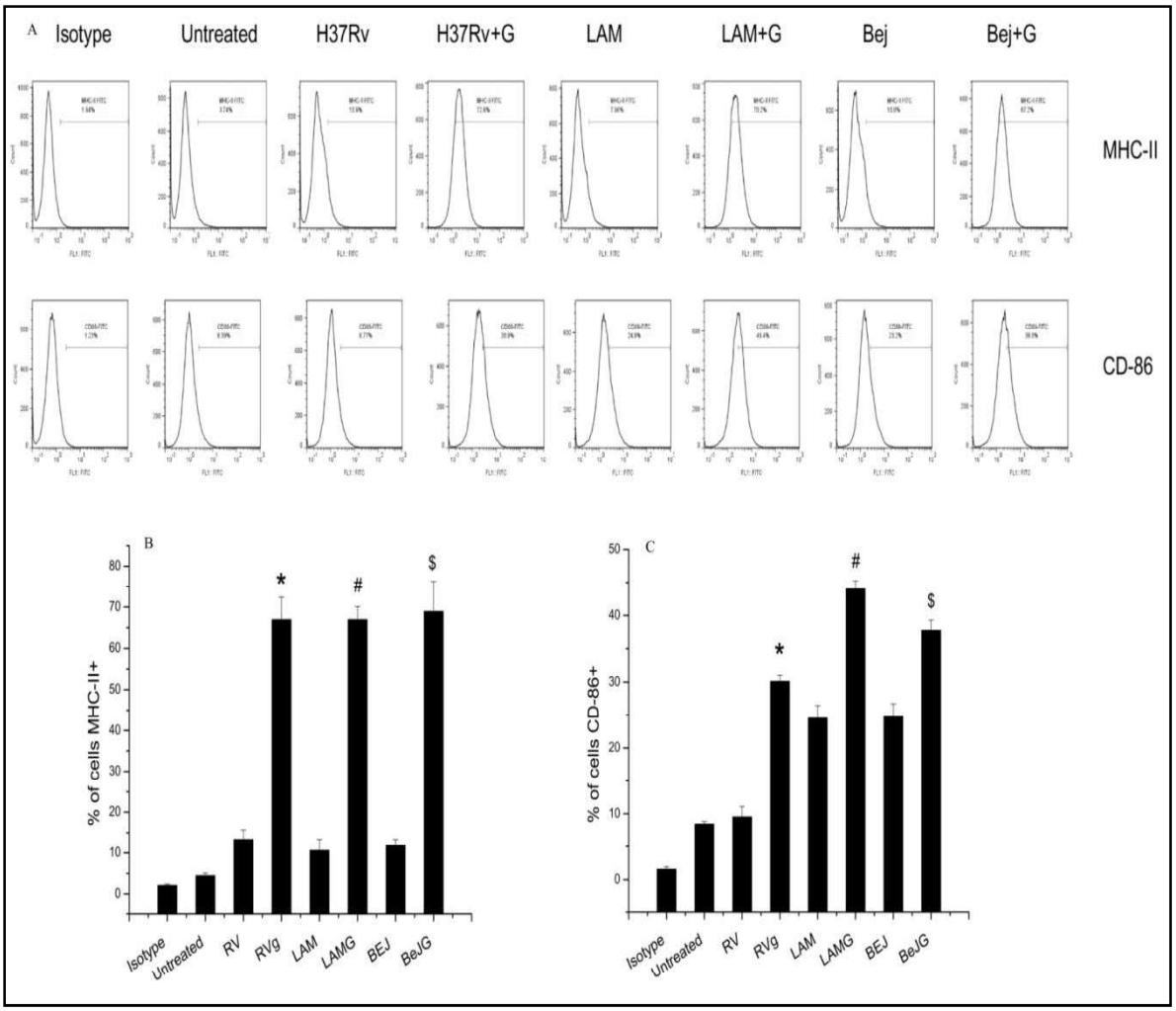


Figure 3.16. Effect of G1-4A on CD-86 and MHC-II expression in MTB infected RAW cells.

*(A)RAW cells were treated with G1-4A and infected with three strains of MTB. Post G1-4A treatment, cells were incubated with FITC-I-A^d, FITC anti–CD86 and FITC conjugate isotype Ab and surface expression was evaluated by flow cytometry. (B) and (C) are quantitative representation of percentage of cells MHC-II+ and CD-86+(*p<0.01 as compared to untreated H37Rv infected control, #p<0.01 compared to untreated LAM infected control and $p<0.01 compared to Beijing infected control, values of at least three independent experiments presented as Mean ± SD).*

3.1.2.4. G1-4A treatment augments the phagocytosis of MTB in murine macrophages

Activated macrophages are known to have enhanced phagocytic ability; therefore, we evaluated the effect of G1-4A on phagocytosis of GFP tagged MTB. RAW cells

seeded on coverslips, were treated with G1-4A or LPS and infected with GFP-H37Rv. Subsequently cells were fixed using 4% para formalin and visualized under Nikon TE 2000E laser scanning confocal microscope. It was observed that phagocytic uptake of GFP-MTB was significantly higher in cells treated with G1-4A or LPS compared to untreated cells (Fig 3.17A). Phagocytic indices of G1-4A and LPS treated cells were comparable and were significantly higher as compared to that of untreated control ($p<0.05$, Fig 3.17B). Flow-cytometry data demonstrated that percentage of GFP positive cells increased significantly, 56.4±6.6 percent, ($p<0.01$) in G1-4A treated cells, compared to untreated, 30±3.8 percent in uninfected control (background control), 6.2±1.2 percent (Fig 3.17C).

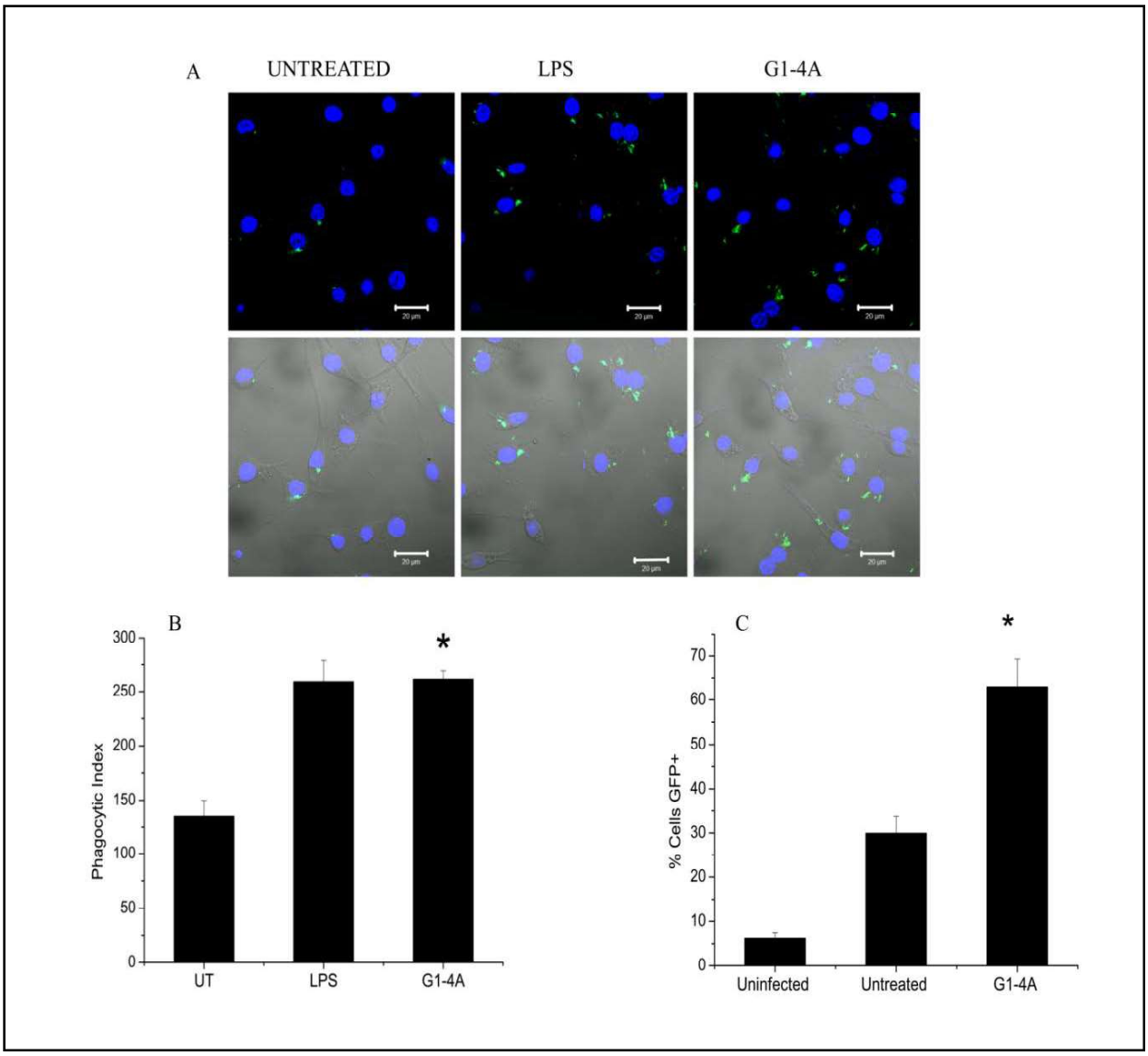


Figure 3.18. Effect of G1-4A on phagocytic uptake of MTB by RAW cells.
*(A) Confoacal microscopy images of RAW cells infected with GFP tagged MTB H37Rv either treated with G1-4A/LPS or left untreated (DAPI counter stain) (B) Phagocytic index (*p<0.01 compared to untreated control) (C) Quantitative representation of percentage of GFP+ cells as analyzed by Flow cytometer. Values of at least three independent experiments presented as Mean ± SD).*

3.1.2.5. Inhibition of the intracellular survival of drug-sensitive and MDR strains of MTB in macrophages by G1-4A treatment partially through nitric oxide production in TLR4-MYD88 dependent manner

Since G1-4A was able to augment the expression of pro-inflammatory cytokines and skew the host immune responses towards host protection, we decided to investigate its effect on the survival of intracellular MTB. RAW cells were treated with G1-4A for 8 h prior to infection with MTB H37Rv, LAM and Beijing strains. After removal of non-internalized bacteria, G1-4A was added for 48, and 72 h and intracellular MTB survival was monitored by CFU enumeration. Colony counts were significantly decreased (Fig 3.19A) in case of MTB H37Rv ($p<0.05$), LAM ($p<0.05$) and Beijing ($p<0.05$) and a marked decrease in percent bacterial burden (Fig 3.19B) was observed i.e. 64±5 percent in case of H37Rv ($p<0.05$), 62±9 percent in case of LAM ($p<0.05$) and 65±9 percent in case of Beijing ($p<0.05$), after 48 h of G1-4A treatment and 78±6 percent in case of H37Rv, 76±5 percent in case of LAM and 73±4 percent in case of Beijing after 72 h of G1-4A treatment as compared to untreated MTB infected cells.

As nitric oxide produced by activated macrophages is known to confer bactericidal activity on macrophages, we used L-NAME, a pharmacological inhibitor of NO to determine the role of NO in G1-4A mediated inhibition of intracellular survival of MTB strains. Treatment of L-NAME partially abrogated the G1-4A mediated intracellular inhibition of MTB (Fig 3.19C) and led to increase in the CFU counts, though counts were comparably lower than control, which suggested that G1-4A induced NO, was partially responsible for G1-4A mediated inhibition of intracellular survival of MTB strains. We had already established that G1-4A induced NO in RAW cells by up-regulating NOS2 expression in TLR4-MyD88 dependent manner, therefore, to determine the involvement of TLR4-MyD88 pathway in G1-4A mediated intracellular inhibition of MTB. RAW cells were first infected with MTB H37Rv followed by transfection with siRNA against TLR4, MyD88 and scrambled siRNA to avoid the interference by siRNAs in uptake of MTB. After treatment of G1-

4A for 48 and 72 h, cells were lysed, diluted and plated on Middlebrook7H11 plates for CFU enumeration. In presence of TLR4 and MyD88 siRNA, CFU counts were increased compared to CFU in presence of G1-4A alone or scrambled siRNA (Fig 3.19D). This suggested a role of TLR4 and MyD88 in G1-4A mediated intracellular inhibition. It was further confirmed by addition of anti-TLR4 blocking antibodies in MTB infected RAW cells prior to G1-4A treatment. As shown in Fig 3.19D, CFU counts increased in presence of anti-TLR4 blocking antibody compared to isotype antibody control or G1-4A alone, corroborating the involvement of TLR4 in G1-4A mediated intracellular inhibition of MTB growth as obtained by knocked down experiments. Additionally, decrease in the expression of TLR4 and MyD88 in RAW cells after siRNA treatment was shown by immunoblotting (Fig 3.19E). Hence, collectively all the results obtained so far, prove that G1-4A inhibits the intracellular survival in macrophages partially through the induction of NO in TLR4-MyD88 dependent manner.

REMA assay was used to determine the direct anti-mycobacterial property of G1-4A and results of this assay confirmed that G1-4A did not target MTB directly (Fig 3.20) but its anti-mycobacterial property is result of host immunomodulatory activity.

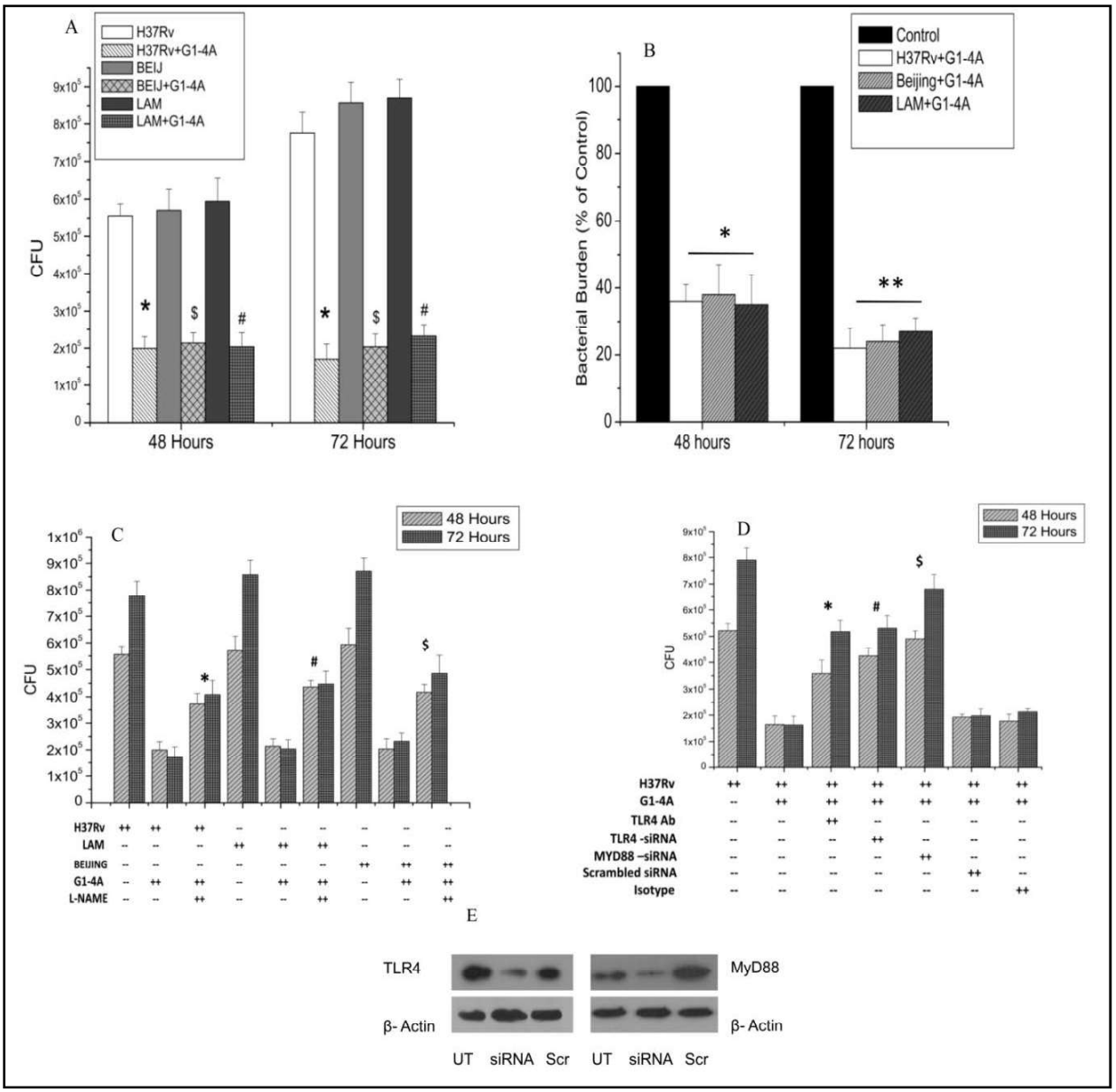


Figure 3.19. Inhibition of intracellular survival of MTB strains in macrophages by G1-4A.

*(A) CFU counts of three strains of MTB in presence or absence of G1-4A (*p<0.01 as compared to untreated H37Rv infected control, #p<0.01 compared to untreated LAM infected control and $p<0.01 compared to untreated Beijing infected control). (B) Bacterial burden (Percentage of control, *p<0.05, **p<0.01). (C) CFU assay of RAW cells infected with MTB strains and treated with G1-4A in the presence or absence pharmacological inhibitor of NOS2, L-NAME (100 µM), to block nitric oxide production and incubated for 48 and 72 h. (D) Cells were infected with MTB H37Rv and incubated in the presence of anti TLR-4 blocking antibodies or isotype control antibodies for 2 h followed by treatment with G1-4A for 48 and 72h. In other set of experiments, expression of TLR4 and MyD88 was transiently knocked down by adding siRNA against TLR4 and MyD88. Scrambled siRNA was used as control. Later, cells were infected with H37Rv and treated with G1-4A for 48 and 72 h. Figure depicts*

*CFU data for both the sets of experiments (E) Immunoblotting of TLR4 and MyD88 in RAW cells after siRNA treatment. Results shown are representative of at least three independent experiments and presented here as mean ± SD, *p<0.05 as compared to only G1-4A treated H37Rv infected control, #p<0.05 compared to only G1-4A treated LAM infected control and $p<0.05 compared to only G1-4A treated Beijing infected control).*

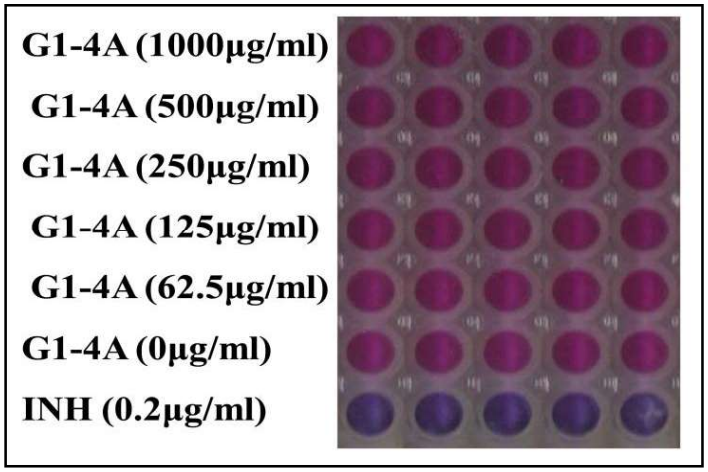


Figure 3.20. Effect of G1-4A on MTB growth in Middlebrook broth by Resazurin microtitre plate assay (REMA).
Resazurin microtitre plate assay (REMA) was performed to evaluate the effect of G1-4A on MTB growth in Middlebrook 7H9 broth supplemented with ADC. Briefly, dilutions of G1-4A (62.5µg/ml-1000µg/ml) were prepared in 7H9 broth and dispensed in sterile 96 well plates. Isoniazid was taken as positive control. Inocula of 10^4 MTB cells were added to each well. Sterile water was filled to periphery wells to minimize evaporation. The plate was sealed with paraffin, and incubated a 37°C for one week. Resazurin solution (30 µl/well) was added to each well and incubated overnight. A change in color from blue to pink indicated the growth of bacteria. Data shown here are from a single representative experiment out of three independent experiments.

Upto this point we could prove that G1-4A inhibited intracellular survival of MTB in murine macrophages and investigated the underlying mechanism in MTB infected RAW 264.7 cells. The detailed mechanism is summarized in the Fig 3.21

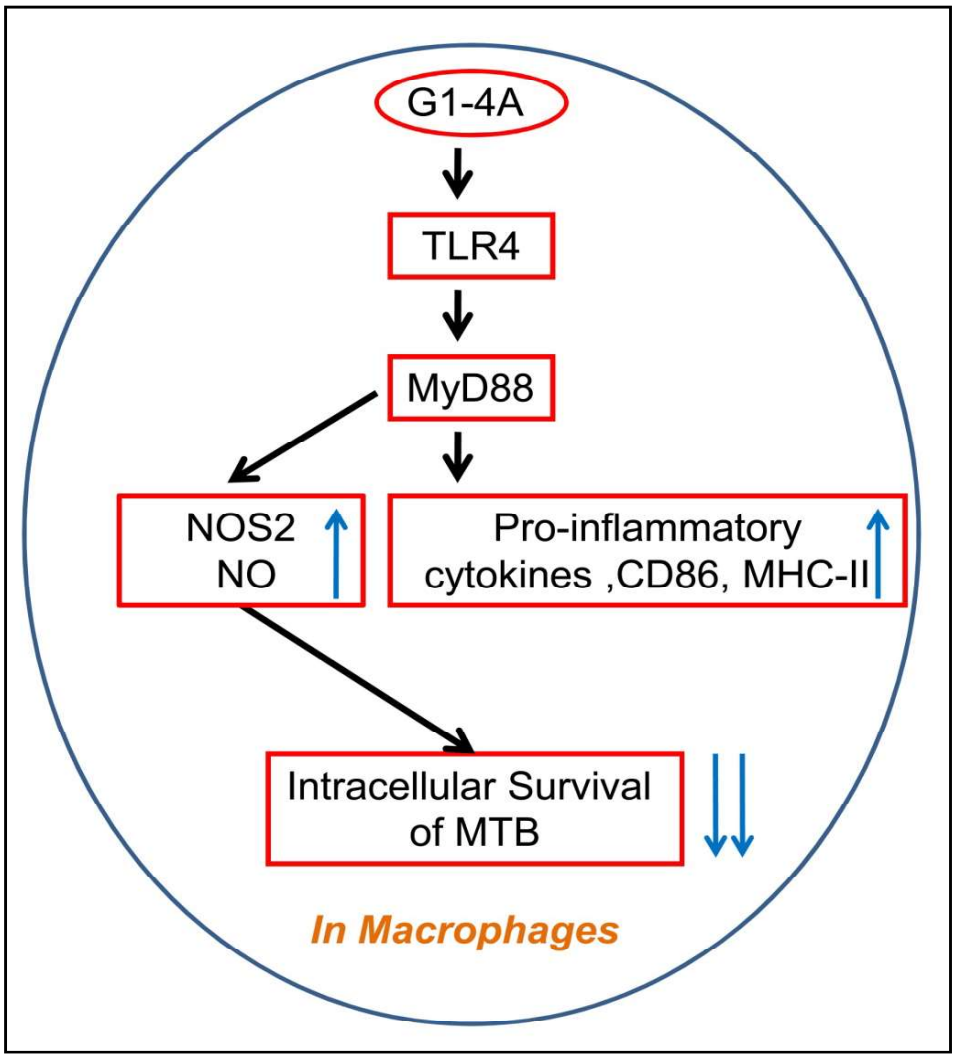


Figure 3.21. Underlying mechanism of G1-4A mediated inhibition of intracellular survival of MTB in RAW 264.7 cells

3.1.3. Evaluation of immunomdulatory and anti-mycobactrial effects of Polysaccharide Rich Extract (PRE) of *Tinospora cordifolia*

Due to the difficulty in the isolation of G1-4A, we decided to investigate the effects of PRE (Polysaccharide Rich Extract) on macrophage activation and intracellular survival of MTB in RAW cells. PRE is the crude extract from which the G1-4A is isolated by gel filtration chromatography. Results obtained are given below.

3.1.3.1. PRE treatment up-regulates the expression of cytokines in RAW 264.7 cells

To investigate the effect of PRE on cytokine expression in RAW cells, we treated the cells with two doses of PRE (1mg/ml and 2mg/ml) for 24 and 48 h. Supernatant was collected and levels of cytokines such as IL-6, IL-1β, IFN-γ, IL-12 and TNF-α in supernatant were estimated by ELISA. It was observed that PRE treatment up-regulated the expression of all cytokines in time dependent manner. IL-6 and IL-1β levels peaked up to 24 h of treatment and declined at later time point (Fig 3.22A-E). Levels of IFN-γ and IL-12 increased after 24 h of treatment and further increased at time point of 48 h. TNF-α level peaked at 6 h and 12 h of time point and declined thereafter. There was no significant difference between the expression of cytokines obtained with treatment of PRE 1mg/ml and 2 mg/ml. Data obtained with the treatment of PRE exhibits similarity with G1-4A treatment results.

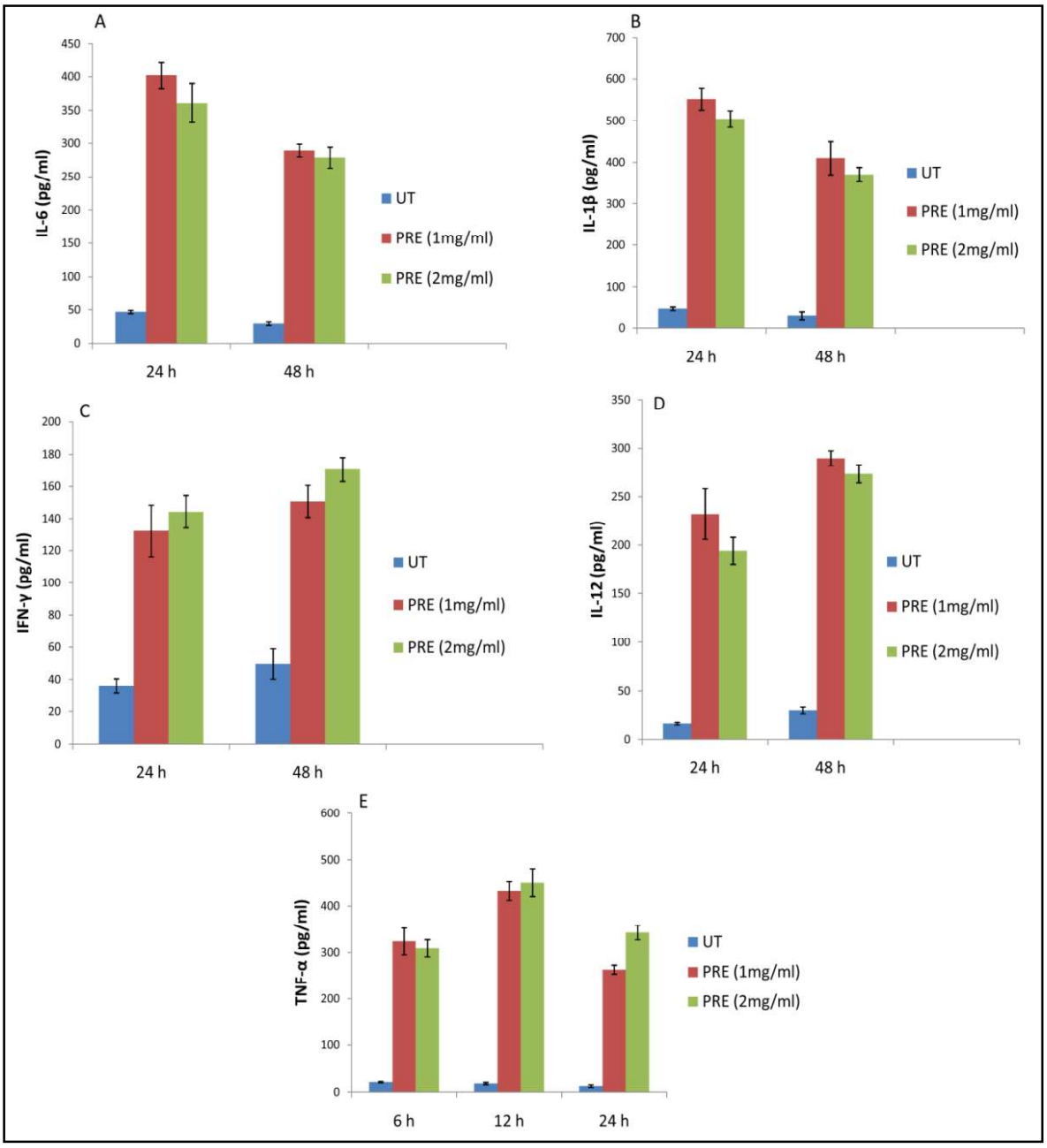


Figure 3.22. Effect of PRE on cytokine production in RAW 264.7 cells
RAW 264.7 cells were treated with two doses of PRE (1mg/ml and 2mg/ml) for 24 and 48 h. Levels of cytokines were detected in supernatants by ELISA. (A) IL-6 (B) IL-1β (C) IFN-γ (D) IL-12 (E) TNF-α. Results represent three independent experiments and presented here as mean ± SD.

3.1.3.2. PRE augments nitric oxide production in RAW 264.7 cells

To investigate the effect of PRE on nitric oxide production in RAW cells, we treated the cells with two doses of PRE (1mg/ml and 2mg/ml) for 24, 48 h and 72 h.

Supernatants were collected at each time point and nitric oxide estimation was carried out by using Griess' Reagent which measures the nitrite concentration. Data demonstrated that PRE treatment increased the level of Nitric oxide significantly after 24 h of treatment ($p<0.05$) and it further increased after 48 ($p<0.01$) and 72 h ($p<0.01$) of treatment, however there was no dose dependent difference observed. This data is in the concurrence with the earlier observation obtained with G1-4A treatment (Fig 3.23).

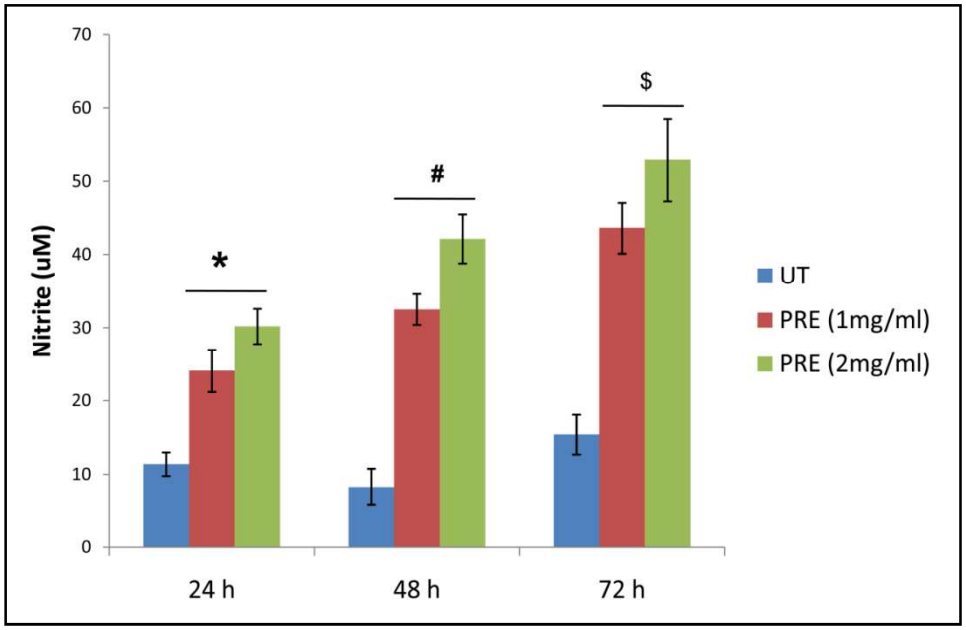


Figure 3.23. Effect of PRE on the secretion of nitric oxide in RAW 264.7 cells
*RAW 264.7 cells were incubated in the presence or absence of PRE (1mg/ml and 2mg/ml) for 24, 48 and 72 h. Nitric oxide estimation in supernatants was carried out by using Griess' Reagent. Results represent three independent experiments and presented here as mean ± SD. (*p<0.05, #p<0.01, $p<0.01 as compared to untreated control, student's t-test, values presented as Mean ± SD).*

3.1.3.3. Modulation of surface expression of MHC-II and CD86 in RAW cells by PRE treatment

RAW 264.7 cells were treated with PRE (1mg/ml and 2mg/ml) for 48 h, harvested and incubated with FITC anti I-A^d, FITC anti–CD86 and FITC IgG2a Isotype control Ab. Later labeled cells were analyzed by flow-cytometer. Data revealed a significant

increase in MHC-II+ and CD-86+ population which indicated the activation of macrophages due to PRE treatment at both the doses (Fig 3.24).

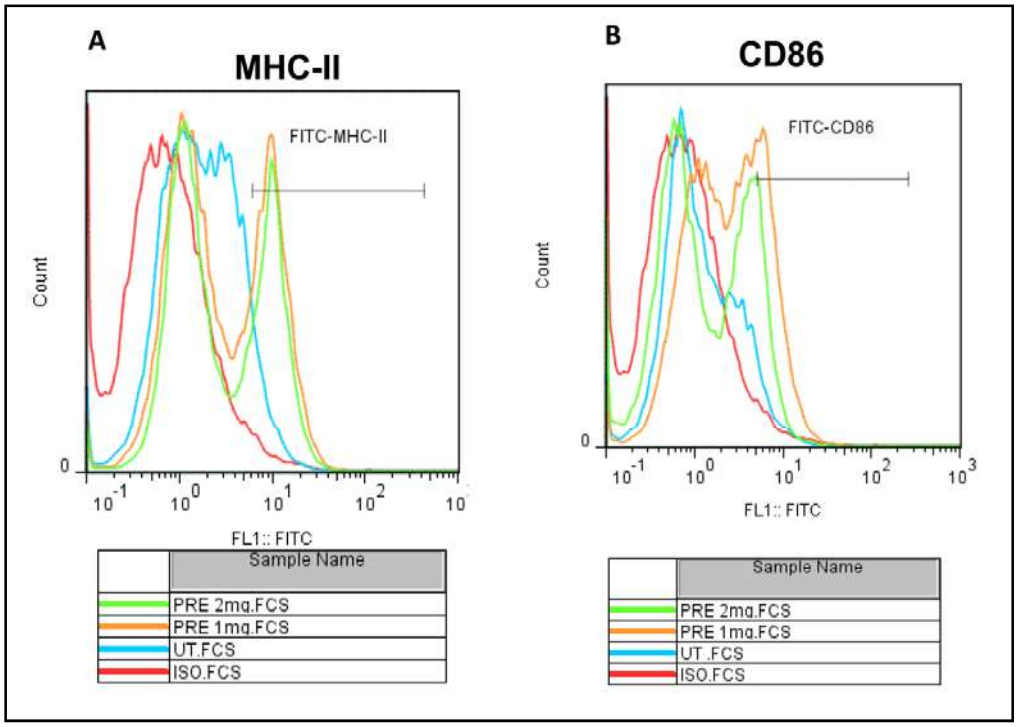


Figure 3.24. Effect of PRE on surface expression of MHC-II and CD-86
RAW 264.7 cells were incubated in the presence or absence of PRE (1mg/ml and 2mg/ml) for 48h. Cells were stained with FITC anti I-A^d, FITC anti CD86 and FITC IgG2a Isotype control Ab. (A) MHC-II and (B) CD86. Each panel is representative of three or more independent experiments.

Taking all above results into the account we could conclude that PRE treatment activates RAW cells through classical pathway like G1-4A.

3.1.3.4. PRE treatment activates p38, ERK1/2 and JNK1/2 MAPK in RAW 264.7 cells

G1-4A activates all the three MAPKs in RAW cells therefore we investigated the effect of PRE on MAPK activation in RAW cells. RAW cells were treated with PRE for 0, 15, 30, 60 and 120 min, and level of phosphorylated and total MAPK i.e. p-38, JNK1/2 and ERK1/2 were determined by immunoblotting. It was observed that PRE treatment phosphorylated all the three MAPKs and phosphorylation started after 15

min of treatment, reached to maximum 30 min post treatment and declined gradually at further time points. There was no significant difference observed between two doses of PRE (Fig 3.25).

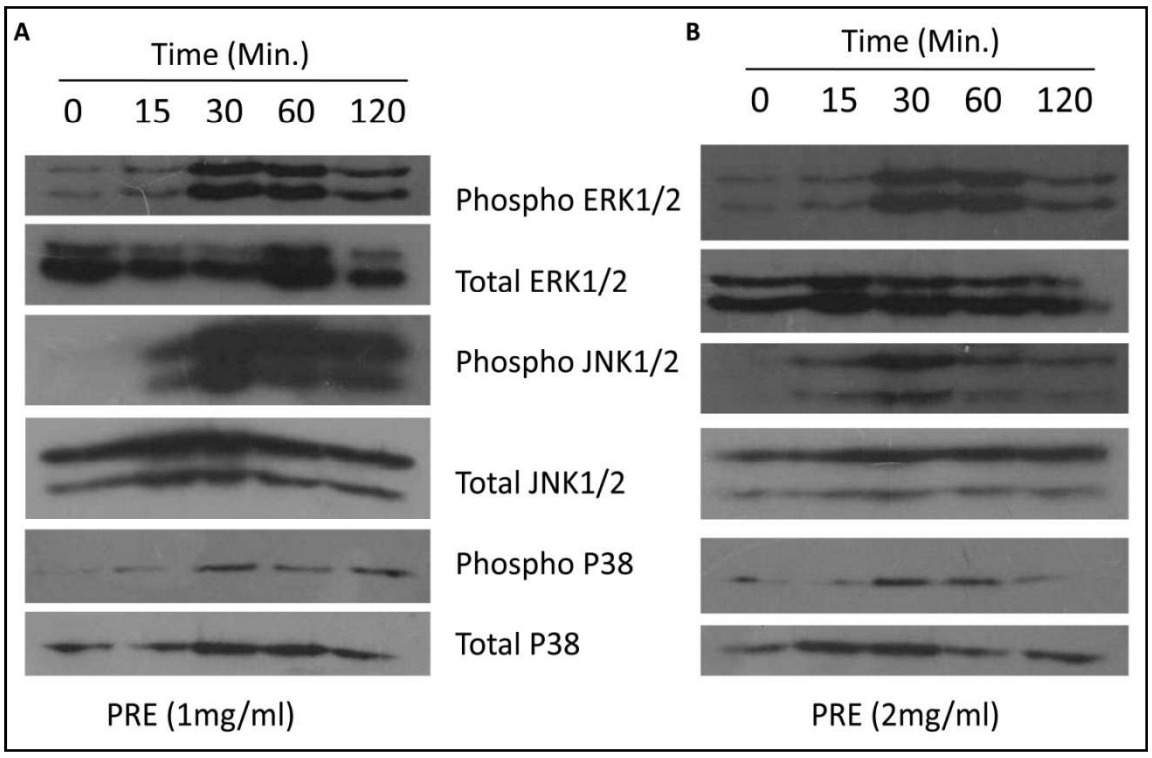


Figure 3.25. Effect of PRE on MAPK activation in RAW cells
RAW 264.7 cells were treated with PRE (1mg/ml and 2mg/ml) for 0, 15, 30, 60, or 120 min. Macrophages were lysed, and subjected to SDS PAGE followed by immunoblotting using monoclonal antibodies to phospho-JNK, phospho-ERK, and phospho-p38. Parallel blots were also run, using the antibodies against total JNK, ERK and p-38. (A) Represents MAPK activation at PRE dose 1mg/ml and (B) PRE dose 2mg/ml.

3.1.3.5. PRE inhibits the intracellular survival of MTB in murine macrophages

RAW 264.7 cells were seeded onto 24 well tissue culture plates at a density of 2 x10^5 cells/well and treated with PRE, 8-12 hrs prior to infection with MTB at an MOI of 5. Four hour post infection, cells were washed and incubated with medium containing amikacin to kill extracellular bacteria and again treated with PRE (1mg/ml and 2mg/ml) for 48 and 72 hrs. For CFU assay, infected cells were washed with PBS and

lysed with SDS in PBS at different time points. Serial dilutions were prepared in PBS and plated on 7H11 medium supplemented with OADC. Colonies were counted after 3 weeks. CFU data demonstrated a significant decrease in counts in PRE treated groups irrespective of their strains. It was observed that bacterial burden was reduced by 51±6.4 percent after 48 h of treatment and 67±7.3 percent after 72 h of treatment of PRE (Fig 3.26).

Thus from overall results obtained we could conclude that PRE inhibits the intracellular survival of MTB in RAW cells.

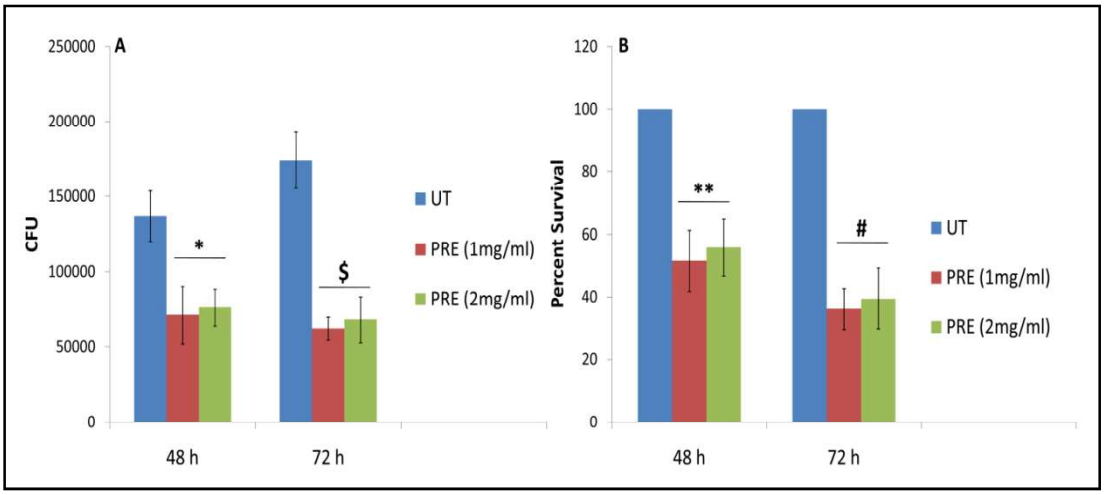


Figure 3.26. In vitro Evaluation of effect of PRE on survival of MTB in macrophages

*(A)CFU counts of MTB H37Rv in presence or absence of PRE (1mg/ml and 2mg/ml) (*p<0.05 as compared to untreated H37Rv infected control after 48 h treatment and $p<0.05 compared to untreated H37Rv infected control after 72 h treatment. (B) Bacterial burden (Percent survival of MTB after PRE treatment, **p<0.05, #p<0.01). Results represent three independent experiments and presented here as mean ± SD.*

3.1.4. Effect of G1-4A in infected human macrophages

Further, to strengthen our earlier results obtained in RAW cell Line (mouse macrophage cell line) and to evaluate the effect in human cell line, we used THP-1 as model and obtained similar results as we observed in case of RAW and PEM cells. Results of these experiments are given below:

3.1.4.1. G1-4A inhibits the intracellular survival of drug-sensitive and MDR strains of MTB in THP-1 cells

In the next step, we investigated the effect of G1-4A on intracellular survival of MTB in THP-1 cells. Cells were treated with G1-4A overnight followed by infection with MTB H37Rv, LAM and Beijing strains. After 4 h of infection, cells were retreated with G1-4A for 48 and 96 h. and CFU assay was performed at both the time points. Colony counts were significantly decreased (Fig 3.27A) in case of MTB H37Rv ($p<0.05$), LAM ($p<0.05$) and Beijing ($p<0.05$). A marked decrease in percent bacterial burden (Fig 3.27B) was observed i.e. 36.73±3.2 percent in case of H37Rv ($p<0.05$), 38.26±4.9 percent in case of LAM ($p<0.05$) and 33.72±4.3 percent in case of Beijing ($p<0.05$), after 48 h of G1-4A treatment and 57.17±7.9 percent in case of H37Rv ($p<0.01$), 53.21±4.4 percent in case of LAM ($p<0.01$) and 55.55±8.5 percent in case of Beijing ($p<0.01$)after 96 h of G1-4A treatment as compared to untreated MTB infected cells. Thus CFU data in THp-1 cells corroborated with earlier CFU data in murine macrophages.

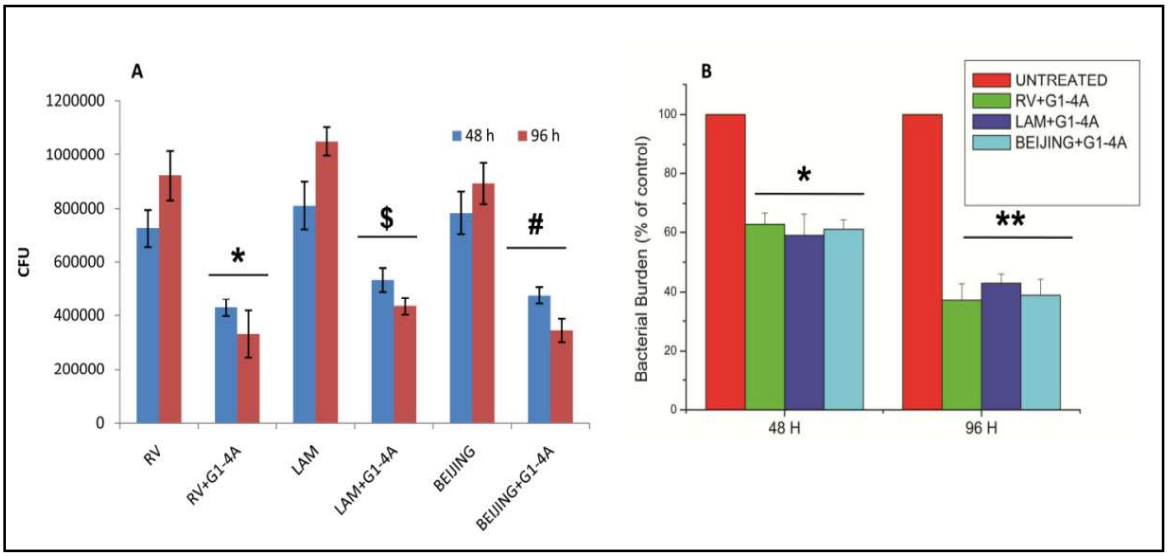


Figure 3.27.Inhibition of intracellular survival of MTB strains in THP-1 cells by G1-4A.*(A)CFU counts of MTB H37Rv in presence or absence of G1-4A (1mg/ml) in*

*MTB infected THP-1, (*p<0.05 as compared to untreated H37Rv infected control after 48 and 96 h of treatment, $p<0.05 compared to untreated LAM infected control after 48 and 96 h treatment and #p<0.05 compared to untreated Beijing infected control after 48 and 96 h treatment. (B) Bacterial burden (Percent survival of MTB after G1-4A treatment, *p<0.05, **p<0.01). Results represent three independent experiments and presented here as mean ± SD.*

3.1.4.2. G1-4A treatment induces apoptosis in MTB H37Rv/BCG infected THP-1 macrophages.

By virtue of the immunomodulatory effects of G1-4A to inhibit the intracellular survival of MTB in RAW cells, we decided to investigate the effects of G1-4A in MTB infected human monocytic cell line THP-1. It is well known that avirulent strains of MTB induce apoptosis in MTB infected macrophages which is host favorable phenomenon while virulent strains inhibit apoptosis in infected macrophages to avoid anti-mycobacterial effects of macrophages. Hence, we investigated the effect of G1-4A treatment on the cell death in MTB infected THP-1 cells. THP-1 cells were treated with G1-4A for overnight followed by infection with MTB H37Rv and BCG. After 4 h, infection was terminated; cells were retreated with G1-4A for 48 h and were subjected to PI-staining. PI-staining data revealed that G1-4A treatment enhanced the cell death in MTB H37Rv and BCG infected cells compared to untreated controls. Cell death in G1-4A treated H37Rv infected cells was 40.13±5.71 percent as compared to 22.96±2.47 percent in case of untreated infected cells after 24 h. After 48 h, cell death in G1-4A treated H37Rv infected THP-1 cells was 56.93±5.85 percent as compared to 38.93±1.82 percent in case of untreated infected cells (Fig. 3.28). In case of BCG infected cells, cell death after 24 h of G1-4A treatment was 60.53±2.32 percent as compared to 42.43±2.15 percent in untreated infected control. After 48 h, cell death in G1-4A treated infected THP-1 cells was 72.26±1.89 percent as compared to 49.3±1.05 percent in untreated control cells (Fig

3.29). To further prove that cell death induced by G1-4A is apoptosis and not necrosis, THP-1 cells were infected with H37Rv and BCG and treated with G1-4A as described earlier for 48 h. Later, cells were labeled with FITC-Annexin-V and analyzed by flow cytometer. Our data demonstrated induction of apoptosis in G1-4A treated MTB infected cells. In H37Rv infected cells percentage of Annexin V positive cells was 30.85±3.2 percent after G1-4A treatment compared to 13±0.14 percent in untreated H37Rv infected cells. Similarly BCG infected cells exhibited 39.3±2.12 percent Annexin V positive population after G1-4A compared to 15.4±0.84 percent in untreated BCG infected cells (Fig 3.30). Above data suggest that in addition to other immunomodulatory properties of G1-4A; it also enhances apoptosis in MTB infected cells which is beneficial for host.

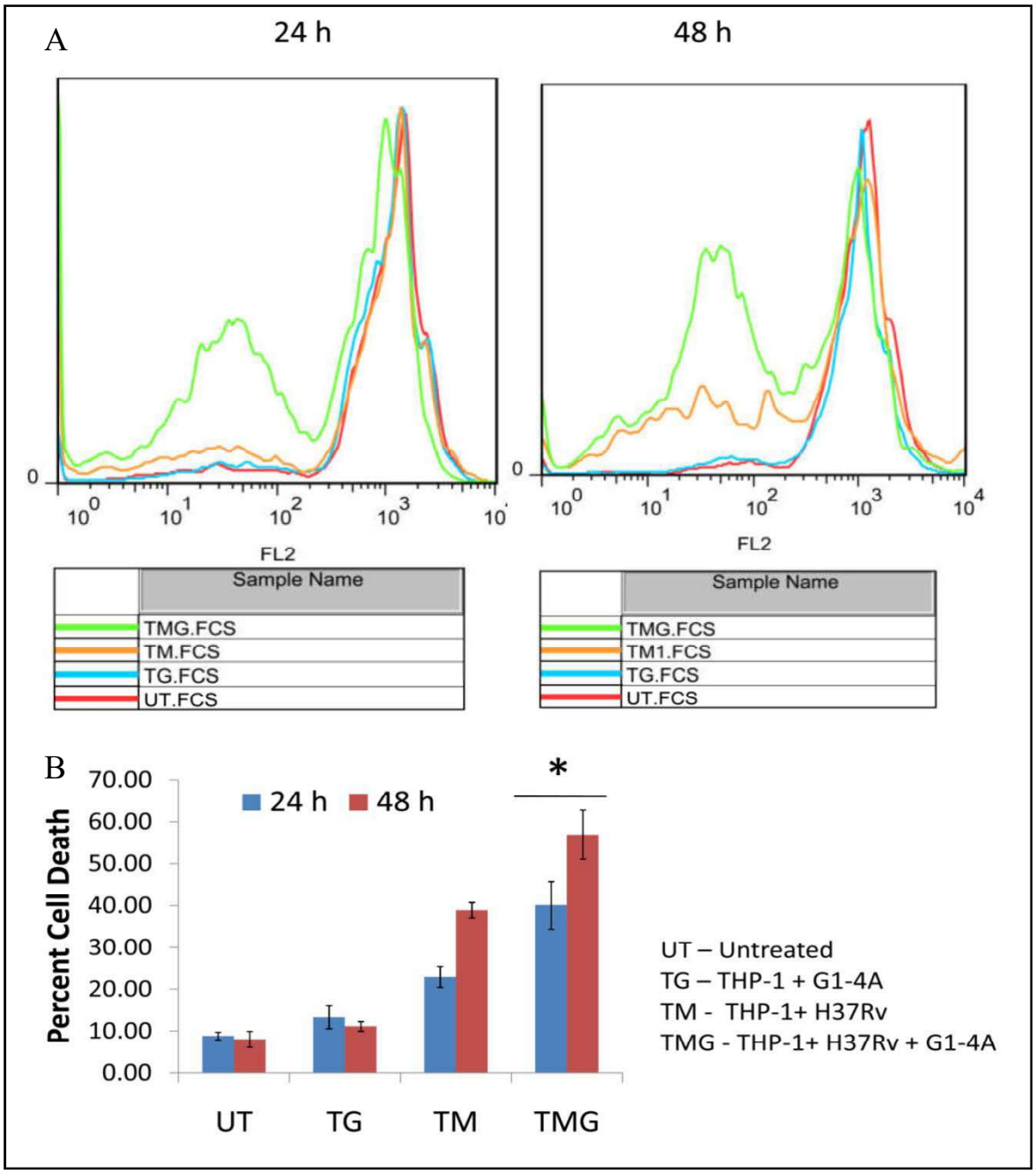


Figure 3.28. Induction of apoptosis in G1-4A treated MTB H37Rv infected THP-1 cells

THP-1 cells infected with H37Rv were incubated in the presence or absence of G1-4A (1mg/ml) for 24 and 48 h. Cells were stained with PI. (A) Each panel is representative of three or more independent experiments and (B) Quantitative representation of percent cell death. Results represent three independent experiments and presented here as mean ± SD.

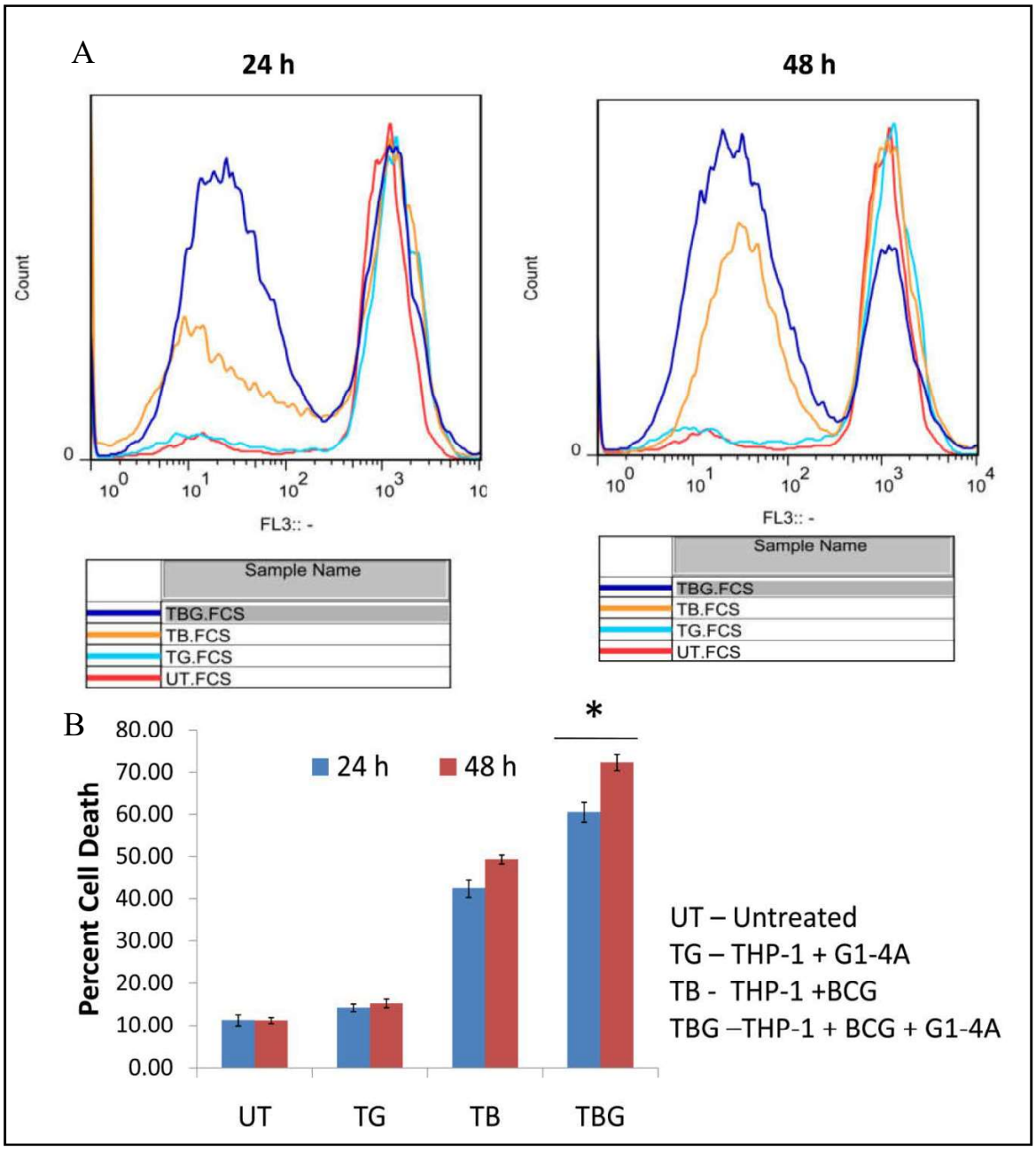


Figure. 3.29: Induction of apoptosis in G1-4A treated BCG infected THP-1 cells
THP-1 cells infected with BCG were incubated in the presence or absence of G1-4A (1mg/ml) for 24 and 48 h. Cells were stained with PI. (A) Each panel is representative of three or more independent experiments and (B) Quantitative representation of percent cell death. Results represent three independent experiments and presented here as mean ± SD.

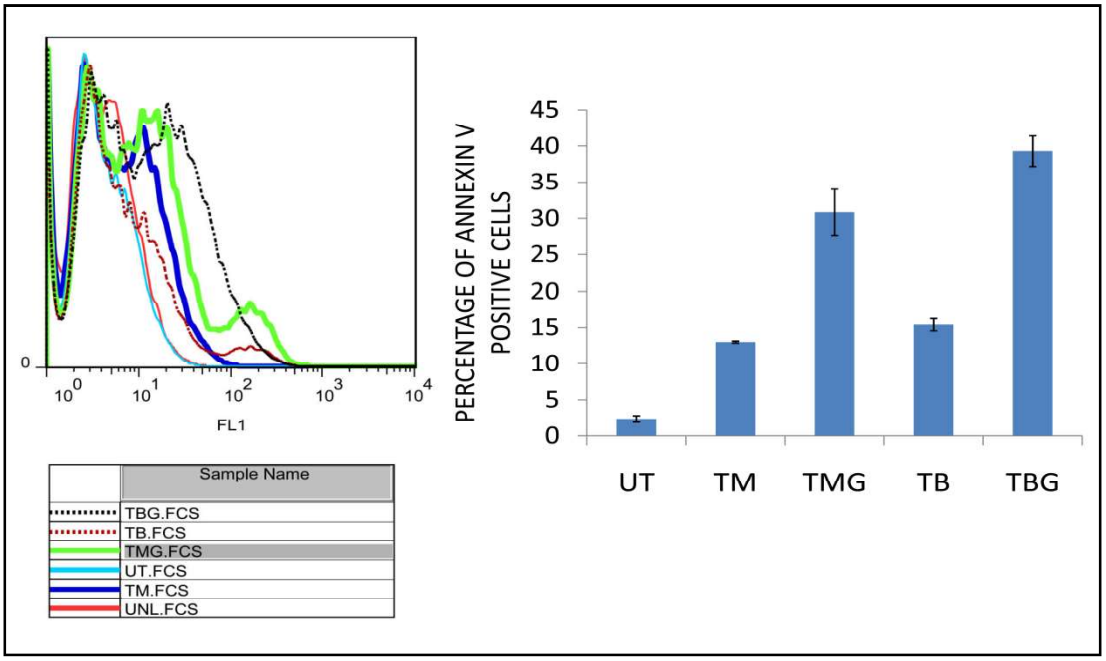


Figure 3.30. FITC-Annexin V staining in G1-4A treated BCG and H37Rv infected THP-1 cells.*THP-1 cells infected with H37Rv and BCG were incubated in the presence or absence of G1-4A (1mg/ml) for 48 h. Cells were stained with FITC-Annexin V. (A) Each panel is representative of three or more independent experiments and (B) Quantitative representation of percent of FITC- Annexin-V positive cells. Results represent three independent experiments and presented here as mean ± SD.*

3.1.4.3. G1-4A modulates cytokine levels in MTB infected THP-1 cells

Pro and anti-inflammatory cytokines play very important role in pathology of tuberculosis. We studied the effect of treatment of G1-4A on the expression of cytokines MTB infected in THP-1 cells. We found that treatment of G1-4A augments the level of pro-inflammatory cytokines (TNF α, IL-1β and IL-6) in infected cells as compared to untreated infected cells. It suggests that G1-4A treatment skews the cytokine profile in favor of host (Fig. 3.31).

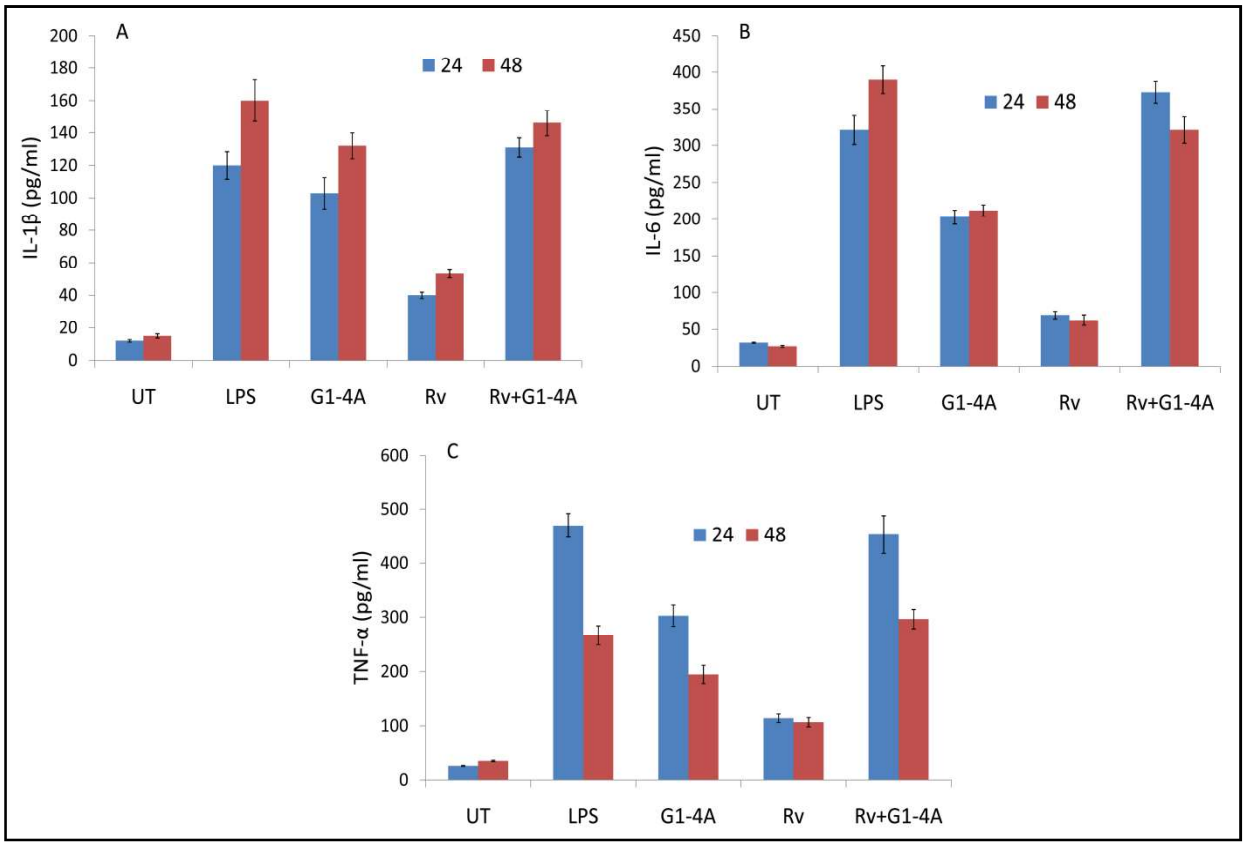


Figure 3.31. Cytokine expression in G1-4A treated H37Rv infected THP-1 cells *THP-1 cells were treated with G1-4A (1mg/ml) for 24 and 48 h. Levels of cytokines were detected in supernatants by ELISA. (A) IL-1β (B) IL-6 (C) TNF-α. Results represent three independent experiments and presented here as mean ± SD.*

3.1.4.5 G1-4A inhibits the intracellular survival of drug-sensitive and MDR strains of MTB in human PBMCs

As the THP-1 cells are malignant in nature, effect of G1-4A was investigated on the intracellular clearance of MTB in human PBMCs which are primary cells. PBMCs were isolated from blood of two individuals by Ficoll hypaque method. Buffy coat was isolated, plated for 2 h, followed by washing of non-adherent cells. Later adherent cells were seeded in the 24 well plates and treated with G1-4A overnight prior to infection. Post infection, cells were retreated with G1-4A for 48 and 96 h and CFU assay was performed at both the time points. Colony counts were significantly decreased (Fig 3.32A) in case of MTB H37Rv ($p<0.05$), LAM ($p<0.05$) and Beijing ($p<0.05$). A marked decrease in percent bacterial burden (Fig 3.32B) was observed i.e.

36.91±9.9 percent in case of H37Rv ($p<0.05$), 45.51±7.9 percent in case of LAM ($p<0.05$) and 40.20±9.7 percent in case of Beijing ($p<0.05$), after 48 h of G1-4A treatment and 61.27±9.1 percent in case of H37Rv ($p<0.01$), 55.65±11.3 percent in case of LAM ($p<0.01$) and 60.84±12.1 percent in case of Beijing ($p<0.01$)after 96 h of G1-4A treatment as compared to untreated MTB infected cells. Thus CFU data in THp-1 cells corroborated with earlier CFU data in murine macrophages**.** Therefore, we can conclude that G1-4A inhibits the intracellular survival of MTB in PBMCs also.

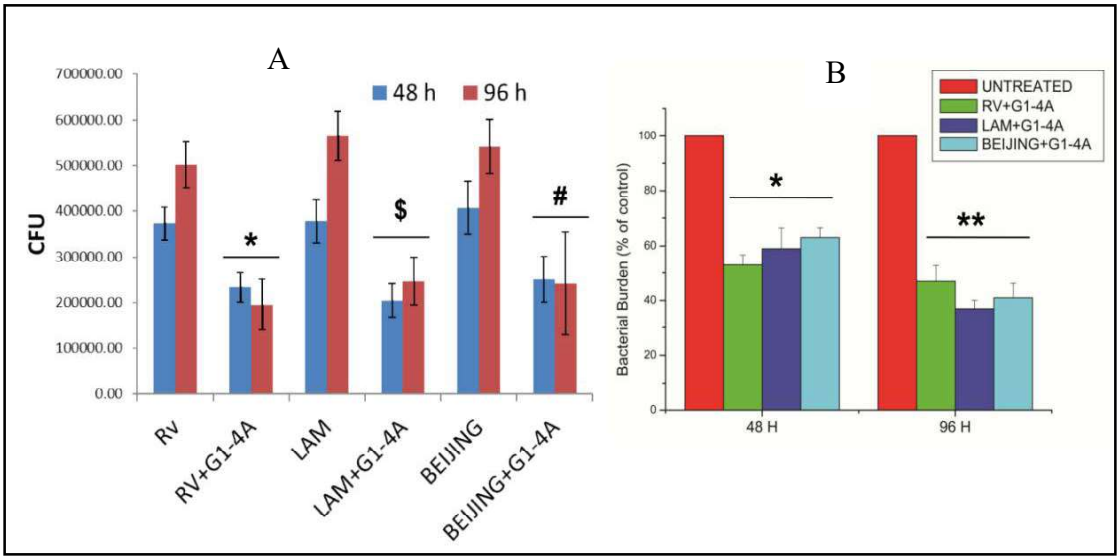


Figure 3.32. Inhibition of intracellular survival of MTB strains in human PBMC by G1-4A. *(A)CFU counts of MTB H37Rv in presence or absence of G1-4A (1mg/ml) in MTB infected human PBMCs) (*p<0.05 as compared to untreated H37Rv infected control after 48 and 96 h of treatment, $p<0.05 compared to untreated LAM infected control after 48 and 96 h treatment and #p<0.05 compared to untreated Beijing infected control after 48 and 96 h treatment. (B) Bacterial burden (Percent survival of MTB after G1-4A treatment, *p<0.05, **p<0.01). Results represent three independent experiments and presented here as mean ± SD.*

Sub-chapter 3.2. Differential activation of BMDCs by MTB strains and effect of G1-4A on the intracellular survival of MTB in BMDCs

3.2.1. Effect of MTB strains on the activation of bone marrow derived dendritic cells (BMDCs) of BALB/c mice

Although macrophages are primary hosts for MTB, it infects DCs as well. Owing to their ability to prime naïve and memory T cells, Dendritic cells (DCs) play a key role

in the host defense against MTB infections. Dendritic cells being the part of the innate immune system trigger T-cells to polarize towards Th-1 or Th-2 type of immune response depending upon their maturation status. It has been reported that H37Rv partially inhibits the maturation of human monocyte derived DCs but the impact of drug resistant clinical isolates on the maturation of DCs is poorly understood.

Hence, we investigated effect of sensitive and MDR strains of MTB on the activation and maturation of BMDCs. BMDCs were isolated from the bone marrow of BALB/c mice. Briefly bone marrow cells were cultured in RPMI-1640 containing 10% FBS and GMCSF (20 ng/ml) for 7 days. Media was changed on day 3 and 7. Immature DCs were isolated with the help CD-11c magnetic beads. Later CD-11c positive cells were seeded and infected with BCG, H37Rv and Beijing and LAM for 24 and 48 h. Supernatants obtained at both the time points were stored for cytokine estimation and cells were stained with FITC labeled anti-CD86, CD-40 and IA^d antibodies followed by flow cytometric analysis. Flow cytometry data suggested a significant increase in the surface expression of MHC-II and CD86 after 24 h in LPS treated BMDCs and BCG infected BMDCs as compared to immature BMDCs (IMDCs) which indicate the activation of BMDCs. No significant increase in the expression of CD40 was observed at 24 h time point. However, the expression level of MHC-II and CD86 in BMDCs infected with virulent strains H37Rv, LAM and Beijing was significantly low compared to LPS treated of BCG infected BMDCs at 24 h time point. Similarly at 48 h time point, significant increase was observed in the surface expression of MHC-II, CD86 and CD40 in LPS treated BMDCs and BCG infected BMDCs as compared to immature BMDCs (IMDCs). However, the expression level of MHC-II, CD40 and CD86 in BMDCs infected with virulent strains H37Rv, LAM and Beijing was significantly low compared to LPS treated of BCG infected BMDCs at 48 h time

point which suggested suboptimal maturation of BMDCs by virulent strains of MTB (Fig 3.33).

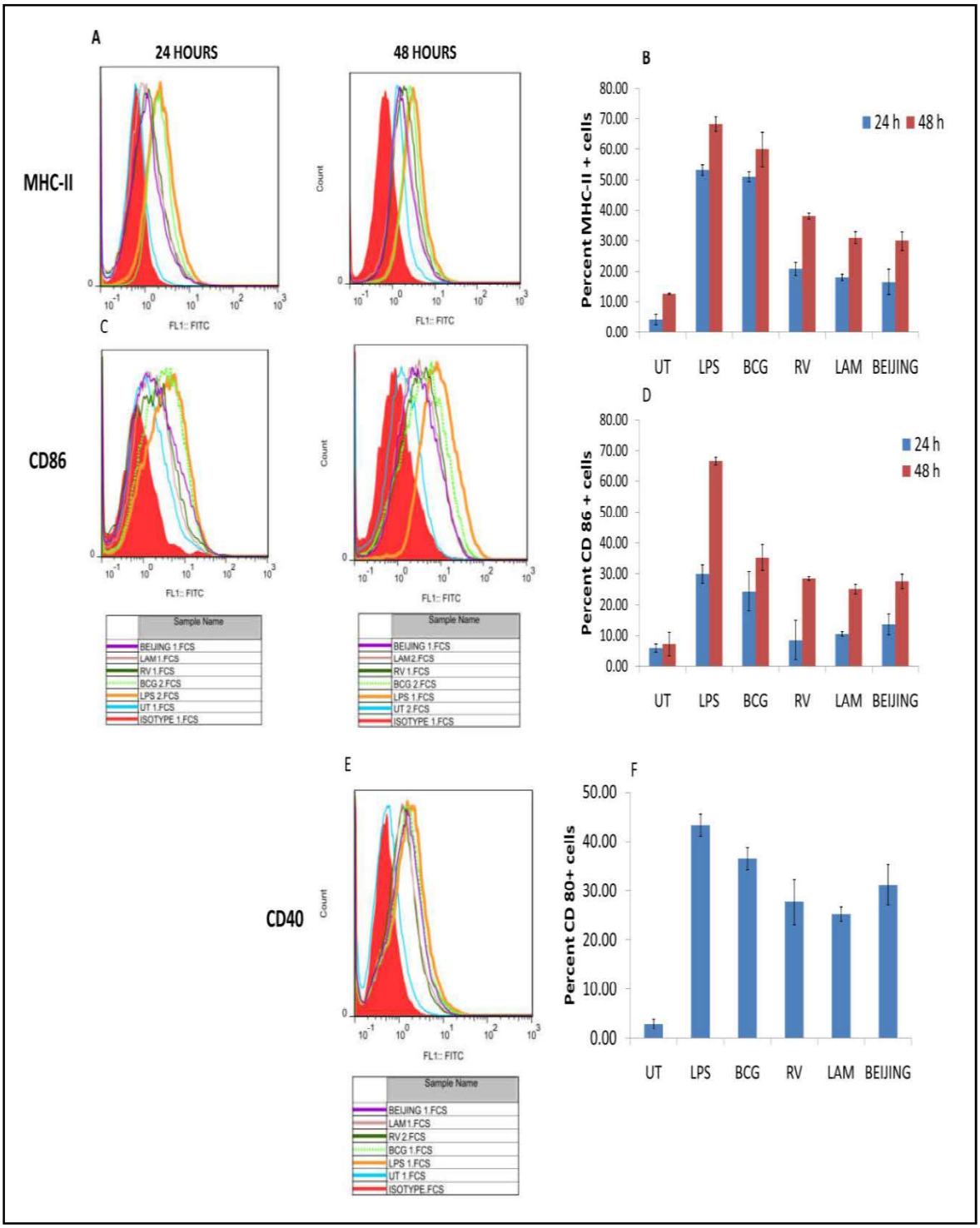


Figure 3.33. Differential expression of MHC-II, CD86 and CD40 in BMDCs
BMDCs were infected with H37Rv, BCG, LAM and Beijing for 24 and 48 h and cells were stained with FITC anti I-A^d, FITC anti CD86, FITC anti CD40 and FITC IgG2a Isotype control Ab. (A-B)MHC-II (C-D) CD86 and (E-F) CD40. Each panel is

representative of three or more independent experiments. Data presented as mean±SD.

3.2.2. Cytokine levels in bone marrow derived dendritic cells (BMDCs) of BALB/c mouse infected with MTB strains

We observed significant increase in the level of TNFα (24 h) in all the groups except untreated BMDCs but it exhibited within the group variation. Expression of IL-12 in BMDCs infected with all the virulent MTB strains; is marginally increased at both time points, as compared to BMDCs but in comparison to BCG infected and LPS treated BMDCs, level of IL-12 is insignificant. This suggests lack of Th1 response in BMDCs infected with virulent strains of MTB. IL-6 expression in all groups except BMDCs, has increased at both time points and 24 h post infection and its levels among the groups exhibit minor variation, however, 48 h post infection no variability among the groups was observed (Fig 3.34).

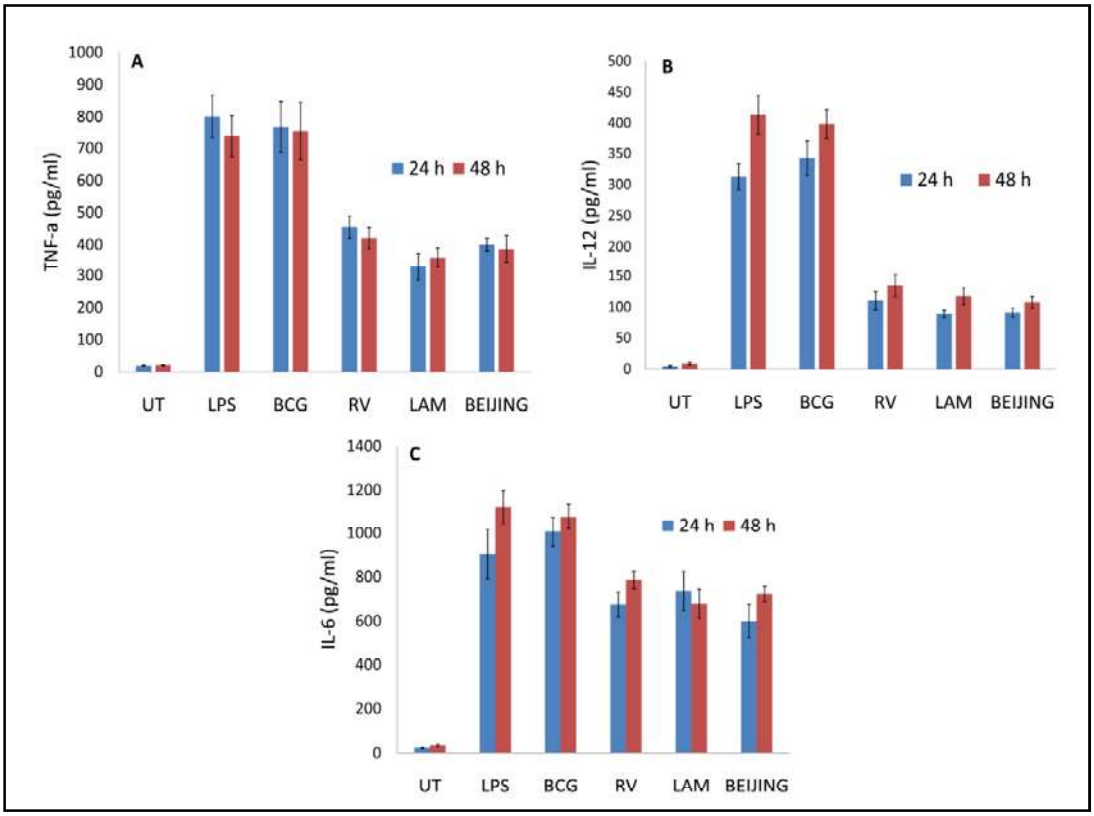


Figure 3.34.Cytokine levels in BMDCs of BALB/c mouse infected with MTB strains
BMDCs were infected with H37Rv, BCG, LAM and Beijing for 24 and 48 h. Cytokines

were estimated in supernatants by ELISA (A) TNF-α (B) Il-12 and (C) IL-6. Results represent three independent experiments and presented here as mean ± SD.

3.2.3. Effect of G1-4A on intracellular survival of MTB infected BMDCs

Bone marrow cells were isolated from the femur of BALB/c mice. Cells were incubated with GM-CSF (20ng/ml) at 37 °C and 5% CO2. Media was changed at day 3 and day 5. At day 7, cells were harvested and co-cultured with MTB H37Rv at MOI 5 for 5 h. Later infected cells were treated with G1-4A (1mg/ml) for 48 and 72 h and CFU assay was performed. Our data demonstrated reduction in CFU counts by 25% at 48 h and 33% at 72h ($p<0.05$) after G1-4A treatment (Fig 3.35). Though G1-4A inhibited intracellular survival of MTB in BMDCs but it was not comparable to the reduction in CFU counts in macrophages after G1-4A treatment. DCs are most APCs and their intrinsic antimicrobial properties are lower than macrophages. This could be the reason for the above observations.

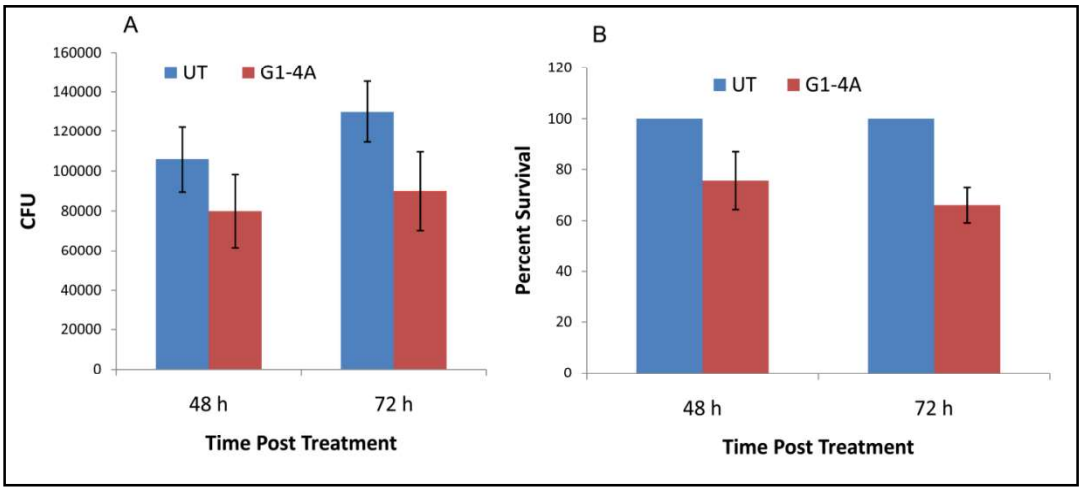


Figure 3.35. Inhibition of intracellular survival of MTB strains in BMDCs by G1-4A. *(A)CFU counts of MTB H37Rv in presence or absence of G1-4A (1mg/ml) in MTB infected murine BMDCs). (B) Percent survival (bacterial burden). Results represent three independent experiments and presented here as mean ± SD.*

Sub-chapter 3.3. Evaluation of *in vivo* efficacy of anti-TB immunomodulatory properties of G1-4A in BALB/c mice

3.3.1. G1-4A inhibits the survival of MTB in mice lungs

Inhibition of intracellular survival of MTB strains in macrophages by G1-4A was attributed to activation of macrophages which enhanced their antimicrobial properties. To further validate the significance of targeting host, we decided to investigate the anti-mycobacterial efficacy of G1-4A in mouse infection model. For this purpose, six to eight weeks old BALB/c mice were included in the study. Mice were pretreated (i.v.) with G1-4A, 96 h and 48 h prior to aerosol infection with MTB strains H37Rv, LAM and Beijing. Seven days post infection G1-4A treatment was started again and given after each 72 h till 15, 30 and 60 days post infection. Animals were sacrificed at each time point described above and lungs were removed, half of it was homogenized and plated on to Middlebrook7H11 plates in appropriate dilutions to determine CFU; whereas the other half was stored in RNALater (Ambion) for RNA isolation. Spleen was removed and used for *in vitro* restimulation assays**.** Fig 3.36A shows a time dependent reduction in CFU counts in lungs of mice treated with G1-4A and subsequently infected with all the three strains, irrespective of their genotype or drug resistance status. Bacterial burden in mouse lung was reduced by 30±11 percent, 48±9 percent and 63±8 percent after 15, 30 and 60 days respectively in case of H37Rv after G1-4A treatment (Fig 3.36B). For LAM, the bacterial burden was reduced by 26±7 percent, 41±8 percent and 54±6 percent after 15, 30 and 60 days respectively after G1-4A treatment. For Beijing, bacterial burden reduced by 23±10 percent, 44±9 percent and 60±6 percent after 15, 30 and 60 days respectively after G1-4A treatment. In another set of experiments mice received adjunct therapy wherein H37Rv infected mice were treated with G1-4A as described earlier. Seven days later they were treated

with both G1-4A and isoniazid (INH) 12.5 mg/kg of body weight, 5 days per week through drinking water. Animals were sacrificed after 15, 30 and 60 days of treatment. Animals receiving treatment with both INH and G1-4A, exhibited significantly lower levels of bacillary load in their lungs at each time point, compared to the animals receiving only G1-4A or only INH treatment (Fig. 3.36C). Bacterial burden was reduced by 51±14 percent in animals receiving INH +G1-4A as compared to 40±17 and 30±11 percent in animals receiving INH and G1-4A alone respectively, for 15 days (Fig. 3.36D). Further, the percent reduction in bacterial burden after 30 days was 70±5 percent with INH+G1-4A, 57±6 percent with INH and 48±9 percent with G1-4A treatment alone, after 30 days, and most remarkably, 89±6 percent with INH+G1-4A, 73±8 percent with INH and 63±8 percent with G1-4A alone, after 60 days of treatment. The time dependent inhibition of bacterial burden in mice lungs treated with INH+G1-4A demonstrates the potential of G1-4A for use in adjunct therapy with current drug regimen.

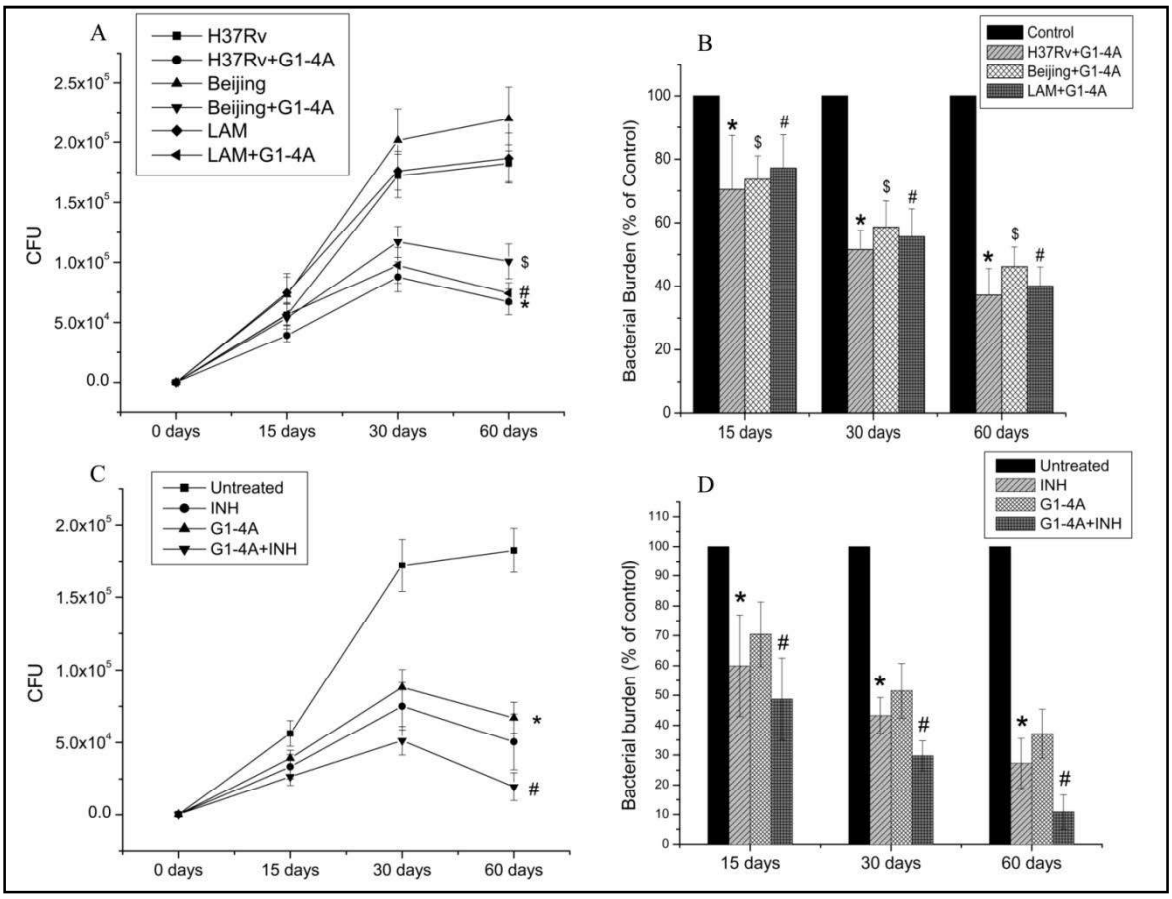


Figure 3.36. Effect of G1-4A on bacterial burden in MTB infected mice.
*BALB/c mice infected with MTB strains H37Rv, LAM and Beijing in the presence or absence of G1-4A were sacrificed at 15, 30 and 60 days post infection. Lungs were homogenized and plated on to Middlebrook7H11 plates after appropriate dilutions. (A) CFU counts (B) Bacterial burden as percentage of untreated controls were plotted, *p<0.05, $p<, 0.05 #p<0.05 compared to untreated, paired t test. (C) In another set of experiment mice were infected only with H37Rv and treated with G1-4A, INH alone or in combination. Animals were sacrificed and lung homogenates were plated onto Middlebrook7H11 plates for CFU enumeration (D) Bacterial burden as percentage of untreated controls *p<0.05, $p<, 0.05 #p<0.01 compared to untreated. Results shown here are representative of at least three independent experiments performed and presented as mean ± SD.*

3.3.2. Immunomodulation by G1-4A in MTB infected mice

Further investigations were carried out to elucidate the effect of G1-4A on modulation of immune responses in MTB infected mice.

3.3.2.1. mRNA expression of TNF-α, IFN-γ and NOS2 in lungs of MTB infected BALB/c mice

Total RNA from the lung of MTB infected mice was isolated and the expressions of NOS2, TNF-α and IFN-γ were quantitated by real time RT PCR. Fig 3.37 shows that in presence of G1-4A, expression of TNF-α, IFN-γ and NOS2 is up-regulated in lungs of MTB infected animals. In case of TNF-α, all the three MTB strains induced differential pattern of gene expression in absence of G1-4A but treatment of G1-4A up-regulated the expression of TNF-α after 15 days which peaked at 30 days and declined later on (Fig 3.37A). Similar pattern was visible in case of IFN-γ (Fig 3.37B) and NOS2 expression (Fig 3.37C). Though, G1-4A augmented the expression of above mentioned genes, a marked variation in the fold changes induced by different strains was evident, which could be due to differences in their genotypes or lineage.

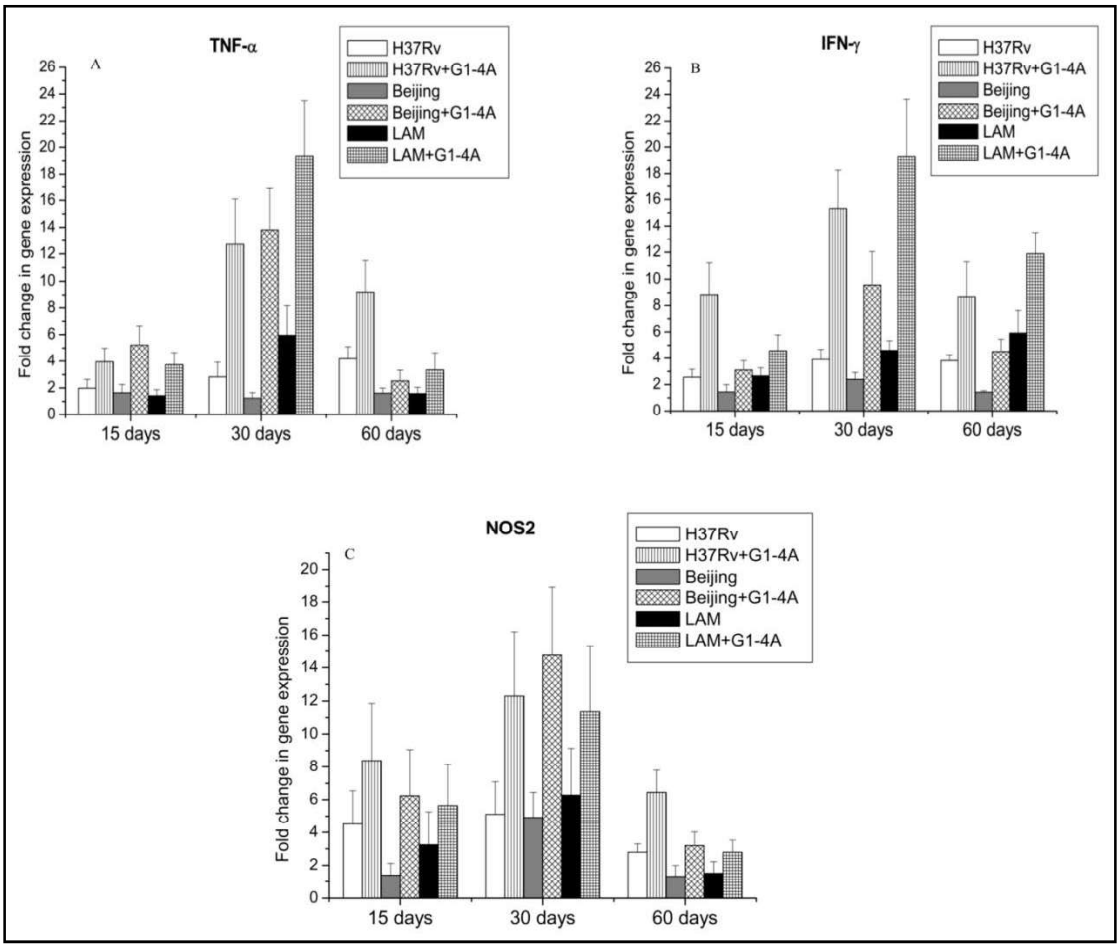


Figure 3.37. Relative quantitation of gene expression of TNF-α, IFN-γ and NOS2 in lung of MTB infected mice after G1-4A treatment.

BALB/c mice were infected with MTB strains and treated with G1-4A as in Fig 6. Half of the infected lungs were used to isolate total RNA subjected to real time RT

PCR for the quantitative gene expression analysis. (A) TNF-α (B) IFN-γ (C) NOS2. Results shown here are representative of at least three independent experiments performed and presented as mean ± SD.

3.3.2.2. Cytokine expression in serum of MTB infected BALB/c mice

Since G1-4A modulated the cytokine responses *in vitro*, we determined the concentration of different cytokines in the serum of infected animals. Blood was collected from the mice by retro-orbital bleeding at 15, 30 and 60 days post treatment, serum was then separated and level of cytokines such as TNF-α, IL-1β, IL-12, IFN-γ, IL-10 and IL-4 was determined by ELISA. Levels of IL-1β and TNF- α (Fig 3.38A-B), in the sera of mice infected with all MTB strains and treated with G1-4A, started increasing at 15 days post infection, peaked to maximum at 30 days and later declined on 60 days post infection. IL-10 levels were highest at 30 days post infection in untreated mice which is a known mechanism by which MTB evades immune system to establish long term infection [190]. Interestingly, however, in animals treated with G1-4A, IL-10 levels fell down substantially, which is beneficial for host (Fig 3.38C). IL-12 and IFN-γ levels (Fig 3.38D and 3.38E) also started increasing at 15 days and remained at the maximum at 30 and 60 days post infection. IL-4 levels were measured at all the time points but it could be detected only at 60 days post infection. IL-4 levels were also higher in all the strains in absence of G1-4A, though a minute variation among the strains was visible but treatment with G1-4A reversed the pattern with decrease in IL-4 levels in animals infected with all strains in presence of G1-4A (Fig 3.38F). Observations described above, therefore, demonstrate up-regulation of Th1 response which is beneficial for the host for the containment and eradication of MTB.

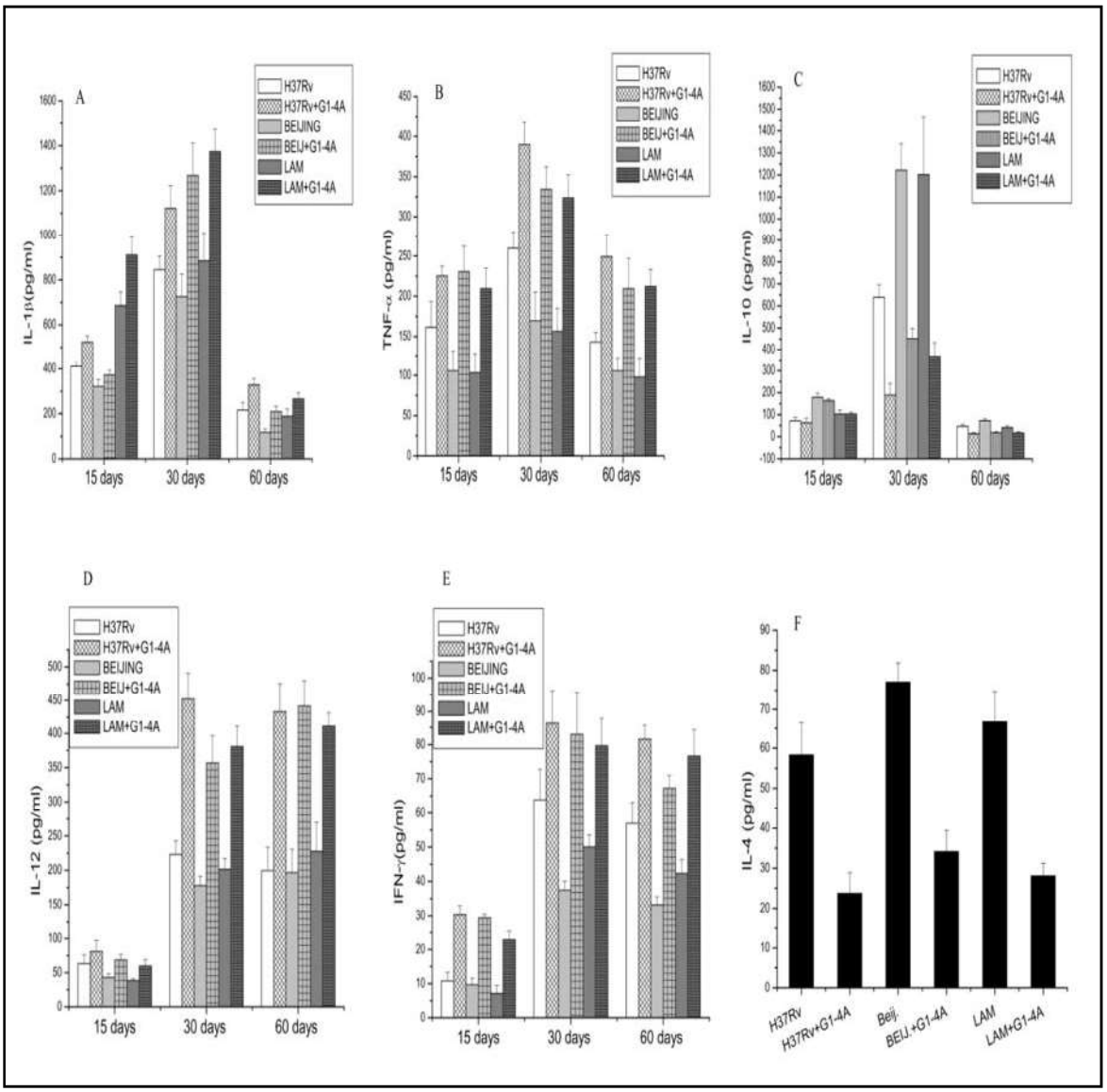


Figure 3.38. Evaluation of serum cytokines in MTB infected mice after G1-4A treatment. *BALB/c mice were infected with MTB strains and treated with G1-4A as in Fig 6. Blood was collected from the animals prior to their sacrifice at 15, 30 and 60 days.. Serum was used to determine the concentration of given cytokines by ELISA. (A) IL-1β (B) TNF-α (C) IL-10 (D) IL-12(E) IFN-γ (F) IL-4. Results shown here are representative of at least three independent experiments performed and presented as mean ± SD.*

3.3.2.3. Effect of G1-4A treatment on IFN-γ/IL-4 ration in MTB infected BALB/c mice

To further corroborate that G1-4A treatment leads to Th1 stimulation we restimulated splenocytes from infected mice; of both treated and control groups, with PPD and concavalin A (con A) and monitored the level of IFN-γ and IL-4 in supernatants after 72 h by ELISA. It was observed that level of IFN-γ in splenocytes from G1-4A

treated animals was significantly higher than that from spleen cells of untreated animals. However, level of IL-4 was lower in case of G1-4A treated animals than untreated animals (Fig 3.39A-B). As Th1 cells produce IFN-γ and Th2 cells produce IL-4 and the ratio of IFN-γ/IL-4 determines the outcome, higher IFN-γ/IL-4 ratio indicates shift towards Th-1 response and lower IFN-γ/IL-4 ratio indicates Th-2 response. IFN-γ/IL-4 ratio in the G1-4A treated animals was significantly greater than that in untreated control mice (Fig 3.39C). Additionally, to ensure that IFN-γ and IL-4 were produced specifically by Th cells, splenocytes from each mouse were divided into two groups: one group was incubated with anti-MHC-II (IA^d) blocking antibodies whereas other group was not incubated with any antibody before restimulation with purified protein derivative of MTB (PPD). IFN-γ and IL-4 were undetectable in the splenocytes pre incubated with anti-IA^d blocking antibodies, while control exhibited appropriate IFN-γ and IL-4 response (Fig 3.40). Hence, it was confirmed that in the present study, IFN-γ and IL-4 were produced by T cells only.

Thus our studies establish that in a mouse model G1-4A is effective in the inhibition of intracellular survival of MTB strains irrespective of their drug resistance status and its efficacy may be attributed to up-regulation of NOS2 and Th1 response elicited by G1-4A.

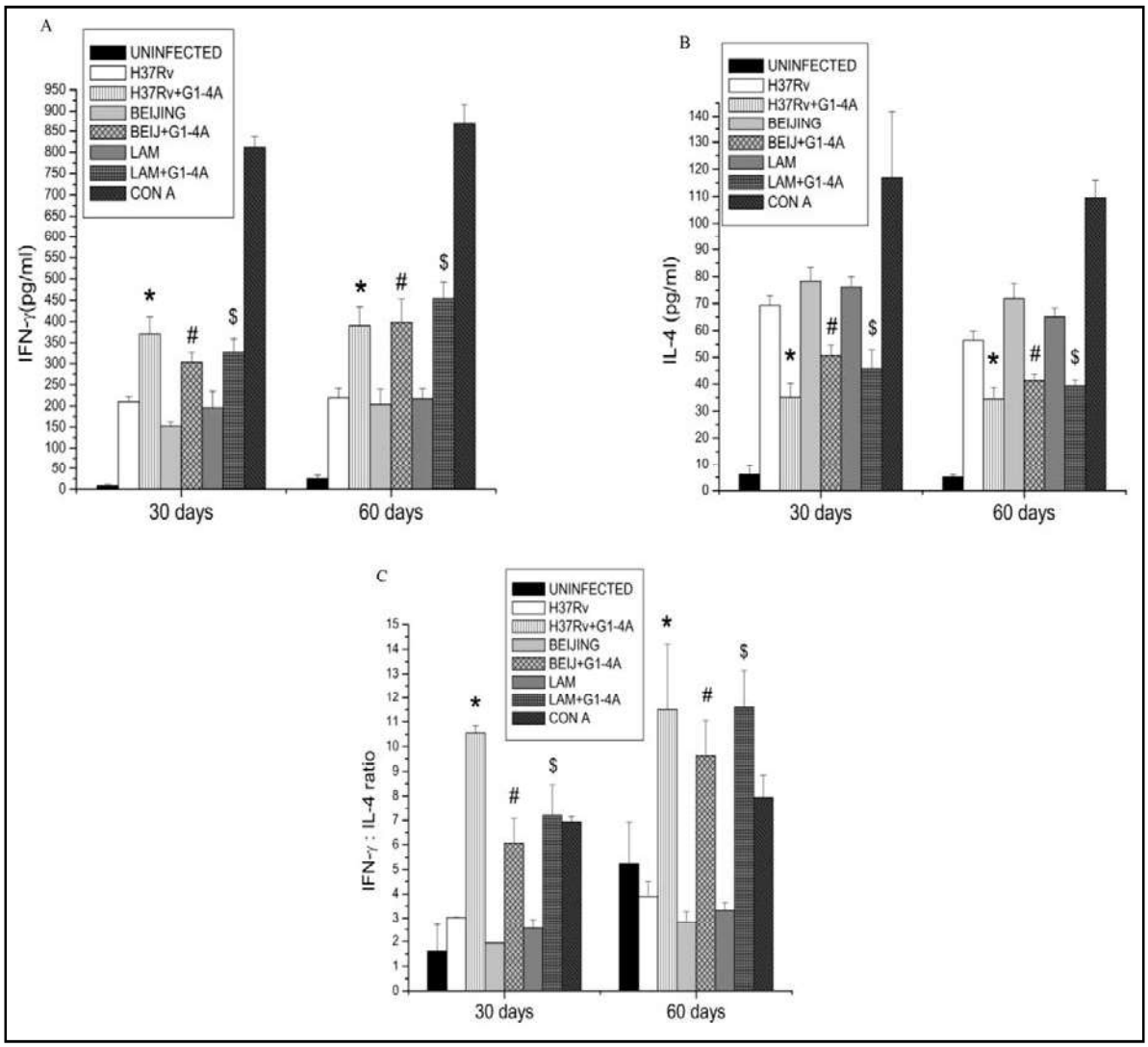


Figure 3.39. G1-4A enhances IFN-γ and IL-4 ratio in MTB infected mice.
Splenocytes isolated from BALB/c after 30 and 60 days of infection either in presence or absence of G1-4A, were treated with PPD and conA for 72 h. Levels of IFN-γ and IL-4 were determined in supernatant. (A) IFN-γ (B) IL-4 (C) IFN-γ/IL-4 ratio. Results shown here are representative of at least three independent experiments performed and presented as mean ± SD.

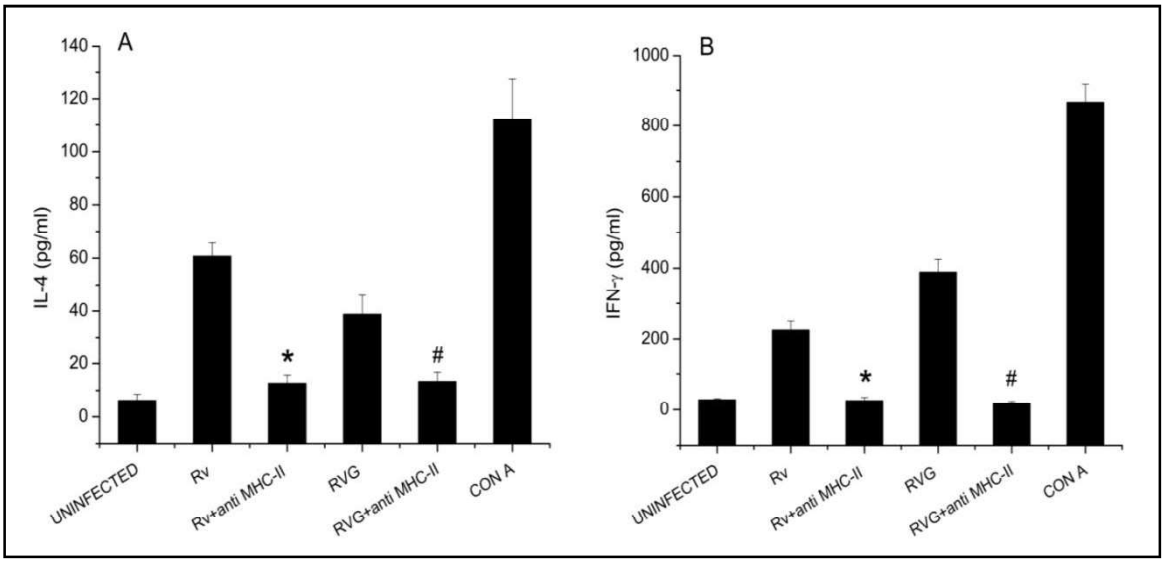


Figure 3.40. In vitro restimulation assay using anti MHC-II (IA^d) blocking antibodies

Splenocytes were isolated from the mice infected with H37Rv, infected with H37Rv receiving G1-4A treatment and uninfected. Cells from H37Rv infected mice, either untreated or treated with G1-4A were divided into two groups. Cells of one group were blocked with anti-MHC-II blocking antibodies while cells of other group were not blocked. Later, cells were treated with con A, PPD or left untreated and incubated in humidified environment containing 5% CO2 for 72 h. Levels of IL-4 and IFN-γ were detected by ELISA. (A) IL-4 (B) IFN-γ. Values of at least three independent experiments presented as Mean ± SD).

CHAPTER 4

4. *Discussion and conclusions*

When pathogenic microbes infect their hosts, it is followed by activation of innate and adaptive immune responses which may result in the symptoms of the disease. Adaptive immune system often neutralizes the invading pathogen if pathogen survives this initial immune interaction [191]. However, several pathogens are capable of subverting the robust immune response and specific antimicrobial mechanisms to ensure their survival in the hostile environment of host and therefore resulting into establishment of persistent or chronic infection. MTB, the causative agent of tuberculosis, is one such pathogen which successfully parasitizes the macrophages, due to its capacity to adapt to the changing host environment by utilizing the resources available to it within host and inhibiting the host responses directed against it [192]. MTB inhibits several host processes such as phagosome and lysosome fusion [147,193], Ag processing [194], responsiveness to IFN-γ [195], production of cytokines, reactive oxygen intermediates, reactive nitrogen intermediates [196] and host cell apoptosis [159,162,197]. It has also been reported that MTB up-regulates the expression of anti-inflammatory cytokine IL-10 to suppress macrophage activation [190] and host cell apoptosis [162].

Tuberculosis constitutes one of the leading cause of mortality and kills ~ 1·4 million people every year. Past ten years have witnessed a significant surge in the number of MDR and XDR tuberculosis cases, especially in Asia, eastern Europe and southern Africa [198]. A combination of antibiotics has been used for the eradication of active tuberculosis, prevention of relapse and resistance for the past 4 decades. However, there are several unmet needs for the treatment of tuberculosis which include shorter

treatment duration for sensitive and drug resistant tuberculosis, improved drug regimen without substantial side effects and effective treatment for latent tuberculosis [199].

Development of new anti-TB drugs couldn't keep pace with the advent of drug resistance among MTB strains. In last 40 years only two drugs have received FDA approval and rapid development of drug resistance in MTB strains has worsened the scenario. Innovative alternative interventions for prevention and management of MDR and XDR TB are therefore required urgently. One alternative approach involves selective modulation of host immune responses to enhance the therapeutic potential [200]. This approach falls under a broad category of **"Host Directed Therapeutics"** (HDTs) which is being developed to refocus the host immune responses subverted by MTB towards the host. This approach is useful for the management of patients of MDR and XDR TB as it targets the host pathways, in contrary to antibiotics which target pathogen pathways; hence this strategy minimizes the risk of development of drug resistance. HDTs, target elements of host pathways, with an objective to reduce the treatment duration, minimize the damage due to chronic pulmonary inflammation and to restrict the risk of reinfection with MTB [201].

Targeting the host pathways, offers one potential solution for the treatment of drug resistant TB and eradication of non-replicating bacilli. Usually, HDTs interfere with MTB survival in two ways: First class of HDTs inhibit the MTB survival by disrupting the macrophage signaling pathways used by MTB during infection, thereby leaving the bacteria more vulnerable to antimicrobial host defenses or to antibiotics. These HDTs include drugs with ability to interfere bacterial uptake, autophagy, and induction of antimicrobial mechanisms [e.g. reactive oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs), antimicrobial peptides like β-defensins and

cathelicidins]. Other class of HDTs have the ability to augment the host immune response against MTB by skewing the equilibrium between MTB and host; required for the survival of MTB in hostile microenvironment of macrophage, in the favor of host [46]. HDTs with immunomodulatory potential may induce novel immune responses to facilitate the clearance of MTB, which either rarely occur or, are vigorously subverted by the MTB. These immunomodulatory HDTs include agents that can affect cytokines, inflammatory pathways, Ag processing and presentation and activation of T cells. Compared to antibiotics, HDTs offer several benefits. First, HDTs minimize the risk of evolution of drug resistance in pathogens as it would be very difficult for the bacteria to develop entirely new modes of interaction with hosts, though it is not an impossible task but very unlikely. Second, HDTs, due to their potential to stimulate host immune responses, exhibit anti-TB activity against drug resistant strains of MTB. Though HDTs have many advantages to offer, some negative points are also attached with its uses. For example, considerable amount of functional redundancy exists in mammalian systems which microbes have exploited for their benefit by interacting with different families of host proteins. Such interactions are usually non specific in nature, hence targeting such interactions by HDTs may require that HDTs work in somewhat non specific ways and impinge on family of proteins rather than a specific protein [202].

Modulation of immune responses has been proven to work as useful therapeutic approach in many cases such as prevention and treatment of various infections, augmentation of anti-tumor immunity in cancer and suppression of autoimmune and inflammatory conditions [203,204]. This approach is largely used as adjunct therapy to support and enhance the efficacy of antibiotics and antivirals [200]. Many molecules which fall under category of HDTs are in clinical or preclinical stages of

development for the treatment of infectious diseases [205]. Blum *et al* reported that prednisone reduces tissue damaging inflammation in lungs by activation of glucocorticoid pathway, and thus can be used as adjunct to the current drug regimen in pneumonia [206]. Vitamin D3 and monoclonal antibodies against IL-1β and TNF-α have been reported to be used as HDTs in *H. pylori* infections by reduction in tissue damage and cytokine neutralization [205]. Sulforaphane has shown antibacterial effect in sensitive as well as drug resistant *H. pylori* infection and it prevented stomach tumors induced by benzo[a]pyrene in a mouse model [207]. Several molecules like imatinib, verapamil, metformin and ibuprofen have shown their anti-mycobacterial effects by targeting host pathways [205]. Monoclonal antibodies like Anti-PD-1, anti-LAG3, anti-CTLA-4 are in preclinical stage for the treatment of tuberculosis. Vitamin D3 also exhibits immunomodulatory role in tuberculosis and it is in late clinical phase as adjunct therapy for tuberculosis [205]. Prochlorperazine, lithium, nortriptyline, haloperidol and desipramine are some HDT candidate molecules for TB, which induce autophagy in MTB infected macrophages and thus inhibit its survival [202]. In the present study, we report that G1-4A, a polysaccharide from *T. cordifolia,* modulates the host immune responses in macrophages as well as BALB/c mice leading to the inhibition of intracellular survival of MTB.

The discovery of the pathogen recognition receptors of innate immunity, particularly the TLRs, has opened new possibilities for modulation of the innate immune system. Different TLR agonists are being investigated as potential therapeutic agents for the treatment of cancer and infectious disease. Imiquimod, one of the earliest and successful drugs, acts in TLR7-MYD88 dependent manner to cause secretion of IFN-α, having antiviral and anti-tumor properties [208]. Immunomodulatory oligonucleotide (IMOs) eg. IMO-2055, which is TLR9 agonist, exhibits anti-cancer

activity by enhancing T cell responses [209-211]. IPH-3102, a dsRNA mimic which acts through TLR3 signaling, has shown anti-cancer properties in breast cancer cells and melanoma by induction of IFN-α secretion [212]. Lipid-A derivative OM-174 which is a TLR4 agonist has been shown to have anti-tumor effects through the expression of IFN-γ [213]. SMP-105, a TLR2 agonist derived from *Mycobacterium bovis* BCG is approved for bladder cancer treatment [214,215]. DIMS0150, a TLR9 agonist is being clinically used for the treatment of steroid resistant or dependent ulcerative colitis [216]. Many TLR agonists are in development phase for the treatment of allergy and asthma. AVE-0675 and SAR-21609 are CpG DNA based molecules which act through TLR9. These molecules are being investigated for their role in treatment of asthma and viral respiratory tract infections respectively [217]. Further, agonists of TLR 4/5/7/8/9 are being developed as prophylactic and therapeutic vaccines and vaccine adjuvants for the treatment of cancer and viral infectious diseases [218]. Resiquimod (R-848) which acts through TLR7-TLR8 has shown therapeutic potential for the treatment of Hepatitis C and other viral infections [219,220]. CpG DNA, a TLR9 agonist has ability to control the infection by boosting the innate immune responses which indicates the potential to use such compounds as antimicrobials by activating or enhancing innate responses [221]. Cadi-05, a poly TLR agonist which is derived from autoclaved mycobacterium, is in phase III clinical trial for its anti-TB effects [217]. Thus, taking account of all the studies, it is evident that HDTs using TLR agonists having therapeutic potential against MTB; have been poorly investigated so far.

To the best of our knowledge, the present study is the first study to demonstrate therapeutic potential of plant based TLR4 agonist against MTB infections.

Macrophages are one of the most important sentinels of innate immune system that play crucial role in host defense and resistance to microbial invasion. Activated macrophages are the key source of ROS, RNI and inflammatory cytokines that are used for defense against microbial invasion [222]. Lipopolysaccharide (LPS), the cell wall component of gram negative bacteria, is known to activate the macrophages by binding to TLR4 which leads to the production of inflammatory mediators such as TNF-α, IL-1β, IL-6, RNI and ROI. It also enhances the Ag presentation and processing of macrophages by up-regulating the surface expression of MHC-II and CD86. Activated macrophages have been broadly classified in two categories: classically activated macrophages or M1, and alternatively activated macrophages or M2. In general M1 macrophages are characterized by increased expression of cytokines TNF-α, IL-1β, IFN-γ, IL-12 and IL-6, enhanced production of NO, up-regulation of MHC-II and CD-86 and acquisition of microbicidal properties. Conversely, M2 macrophages are characterized by increased expression of IL-4, IL-10, TGF-β [223] and are poorly microbicidal [67]. Apart from microbial components, TLRs also display a broad affinity for polysaccharides isolated from various sources of non-microbial origin [224]. It was demonstrated that polysaccharides, isolated from various plants like *Schisandra chinensis, Paecilomyces cicadae* and *Platycodongra diflorus,* activate macrophages through TLR4 dependent signaling pathways [225]. Further, several reports have demonstrated the effects of polysaccharide on various immune regulatory functions such as activation of macrophages, lymphocytes and NK cells, induction of cytokine expression, production of RNI and ROS, antibody production and activation of the complement system [224,225]. In agreement with these reports, the present study has shown that treatment of G1-4A, a polysaccharide from *Tinospora cordifolia,* activates murine macrophages through classical pathway

in TLR4 dependent manner. Our data further indicate that G1-4A treatment induces expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-12 and IL-6 in RAW 264.7 cells as well as peritoneal macrophages of BALB/c mice. G1-4A treatment down regulated the expression of IL-10 in MTB infected BALB/c mice however, surprisingly G1-4A treatment up-regulated the expression of anti-inflammatory cytokine IL-10 in RAW 264.7 as well as peritoneal macrophages however, the levels were lower compared to the LPS. The above difference in the expression pattern of IL-10 could be due to variation in the treatment duration as well as cell types involved. Further, we have shown that G1-4A treatment, up-regulates the expression of NOS2 which results into enhanced production of nitric oxide in both types of cells, thus enhancing the microbicidal properties of the macrophages. Additionally, it also up-regulates the surface expression of MHC-II and CD86. These data suggest that G1-4A treatment induces classical activation (M1 activation) in murine macrophages exhibiting enhanced antimicrobial properties and Ag presentation potential.

Extraction of purified G1-4A is a cost intensive affair; hence we extracted total polysaccharide rich fraction (PRE) from stem of *Tinospora cordifolia*. PRE is the second purest form of polysaccharide fraction during the extraction of G1-4A. Following gel filtration chromatography of PRE on sephacryl column S-400, G1-4A is obtained in the elute. When RAW 264.7 macrophages were treated with PRE, we observed similar effects like G1-4A treatment. PRE treatment up-regulated expression of pro-inflammatory cytokines and nitric oxide. Further, it up-regulated the surface expression of CD-86 and MHC-II in RAW 264.7 cells, hence we conclude that like G1-4A, PRE too activated macrophages through classical pathway.

Due to the vital role played by macrophages in the induction of host immune response, several pathogens have developed the strategies to perturb the macrophage differentiation program in their favor. Thus, as the M1 phenotype of macrophage is associated with defense against intracellular pathogens like *Salmonella typhimurium* and *Mycobacterium turbeculosis*, they have evolved strategies to avoid the M1 activation of macrophages to ensure their survival [226,227]. Kyrova *et al* have shown that the production of nitric oxide (NO) by M1 cells play a critical role in the intracellular clearance of *Salmonella* infection [228]. Our data demonstrate that G1-4A induced NO production in MTB infected macrophages, was responsible for intracellular clearance of the pathogen. Recent reports have also demonstrated that IFN-γ mediated M1 polarization leads to the development of immune responses required for host protection during chlamydial and mycobacterial infections [229-232]. Apart from infectious diseases, macrophages play a crucial role in different types of cancer as these cells are recruited to all solid tumors [233]. M1 activation of macrophages is considered to be advantageous to the host as cytokines such as IFN-γ, IL-1β, and IL-6 produced by peritumoral macrophages exhibit anti-tumoral responses [234-237]. On the other hand, tumor-associated macrophages (TAM) with M2 phenotype are often associated with pro-tumor effects like tumor growth, metastasis and angiogenesis [238]. Recent report suggested that G1-4A treated dendritic cells exhibited anti-tumor properties by the production of peroxynitrite [239]. By virtue of M1 polarizing properties of G1-4A, it may also have anti-tumor properties, which needs to be investigated.

In the next step, use of TLR4 blocking antibodies and siRNAs against TLR4 and MyD88 revealed the involvement of TLR4 and MyD88 in G1-4A mediated up-regulation of above mentioned molecules, therefore we conclude that G1-4A activates

macrophages by classical pathway and imparts M1 phenotype to them in TLR4-MyD88 dependent manner. Signaling through TLRs, triggers the activation of all the three MAPKs; extracellular signal regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) [240]. Extracellular signals are transmitted by MAPKs to the nucleus which leads to activation and phosphorylation of transcription factors resulting into transcription of specific genes. It was demonstrated by Chen *et al* that MAPKs such as p38, ERK1/2, and SAPK/JNK are activated in macrophages by LPS [222] and this activation results into generation of inflammatory responses. Further, it was reported that p38 MAPK is involved in LPS induced NO production in macrophages [222,241], whereas PGN, peptidoglycan from gram positive bacteria induces NO production through the activation of c-Jun N-terminal kinase (JNK) [242].

In the present study we have shown that G1-4A treatment caused the phosphorylation of p38, ERK1/2, and SAPK/JNK in RAW 264.7 murine macrophage leading to their activation. Similarly, PRE treatment also caused activation of p38, ERK1/2, and SAPK/JNK in RAW 264.7 cells. The activation of MAPKs leads to the phosphorylation of other substrates and transcription factors such as NF-κB (nuclear transcription factor kappa B), which in turn induces the expression of genes responsible for the pro-inflammatory cytokines and reactive oxygen and nitrogen species [243]. These innate mediators help in activation of adaptive immune system and clearance of invading pathogen. Several bacterial pathogens such as *Shigella flexneri*, *Pseudomonas syringae*, *Salmonella spp* and *Yersinia spp* etc have evolved several mechanisms to intercept the MAPK signaling cascade to promote their pathogenecity and virulence [244]. We used pharmacological inhibitors of MAP kinases i.e. UO126 (ERK inhibitor), SB203580 (p-38 inhibitor) and SP600125 (JNK inhibitor) to suppress the activities of MAPKs to delineate their involvement in G1-

4A mediated macrophage activation and the data indicated that p38 and JNK1/2, MAPKs were involved in G1-4A mediated NO induction and NOS2 expression, whereas p38 and ERK1/2 MAPKs were involved in G1-4A mediated expression of IL-6, TNF-α, IL-1β and IL-12 in macrophages, which is in concurrence with other reports [241,245-247]. PDTC, inhibitor of NF-κB was used to investigate the involvement of G1-4A mediated MHC-II expression. In presence of PDTC, the expression of MHC-II was inhibited that indicates the activation of NF-κB after G1-4A treatment. Finally, our data demonstrates that G1-4A activates TLR4 which leads to the activation of p38, ERK1/2 and JNK1/2 resulting into M1 activation of macrophages.

Pathogenic microbes are recognized by TLRs present on the macrophages and outcome of this interaction has very important role in deciding the fate of macrophage activation and subsequent immune response, therefore it is logical to think that TLRs mediated signaling can be modulated by suitable TLR agonists for the benefit of the host. Targeting TLRs for enhancing the overall immune response for protection against infectious and inflammatory diseases have been recently reported [248]. In addition to its application in TB, G1-4A a TLR4 agonist, may have beneficial effects in other infectious diseases. Unlike TLR agonists of microbial origin, the plant polysaccharide G1-4A is rather safe for the immunomodulation in various host systems.

It has been observed that successful pathogens have developed several strategies to circumvent the host immune response for their survival within the host and perturbation of macrophage polarization is one such strategy to undermine the adaptive immunity since macrophages are professional Ag presenting cells [67]. MTB which resides inside macrophages also adapts several strategies to alter macrophage

activation. Classically activated macrophages are associated with tuberculostatic and tuberculocidal properties due to production of ROS, NO and other antimicrobial peptides [249]. MTB escapes this hostile environment within the macrophages by creating an environment that supports the macrophage polarization through alternative pathway (M2) resulting in inhibition of NO production, down regulation of TNF-α, IL-6, IL-1β, IL-12 and MHC-II expression, which renders macrophages poorly microbicidal. MTB employs several strategies to achieve this goal, crucial among which is induction of IL-4, TGF-β and IL-10 in macrophages which interferes with IFN-γ signaling and drive the alternative activation of macrophages [250].

Protective responses inhibited by MTB induced IL-10, include blockade of production of pro-inflammatory cytokine TNF-α and Th1 cytokine IL-12, by macrophages and dendritic cells [251,252]. IL-10 also exerts its inhibitory effect on phagocytosis and bactericidal activities through the inhibition of the production of RNI and ROS by macrophages and down regulation of expression of MHC-II and CD86, both having important role in specific immunity [253-255], and all of these are key to host immune response to contain or eradicate intracellular pathogen. Another study reported that in absence of IL-10 i.e. in IL-$10^{-/-}$ mice, MTB infection resulted into better protection with reduced MTB load in lungs followed by enhanced IFN-γ and Th1 response [256]. MTB disrupts Ca2+ homeostasis by altering the ER stress in macrophages which alters the polarization of macrophages. Macrophages infected with non-virulent MTB strain H37Ra exhibit M1 phenotype, whereas in case of virulent strains of MTB, M2 phenotype is predominant in macrophages [257].

Reversal of such inhibitory responses employed by MTB in macrophages may have a promising role in intracellular killing of MTB. Like uninfected macrophages, MTB infected macrophages, when treated with G1-4A, exhibited the up-regulation of TNF-

α, IL-6, IL-1β, and IL-12 expression, with concomitant increase in NO production by up-regulating the NOS2 expression. Additionally, MHC-II and CD86 surface expression was also up-regulated in G1-4A treated MTB infected macrophages compared to untreated MTB infected macrophages. G1-4A treatment, also up-regulated the phagocytosis of MTB by macrophages. These responses indicate that G1-4A treatment modulated the activation of MTB infected macrophage and caused the classical activation of MTB infected macrophages which is a host protective phenotype. Interestingly, MTB strains exhibited a variation in their immune responses induced in macrophages but G1-4A treatment exhibited similar effect in activation of macrophages infected with different MTB strains irrespective of the lineage or drug resistance status of the strains. Consequently, G1-4A treatment inhibited the intracellular survival of MTB H37Rv a drug sensitive strain as well as two clinical isolates with MDR status. Nitric Oxide (NO), one of the inflammatory mediators produced by activated macrophages in response to microbial invasion, contributes to the eradication of microbes including MTB. IFN-γ activated macrophages kill MTB through NO dependent mechanisms [258]. Using L-NAME, a pharmacological inhibitor of NO, we established NO dependent killing of MTB in macrophages treated with G1-4A. Further, we also demonstrated that intracellular killing of MTB by G1-4A is TLR4-MyD88 dependent. Uszynski *et al* have similarly reported that TLR2 dependent NO production in murine macrophages by 19-kDa lipoprotein of MTB was responsible for intracellular killing of MTB [259]. In concurrence with these reports, our data demonstrated that G1-4A treatment inhibited the intracellular survival of MTB strains in RAW 264.7 cells.

Since tuberculosis is predominately a human disease, therefore after studying the effects of G1-4A in murine macrophages, we decided to investigate the effects of G1-

4A in human macrophages. THP-1, a human monocytic cell line is a preferred *in vitro* model for such studies hence the effects of G1-4A in MTB infected THP-1 cells were investigated.

Apart from earlier mentioned immune responses subverted by MTB strains, there are several other host responses, perturbed by MTB strains for it survival inside macrophages. Apoptosis of MTB infected macrophages is one such mechanism which is inhibited by virulent MTB strains to ensure its survival. There are two cell death pathways which are commonly observed during the death of MTB infected macrophages: a) necrosis, a form of cell death characterized by cell lysis destroying the integrity of plasma membrane and b) apoptosis, a form of death which maintains the integrity of plasma membrane and thus immunologically silent [260,261] . Induction of necrosis by MTB strains is an escape mechanism used by bacteria to evade the host immune responses directed against it and spread the infection. On the other hand induction of apoptosis is detrimental to the pathogen as it helps the host in the eradication of pathogen [261]. Apoptosis prevents spread of MTB infection by impeding the escape of intracellular MTB. Further it activates innate and adaptive immune response against the pathogen and thus limits the MTB infection [262]. Subsequent to the apoptosis in MTB infected macrophages, apoptotic bodies which contain pathogens and other components of cytoplasm are engulfed by macrophages and dendritic cells, followed by degradation and presentation of MTB Ag to T cells via MHC-II complexes resulting into activation of innate and adaptive immune responses. Molloy *et al* reported that induction of apoptosis in MTB harboring macrophages reduced the viability of MTB where as necrosis had no effect on the MTB viability [263]. Therefore it is clear that macrophage death by necrosis or apoptosis results into different outcomes, apoptosis leads to activation of T cell

response via Ag presentation whereas necrotic death is associated with exacerbation of MTB infection [264].

Avirulent strains of mycobacteria like MTB H37Ra or BCG induce apoptosis in macrophages whereas virulent strains of MTB like H37Rv induce necrosis instead of apoptosis to avoid bacterial killing [265]. Virulent strains of MTB possess several mechanisms to evade apoptosis. Virulent MTB manipulate the structure of endoplasmic reticulum (ER) in macrophages to predominately rough ER (RER) phenotype compared to the macrophages infected with non-virulent MTB, which exhibit smooth ER phenotype. This change in the phenotype of ER results into enhanced cytosolic Ca2+ levels and induction of phosphatidyl choline/phosphatidyl ethanolamine (PC/PE) expression in macrophages infected with non-virulent strain leading to facilitated apoptosis. Virulent strains alter the cholesterol homeostasis in macrophages leading to the inhibition of apoptosis [266]. In the present study THP-1 cells were infected with MTB H37Rv and BCG followed by G1-4A treatment. Our data suggested that G1-4A treatment enhanced the apoptosis in macrophages infected with MTB H37Rv and BCG both, compared to untreated infection control. Strangely, uninfected THP-1 cells when treated with G1-4A, exhibited cell death comparable to untreated uninfected cells, which is a desirable property as it means that G1-4A is not toxic to uninfected cells. The reason for this selective induction of apoptosis of MTB infected macrophages after G1-4A treatment is not yet known. Further, TNF-α plays a significant role in apoptosis of macrophages and our data suggest that G1-4A treatment up-regulated the expression of TNF-α in MTB infected cells compared to untreated, however, the link between G1-4A mediated TNF-α secretion and apoptosis has not been established in the present study. In addition, G1-4A treatment up-regulated the expression of IL-1β and IL-6 in MTB H37Rv infected THP-1 cells. The

cytokine data obtained here with THP-1 cells supports our previous cytokine data obtained in MTB infected RAW264.7 and PEMs.

Further, when THP-1 cells were infected with H37Rv, Beijing and LAM and treated with G1-4A, CFU counts of all the strains were reduced indicating the inhibition of intracellular survival of MTB in macrophages after G1-4A treatment. Similar reduction in CFU counts were observed in MTB infected human PBMCs after G1-4A treatment. Since G1-4A doesn't have direct anti-TB activity, (evident by REMA assay results) thus intracellular clearance of MTB may be attributed to the modulation of host immune responses. Though the underlying mechanism is not clearly understood, the induction of apoptosis after G1-4A treatment seems a plausible reason for anti-TB effect of G1-4A in MTB infected THP-1 cells.

Hence, our study suggests that G1-4A inhibits the intracellular survival of MTB strains (either drug sensitive or resistant) in human macrophages and murine macrophages both by the modulation of host responses.

Host immunity against any pathogen including MTB involves two forms of defenses: innate and adaptive [267]. We discussed about innate defenses so far. The other arm of host defense i.e. adaptive immunity is specific form of immune defense to combat pathogens, however unlike innate immunity, several complicated steps are involved before its activation, as Ag recognition by lymphocytes involves processing and presentation of Ag by Ag presenting cells (APCs) like macrophages and dendritic cells [268]. Dendritic cells are considered as link between innate immunity and adaptive immunity. As a part of the innate immunity DCs stimulate polarization of T cells towards Th1 or Th2 phenotype [269,270], according to their maturation status [271]. DCs exists in two functional states: immature DCs which display high phagocytosis, low expression of CD40, CD80, CD86 and MHC-II, and mature DCs

with high Ag presentation, high expression of CD40, CD80, CD86 and MHC-II [269]. Immature DCS give rise to T cell tolerance while mature DCs lead to activation of naïve T cells. Although macrophages are the most important cells for MTB infection other leukocytes such as DCs also play a critical role in host immunity against MTB infections. DC-SIGN a lectin-surface receptor is expressed on the surface of DCs for Ag uptake [272,273]. MTB predominately interacts with DC-SIGN, though CR3 and MR are also expressed on the surface of human DCs, MTB seems to neglect these receptors [48]. It has been reported that infection of monocyte derived dendritic cells (MoDC) with *M. bovis* BCG or *M. avium* resulted into maturation of MoDC and subsequent activation of T cells [274]. However, when MoDC were infected with virulent H37Rv strain, it resulted into inhibition of maturation of MoDC [58]. Though the information about interaction of MTB H37Rv and DCs is available, interactions of DCs and clinical isolates of MTB are poorly understood. Clinical isolates of MTB differ in their pathogenicity, transmission potential and immunogenicity [22,275].

In the present study, DCs were isolated from bone marrow of BALB/c mice and infected with two clinical isolates of MTB belonging to two different genetic lineages Beijing and LAM, lab strain H37Rv and BCG. To assess the effects on maturation, expression of cytokines and surface expression of MHC-II, CD86 and CD40 were investigated. Our data demonstrated that virulent strains, H37Rv, Beijing and LAM exhibited reduced expression of TNF-α, IL-6 and Il-12, compared to BCG or LPS treated BMDCs, which suggested the suboptimal maturation of BMDCs by virulent clinical isolates. Further, our data demonstrated a decreased surface expression of MHC-II, CD86 and CD40 in BMDCs infected with virulent strains compared to BCG infected and LPS treated BMDCs. Collectively, our data suggested that clinical isolates altered the maturation of BMDCs which may eventually lead to the impaired

T cell mediated immune response. Further, studies are needed to establish the effects of these MTB strains on overall T cell immunity. Since G1-4A inhibited the intracellular survival of MTB in murine and human macrophages, we also investigated the effect of G1-4A on the intracellular survival of MTB in BMDCs and our data demonstrated that G1-4A treatment inhibited the survival of MTB in BMDCs too, however, the net decrease in the percent survival was in the range of 25-35 percent. Intracellular survival of MTB in macrophages and DCs is different due to the difference in the nature of both the cells. Macrophages being the most important sentinel of innate defense are armed with antibacterial mechanism like antimicrobial peptides, ROI and RNI. On the other hand DCs being the most potent APCs are armed with Ag presentation machinery but lack potent antimicrobial peptides, ROI or RNI. This could be the reason for the difference in percent survival of MTB in macrophages and DCs after G1-4A treatment. Further, studies are required to find out the mechanism of suboptimal activation of BMDCs by MTB strains.

So far, we discussed the effects of G1-4A in *in vitro* models and concluded that G1-4A may be a potential agent for the HDT in tuberculosis, hence to assess whether similar effects of G1-4A would be seen in an *in vivo* set up; the efficacy of G1-4A was evaluated in a mouse infection model. G1-4A treatment of BALB/c mice inhibited the MTB burden in mice lungs irrespective of genotype or drug resistance status of MTB strains.

Cell mediated immunity (CMI), specifically Th1 response is critical to the control and eradication of mycobacteria in tuberculosis [276]. Th1 cells express IFN-γ, which plays a critical role in the host defense against MTB infection; on the other hand Th2 cells inhibit the Th1 response by secreting IL-4, IL-5, and IL-13 [118,277,278]. Further regulatory T cells (Tregs) are also involved in the suppression of Th1 immune

response against MTB by producing TGF-β and IL-10 which prevent the production of IFN-γ [277-279]. The aim of immunomodulation as HDT for TB is to refurbish the Th1/Th2 balance either by suppressing Th2 response or by inducing Th1 response [280]. Therefore immunomodulators should have the ability to either evoke a pro-inflammatory response or suppress anti-inflammatory responses for better control of MTB infection [280]. Th1 cytokines particularly IFN-γ, is a key to the control of MTB infection, which activates infected macrophages to initiate the bactericidal response including production of TNF-α and nitric oxide [281]. In our study up-regulation of mRNA expression of IFN-γ, TNF-α and NOS2 was observed in lungs of MTB infected mice that received G1-4A treatment. The reduction in bacilli count in mice lungs after G1-4A treatment may be attributed to increased expression of IFN-γ, TNF-α and NOS2. In addition to this, the levels of cytokines like IFN-γ, TNF-α, IL-1β and IL-12 in the serum of G1-4A treated MTB infected mice were increased. IL-12, another crucial cytokine in controlling MTB, is secreted from Ag presenting cells and drives the Th1 response [281]. Increase in the IL-12 and IFN-γ levels after G1-4A treatment suggests Th1 polarizing potential of G1-4A. Level of IL-10, an anti-inflammatory cytokine, which alters the bactericidal properties of macrophages is increased during MTB infection to enhance the mycobacterial survival. Additionally, MTB is known to manipulate the equilibrium of T cell polarizing cytokines by enhanced expression of IL-4 in infected cells to promote Th2 response which supports the MTB survival [282]. Reduction in the level of IL-10 and IL-4 may have beneficial effects on host and our data demonstrated that G1-4A treatment reduced the serum IL-10 and IL-4 levels in MTB infected mice and this reduction seems to be related with reduced bacterial burden in mice lung after G1-4A treatment. Further, *in vitro* restimulation of splenocytes from MTB infected and G1-4A treated mice with PPD

led to increase in IFN-γ levels and decrease in IL-4 levels resulting into higher IFNγ/IL-4 ratio. This indicates the presence of Th1 polarized cells in G1-4A treated splenocytes, which further support our data that G1-4A treatment induces Th1 immune response in MTB infected BALB/c mice. Therefore, we concluded that G1-4A inhibits the bacterial burden by inducing Th1 response in mice.

One of the main targets of HDT in tuberculosis is to establish the therapeutic candidate as adjunct therapy, as targeting of host immune response has very rare possibility to yield sterile immunity against tuberculosis. In the recent times several groups have reported various adjunct therapies against tuberculosis. Singhal *et al* reported that anti-diabetic drug Metformin (MET) in combination with Isoniazid (INH) or Ethionamide (ETH) reduced the bacillary burden in the lungs of mice [283]. In another study Verapamil, an efflux pump inhibitor, was used in combination with first line anti-TB drugs in BALB/c mice infected with MDR strain of MTB and the treatment reduced the lung bacilli loads [284]. Further, Verapamil also exhibited synergistic effect with bedaquiline in a mouse model, consequently use of lower doses of bedaquiline may be allowed to reduce the dose related toxicity [285].

In concurrence with above reports we also observed enhanced bacterial clearance in mice lungs, when G1-4A was given in combination with INH compared to INH alone or G1-4A alone which indicated the potential of G1-4A to be used as adjunct to current drug regimen. MTB has evolved to coexist with the human host since thousands of years, its rapid eradication needs a paradigm shift in our therapeutic approaches which may utilize adjunct therapy as tool [286].

In summary our results supports a novel hypothesis that modulation of host immune responses by immunodulators or TLR agonist may have the potential to inhibit the MTB survival both *in vitro* and *in vivo* by activation of macrophages and induction of

Th1 response. Our intention here is not to establish G1-4A as therapeutic agent but our data provide a 'proof of concept' demonstration of the above mentioned hypothesis. This clearly shows that G1-4A may offer an attractive option of adjunct therapy for tuberculosis treatment along with current anti-TB drugs. Needless to say, further validation in other models like guinea pigs, rabbits and primates will be necessary before this promise can be realized and human clinical trials are carried out.

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